Journal Pre-proof

Use of Alcalase in the production of bioactive peptides: A review

Veymar G. Tacias-Pascacio, Roberto Morellon-Sterling, El-Hocine Siar, Olga Tavano, Ángel Berenguer-Murcia, Roberto Fernandez-Lafuente

PII: S0141-8130(20)34691-2

DOI: https://doi.org/10.1016/j.ijbiomac.2020.10.060

Reference: BIOMAC 16950

To appear in: *International Journal of Biological Macromolecules*

Received date: 16 July 2020

Revised date: 5 October 2020

Accepted date: 8 October 2020

Please cite this article as: V.G. Tacias-Pascacio, R. Morellon-Sterling, E.-H. Siar, et al., Use of Alcalase in the production of bioactive peptides: A review, *International Journal of Biological Macromolecules* (2018), https://doi.org/10.1016/j.ijbiomac.2020.10.060

Use of Alcalase in the production of bioactive peptides: a review

Veymar G. Tacias-Pascacio [a, b, +,*], Roberto Morellon-Sterling [c, +], El-Hocine Siar [c,d], Olga Tavano [e], Ángel Berenguer-Murcia [f] and Roberto Fernandez-Lafuente [c, *]

[a] Facultad de Ciencias de la Nutrición y Alimentos, Universidad de Ciencias y Artes de Chiapas, Lib. Norte Pte. 1150, 29039 Tuxtla Gutiérrez, Chiapas, México.

[b] Tecnológico Nacional de México/Instituto Tecnológico de Tuxtla Gutiérrez, Carretera Panamericana Km. 1080, 29050 Tuxtla Gutiérrez, Chiapas, México.

[c] Departamento de Biocatálisis. ICP-CSIC. Campus UAM-CSIC. Madrid. Spain.

[d] Equipe TEPA, Laboratoire LNTA, INATAA, Université des Frères Mentouri Constantine 1, Constantine, 25000, Algeria

[e] Faculty of Nutrition, Alfenas Federal Univ., 700 Gabriel Monteiro da Silva St, Alfenas, MG 37130-000, Brazil

[f] Departamento de Química Inorgánica e Instituto Universitario de Materiales, Universidad de Alicante, Alicante, Spain.

+ Both authors contributed evenly in this paper.

* Co-corresponding authors: rfl@icp.csic.es; Tel: +34-91 585 49 41 (R. F-L.); veymar.tacias@unicach.mx; Tel: +52-961 269 79 60 (V.G. T-P)

Abstract

This review aims to cover the uses of the commercially available protease Alcalase in the production of biologically active peptides since 2010. Immobilization of Alcalase has also been reviewed, as immobilization of the enzyme may improve the final reaction design enabling the use of more drastic conditions and the reuse of the biocatalyst. That way, this review presents the production, via Alcalase hydrolysis of different proteins, of peptides with antioxidant, angiotensin I–converting enzyme inhibitory, metal binding, antidiabetic, anti-inflammatory and antimicrobial activities (among other bioactivities) and peptides that improve the functional, sensory and nutritional properties of foods. Alcalase has proved to be among the most efficient proteases for this goal, using different protein sources, being especially interesting the use of the protein residues from food industry as feedstock, as this also solves nature pollution problems. Very interestingly, the bioactivities of the protein hydrolysates further improved when Alcalase is used in a combined way with other proteases both in a sequential way or in a simultaneous hydrolysis (something that could be related to the concept of combi-enzymes), as the combination of proteases with different selectivities and specificities enable the production of a larger amount of peptides and of a smaller size.

Key words: protease immobilization, protein hydrolysis, bioactive peptides, combienzymes, enzyme selectivity, enzyme specificity.

Layout

1. Introduction

Proteases are recognized as widely applicable enzymes, standing out for their uses in the pharmaceutical, cleaning, and food industries [1]. More recently, their application in the area of nutraceuticals has been highlighted, finding a wide application in the liberation of bioactive peptides. Peptides, even more so than proteins, have been showing potential for bioactivities that were not detected or occurred with less intensity in the intact protein [2, 3]. These bioactivities have been highly related to the type of protein used as raw material for the hydrolysis, since its size (number of amino acids) and the terminal amino acids (amino and carboxyl-terminal amino acids) can determine the potential bio-activity of the produced peptides. The final properties of the hydrolysate will be also determined by the specificity and selectivity of the utilized enzymes [4]. Alcalase has been shown to be one of the most efficient enzymes in the release of bioactive peptides from different protein sources. This review addresses these characteristics and potentialities of Alcalase, especially related to applications in the release of peptides with outstanding bio-functionalities.

1.1. Proteases

The most striking function of proteases is their role in promoting proteolysis, which classifies them as “Hydrolases” into the international system for the classification and nomenclature of enzymes (EC number), class 3, and subclass 3.4. - hydrolysis of peptide bonds [5]. Due to the different hydrolysis selectivity, proteases are classified as endopeptidases and exopeptidases, a characteristic that indicates the position in which the protease exercises its function in the substrate protein chain, but also indicates the

characteristic of the final products. Using endoproteases, for example, the researcher can generate products with larger peptides than using exoproteases. Amino exopeptidases are generally associated with the release of products with one, two, or three amino acid residues from the N-terminus, while carboxy exopeptidases are able to release free amino acids or dipeptides from the C terminus [6]. Endopeptidases are not restricted to terminal peptide linkages and find a much wider range of options for cleaving sites, and may also be more selective.

This hydrolytic function of proteases is not exercised randomly and it is usually not coincident between different proteases. Proteases have distinct specificities and selectivities, and this fact makes the final product of protein extract hydrolysis extremely varied depending on the enzyme even using the same substrate protein extract. This difference between the actions of different proteases can be seen as an advantage in the sense of having a huge variety of "tools" to be chosen and thus obtaining a wide range of final products from the same hydrolyzed protein source [1, 7]. These protease features determine how the enzyme active center interacts with the protein substrate chain, which largely depends on the configuration of the enzyme active site. In this way, proteases can also be divided into clans that highlight the particularities of their tertiary structure and catalytic sites, classifying proteases according to the iconic amino acid in the active site or metal present in its structure. That way, proteases are classified as: aspartic peptidases, cysteine peptidases, metallo peptidases, or serine peptidases, in addition to those with mixed catalytic type or unknown catalytic mechanism - unknown type [8, 9].

1.2. Alcalase

In the context of proteases, Alcalase is considered a “serine endopeptidase”, which provides information about the catalytic structure known for the classical catalytic triad of amino acids, being serine one of them. This enzyme also cleaves proteins in the middle of the amino acid chain [8, 9]. It was initially obtained from *Bacillus subtilis* and called “Subtilisin Carlsberg”. It was discovered by Linderstrom-Lang and Ottesen and purified by Gtintelberg and Ottesen [10]. Other proteases were produced from different strains of *Bacillus subtilis.* They presented broad specificity with an alkaline pH optimum. This enzyme has also been called subtilisin A, subtilopeptidase A, and when launched by Novozymes, "Alcalase". Nowadays this enzyme is produced by submerged fed-batch fermentation using *Bacillus licheniformis*.

Alkaline proteases are very significant from an industrial point of view, because of their activity and stability at alkaline pH values, having been used primarily as additives in detergent formulations. But their applications are increasingly broadly. They can be employed in the dehairing and bating leather, meat tenderizing, cheese flavor development, baked manufacture, or improving digestibility of animal feeds [11-14].

Alcalase, like other alkaline proteases, was first applied widely as a component of cleaning products, being the first detergent protease developed by Novozymes during the 1960s [15]. Later, other applications of Alcalase have been proposed, such as auxiliary in degumming of silk fibers process [16] or other fabric processes such as the enzymatic surface modification of polyamide [17]. Alcalase found a wide field of application in the production and modification of food. This application gained a huge impact, as it will be exposed in this review, with its use in the production of protein hydrolysates. These applications were highlighted in the 70s, as in the report of Hale (1972) with its application in making fish protein concentrates after Alcalase catalyzed hydrolysis [18].

Commercial "Alcalase®" is a registered trademark of Novozymes Corp. and consists of a liquid enzymatic preparation composed of about 50% (w/w) glycerol, 41% (w/w) water and 9% (w/w) protease extract from *Bacillus licheniformis*. Its activity is expressed in Anson Units (AU) and the most typically activity is ≥2.4 U/g, which may have purity specifications for food-grade product, according to conditions by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC).

The positions of the amino acids in the substrate around the hydrolysis site are conventionally numbered in P_1, P_2, P_3, etc., to the left of the scissile bond and as P_1', P_2', etc., those to the right of the hydrolysis site. Considering that in a protein chain it is assumed that the first amino acid has the N-terminus and the last the C-terminus, when the peptide bond is broken, the amino acid corresponding to P_1 will be the one that presents the radical carboxyl terminal of the new fragment or newly generated peptide. Equally, the amino acid present in P_1' will be the new terminal amino of the second fragment released [19].

Alcalase specificity is described as preferential for a large uncharged residue in P_1, but other specifications have already been pointed out. Adamson and Reynolds (1996) observed the cleavage of peptide bonds when the amino acids Glu, Met, Leu, Tyr, Lys, and Gln are positioned at P_1, preferentially if Glu was at P_1 and also another hydrophobic residue in P_2' or P_3' [19]. . In this way, Alcalase can be used to obtain peptides with general hydrophobic characteristics. Due to the wide range of amino acids that it can recognize, the reaction of protein hydrolysis catalyzed by Alcalase has a strong tendency to give a hydrolysate with many peptides of small size.

This broad enzyme selectivity and specificity permit the use of Alcalase in a wide variety of protein substrates always yielding a high protein hydrolysis degree, either applied individually or in association with other proteases. When Ahmadifard *et al.* (2016) performed a comparison between the efficiencies of Alcalase, papain and a commercial cocktail containing trypsin, thymotrypsin, and aminopeptidase in the enzymatic hydrolysis of rice bran protein concentrate and soybean protein the results showed that Alcalase presented a higher capability for hydrolysis (about 10 times higher than the other tested enzymes) [20].

In the study by Kula *et al*. (2020), when the enzymes Trypsin and Alcalase were used separately, they reached a degree of hydrolysis of *Trachinus draco* proteins of about 44%, but when they were used in a sequential way (2h for trypsin hydrolysis and, after, 2h of hydrolysis using Alcalase) the hydrolysis degree reached a value of about 78%. These results illustrate the synergy effect of two enzymes that act in different sites of the protein to be digested, since they present different specificities [21].

The interest in protein hydrolysates has been progressively growing in the last times, being an interesting alternative to the use of intact proteins both in foods [22] and feed applications [23]. The interest in protein hydrolysates is founded on improving the characteristics of the protein, increasing its digestibility or reducing its allergenic characteristic. Moreover, the production of new compounds, free amino acids and peptides of varying sizes and sequences, may improve the functional properties of the original protein, depending on the source the substrate protein and of the employed protease [1, 7]. Among these peptides, many can have bio-functions, that is, they can positively impact human and animal metabolism and health, which has intensively attracted research in order

to establish protocols for the production of these peptides [24]. Alcalase has found a broad field of application in this matter. That is in fact the main objective of the current review.

Alcalase is remarkably stable at moderately alkaline pH values. This has made it a very adequate enzyme for detergents [25]. However, it is not so stable under other pH conditions. The company reports that optimal activity may be found at pH 10, maximum activity at 70ºC and that the enzyme maintains full activity at room temperature in the pH range 5 to 11, with a deeper decrease in activity at more acidic pH vales than at more alkaline pH values [26]. The enzyme is also quite stable in organic medium, and this has permitted to use it in many different reactions [27]. For example, in 2- methyl-2-propanol and ethanol, 50% of the initial activity was retained after 5 days, and in tert-amyl alcohol, Alcalase remained fully active for weeks. However, in methanol, only 50% of the initial activity was retained after 35 min. The stability increased as the polarity or dipole moment of the solvents decreased. This means that Alcalase was 4 fold more stable than Subtilisin Carlsberg in ethanol [27]. That is, even if the enzyme is very stable, further stabilization may enlarge the range of operational conditions.

1.3. Bioactive peptides

Proteins are important health promoting agents due to their nutritional and nutraceutical potential. Both the intact form of proteins and their free amino acids or peptides can perform these functions, but it has been shown that peptides present a greater potential to exhibit bioactivities, due to their particular potential to be better absorbed through the small-intestinal epithelium by passive transcellular mechanisms, carrier-mediated transport via PEPT1, transcytosis, or via paracellular mechanisms [4]. This way,

peptides are effectively able to be applied as health promoters, whether in curative and/or preventive metabolic aspects *in vivo* [3].

Many bio-functionalities of peptides have been demonstrated on *in vitro* and *in vivo* studies, including positive impacts on cardiovascular, immune or nervous systems, such as inhibitors of the angiotensin-I-converting or dipeptidyl peptidase IV enzymes, antioxidant, antithrombotic, opioid, hypocholesterolemic or immunomodulating activities [2]. One fact that is usually unexplored is that some of the bioactivities of peptides may be negative, as the possibilities of sequence, confirmation, size, etc. are huge. These negative peptides will be presented when analyzing the whole protein hydrolysate, and they can hide the effects of some beneficial peptides. That way, a purification (at least a fractioning) of the peptides may help detect positive bioactive peptides. The negative ones could be useful to understand some mechanism of action.

The combination of these potential activities and their capability to reach the site of action makes these molecules extremely interesting. These capabilities seem to be related to specific characteristics of the peptides, such as size and sequence [4]. Some of the relationships between the concentration and/or certain characteristics of the peptide that make it preferable (or not) for a given adsorption route are already evident. For example, when peptides are in low concentration, the route through absorption transport by PepT1 is the major contributor to the total transport rate, with passive transport being favored when high peptide concentrations are available in the absorption environment [28]. But the concentration of the peptide is not the defining factor of the absorption pathway. The pathway through PepT1 is preferably used by small peptides, as di- or tri-peptides with neutral charge and hydrophobic nature, with special affinity for peptides containing nonpolar amino acids. The same may be said for peptide transport by transcytosis, while the

paracellular route preferably transports low molecular weight and hydrophilic peptides [4, 28, 29]. Regarding the bioactivities exhibited by the peptides, these are also related to their characteristics. As highlighted by Nwachukwu and Aluko, low molecular weight peptides exhibited antioxidant potential activity, mainly if they present hydrophobic amino acids such as Leu or Val in their N-terminal regions, and even stronger antioxidant activity may be found if they presented a sulfur- (Cys and Met) , aromatic amino (Phe, Trp, and Tyr) or His residues [30]. Lee and Hur showed that the presence of proline, isoleucine or leucine at the N-terminus of the peptide increased the ACE-inhibitory activity of the peptides [31].

The characteristics of the peptides are closely related to the protein chain from which they were released and the protease used for this hydrolysis. With regard to the protein chain, different proteins, with their different sequences of amino acids and sizes, may be susceptible to hydrolysis, and they can release very different peptides, even if the same enzyme is used in the hydrolysis process. That way, different protein sources have been used in the generation of protein hydrolysates such as plants, fish, milk, egg or even insects [32, 33]; which produce a very wide repertoire of final products. Although the source of the protein does not exactly define the characteristic of the protein itself, the matrix where this protein is immersed may be very different depending on the enzyme source, and some characteristics of this matrix can influence the performance of the enzyme chosen for the protein hydrolysis [34-38]. The cellular structure of the material, tissue integrity, presence or absence of protease inhibitors, are some examples of characteristics that make the environment in which the protein is more or less adequate to be hydrolyzed by a specific enzyme. The selection among the different protein fractions of a material, or types of previous processing of the protein substrate material before the hydrolysis step, can make a protein source more or less suitable for hydrolysis and release of bioactive peptides

[32, 33, 39]. Alcalase stands out as a protease able to release peptides with potential for bioactivities [40]. These matters will be the subject of the current review.

2. Immobilization of enzymes

Enzymes have some properties that make them highly desirable catalysts with very good prospects for industrial implementation; they are very active under mild conditions, very selective and specific [41-45]. This is stressed nowadays with the huge public demand for green chemistry [45-48]. However, their biologic origin means that they have evolved under natural selection to give a rapid answers to stress conditions, making some enzyme properties not desirable for industrial use: enzymes are inhibited, unstable, present saturation kinetics, etc. [49]. Moreover, enzymes are water-soluble molecules, making their recovery and reuse difficult [50]. Mainly in food uses, enzyme solubility causes the enzyme or enzyme fragments to be incorporated to the aliment, and this is not always desired as it can give rise to some allergic reactions. Enzyme immobilization solved this problem, enabling the preparation of heterogeneous biocatalysts [51, 52].

Together with enzyme reuse, an immobilized enzyme may be utilized in many reactor configurations and permits a stricter control of the reaction [53, 54]. Moreover, modern enzyme immobilization pursues other objectives [55]. The most usual is the improvement of enzyme stability [56-58]. Enzyme operational stabilization may be accomplished just by having the enzymes immobilized on the surface of the pores of porous particles, that will prevent enzyme intermolecular interactions (preventing enzyme proteolysis or enzyme aggregation) or interactions with external surfaces (e.g. gas bubbles or drops of solvents) [59, 60] that can lead to enzyme inactivation [61]. More interestingly,

immobilization of enzymes via multipoint covalent attachment may permit the rigidification of the enzyme structure, limiting the possibility of conformational changes and improving enzyme stability caused by any distorting agent. This can allow extending the range of conditions where the enzyme is utilized [62-64]. When the enzyme is a multimeric enzyme, and the first step of enzyme inactivation is subunit dissociation, immobilization via all enzyme subunits will fully prevent this inactivation cause [65], once again permitting the use of the immobilized enzyme under conditions where the free enzyme cannot be used [66]. Moreover, a proper immobilization can allow purifying the enzyme during the immobilization process [67].

Immobilization may also improve enzyme activity (e.g., that is the case of lipases immobilized on hydrophobic supports via interfacial activation at very low ionic strength) [68-71], reduce inhibition and tune enzyme selectivity or specificity [56]. That way, enzyme immobilization is not just a simple way to enable enzyme reuse, but it may become a powerful tool in the design of an industrial biocatalyst. However, this can only be obtained if the support, active group and immobilization protocol are properly designed [72-75] .

2.1. Immobilization of proteases

Proteases, as stated before, have many possible applications [76-81]. Proteases immobilization and all advantages derived therefrom may be also a very important tool to permit the use of these enzymes in industry [1, 82]. For example, in many instances proteases are used to hydrolyze precipitated proteins (e.g., after oil extraction with benzene) that need to be redissolved using chaotropic agents [83, 84]. An extensively rigidified enzyme by multipoint covalent attachment may be used even in these media [85-87]

(Figure 1). Using proteases, one of the additional advantages of enzyme immobilization is the prevention of autolysis, a general phenomenon using proteases [88-91]. This protection mainly occurs if the enzymes are immobilized on porous supports (using non-porous supports, the enzymes on one particle can hydrolyze the enzyme molecules located on another particle) [61] (Figure 2). When the enzymes are used in the hydrolysis of proteins, in many instances the hydrolysis degree is a key point to reach the desired properties in the product [92-96], and the use of immobilized proteases may facilitate the control of the hydrolysis degree. Using free enzymes, the only way to stop the reaction is protease inactivation, and this inactivated enzyme will become part of the final product. Some new applications of immobilized proteases have been reported. For example, immobilized proteases may be utilized in the two-step coagulation of milk proteins, using during the hydrolysis step a temperature at which the hydrolysate precipitation does not occur, and then, changing the conditions after filtration to recover the immobilized enzyme, where the hydrolysate precipitation step takes place [97-102]. Immobilized proteases have been used to produce antimicrobial packages, as they can destroy bacteria and some fungi [103-107].

However, even with the many advantages and applications of immobilized proteases; there are some specific problems that need to be considered in protease immobilization. If they are going to be employed in fine chemistry using small substrates, enzyme orientation will not be a key point in the final immobilized enzyme performance. However, if the enzyme is used in the hydrolysis of proteins some additional problems may appear [1, 61] (Figure 3). Only properly oriented enzyme molecules can attack these large substrates, any enzyme molecule with the active center oriented towards the support surface will be fully inactive at least in the first hydrolysis steps, although perhaps it may attack to

the successively smaller protein fragments generated in the hydrolysis [108]. Moreover, the enzyme support loading determines the requirements for a proper enzyme orientation [85, 86, 109]. A lowly loaded enzyme biocatalyst, with the enzyme molecules dispersed on the support surface, may have no steric hindrances to hydrolyze the protein substrate even if the enzyme molecules have not the active center fully oriented opposite to the support surface (Figure 4). However, only perfectly well oriented enzyme molecules will be active versus the proteins using a fully loaded protease biocatalyst. This is valid for porous and non-porous supports (Figure 4).

The problems will be also influenced by the size of the substrate protein. If the substrate protein is much larger than the protease, this can result in the pore diameter of the support having a significant proteolytic activity, as pore diameters that permit the entry of the enzyme may not permit the entry of the protein substrate (Figure 5). The use of supports with larger pores reduces the volumetric loading capacity of the support and also their mechanical resistance, both undesired effects [72]. If this is not considered, it may be that an immobilized protease biocatalyst with perfectly oriented enzyme molecules may be almost fully inactive in the target process.

The situation using porous supports becomes more dramatic using insoluble substrates, such as textile materials, as only the enzyme immobilized on the support external surface will be able to hydrolyze the substrate [61] (Figure 6). This may be under 0.1% of the enzyme molecules immobilized on a porous support. Using solids as substrates, only non-porous nanoparticles can be utilized as catalysts, as in this case at least a significant proportion of the enzyme molecules can to the solid (if properly oriented) (Figure 7). Magnetic nanomaterials may permit the handling of these small particles [82].

However, it should be stressed that now the enzyme is neither protected from interfaces nor proteolysis [110].

All the steric problems are critical at the beginning of the reaction. However, the expected reaction course may be quite different from those when these steric problems do not exist. The initial protein substrate is very large, but the smaller fragments produced by the hydrolysis caused by the few available enzyme molecules that can attack the substrate, may be later subject of hydrolysis by more enzyme molecules no so favorably immobilized, and when the size is very reduced, by all enzyme molecules (Figure 8). That is, a progressive acceleration of the reaction may be found when the protein hydrolysis reaction advance.

There are some problems when analyzing the effects of immobilization on protease stability. The first one is that if autolysis plays an important role in protease inactivation [110], an enzyme just immobilized and dispersed in a porous support may, apparently, greatly increase enzyme stability, as this autolysis is no longer possible [61]. In this instance, the protease concentration in free form may determine the apparent stability: the more concentrated the enzyme is, the more autolysis occurs. Mixing the enzyme with some inert protein, or competitive inhibitors, may reduce this problem, enhancing proteases storage stability. Another problem is that the liquid formulations of proteases may have some agents to prevent this autolysis, usually presenting stabilizing effects on the enzyme [111]. That way, the use of the concentrated enzyme solutions will have a high concentration of these reagents, while diluted concentrations of this extract will reduce their concentration. That is, the protease stability may increase when the crude protease solution concentration increases. To prevent this, the best solution is to compare the immobilized

enzyme with one-point covalently immobilized enzyme, as this should have stability properties very similar to those of the free enzyme, but in the absence of any intermolecular process [112, 113] (Figure 9). Next, we will focus on the examples of immobilization of Alcalase.

2.2. Immobilization of Alcalase

We will next review the different strategies applied to immobilize Alcalase since 2010. Alcalase immobilization will be important to facilitate its reuse. Moreover, although the enzyme is very stable compared to other proteases, (mainly under alkaline conditions and also in some organic solvents (see a section 1.2), further stabilization of the enzyme may permit to enlarge the range of conditions where the enzyme may be used [63, 64].

In a first example, Alcalase was immobilized on glyoxyl agarose and utilized to produce hydrolysates of chickpea protein [114]. This biocatalyst was selected due to the high stabilization achieved when the enzyme was immobilized [115-117]. The protein hydrolysates feature improved when compared to the intact proteins, being this more remarkable at pHs near the isoelectric point of the intact chickpea proteins. Although the emulsifying activity did not improve, this treatment improved many other functional chickpea protein properties [114]. This biocatalyst was used by another group in the hydrolysis of whey protein isolate to reduce its antigenicity [118]. However, the immobilized enzyme did not reduce α- and β-lactoglobulins as efficiently as the free enzyme.

In another research, lauroyl glycine lipoaminoacid was synthetized using a kinetically controlled strategy, comparing the performance of the octyl-agarose

immobilized lipase from *Pseudomonas stutzeri* and Alcalase immobilized on glyoxyl-silica supports [119]. Both enzymes favor the lauroyl glycine synthesis over the Gly-Gly peptide synthesis, but the immobilized protease gave the best yield and selectivity balance: less than 5% for dipeptide and 40% yield for lauroyl glycine [119].

Later, Alcalase was immobilized in another research report using glass sol-gel matrices and tetramethoxysilane and the biocatalysts were used for catalyzing C-terminal amidation of Z-Ala-Phe-OMe [120]. The immobilized biocatalyst prepared with dimethyldimethoxysilane gave the best performance in the ammoniolysis of Z-Ala-Phe-OMe. 115 mg of proteins could be immobilized per gram of dry silica xerogel. The immobilization improved the enzyme thermal stability at 70 °C threefold [120].

Vossenberg and coworkers were very active in the immobilization and use of immobilized Alcalase in this time period. In an interesting paper, they tried to simultaneously utilize a lipase and Alcalase as catalysts for the one-pot enzymatic synthesis of peptides [121]. The lipase could be hydrolyzed by the Alcalase if both enzymes were used in free forms. To avoid this, the lipase and the proteases were immobilized onto macroporous beads, showing that immobilization of either the lipase or the protease (and even better both enzymes) reduced this problem [121]. In another research, this research group studied the Alcalase catalyzed coupling of the carbamoylmethyl ester of N-protected phenylalanine with phenylalanine amide in tetrahydrofuran, using different immobilized Alcalase forms [122]. This is a kinetically controlled process, where the yields are determined by the kinetic properties of the enzyme and they are transient, as the product may be the substrate of the enzyme [123]. In this reaction, the maximum yields are determined by the enzyme properties and even by the way the enzyme is immobilized [62,

66, 124]. In this new research effort, the authors analyzed the effect of enzyme hydration prior to drying, and found a significant increase in the activity of the enzyme in this reaction by the hydration treatment. The best activity was obtained using dicalite activated using glutaraldehyde as immobilization support, but their low stability led the authors to conclude that the most promising Alcalase covalently immobilized biocatalyst for these reactions was the one prepared utilizing macroporous acrylic beads [122]. In a new research effort, this group studied the same reaction using the optimal Alcalase catalysts, and controlling the water activity during the reaction [125]. The kinetics of the process was analyzed, and they found that it followed a two-substrate kinetic model with two competitive product inhibition terms. The authors proposed the continuous removal of the strongest inhibitor (the glycol amide) to improve the reaction course [125]. Later, they focused their efforts on the stability of the immobilized Alcalase in this reaction [126]. The addition of molecular sieve beads reduced the operational stability of the immobilized enzyme (mainly because of the mechanical breakage of the biocatalyst particles), and intermediate rehydration of the immobilized enzyme also promoted some activity losses. The inactivation produced by the molecular sieves was studied in more detail in a further paper [127]. Enzyme inactivation followed three phases, a fast and initial enzyme inactivation induced by the dehydration, an inactivation that follows first-order kinetics, and a plateau. This was used to build a model that predicted the enzyme behavior in a reactor. Then, they moved the immobilization technique to the immobilization via crosslinking of enzyme aggregates. This technique is a carrier-free immobilization method that consists in the chemical crosslinking of enzymes that have been previously precipitated, permitting the use of the enzyme aggregates under any experimental condition [128, 129]. This research group, using the coupling of carbamoylmethyl ester of N-

protected phenylalanine and phenylalanine amide as model reaction, employed Alcalase CLEA-OM (commercially available from CLEA Technologies). This catalyst was used again to analyze the effect of the water activity on the hydrolysis reaction (of the activated acyl donor) [130]. Results suggested that hydrolysis was relevant only if the water activity was over 0.2. This commercial biocatalyst was used later by another research group, to analyze the promiscuous capacity of the enzyme to produce C-C bonds (aldol, Henry and Mannich reactions) [131]. Moreover, Bayllis-Hillman reaction between methyl vinyl ketone and 4 -nitrobenzaldehyde happened through unspecific catalysis. Aza- Michael addition reactions of pyrrolidine, piperidine, and more efficiently using diethylamine to acrylonitrile were catalyzed by this commercial preparation [131]. This immobilized catalyst was also utilized to prepare (S)-clopidogrel, by resolving the building block(RS)-N-Boc-2-chlorophenylglycine methyl ester [132].

Alcalase has also been immobilized on magnetic nanoparticles, for example in chitosan-coated magnetic nanoparticles activated with glutaraldehyde [133]. This immobilization broadened the pH and temperature range where the enzyme could be utilized. The biocatalyst was employed in a proteolysis reaction, obtaining a hydrolysis degree of 18.38 %, versus the 17.50 % obtained using the free enzyme [133]. Immobilized Alcalase was found to be useful in the resolution of racemic mixtures of N-benzyl-3-hydroxypyrrolidine and N-benzyl-3-hydroxypiperidine, as it exhibits the opposite enantiospecificity to other enzymes [134]. Alcalase was hydrophobically adsorbed onto macroporous silica gels submitted to diverse modifications [135]. The biocatalysts were stable in the dynamic kinetic resolution of racemic N-Boc-phenylalanine ethyl thioester via aminolysis with benzylamine producing (S)-N-Boc-phenylalanine benzylamide in high

enantiomeric purity. To reach this goal, the researchers coupled alternatively six biocatalyst-filled and five grafted silica gel-filled reactors, the enzymatic reaction was performed at 50 °C and the base-racemization was carried out at 150 °C [135].

In another research, the profiles of produced peptides in the hydrolysis of whey protein were studied using free and immobilized Alcalase under different conditions [136]. The proposed conditions were a substrate concentration of 7%, pH between 8 and 9 and 50°C, producing a hydrolysate with very good organoleptic features to be added in commercial desserts. In another paper, Alcalase was immobilized on alginate beads, and used to analyze the effect of ultrasounds in the hydrolysis of rapeseed protein [137]. The hydrolysis degree increased by almost 75% with ultrasound irradiation under optimal conditions. The same research group used that biocatalyst to hydrolyze casein [138]. This group later used triple-frequency ultrasound treatment to study its effects on the performance of immobilized Alcalase in the hydrolysis of corn gluten meal [139]. This improved the peptide concentration by 34.4 %, the degree of hydrolysis by 20.6 %, the relative enzyme activity by 25.0 %, and the ACE inhibitory activity by 24.1 % [139].

In another paper, Alcalase was immobilized by physical adsorption, enzyme crosslinking with glutaraldehyde or covalent enzyme binding to activated chitosan microbeads and used to hydrolyze soy protein and egg white [140]. A hydrolysis degree of almost 30% in 180 min was obtained by the enzyme immobilized on activated chitosan. In another research, mesoporous silica nanoparticles were coated with acrylic acid or chitosan, and employed to immobilize Alcalase [141]. The coated nano-particles gave better results in terms of Alcalase activity, stability and reusability. In another research, sol-gel immobilized Alcalase was used to hydrolyze proteins from seeds from *Gnetum gnemon*

[142]. After 2 hours at 50°C, around 23% of hydrolysis degree was obtained, with a profile showing low molecular weight peptides. These peptides presented a very good antioxidant activity [142]. In another example, Alcalase was immobilized on carboxyl-functionalized magnetic beads using the carbodiimide route and used to reduce the allergenicity of egg white protein [143]. The immobilization improved Alcalase thermal and storage stabilities and the obtained hydrolysates reduce IgE and IgG binding [143]. Other research reports used Alcalase and Flavourzyme immobilized on sodium alginate to hydrolyze seed proteins from *Linum usitatissimum* [144]. Among the produced peptides, those with a molecular weight over 1,000 Da improved the stability and mouthfulness of umami soup; while smaller peptides presented a significant effect on umami taste and bitterness [144]. Later on, amino silane modified yttria stabilized zirconia capillaries was used to immobilize Alcalase [145]. The degree of hydrolysis of lupin sunflower and casein protein isolates was controlled by adjusting the residence time and that way altering the enzyme specific peptide fingerprint [145].

Alcalase was also immobilized using a nanoflower strategy [146, 147], using calcium hydrogen phosphate to trap the enzyme [148]. The biocatalyst increased by 57% the activity of the free enzyme in the hydrolysis of soybean protein isolates. The hydrolysates presented a good calcium-binding and radical-scavenging capacities [148].

In another paper the effect of the immobilization on glyoxyl agarose of Alcalase on the activity versus a small substrate (Boc-L-alanine 4-nitrophenyl ester) and versus casein were compared [149]. While with the small substrate the recovered activity was 50%, the recovered activity versus casein was under 20% at 50ºC. However, at 60 °C, the activities of free and immobilized enzyme became similar. Using the advantages of the solid phase

chemical modification [150, 151] the immobilized enzyme was treated with glutaraldehyde or was chemically aminated, these treatments only doubled the enzyme stability with high losses of enzyme activity. However, the modification with glutaraldehyde of the previously aminated enzyme greatly stabilized the immobilized enzyme and permitted to use the biocatalyst in the hydrolysis of casein at pH 9 and at 67 °C. The enzyme could be reused under these drastic conditions for 5 hydrolytic cycles maintaining 50% of the activity, while the non-chemically modified immobilized preparation was almost inactive after 3 cycles. At 45 °C and pH 9, the modified enzyme could be used for six cycles of 6 h without a detectable decrease in enzyme activity [149]. The same group showed the synergy of different immobilization causes in the Alcalase immobilization on amino-glutaraldehyde: the enzyme was readily immobilized on amino-glutaraldehyde at low ionic strength while it was not immobilized on the amino support, and neither on amino glutaraldehyde at high ionic strength [152]. The immobilization pH value determined the activity versus casein. While when immobilizing the enzyme at pH 5 the activity versus casein decreased by 50%, after immobilization at pH 9 the activity increased to 140% and at pH 7 the immobilized enzyme doubled the activity of the free enzyme [149].

That way, Alcalase immobilization has been a topic of great interest in this time-period, showing how it can greatly improve enzyme performance in diverse reactions.

2.3. Coimmobilization of Alcalase with other proteases

When two or more enzymes are used in a cascade reaction, the use of coimmobilized biocatalysts may give some kinetic improvements, mainly in the first stages of the reaction [61, 153, 154]. These advantages may be a key point in some instances, mainly if the intermediate product is unstable. However, enzyme coimmobilization has

some problems, which have been recently reviewed [155]. This makes that coimmobilization may only be recommended if the advantages outweigh the problems. Unfortunately, these drawbacks are hardly considered.

The hydrolysis of proteins catalyzed by several proteases may be considered a cascade reaction [156, 157]. Thus, Alcalase and trypsin were coimmobilized in calcium alginate-chitosan [158]. The new coimmobilized biocatalyst gave a hydrolysis degree of 65.8% while each single immobilized enzyme gave as maximum 45.5% or the free enzyme yielded 49.3% [158].

In another research, Alcalase and trypsin were coimmobilized using magnetic nanoparticles that were first coated with chitosan, then with sodium tripolyphosphate, and finally treated with glutaraldehyde [159]. Enzyme stabilities were improved after coimmobilization. When used in various proteins hydrolyses, the catalysts yielded suitable degrees of hydrolysis, yields and antioxidant activities of the hydrolysates [159]. However, a comparison with the individually immobilized enzymes is lacking.

The coimmobilization of several proteases may have a great interest, but the studies that we have found in this time-period are limited.

3. Production of bioactive peptides by Alcalase hydrolysis of proteins from different sources

Next, we will review the use of Alcalase in the production of bioactive peptides from 2010, as the amount of available papers is huge to make a full review of the uses of this enzyme even in this specific topic. We will revise the hydrolysis of proteins from different sources, using Alcalase, comparing Alcalase with other proteases, using Alcalase

and other proteases sequentially or using simultaneously Alcalase and other proteases (Figure 10).

3.1. Production of multifunctional peptides

Through the previous topics it was possible to observe that Alcalase has a high potential for the release of peptides with different bioactivities from different protein sources.

In many instances, only one bioactivity of the protein hydrolysates is analyzed. However, it is very likely that these hydrolysates, containing many different peptides, can contain multiple bioactivities. Thus, a large number of studies find two or more potential bioactivities for the same hydrolysate produced by Alcalase hydrolysis. For example, Sutthiwanjampa and Kim produced a *Venus clam* hydrolysate using Alcalase , and this presented antioxidant, anti-tyrosinase and immunomodulatory activities [160]. Xie *et al.* showed that the mung bean hydrolysate, produced via Alcalase hydrolysis, exhibited the highest degree of hydrolysis and excellent antioxidant and ACE inhibitory activities, compared to the products obtained using other tested proteases [161]. Santos Aguilar *et al.* demonstrated the efficiency of using Alcalase in conjunction with Flavourzyme in the hydrolysis of chicken viscera producing an interesting hydrolysate with antioxidant and also antihypertensive properties [162].

Using hydrolysates, this multiple function can be expected due to the wide variety of peptides that are released, especially in the case of enzymes such as Alcalase, whose broad specificity allows it to break many peptide bonds and generate a large number of different peptides. This number of fragments is even greater if an unpurified protein source is utilized as substrate, where countless chains of different proteins may be present.

In the case of hydrolysates, the tests reveal the potential of the mixture of peptides as a whole, and among the various peptides it may be those with different specific functions that give a multifunctional characteristic to the hydrolysis product. In the case of a peptide identified as multifunctional, it is the peptide itself that exhibited two or more activities. As described by Lammi *et al*., multifunctional peptides are those peptides "*which have the capacity to impart more than one physiological outcome by affecting different targets*" and "*may be considered an improvement in respect to monofunctional peptides*"[163].

As an example, Kula *et al.* observed that the myofibrular hydrolysate of *Trachinus draco* proteins after trypsin and Alcalase treatment were inhibitors of ACE and DPP4, beside they presented antioxidant and metal chelating activities. After isolating some of the peptides present in this hydrolysate, they observed that there were peptides with a single bio-function, such as Ala-Ala-Gly-Asn-Ser-Gly-Ser-Ser-Gly-Asn-Thr-Asn-Thr-Leu-Gly-Tyr-Pro-Ala-Tyr-Lys, that was a peptide with ACE inhibition, and also peptides with multifunctions such as Asn-Ala-Ser-Gly-Ser-Thr-Ala-Met-Lys-Gln-Ala-Val-Asp-Asn- Ala-Tyr-Ala-Arg, presenting ACE inhibition, metal chelating and antioxidant activities, or Phe-Pro-Gly-Asp-His-Asp-Arg presenting DPP4 inhibition, metal chelating, and antioxidant activities [21].

Other studies demonstrate Alcalase efficiency in releasing multifunctional peptides from different protein sources. Karamia *et al.* observed that peptides released from wheat germ protein by the action of Alcalase had different functions, such as GNPIPREPGQVPAY, an efficient radical scavengers and anti-hypertensive peptide. In the same hydrolysate, the authors identified the peptides TVGGAPAGRIVME and VGGIDEVIAK presenting both anti-hypertensive and anticancer activities [164]. Montone *et al*. characterized peptides released by Alcalase from cauliflower by-products with both

ACE inhibition and antioxidant functions, such as SKGFTSPLF peptide. Alcalase has also been used in combination with other enzymes to release these multifunctional peptides [165]. Zheng, Li, and Li , for example, identified three peptides with multifunctional function of coconut cake albumin after sequential digestion with Alcalase, Favourzyme, pepsin and trypsin presenting ACE-inhibitory and antioxidant activities [166].

Due to their multifunctionality, these hydrolysates, and even more especially these specific peptides, could be more efficiently applied to control certain complex diseases. For example, in the treatment of cardiovascular diseases, which is a multifactor disease in itself [163], peptides that combine actions such as anti-inflammatory, hypotensive, hypocholesterolemic, anti-diabetic and / or antioxidant can act more broadly and effectively [163].

It is important to highlight that these multiple functions must be assumed to be even more present among hydrolysates and even among isolated peptides, than the literature presents, bearing in mind that these works often present tests with clearly complex hydrolysates in peptide composition but where unique bioactivities are tested. In other words, care must be taken not to admit that a single tested bioactivity represents the only monofunctionality covered by a given material.

3.2. Production of peptides with antioxidant activity

Free radicals affect both human health and food quality; in the body these unstable radicals react easily with biological macromolecules such as unsaturated lipids, nucleic acids (DNA and RNA) and carbohydrate polymers, which can cause oxidative stress, generating many health disorders such as neurodegenerative diseases, arteriosclerosis, cancer, diabetes mellitus and inflammatory diseases associated with tissue injuries [167-

169]. In foods, the presence of free radicals causes their oxidation which directly affects food quality by alterations of flavor, color, texture and loss of nutritive value [168, 170]. For this reason, in order to preserve food quality, many synthetic antioxidants such as butylated hydroxyanisole, propyl gallate and butylated hydroxytoluene have been used in the food industry [171]. Nevertheless, it has been reported that, the use of large quantities of synthetic antioxidants causes stability problems in foods and can be a potential health hazard [172-174]. This has led to a strong demand and search for natural antioxidants that can replace synthetic compounds [175]. It is currently known that many natural products such as flavonoids, carotenoids, phenolic acids, vitamin E, ascorbic acid, proteins and their respective hydrolysates and peptides, possess antioxidant activity. Furthermore, they can be used as food additives and pharmaceutical excipients [176]. In this context, hydrolysates and peptides with high antioxidant activity have been prepared from many sources of proteins [177], and they represent an excellent option to be used as nutritional supplements and natural antioxidants in oxidative stress management [178].

As previously mentioned, enzymatic hydrolysis of proteins is an effective method to prepare antioxidant peptides, and in general, it is widely applied to improve and upgrade the nutritional and functional properties of proteins [179]. Next, examples of Alcalase utilization to produce antioxidant peptides from different protein sources are presented.

3.2.1. Hydrolysis of vegetable proteins

3.2.1.1. Use of stand-alone Alcalase

There are many reports in which the enzyme Alcalase is used to hydrolyze proteins of vegetal origin to get antioxidant peptides. In this regard, one of the most reported

proteins for its conversion into peptides with antioxidant activity by hydrolysis with Alcalase is soybean protein [180]. It has been reported that 72 h of germination in combination with 1 h of Alcalase hydrolysis of Brazilian soybean cultivar BRS 133 generated peptides with potent antioxidant activity and which are effective in the reduction of some inflammation markers [181]. Furthermore, optimized operational conditions of Alcalase hydrolysis (50 °C, pH 10.32, and enzyme/substrate ratio of 12%) of soybean protein produced hydrolysates with strongest antioxidant capacity [182]. Besides, the scavenging activity (43.6% on 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical *in vitro*) of Alcalase hydrolysate of soybean protein isolate can be improved by its modification with the plastein reaction catalyzed by Alcalase [183].

A potent antioxidant peptide has been purified from soy protein hydrolysates obtained by Alcalase hydrolysis [184], and it has been observed that the Alcalase soybean protein hydrolysate which displayed 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (IC50 = 4.22 mg/mL), ABTS radical scavenging (IC50 = 2.93 mg/mL), reducing power and metal ion-chelating activities (IC50 = 0.67 mg/mL), significantly inhibited the generation of intracellular reactive oxygen species in Caco-2 cells [185]. In another paper, soybean protein hydrolysate prepared with Alcalase was subsequently ultrafiltered and separated into four peptide fractions [186]. Results showed that fraction SPH-I (< 3 kDa) exhibited the strongest DPPH radical scavenging activity and reducing capacity. It also showed dose-dependent suppressed intracellular reactive oxygen species accumulation induced by H_2O_2 in Caco-2 cells. It also protected Caco-2 cells from H_2O_2-induced oxidative stress via inhibiting lipid peroxidation and stimulating antioxidant enzyme activities [186]. In addition, the antioxidant peptides from the low molecular

weight fraction of Alcalase soybean hydrolysate presented cyto-protective effects against oxidative stress in human intestinal Caco-2 cells [187].

Corn and its zein protein Alcalase hydrolysates have also been investigated for their antioxidant activity [188]. In this respect, Tang *et al*. evaluated the antioxidant properties of the purified fraction of Alcalase-treated zein hydrolysate and the results showed that free radical scavenging activity of zein depended on the radical species and was strongly related to the molecular weight and hydrophobicity of the constituting peptides [189]. In addition, corn protein hydrolysates prepared using Alcalase, exhibited excellent antioxidant activity after simulated gastrointestinal digestion, which was higher than the undigested hydrolysate activity [190].

Alcalase has also been employed to hydrolyze *Amaranthus* protein isolates which led to the improved scavenging activity of the samples [191]. Among the different processes to treat this isolates, such as defatting, protein concentration, thermal treatment, hydrolysis with Alcalase and *in vitro* digestion [192], it was found that the combination of protein concentration and hydrolysis with Alcalase produced hydrolysates from amaranth seeds with higher antioxidant activity [192]. Furthermore, the application of Alcalase hydrolysate of amaranth proteins showed antioxidant properties in restructured fish products [193].

Chickpea protein hydrolysate obtained by Alcalase hydrolysis has also been studied as a potential source of natural antioxidants. In one report, the hydrolysis efficiency and antioxidant activity of Alcalase hydrolysate from chickpea protein was improved by ultrasonic pretreatment [194], while another study reports the modification by plastein reaction of the chickpea protein hydrolysates prepared by Alcalase with a hydrolysis degree

of 20.03%, which enhanced their reducing power and hydroxyl radical scavenging activity [195]. Moreover, a novel peptide was isolated by chromatographic fractionation of the Alcalase chickpea protein hydrolysate which displayed a DPPH radical-scavenging activity of 67% at 200 μg/ml and did not show hemolytic activity towards bovine erythrocytes [196].

Literature reports dealing with the Alcalase hydrolysis of rice proteins are also frequent. In this context, rice bran protein extract hydrolysate prepared with Alcalase showed DPPH free radicals scavenging activity and a FRAP (Ferric Reducing Antioxidant Power) value of 32.1-35.5% and 951-1,018 μmol $FeSO_4$/mL of hydrolysate, respectively [197]. In addition, the Alcalase hydrolysis of glutinous rice bran, a byproduct of milling rice, under optimal conditions (enzyme/substrate ratio of 2.84% and 480 min) produced a protein hydrolysate with an IC50 value of 0.87 ± 0.02 mg/ml in the DPPH assay [198]. It has also been reported that when rice protein was pretreated at high pressures, peptides with improved antioxidant properties were obtained [199].

Alcalase has also been used to produce antioxidant peptides from pea protein [200]. The obtained hydrolysate showed a DPPH radical scavenging activity of 37.94 ± 1.24% and a hydroxyl (OH) radical scavenging activity of 28.43 ± 1.54% [200]. Besides, in order to improve the oxygen radical absorption capacity, 2,2-Diphenyl-1-picrylhydrazyl, superoxide radical and hydroxyl radical scavenging activities of pea protein hydrolysates, isolated pea protein dispersions were pretreated at high pressure (400 and 600 MPa) before being subjected to Alcalase hydrolysis [201].

It is important to highlight that within the vegetable proteins, the proteins from different seeds have a central role as raw material for hydrolysis with Alcalase to obtain

antioxidant hydrolysates, proof of which are the numerous articles that have been published in this regard. For example, Alcalase rapeseed protein hydrolysates with a degree of hydrolysis of 25% exhibited notable reducing power (0.51 at 2.00 mg/mL) and showed scavenging activity against free radicals such as DPPH, superoxide, and hydroxyl radicals with EC 50 values of 0.71, 1.05, and 4.92 mg/mL, respectively [202]. In addition, when Alcalase rapeseed protein hydrolysates were fractioned by membrane ultrafiltration [203], they showed an oxygen radical absorbance capacity value of 1610 ± 113 μmol TE/(g sample), a peroxyl radical-scavenging capacity value of 622 ± 30 mg VC/(100 g sample), and a cellular antioxidant activity value of 25 ± 2 μmol QE/(g sample) and a corresponding EC50 value of 58 ± 3 μg/mL [203]. The hydrolysis of a flaxseed protein isolate with Alcalase was also performed as a strategy to generate antioxidant peptides [204]. The peptide GFPGRLDHWCASE showed a notable ORAC activity of 3.20 μmol Trolox equivalents/μmol of peptide [204]. Additionally, after *in vitro* simulated gastrointestinal digestion, the antioxidant capacities of flaxseed protein isolate and their Alcalase hydrolysate were compared [205]. It was found that the hydrolysate had the highest antioxidant capacity, measured by FRAP [205]. The Alcalase hydrolysis of African yam bean seed protein and further fractionation using membrane ultrafiltration showed that the <1 kDa peptides exhibited significantly better ferric reducing power, diphenyl-1-picryhydradzyl (DPPH) and hydroxyl radical scavenging activities when compared to peptide fractions of higher molecular weights [206]. In another paper, tea seed (*Camellia oleifera Abel*.) protein was hydrolyzed using Alcalase at different degrees of hydrolysis [207]. It was found that as the degree of hydrolysis value increased, the hydrolysate antioxidant activities increased, so that hydrolysates obtained at 20 and 30% of degree of hydrolysis exhibited higher superoxide radical scavenging and stronger iron chelating

activities respectively, than other hydrolysates [207]. Lead tree (*Leucaena leucocephala*) seed protein was also subjected to Alcalase hydrolysis at pH 9, using an enzyme to substrate ratio of 2%, for 90 min at 55°C [208], which allowed to obtain a hydrolysate with a ferrous ion chelating activity of 92.79%, high DPPH radical scavenging activity of 76.21% and hydroxyl radical scavenging activity of 66.72% [208]. In another paper the optimization of the Alcalase hydrolysis conditions of fenugreek seed protein by response surface methodology was carried out [209]. The optimal conditions were an enzyme to substrate ratio of 2.32%, a temperature of 47.04 °C and a reaction time of 198.21 min, producing a hydrolysate with a hydroxyl radical scavenging activity of 69.49 % and a maximum DPPH radical scavenging activity of 50.99 % at the concentration of 40 mg/mL and 50 mg/mL, respectively [209]. On the other hand, it was demonstrated that Sorghum kafirin Alcalase hydrolysates had a good balance of antioxidant activity, yield, and economic efficiency [210]. Another studies report the isolation of a peptide with potent antioxidant activity from walnut protein hydrolysate [211], a novel antioxidant peptide with an amino acid sequence of SMRKPPG from peony (*Paeonia suffruticosa Andr*.) seed protein isolate [212], four antioxidant peptides identified as PMPVR, FETLPF, KMRDNL, and LDESKRF from Semen cassia (seeds of *Cassia obtusifolia*) hydrolysate [213], and an antioxidant peptide from oats globulin hydrolysate with the strongest hydroxyl and DPPH radical scavenging ability value of 58.38 ± 0.87% and 24.53 ± 0.53%, respectively [214], all of them produced by Alcalase hydrolysis.

One interesting work reports that the Alcalase hydrolysis of melinjo seeds (*Gnetum gnemon*) at different stages of maturity (green, yellow and red) generated hydrolysates with different antioxidant activities [215]. Another study shows that the Alcalase hydrolysis of

defatted garden cress (*Lepidium sativum*) seed meal protein improved their antioxidant activity [216]. Hempseed protein isolate was hydrolyzed by Alcalase , and the hydrolysate obtained was subjected to DA201-C macroporous absorption resin, with simultaneous desalting and concentrating of hydrophobic fragments with improved free radical-scavenging activities [217]. The active fraction was further separated to obtain two purified peptides which at a concentration of 10 μg/ml, which possessed protective effects against cell death and oxidative apoptosis [217].

There are many other examples where vegetal proteins were hydrolyzed using Alcalase. For instance, glutelin from cocoa almond was hydrolyzed with Alcalase for the production of hydrolysates and peptide fractions with antioxidant activity [218]. Also, Alcalase hydrolysates from Bambara groundnut protein concentrate provided functional peptides with antioxidant properties which showed DPPH radical scavenging and metal chelating activities that increased with the degree of hydrolysis [219]. Otherwise, an antioxidant hydrolysate was obtained from Douchi protein hydrolyzed by Alcalase under optimal conditions (63℃, 1.4% of enzyme / substrate, and 1.7 h) [220]. In another paper, peptides with OH scavenging activity of 74.52% at a concentration of 1.0 mg/mL were isolated from sweet potato protein hydrolysates prepared by Alcalase [221], and it has been reported that if the Alcalase hydrolysis was performed after high hydrostatic pressure [222] or temperature (at 70, 80 and 90 °C) pretreatment [223], the degree of hydrolysis and the antioxidant activity of peptides from sweet potato protein were improved.

Lupinus mutabilis (Tarwi) protein concentrate was also subjected to the action of Alcalase [224]. The highest radical scavenging activity (TEAC (Trolox Equivalent Antioxidant Capacity) value of 2.7 ± 0.1 μmol Trolox equivalents/mg protein and ORAC

(Oxygen Radical Absorbance Capacity) value of 3.8 ± 0.1 μmol Trolox equivalents/mg protein) was found in hydrolysates produced with an enzyme/substrate ratio of 1.87% after 138 min of hydrolysis [224]. On the other hand, Alcalase hydrolysis of Chinese chestnut (*Castanea mollissima Blume*) protein produced five novel antioxidant peptides which had good antioxidant activity after synthesis and simulated digestion [225], and a novel antioxidative peptide (LAYLQYTDFETR) were successfully purified from pecan meal protein isolate hydrolysate prepared using Alcalase, and it exhibited appreciable scavenging activities on ABTS radical (67.67%), DPPH radical (56.25%) and hydroxyl radical (47.42%) at 0.1 mg/mL [178].

Three antioxidant small peptides, identified as Tyr-Pro-Ala (286 kDa), Ile/Leu-Pro-Ser (315 kDa) and Ser-Pro (202 kDa), were purified from peanut protein isolate hydrolyzed with Alcalase [170], and it was demonstrated that high pressure treatment affected the Alcalase hydrolysis of peanut protein leaving hydrolysates with higher antioxidant activity (reducing power and DPPH radical scavenging) than the non-high pressure treated hydrolysates [226]. In another paper, *Erythrina edulis* (pajuro) protein concentrate hydrolyzed by Alcalase for 120 min showed potent ABTS+ and peroxyl radical scavenging activities [227]. Similarly, wheat bran protein isolate digested with Alcalase produced wheat bran protein hydrolysate and submitted to fractioning using membrane ultrafiltration [228]. The <1 kDa fraction showed significantly higher oxygen radical antioxidant activity with 2044.73 ± 37.45 (μM TE/g protein) when compared to other membrane fractions and wheat bran protein hydrolysates [228].

An important application of hydrolysis with Alcalase is in the recovery of residual proteins generated in the processing or use of some vegetables. For instance, antioxidant

peptides from asparagus wastes [229], and antioxidant hydrolysate from Highland barley brewer spent grain protein [230] were prepared using Alcalase hydrolysis. In addition, proteins of tomato seeds, the main by-product of tomato processing, were extracted and subjected to incubation for 138.62 min with 3% (w/w) Alcalase to produce a tomato seed protein hydrolysate with high antioxidant properties [231]. In the same way, seven potential antioxidant peptides were isolated from Alcalase hydrolysate of plum stones processing byproduct [232]. Additionally, the bioactive peptide production by Alcalase hydrolysis of defatted *Jatropha curcas* flour obtained as by-product of oil extraction for biodiesel production was implemented as a way for the revalorization of this by-product [233]. After 50 min of hydrolysis a protein hydrolysate with a degree of hydrolysis of 31.7% was obtained, and it showed high antioxidant and chelating activities [233].

3.2.1.2. Comparison of Alcalase with other proteases

In addition to studies where Alcalase is used exclusively in the hydrolysis of a particular protein, there are many reports in the literature in which Alcalase is compared with other proteases as biocatalysts to produce hydrolysates or peptides with antioxidant activity. These reports are especially interesting as they show the advantages and drawbacks of each of the used proteases and permit a better selection of the protease depending on the target.

For example, oat flour protein was hydrolyzed with Alcalase and trypsin, and both obtained hydrolysates significantly reduced the generation of lipid hydroperoxides resulting from autoxidation of linoleic acid after 5 days incubation [234].

Using soybean proteins, there are some interesting reports. For instance, ghungkukjang (fermented soybean paste) and soybean powder were hydrolyzed with Alcalase, Protamex and Neutrase [235]. Results showed that Alcalase and Protamex generated greater increases of antioxidant activities of both ghungkukjang and soybean powder hydrolysates than those prepared with Neutrase [235]. Sbroggio *et al.* demonstrated the influence of the degree of hydrolysis and the type of enzyme on the antioxidant activity of okara (by-product of soy milk production) protein hydrolysates using Alcalase and Flavourzyme [236]. It was found that the hydrolysis with Alcalase increased the antioxidant capacity from 36.0 to 202.1, 7.3 to 20.3, and 1.2–5.9 μmol Trolox/g of solids according to the ABTS, FRAP, and DPPH assays, respectively [237].

Comparison of free radical-scavenging activities of sweet potato protein and its hydrolysates prepared by proteolysis catalyzed by Alcalase, Neutrase or Protamex, or in combination with Flavourzyme [238], showed that free radical-scavenging activities of the resulting hydrolysates were all significantly higher than that of the initial sweet potato protein, and Alcalase hydrolysates exhibited the highest superoxide (18.71%), hydroxyl (27.13%) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities (90.10%) [238]. Similarly, among six enzymes (Alcalase, Proleather FG-F, AS1.398, Neutrase, papain and pepsin), sweet potato protein Alcalase hydrolysates exhibited the highest hydroxyl radical-scavenging activity and Fe^{2+}-chelating ability [239]. In addition, different pretreatments significantly increased the degree of hydrolysis and antioxidant activities of sweet potato protein hydrolysates by Alcalase, Protease and Alcalase + Protease [240]. The most effective pretreatment was autoclaving, followed by steaming, microwaving, boiling and the least effective was ultra-sonication [240].

In another paper, cucurbitin extracted from pumpkin (*Cucurbita pepo*) oil cake was enzymatically hydrolyzed by Alcalase, Flavourzyme and pepsin, and the highest antioxidant activity was found in the hydrolysate obtained by Alcalase at hydrolysis degree 25.6 % [241], and in comparison with trypsin hydrolysate, Alcalase hydrolysate showed higher DPPH radical scavenging, total anti-oxidative and ferrous ion chelating activities [242].

Proteins from some bean varieties have been hydrolyzed with several proteases. In one study, concentrates from three cultivars of Azufrado (*sulphur yellow*) beans were obtained and digested with Alcalase, Thermolysin and pancreatin [243]. Regarding the antioxidant activity, Alcalase hydrolysates of Azufrado Higuera and Azufrado Regional '87 showed the highest DPPH scavenging activity (40%) or ABTS scavenging activity (99.89%), respectively [243]. In another work, black bean (*Phaseolus vulgaris L.*) proteins were hydrolyzed for 120 min using pepsin or Alcalase [244]. Results revealed that Alcalase hydrolysate showed higher antioxidant activity for inhibition of the radical ABTS+, while pepsin hydrolysate had higher antioxidant activity for inhibition of the radical DPPH [244]. That is, depending of the main objective, one or the other enzyme should be employed.

Corn gluten meal was hydrolyzed using Alcalase or Protamex [245]. It was found that Alcalase hydrolysis was more efficient, and after ultrafiltration a hexapeptide with potent antioxidant activity was isolated [245]. In another example, Alcalase, Protamex and Flavourzyme at a ratio enzyme/substrate concentration of 13.5% [246] were used to hydrolyze corn gluten meal pretreated by Na_2CO_3, starch removal and cooking, and the hydrolysates obtained in all cases exhibited high antioxidant activity both *in vitro* and *in vivo* [246].

Peanut meal hydrolysates were prepared by digestion using five different peptidases [247]. Among them, Alcalase produced the highest degree of hydrolysis and the hydrolysates with the highest DPPH radical-scavenging activity [247]. In another work, a study was performed on the *in vitro* antioxidant activity of defatted peanut meal hydrolysates produced by hydrolysis with Neutrase (pH 5.0), papain (pH 6.0), Flavourzyme (pH 7.0), and Alcalase (pH 9.0) in a ratio of 1: 500 (enzyme/substrate) at 55°C for 2, 4, 6, 8, and 24 hours, respectively [248]. Results showed that, the Alcalase-treated hydrolysates had the best total anti-oxidative capacity [248].

In another research, Alcalase, Flavourzyme and Neutrase were employed to hydrolyze rice bran protein for 2, 4 or 6 h [249]. The protease had significant effects on the properties of hydrolysates and protein hydrolysis degree, whereas the hydrolysis time was less influent [249]. No major differences were found in terms of ABTS radical scavenging activity between non-hydrolyzed and protease-hydrolyzed rice bran protein, but Alcalase hydrolysis was the most effective providing hydrolysate with the highest protein content and protein yield, concluding that rice bran protein hydrolysate obtained by Alcalase hydrolysis could be a protein source and antioxidant in functional foods and beverages [249].

In another study, Alcalase hydrolysates of barley glutelin showed higher radical scavenging capacity (DPPH/O^{2-}/OH), Fe^{2+}-chelating effect and reducing power than those produced by Flavourzyme [250]. In another research, various proteases were used to hydrolyze rapeseed protein isolate for obtaining hydrolysates that were fractioned by membrane ultrafiltration [251]. It was found that, in general, Alcalase and Proteinase K

were more efficient proteases to release antioxidant peptides than pepsin + pancreatin, Flavourzyme and Thermolysin [251].

Alcalase and Neutrase were used to prepare Chinese cherry (*Prunus pseudocerasus* Lindl.) seed protein hydrolysate [252], which were fractionated by ultrafiltration and chromatographic techniques allowing to obtain two antioxidant peptides identified as Phe-Pro-Glu-Leu-Leu-Ile (731.92 Da) and Val-Phe-Ala-Ala-Leu (520.61 Da) [252]. Other authors reported the hydrolysis of coconut protein using four proteases (Alcalase, Neutrase, Bromelin, papain), among which the Alcalase hydrolysate showed to be the best in terms of degree of hydrolysis and DPPH scavenging activity [253].

These comparisons are also performed using proteins from residues. In this context, the enzymatic hydrolysis of this kind of proteins for obtaining bioactive peptides can contribute to environmental sustainability of processing of fruits, which is characterized by generating a lot of waste material such as fruit stones, skins, etc. For example, Alcalase, Thermolysin, Flavourzyme, and Protease P were used to hydrolyze a protein extract from plum stone (*Prunus Domestica* L.), a by-product of the processing of that fruit [254]. In this study, Alcalase produced the hydrolysates with the highest ABTS radical scavenging and lipid peroxidation inhibition capacities [254]. Cherry stones which contain seeds with a significant amount of proteins were used to obtain bioactive peptides by their digestion with Flavourzyme, Alcalase or Thermolysin, where the last two yielded peptide extracts with the highest antioxidant and antihypertensive capacities [255].

On the other hand, protein hydrolysates were prepared by treatment of olive seed protein isolate with Alcalase, Thermolysin, Neutrase, Flavourzyme and PTN [84]. All hydrolysates presented antioxidant properties, but Alcalase was the enzyme that yielded the

hydrolysate with the highest antioxidant capacity. In this study it was suggested that enzymatic extraction of bioactive peptides from residual materials from table-olive and olive oil production can be a new strategy for the revalorization of these residues [84]. Similarly, hydrolysate of seed cake protein from *Camellia oleifera* produced by Alcalase had the highest hydrolysis degree and antioxidant activity [256], and displayed excellent protein solubility over a wide range of pH, when compared to the hydrolysates obtained using Flavourzyme, trypsin, Neutrase or papain [256]. Alcalase and pancreatin were used in the production of bioactive peptides derived from defatted *Bunium persicum Bioss*. (black cumin) press cake [257]. It was found that DPPH radical scavenging activity was higher using the Alcalase hydrolysates, while the products obtained by using pancreatin had a higher inhibitory effect on the ABTS+ cationic radical scavenging [257]. Papain, trypsin, pancreatin, Alcalase and Flavourzyme were evaluated in the hydrolysis of protein from flaxseed cake and it was found that the hydrolysates obtained using Alcalase and pancreatin had the highest antioxidant activity [258].

In another study, pepsin, trypsin, chymotrypsin, Alcalase and Flavourzyme were used to hydrolyze a protein extract from wild almond (*Amygdalus scoparia*) [259]. Based on radical scavenging activities obtained by 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and ferric-reducing abilities of the hydrolysates, it was found that the hydrolysate from Alcalase had significantly greater antioxidant activity [259]. In addition, protein concentrate obtained from the seed of *Erythrina edulis* (pajuro) was hydrolyzed by Neutrase, Flavourzyme and Alcalase, finding that Alcalase provided hydrolysates with higher radical scavenging activity [260].

In another research, it was shown that the treatment of brown teff with proteases (Protamax, Flavourzyme or Alcalase) affords hydrolysates with significantly increased antioxidant activities [261]; among them, the highest DPPH scavenging activity and FRAP values were observed for the hydrolysates produced by Alcalase and Flavourzyme treatments, respectively [261]. Also, Alcalase was selected among other proteases to hydrolyze fennels seeds (an edible spice) protein [262]. The hydrolysate was fractionated, and it was found that compared to the crude hydrolysate, the fractionated hydrolysate presented a 4.5-fold enhancement in its radical scavenging potential [262]. Besides, the protein fraction of Brewers' spent grain was hydrolyzed by three different proteases to obtain hydrolysates with antioxidant activity [263]. Alcalase hydrolysate presented significantly higher total phenolic content and ferric ion reducing antioxidant power (0.083 mg GAE/mg dw; 0.101 mg TE/mg dw, respectively) than the other hydrolysates [263].

In the hydrolysis of quinoa seeds proteins with Alcalase or pancreatin [264], it could be seen that the antioxidant capacity of the hydrolyzed proteins was significantly higher than that of the non-hydrolyzed proteins [264]. In addition, Alcalase hydrolysate of carrot seed (*Daucus carota L.*) [265] exhibited the strongest DPPH radical-scavenging activity (among that produced using other proteases) and under optimized condition (3.50 h, substrate concentration of 52.8 g/L, and protease dosage of 419.36 U/g), its DPPH radical-scavenging activity was 82.46% at 2 mg/mL [265].

3.2.2. Hydrolysis of fish proteins

3.2.2.1. Use of stand-alone Alcalase

Fish processing by-products represents more than 50% of the starting material in the fish industry, and their disposal can generate additional costs and can cause serious environmental problems [266]. In this sense, Alcalase has played an important role in the recovery of marine fish processing byproducts, as a method for converting fish wastes into valuable products such as bioactive peptides, which can be used for the pharmaceutical and health food industries, such as a way to assist in the efficient management of fishing industry waste. In this regard, antioxidant peptides production from tuna by-products by enzymatic hydrolysis with Alcalase (enzyme to substrate ratio 1: 200 w/w; 60 °C; pH 6.5, 120 min), has been explored with good results [267]. Tuna (*Thunnus obesus*) head protein hydrolysate prepared with Alcalase [268] showed a reducing power of 0.948 at 12.5 $mg \cdot mL^{-1}$ and radical scavenging activity in a dose-dependent manner against 1,1-diphenyl-2-pycrylhydrazyl, superoxide and hydroxyl radicals with EC50 values of 1.34, 1.20 and 2.84 $mg \cdot mL^{-1}$, respectively [268]. Additionally, it was reported that nanofiltration fractioning of the product of Alcalase catalyzed hydrolysis of tuna dark muscle by-product showed the very high 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging activities of 75% and 65%, respectively [269].

Fish skin is one of the most used fish wastes to obtain antioxidant hydrolysates with Alcalase. For example, Alcalase hydrolysis improved the antioxidant properties of collagen and gelatin extracted of yellowfin tuna (*Thunnus albacares*) skin waste by their conversion to peptides, which showed antioxidant activities higher than the non-treated material [270]. In another study, Alcalase was used to produce three peptides with potent antioxidant activities from grass carp skin (*Ctenopharyngodon idella*) [271], and to produce gelatin hydrolysates from skin and scale of sole fish (*Cynoglossus arel*) [272]. In addition,

antioxidant peptides production by Alcalase hydrolysis of skin from different fish such as seabass (*Lates calcarifer*) [273], Alaska pollock [274], and tilapia [275], also has been reported.

Little hairtail (*Trichiurus haumela*) proteins have also been hydrolyzed with Alcalase [276]. It was reported that under optimum conditions (3 h, enzyme to substrate ratio of 0.6%, 55°C and pH 7.5), the resulting little hairtail protein hydrolysate showed an ABTS radical scavenging activity of 76.5% [276], while in another study the little hairtail protein Alcalase hydrolysate had a value of reducing power and radical scavenging activities of 1.89, 46.15% (DPPH radical), 75.65% (hydroxyl radical) and 82.5% (superoxide anion radical), respectively [277].

On the other hand, antioxidant peptides from *Pseudosciaena crocea* protein viscera with scavenging activity of DPPH and OH of 85.97% and 75.79%, respectively [278], were prepared by Alcalase hydrolysis under optimal hydrolysis conditions (62℃, pH 9, enzyme concentration of 4.26%, substrate concentration of 8 g/100 mL and 3.7 h) [278]. Also, an antioxidant peptide (Ala-Thr-Ser-His-His) was purified from Alcalase hydrolysate of *Arctoscopus japonicus* sandfish protein extract [168]. The DPPH radical scavenging activity of the peptide was above 90% at a concentration 1.0 mg/mL, which remained at around >66% and >79% after treatment at various temperatures with intestinal proteases and different pH conditions [168]. Similarly, *Arctoscopus japonicus* meat was used as natural material for the preparation of antioxidant peptides using Alcalase hydrolysis [279]. Under optimal conditions (pH 6.0, 70 °C, enzyme concentration of 5% (w/w), and 3 h) the obtained hydrolysate presented a DPPH radical scavenging activities of 60.04% [279].

In another paper, squid protein hydrolysates with a degree of hydrolysis of 13.7% were prepared from *Uroteuthis (Photololigo) duvaucelii*, using Alcalase [280]. Hydrolysates had 89% 2, 2-diphenyl-1- picrylhydrazyl inhibition, 94% 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) inhibition and 96% hydroxyl inhibition at 10 mg/ml concentration [280]. In another report, 8 h of Alcalase hydrolysis produced a Stone fish (*Actinopyga lecanora*) flesh hydrolysate with potent antioxidant activity in terms of DPPH radical scavenging activity (77.43%, IC50 of 0.5 mg/mL), ABTS radical scavenging activity (92.73%, IC50 of 0.33 mg/ mL) and FRAP value (39.2 mmol/100 mL $FeSO_4$) [281]. Hydrolysate of shortfin scad (*Decapterus macrosoma*) myofibrillar protein with DPPH antioxidant activity of 56.10%, were prepared under optimized Alcalase hydrolysis conditions (180 min, 59.49°C, pH of 6.93 and 1% enzyme concentration) [282]. Similarly, Alcalase hydrolysis under optimal conditions (pH 8.5, 55 °C, enzyme concentration of 1.5% w/w and 3 h) was performed to recover the fish protein from Caspian kutum (*Rutilus frisii kutum*) by-product, which resulted in an antioxidant hydrolysate with a degree of hydrolysis of 19.08% [283].

Whitemouth croaker (*Micropogonias furnieri*) protein hydrolysates were prepared by Alcalase hydrolysis varying the reaction time, finding that the hydrolysate from the longest studied hydrolysis time (8 h) showed the highest degree of hydrolysis (32.1%) and oxidation inhibition using the ABTS and DPPH methods (98.35% and 54.11%, respectively) [284].

Two peptides (WAFAPA and MYPGLA), with stronger antioxidant activity than glutathione, were isolated from the Alcalase hydrolysate of the blue-spotted stingray [285]. In another interesting study, common carp (*Cyprinus carpio*) protein by-products were

hydrolyzed by Alcalase [286]. The hydrolysate showed antioxidant properties which led to a reduction in muscle lipid peroxidation and a decrease in brain lipid peroxidation in different organs of zebrafish (*Danio rerio*) [286]. Also, it was demonstrated that Alcalase *hippocampus abdominalis* protein hydrolysate contains antioxidant peptides that exhibit a strong antioxidant activity which reduced dose-dependently both intracellular reactive oxygen species levels in 2,2-azobis hydrochloride -induced cells and cell death in 2,2-azobis hydrochloride -induced zebrafish embryos [287]. Besides, the feeding of *Caenorhabditis elegans* with antioxidant peptides isolated from Alcalase hydrolysates of residue rich in protein obtained from round scad (*Decapterus maruadsi*) after oil extraction, led to longer lifespan, higher survival rate, and high superoxide dismutase and catalase activities [288].

3.2.2.2. Comparison of Alcalase with other proteases

There are many reports comparing various proteases to obtain antioxidant peptides from fish proteins. Nile tilapia (*Oreochromis niloticus*) scale gelatin was hydrolyzed using Alcalase, Pronase E, trypsin or pepsin [289]. Among the obtained hydrolysates, Alcalase-derived hydrolysate exhibited the highest antioxidant activity [289]. In another paper, gelatin extracted from Nile tilapia skin was independently hydrolyzed by several proteases [290]. Among the obtained products, Flavourzyme hydrolysate had potent activity on ABTS radical scavenging and also inhibits the oxidation of linoleic acid at a high level, while Alcalase hydrolysate showed the greatest reducing power, and bromelain hydrolysate had the highest ferrous ion chelating activity [290]. In addition, red tilapia (*Oreochromis niloticus*) protein hydrolysates were prepared by the enzymatic hydrolysis with Alcalase,

Flavourzyme and Protamex for 5h, finding that Alcalase hydrolysate had the highest 2, 2-Diphenyl-1-picryhydrazyl radical-scavenging activity [291].

The antioxidant activities of grass carp (*Ctenopharyngodon idellus*) protein hydrolysates prepared with Alcalase or papain were investigated [292]. In this case, it was observed that at the same degree of hydrolysis, papain hydrolysate possessed higher DPPH scavenging activity and reducing power than Alcalase hydrolysate [292]. In another study, peptide fractions of protein hydrolysates from underutilized silver carp (*Hypophthalmichthys molitrix*) prepared using Flavourzyme and Alcalase for 30 and 60 min, respectively, showed higher cell-based antioxidant activity under stress and non-stress conditions among other hydrolysates [293].

Common carp (*Cyprinus carpio*) roe (egg) protein hydrolysates were prepared by treatment with pepsin, trypsin or Alcalase [294]. The hydrolysates showed excellent antioxidant activity in a dose dependent manner in various *in vitro* models such as DPPH radical scavenging activity, ABTS+ radical scavenging activity, ferric reducing antioxidant power and ferrous ion chelating ability [294]. Also, common carp by-product was hydrolyzed using Alcalase and Protamex [266], and the results revealed that the Alcalase hydrolysate exhibited significantly higher antioxidant activity against the DPPH radical and the highest *in vitro* antioxidant competence against peroxyl radicals, whereas Protamex hydrolysate showed the lowest activity against peroxyl radicals [266]. Also, hydrolysates of fin from silver carp (*Hypophthalmichthys molitrix*) produced by trypsin or Alcalase exhibited stronger *in vitro* scavenging activity against 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals and chelating activity to ferrous ions [295], and inhibited the freeze-thaw-induced protein oxidation (the formation of carbonyls

and disulfide bonds) and degradation (the loss of Ca^{2+}-ATPase activity) in freeze-thawed bighead carp (*Hypophthalmichthys nobilis*) fillets, than papain and Neutrase [295].

Fish protein hydrolysates were prepared from anchovy sprat (*Clupeonella engrauliformis*) using endogenous enzymes and diverse commercial proteases [296]. Alcalase and papain gave the highest degree of hydrolysis; Alcalase and bromelain had the highest protein recovery, and the highest ABTS activity was observed in Alcalase hydrolysate, followed by Promod and Protamex hydrolysates [296]. In another work, hydrolysates of Argentine anchovy were produced with Alcalase, Flavourzyme and Protamex, being Alcalase the one which led to the hydrolysate with a maximum value of the degree of hydrolysis (78.26 ± 1.66%) that also showed the greatest inhibition of lipid peroxidation (23.38%) and reducing power [297].

Salmon processing byproducts were hydrolyzed using Alcalase, Flavourzyme, Neutrase, pepsin, Protamex, or trypsin obtaining hydrolysates with different antioxidant activities where pepsin hydrolysate possessed the highest DPPH scavenging [298]. Similarly, two forms of salmon frames named "chunk" and "mince" were hydrolyzed using Alcalase and papain at 1%–3% (w/w protein) for 0–240 min [299]. It was showed that different hydrolysates exhibited different antioxidant capacities, and the authors suggested that to produce the hydrolysate with less time consumption, the use of frame chunk instead of minced frame and Alcalase instead papain, can be the best choices [299]. Hydrolysates from chum salmon (*Oncorhynchus keta*) skin gelatin were prepared by Alcalase or papain hydrolysis [300]. It was found that hydrolysates generated by the two proteases had quite strong scavenging activity toward superoxide radicals and weak activity toward DPPH and hydroxyl radical [300].

In another research, Alcalase and Flavourzyme were evaluated in the production of antioxidant hydrolysates dark muscle and skin from Skipjack tuna (*Katsuwonus pelamis*) [301]. Also, dark muscles from skipjack tuna were hydrolyzed using pepsin, trypsin, Neutrase, papain or Alcalase [302]. The hydrolysates prepared using Alcalase and Neutrase, showed the strongest antioxidant capacities which were attributed to the presence of peptides with smaller molecular size, bearing hydrophobic and aromatic amino acid residues, and the specific amino acid sequences [302]. Additionally, among pepsin, papain, trypsin, Neutrase and Alcalase, the last one produced peptides with the highest antioxidant activity from scale gelatin of skipjack tuna (*Katsuwonus pelamis*) [303].

Stone fish (*Actinopyga lecanora*) ethanolic and methanolic tissue extracts were hydrolyzed using papain, Alcalase, trypsin, pepsin, bromelain, and Flavourzyme, which considerably enhanced its antioxidant activity, especially when papain and Alcalase were used [304]. Also, heads and/or viscera of sardine (*Sardinella aurita*) were treated with different proteases [305]. All obtained hydrolysates had different degrees of hydrolysis and varying degrees of antioxidant activity, but the hydrolysates obtained with crude enzyme from *Mustelus mustelus* intestines showed the highest radical- scavenging activity, while Alcalase hydrolysates exhibited the greater reducing power activities. [305].

Patin (*Pangasius sutchi*) sarcoplasmic protein was hydrolyzed with Alcalase and papain. Alcalase hydrolysate showed the highest DPPH radical-scavenging activity [306]. In another research, two novel antioxidant peptides were isolated from round scad (*Decapterus maruadsi*) hydrolysate prepared with Alcalase which showed higher antioxidant activity than the hydrolysates obtained by neutral protease, papain, pepsin or trypsin [307]. Conversely, hydrolysate from croceine croaker (*Pseudosciaena crocea*)

muscle protein prepared using pepsin exhibited higher antioxidant activities that the ones prepared with Alcalase [177].

It was showed that Alcalase, compared to papain and trypsin, is the best protease for producing hydrolysates with metal chelating and antioxidant activities from blue-spotted stingray proteins [308]. Byproducts from Spanish mackerel (*Scomberomorus*) processing were hydrolyzed by some commercial proteases [309], and it was found that the Alcalase hydrolysate had the highest degree of hydrolysis and DPPH radical scavenging activity, with values of 31.3 and 18.5%, respectively [309]. In addition, Amur sturgeon skin gelatin hydrolysates prepared using either Alcalase or Flavourzyme were effective in preventing lipid oxidation and were able to retard protein oxidation. They also showed cryoprotective effects in unwashed fish mince [310].

Among five proteases, Alcalase was selected to hydrolyze swim bladder of miiuy croaker (*Miichthys miiuy*) proteins [311], and under optimal hydrolysis conditions (3.5 h, 55 °C, pH 9.5, solid-liquid ratio of 1:5 and enzyme dose of 2.5%) it was possible to isolate two peptides with strong scavenging activities on hydroxyl radical, DPPH radical and superoxide anion radical [311]. Alcalase, bromelain or papain were used for obtaining eel protein hydrolysates from whole eel (*Anguilla marmorata*) [312], and the Alcalase hydrolysates had the highest antioxidant activity against 1,1-diphenyl-2-pyridinohydrazinyl (DPPH) and ABTS radicals, and it also presented a higher reducing power than the other hydrolysates [312]. In another paper, both Alcalase and chymotrypsin enzymes were utilized to produce antioxidant hydrolysates from European seabass (*Dicentrarchus labrax, Linnaeus*, 1758) and gilthead seabream (*Sparus aurata, Linnaeus*, 1758) muscles protein [313].

3.2.3. Hydrolysis of seafood proteins

3.2.3.1. Use of stand-alone Alcalase

Seafood proteins resources, mainly byproducts or residues, have been extensively investigated as feedstocks for the production of bioactive peptides such as antioxidant peptides. Alcalase is one of the most widely used proteases in this context.

Alcalase hydrolysis of krill processing byproducts was optimized by response surface methodology in order to improve the degree of hydrolysis and the antioxidant activity of the produced enzymatic hydrolysate [314]. Optimum hydrolysis conditions were pH 9.5 and 62°C and pH 9.1 and 64°C for degree of hydrolysis of 14.1±0.5% and DPPH-scavenging activity of 10.5±0.2% [314]. In another paper, Alcalase was employed to obtain antioxidant hydrolysates from defatted echinoderm byproducts, including viscera of Atlantic sea cucumber (*Cucumaria frondosa*) and digestive tract and non-commercial grade gonads of green sea urchin (*Strongylocentrotus droebachiensis*) [315].

A hydrolysate with antioxidant activity has also been produced by Alcalase hydrolysis of shrimp waste [316]. It was found that the ultra-filtrated fraction with molecular weight below 1 kDa exhibited the highest antioxidant activity among the five fractions obtained, and this activity was stable when the hydrolysate was heated up to 100°C and maintained its activity near 70% at pH 2.0 [317]. In another paper, the carotene-proteins from shrimp (*Parapenaeus longirostris*) processing by-products were submitted to treatment with Alcalase, and their antioxidant activities of the hydrolysate suggested that it is a good source of natural antioxidants [318]. Similarly, a hydrolysate retaining more than 80% of its activity over wide pH ranges (2-11) and temperature (up to 100ºC for 150 min)

was prepared by 90 min of Alcalase hydrolysis of shrimp waste (*Penaeus monodon* and *Penaeus indicus*) [319]. Other authors used response surface methodology to optimize the production of Alcalase hydrolysates from shrimp (*Metapenaeus dobsoni*) head waste [320]. Under optimal conditions, the obtained protein hydrolysates presented a high degree of hydrolysis, 2, 2-diphenyl-1-picrylhydrozyl radical scavenging activity, and ferric reducing antioxidant power of 40.31, 38.93, and 8.21 μM Fe (II)/g of sample, depending on the hydrolysis degree [320].

Alcalase has also been employed to hydrolyze the proteins in the mantle of cuttlefish (*Sepia pharaonis*) [321]. Hydrolysates with a degree of hydrolysis of 20, 30 and 40%, were obtained and showed 2, 2-diphenyl-1-picrylhydrozyl radical scavenging activity, reducing power and total antioxidant capacity that were 2.5, 6.5 and 13.8 times higher, respectively, than that of the initial cuttlefish mantle protein isolate [321]. Under optimal hydrolysis conditions after optimization using response surface methodology (pH 7.88, 50.2°C, 150 min, and enzyme to substrate ratio of 1.5%) [322], it was showed that the reducing power and ability of peptides to quench ABTS radicals in a gastro-intestinal track model system increased during the intestinal stage, while the scavenging ability against 2, 2-diphenyl-1-picrylhydrozyl radicals decreased [322].

In another study, a comparison between the antioxidant properties of oyster meat (*Crassostrea rivularis*) and its Alcalase hydrolysates showed that the hydrolysates displayed a higher antioxidant activity than oyster meat with or without gastrointestinal digestion [323].

This way, the residues of seafood processing seem to be a good material to produce antioxidant peptides using Alcalase. This has not only the value of the product, but also

reduces the environmental impact of the seafood processing, as the materials do not need to be discarded.

3.2.3.2. Comparison of Alcalase with other proteases

Alcalase has been compared to other proteases in the production of antioxidant hydrolysates. For example, oyster (*Crassostrea talienwhannensis*) meat was digested with papain, Neutrase and Alcalase and the obtained hydrolysates were fractionated using a series of ultrafiltration membranes [324]. With all enzymes tested, results indicated that oyster meat hydrolysates possessed DPPH radical scavenging capacity and reducing power in a dose-dependent manner, and the hydrolysate fractions below 1 kDa showed the strongest overall antioxidant activity [324]. In another paper, protein from rushan bay oyster (*Crassostrea gigas*) was hydrolyzed using three proteases, Alcalase hydrolysate presented the highest scavenging activity against 1,1-diphenyl-2-picrylhydrazyl [325].

Shrimp processing byproducts were hydrolyzed using trypsin, pepsin, Neutrase, Protamex, Flavourzyme or Alcalase [326]. The degree of hydrolysis and DPPH radical scavenging activity of the Alcalase hydrolysate were the highest ones [326]. In another paper, Alcalase and Protamex were used to obtain antioxidant protein hydrolysates from white shrimp (*Litopenaeus vannamei*). It was found that all hydrolysates showed dose-dependent antioxidant activities [327]. Alcalase, trypsin, and Flavourzyme produced sea cucumber (*Cucumaria frondosa*) viscera hydrolysates with a higher degree of hydrolysis (19.08, 32.38, and 15.94%, respectively) and better antioxidant activities than those obtained using other proteases [328]. Alcalase and trypsin were compared in the production of antioxidative peptides from Atlantic sea cucumber protein, finding that Alcalase hydrolysates showed 5–35% higher *in vitro* antioxidant activity than the trypsin-produced

ones [329]. In another paper, sea cucumber gut proteins were hydrolyzed using Neutrase, papain, Alcalase or Flavourzyme, and the hydrolysates showed scavenging abilities on hydroxyl and 1,1-diphenyl-2-picrylhydrazyl radical [330]. Similarly, using either Alcalase and Flavourzyme enzymes, it was possible to obtain antioxidant hydrolysates from sea cucumber (*Holothuria leucospilota*) protein with antioxidant activity [331].

Crude enzyme preparations from *Bacillus licheniformis* NH1, *Bacillus mojavensis* A21, *Bacillus subtilis* A26, and commercial Alcalase were used to hydrolyze cuttlefish skin gelatin [332]. Among them, the hydrolysates obtained by Alcalase presented the highest antioxidant activities monitored by β-carotene bleaching, DPPH radical scavenging, lipid peroxidation inhibition and reducing power activity [332]. In another paper, the evaluation of Alcalase, Flavourzyme, Neutrase, and Protamex for hydrolysis of Abalone viscera showed that the hydrolysate produced by Alcalase exerted strong hydrogen peroxide scavenging activity, Fe^{2+} chelating activity, and reducing power [333].

Um *et al.* used six different enzymes to produce hydrolysates from *Octopus ocellatus* meat, which were evaluated for its antioxidant effects using a human liver cell line and zebrafish embryo models [334]. Alcalase hydrolysate showed the highest antioxidant activities, and effectively reduced the hydroxyl radical-induced DNA damage and the production of reactive oxygen species in H_2O_2 treated hepatocytes without showing cytotoxicity. Moreover, it improved the survival rate and reduced the intracellular reactive oxygen species levels in H_2O_2-treated zebrafish embryos [334]. In another research, Alcalase, Neutrase, pancreatin and bromelain were compared in the hydrolysis of protein isolate from crayfish (*Procambarus clarkii*) processing by-products [335]. It was found that

Alcalase had higher digesting efficiency than that of the other enzymes, and some of the ultra-filtrated fractions showed considerable *in vitro* antioxidant activity [335].

3.2.4. Hydrolysis of whey and casein proteins

3.2.4.1. Use of stand-alone Alcalase

Whey, a by-product of the dairy industry, has been studied as a feedstock to obtain antioxidant peptides by Alcalase hydrolysis. For instance, the effect of the time and Alcalase concentrations on the antioxidant activities of whey protein isolate hydrolysates were evaluated, finding that antioxidant activity, measured by peroxide value and thiobarbituric acid-reactive substance values in a liposome-oxidizing system [336]. The results indicated that the antioxidant activity increased when the hydrolysis time increased up to 5 h, and that an increase in Alcalase concentration significantly enhanced antioxidant activities [336]. In another paper, antioxidative peptides (in the size fraction 0.1-2.8 kDa) obtained from gel filtration of Alcalase-hydrolysate whey protein had a significant protection of MRC-5 cells against the toxicity caused by H_2O_2 [337]. Another research showed that the cheese whey mozzarella hydrolyzed with Alcalase and ultra-filtrated under optimal hydrolysis conditions (8 h, pH 9 and 55 °C) produced a whey protein hydrolysate with a maximum antioxidant activity of 1.18 ± 0.015 μmol Trolox mg^{-1} protein [338].

Casein, a milk protein, has also been hydrolyzed with Alcalase. For example, gastrointestinal digested casein and Alcalase hydrolysate were produced and compared [339]. The last one showed higher *in vitro* antioxidant efficacy, especially the low-molecular-weight fraction. This fraction had excellent antioxidant activity as well as hepatic cyto-protection against hydrogen peroxide [339]. In another study, low-molecular-

weight peptides of casein prepared by hydrolysis with Alcalase and fractioned with Sephadex G-25 gel filtration were subjected to simulated gastrointestinal digestion and Caco-2 cell absorption for evaluating gastrointestinal stability [340]. Results suggested that Alcalase produce gastrointestinal resistant peptides [340]. In another research, three hydrophobic chromatography fractions (HC-F1, HC-F2 and HC-F3) were purified from Alcalase-treated casein, and among them HC-F3 had excellent bioavailability and antioxidant activity [341].

3.2.4.2. Comparison of Alcalase with other proteases

The use of Alcalase was compared to the ones obtained using other proteases also in these dairy products to produce antioxidant peptides. For example, the antioxidant activity of whey protein concentrate hydrolysates obtained by its hydrolysis using pepsin, Alcalase, Flavourzyme or trypsin was evaluated [342]. The whey protein was submitted to a pretreatment that consisted in a thermal incubation (95°C for 5 or 10 min). The pretreatment for 5 min increased the degree of hydrolysis of whey protein concentrate in all cases, and Alcalase hydrolysates showed the highest antioxidant activity [342]. In another study, whey protein isolate was hydrolyzed by trypsin, Pepsin, Alcalase, Promatex, Flavourzyme or Protease N [343]. The hydrolysate generated by Alcalase had the highest antioxidant activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, superoxide radicals and in a linoleic acid peroxidation system induced by Fe^{2+} [343]. Also, peptides from whey protein concentrate were generated by the enzymatic hydrolysis using Neutrase, Corolase PP, Alcalase or Flavourzyme [344]. The hydrolysates showed a high antioxidant capacity and they may have a positive effect in the regulation of endothelial cell function [344]. Whey protein hydrolysates were also obtained using Flavourzyme, Alcalase, or their

blend (1:1) [345]. It was found that a maximum degree of hydrolysis of 63% was obtained with Alcalase, and their respective hydrolysates presented the highest antioxidant activities [345].

Oh *et al.* studied the effects on the biological characteristics and antioxidant activity of milk proteins by the combination of the Maillard reaction and enzymatic hydrolysis with commercial proteases Neutrase, Protamex, Alcalase and Flavorzyme [346]. It was found that the hydrolyzed Maillard reaction products generated by Alcalase showed significantly higher antioxidant activity when compared with the other protease products [346]. In another paper, yak milk casein was hydrolyzed using trypsin, pepsin or Alcalase [347]. The yak milk casein hydrolysate prepared with Alcalase or trypsin, had significantly higher DPPH-scavenging capacity than its pepsin counterpart, and compared with intact yak casein, hydrolysate prepared with Alcalase had a more significant effect on attenuating free radicals of DPPH, superoxide and hydrogen peroxide [347].

β-lactoglobulin (other milk protein) was enzymatically hydrolyzed by different proteases under high hydrostatic pressure (100 MPa) and compared with hydrolysates obtained under atmospheric pressure (0.1 MPa) [348]. Results showed that the hydrolysate obtained under high hydrostatic pressure and Alcalase hydrolysis had significantly higher antioxidant properties among the six enzymes examined in this study [348]. In another research, buffalo casein hydrolysate produced by Alcalase showed higher antioxidant activity than that obtained by employing trypsin [349]. In another paper, hydrolysates were obtained using buffalo and bovine casein treated with pepsin, trypsin, Alcalase or papain [350]; Alcalase buffalo casein hydrolysate and trypsin bovine casein hydrolysate showed the best hydrolysates antioxidant properties, with a hydroxyl radical scavenging capacity,

superoxide scavenging activity, oxygen radical absorbance capacity and Fe^{3+} reducing power of 81.16% and 84.55%, 66.84% and 70.30%, 2.45 and 2.23 mM BHA, and 140.73 and 136.59 μM Fe^{2+}/mg protein, respectively [350]. That is, Alcalase is among the most suitable proteases to produce antioxidant peptides using dairy products.

3.2.5. Hydrolysis of blood plasma proteins

Blood is another source of proteins that may be exploited to produce bioactive peptides. Thus, Alcalase has been evaluated in the hydrolysis of porcine blood plasma. For instance, it was reported that porcine blood plasma protein hydrolysates prepared with Alcalase at different degrees of hydrolysis had stronger radical-scavenging ability, Cu^{2+}-chelation ability and a reducing power than the non-hydrolyzed protein [351], and that antioxidant activity of plasma protein hydrolysates, measured by thiobarbituric acid-reactive substance values in a liposome-oxidizing system, increased with increasing of degree of hydrolysis [351]. In another work, porcine plasma protein hydrolysate was prepared by 5 h of Alcalase hydrolysis and fractioned by ultrafiltration [352]. The fraction with the highest antioxidant activity was used to pretreat male rats which later were treated intraperitoneally with a single dose of CCl_4 (2mL/kg of body weight). Oral feeding of the rats with this hydrolysate fraction could significantly lower the serum levels of hepatic enzyme markers (aspartate transaminase and alanine transaminase) [352]. Similarly, another research work showed that porcine plasma protein hydrolysates prepared by Alcalase hydrolysis for 5 h at pH 8.0 and 55°C, produced an antioxidant product able to increase the radical-mediated oxidation system [353].

Blood plasmas from other animals have also been explored for obtaining antioxidative hydrolysates. In this context, the hydrolysis of bovine plasma by Alcalase

hydrolysis increased its scavenging ability on 2,2-azino bis-(3-ethylbenzothiazoline)-6-sulfonic acid free radicals and reduction power, and these activities remained after *in vitro* digestion [354]. In another paper, blood plasma protein and blood cell protein hydrolysates were produced from silkie fowl (*Gallus gallus*) blood by hydrolysis using Alcalase [355]. The hydrolysates showed strong 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity [355]. In another paper, a sheep plasma hydrolysate was produced by Alcalase-hydrolysis [356]. Peptides with high antioxidant properties measured through both the ferric-reducing antioxidant power and the 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability were isolated from this hydrolysate [356].

3.2.6. Hydrolysis of egg proteins

3.2.6.1. Use of stand-alone Alcalase

Egg proteins have also been used as substrate for Alcalase hydrolysis to produce antioxidant peptides. For example, it was reported that hydrolysates of egg white protein powder prepared using Alcalase and fractionated by ultrafiltration membranes possessed strong reducing power ability, particularly the fraction within <1 kDa [357]. Wang *et al.* reported that the fractionation with ultrafiltration membranes and further treatment by pulsed electric field of the peptides from egg white protein powder obtained by Alcalase hydrolysis, improved the antioxidant activity of these peptides (mainly the fraction 1-10. kDa) [358]. On another paper, peptides with strong antioxidant capacity were purified from duck egg white protein hydrolysate prepared with Alcalase with a degree of hydrolysis value of 21% [359].

In another study, it has been shown that some protein pretreatments significantly affected the peptide profiles and antioxidant activity of the hydrolysates obtained by Alcalase hydrolysis [360]. Thus, egg white proteins were submitted to thermal and ultrasound treatments, being the ultrasound pretreatment at 40 kHz - 15 min the one that permitted to get the most effective hydrolysate in scavenging both DPPH and ABTS radicals (28.10±1.38 and 79.44±2.31%, respectively) [360]. Tanasković *et al.* reported an interesting study focused on the influence of operating conditions on the Alcalase hydrolysis of egg white protein performed in a continuously stirred tank reactor equipped with an ultrafiltration module [361], which appears to be a right approach to improve and intensify the enzymatic process, enabling the production of peptides with desired antioxidant activity [361].

3.2.6.2. Comparison of Alcalase with other proteases

Five proteases were employed for the preparation of antioxidant peptides from soluble eggshell membrane protein [362]. Alcalase hydrolysate had the highest free radical scavenging activity and its fraction with an average molecular weight of 618.86 Da, possessed the strongest scavenging activity with IC50 values of the superoxide radicals, hydroxyl scavenging activities, and protective effect on DNA damage caused by hydroxyl radicals generated of 0.10, 0.18, and 0.95 mg/mL, respectively [362].

Other studies deal with the Alcalase hydrolysis of egg white protein powder [363]. For instance, egg white protein powder hydrolysates were prepared using trypsin, Alcalase and pepsin. Alcalase hydrolysate was the one that possessed the strongest reducing power [363]. In another study, Alcalase hydrolysates (compared to trypsin and pepsin hydrolysates) showed the strongest antioxidant activity [364]. Moreover, after high-

intensity pulsed electric field treatment, its reducing power activity was improved [364]. Later, an efficient continuously operated membrane reactor with a polyethersulfone ultrafiltration module was designed for egg white protein hydrolysis [365]. Among the assayed enzymes, Alcalase gave the highest degree of hydrolysis, as well as the best antioxidant properties of the obtained hydrolysates [365].

In another paper, it was found that in the production of hydrolysate with different proteases, Alcalase hydrolysate egg white liquid had the highest radical-scavenging activity compared to the product obtained with other proteases [366].

3.2.7. Hydrolysis of proteins from other sources

3.2.7.1. Use of stand-alone Alcalase

Alcalase has also been used to hydrolyze proteins from many other sources with the goal to produce antioxidant peptides. For example, Alcalase was used to hydrolyze the fresh velvet antler of sika deer (*Cervus Nippon Temminck*) [367]. After reaction optimization (amount of enzyme of 1:150, substrate concentration of 1:13 and 60 min), a hydrolysate with potent antioxidant activity was produced [367]. In another study, golden apple snail (*Pomacea canaliculata*) protein was hydrolyzed using Alcalase [368]. The optimal conditions were established by response surface methodology (45°C, pH 10, enzyme concentration of 2%, and 159 min). These conditions produced a protein hydrolysate with a yield of 9.72% and antioxidant activity of 73.54% [368]. In another paper, Alcalase hydrolysis conditions of *Polyrhachis vicina* Roger protein were optimized by response surface methodology to optimize the antioxidant activity of the hydrolysate [369].

Hydrolysates obtained from the red seaweed *Mastocarpus stellatus* using Alcalase at 50 °C were supplemented with glycerol and directly used as film-forming solution [370]. The obtained films had a high reducing power and radical scavenging capacity, which remained after a heat treatment at 90 °C [370]. *Pinctada fucata* muscles were hydrolyzed by Alcalase and then fractioned using ultrafiltration membranes to obtain peptides with molecular weights smaller than 5 kDa, which exhibits good scavenging capacity against free radicals [371].

Nannochloropsis gaditana (a microalgae) protein hydrolysate produced extracted via hydrolysis using Alcalase under optimum conditions (pH 8.14, 51.4°C, substrate concentration of 5.48 g/L and an enzyme concentration of 0.26 g/L) [372]. The hydrolysate presented a degree of hydrolysis of 55.76%, and an antioxidant activity measured by 1,1-Diphenyl-2-picrylhydrazyl and 2, 2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid assays of 52.19% and 14.13%, respectively [372].

In another paper, two antioxidant peptides were obtained from the Alcalase hydrolysate of *Arca subcrenata* [373]. In another study, two antioxidant peptides (DFTPVCTTELGP and ARFEELCSDLFR) were purified from the Alcalase hydrolysate of housefly (*Musca domestica L.*) pupae [374]. They exhibited strong ABTS and cation radical scavenging activity with EC50 values of 0.39 and 0.35 mM, respectively [374].

Moreover, some animal viscera proteins have been used as substrates for Alcalase hydrolysis, such as sheep visceral protein which produced a hydrolysate with an antioxidant activity of 68.21% [375]. Han *et al*. studied the *in vivo* and *in vitro* antioxidant capacity of porcine splenic hydrolysate prepared using Alcalase [376], suggesting that porcine splenic peptides improve the antioxidant status in rats by enhancing hepatic catalase and

glutathione peroxidase activities [376]. In addition, hydrolysates production from chicken viscera protein were prepared by hydrolysis using Alcalase in an ionic liquid medium (tetramethylammonium bromide) [377]. The hydrolysate presented values of antioxidant activities 40% higher than the hydrolysates obtained produced in the absence of ionic liquid [377].

3.2.7.2. Comparison of Alcalase with other proteases

Silk sericin hydrolysates were obtained by hydrolysis of silk sericin with Neutrase, bromelin, trypsin, papain, Alcalase and Flavourzyme [378]. Alcalase hydrolysate exhibited the highest scavenging activity and exerted the highest peroxidation inhibition [378]. Similarly, silkworm (*Bombyx mori L.*) pupal protein, one of the main by-products of the silk reeling industry was hydrolyzed by several proteases [379]. The Alcalase hydrolysates presented the highest degree of hydrolysis and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity [379].

The comparison of Alcalase with other proteases has been extended to the hydrolysis of larvae protein from different insects. For instance, Alcalase and Neutral proteinase was employed to obtain housefly larvae protein hydrolysates [380]. The results showed that the Alcalase hydrolysate had higher scavenging activities against hydroxyl radical and superoxide anion radical at low concentrations than the Neutral proteinase hydrolysate [380]. In another study, among five different proteases, Alcalase was selected to obtain *Tenebrio molitor* larvae (mealworm) hydrolysates due to its highest production yield (42.05%) of low molecular weight peptides [381]. These were effective as inhibitors on peroxidation of linoleic acid [381]. In addition, *Protaetia brevitarsis* larvae powder was used to obtain protein hydrolysates by enzymatic hydrolysis using Flavourzyme, papain,

Alcalase, bromelain or Neutrase [382]. The Alcalase hydrolysates showed the highest antioxidant activity [382]. In another paper, protein hydrolysates with antioxidant properties were obtained by hydrolysis of *Allomyrina dichotoma* larvae protein using Neutrase, Alcalase, Flavourzyme, bromelain and papain [383]. Alcalase hydrolysate significantly inhibited linoleic acid peroxidation after five days of incubation [383]. Also, antioxidant hydrolysates were produced by Neutrase, trypsin, Alcalase and papain hydrolysis of black soldier fly (*Hermetia illucens L.*) larvae protein [384].

Protein hydrolysates were prepared from *Nitzschia laevis*, *Spirulina platensis* and *Chlorella vulgaris* using trypsin, Flavourzyme and Alcalase [385], and in general, the hydrolysis process enhanced the antioxidant activities, especially those hydrolysates obtained using Alcalase [385]. In another work, antioxidant peptides were produced from *Schizochytrium limacinum* residue obtained by its hydrolysis with Alcalase, Flavourzyme, papain, trypsin and Protamex [386]. It was showed that the Protamex and Alcalase hydrolysates had the highest antioxidant activity, measured as hydroxyl radical scavenging ability (IC50 = 1.66 mg/mL), 1,1-diphenyl-2-picrylhydrazyl radical scavenging ability (IC50 = 1.28 mg/mL) and reducing power (1.42 at 5.0 mg/mL) [386].

In another paper, velvet antler was hydrolyzed using pepsin, trypsin, Alcalase, Neutrase or α-chymotrypsin [387]. Alcalase hydrolysate exhibited the highest peroxyl radical scavenging activity. A peptide was purified and identified (Trp-Asp-Val-Lys), it exhibited strong scavenging activity against peroxyl radical (IC50 value, 0.028 mg/mL), and showed significant protection ability against AAPH-induced oxidative stress by inhibiting the production of reactive oxygen species in Chang liver cells *in vitro* and in a zebrafish model *in vivo* [387].

Solitary tunicate (*Styela clava*) hydrolysates were produced with Thermoase PC10F, Alcalase or pepsin [388]. The hydrolysate produced by Alcalase had the highest antioxidant and anticancer activities [388]. In another study, using either Protamex or Alcalase in the hydrolysis of *Ganoderma lucidum* protein, the antioxidant properties of the protein were increased (from 28.70% to 33.30% and 39.10% respectively) [389]. Skin protein from a bluefin leatherjacket (*Navodon septentrionalis*) processing by-product was hydrolyzed by Flavourzyme, Neutrase, papain, trypsin, Alcalase and pepsin [390]. The Alcalase hydrolysate showed the highest DPPH, HO^{-}, and O^{2-} scavenging activities, and three peptides were isolated from it. The peptides showed strong antioxidant properties which might be attributed to their small molecular sizes and the hydrophobic and/or aromatic amino acid residues in their amino acid sequences [390].

Chicken thigh and breast skin proteins were hydrolyzed using Alcalase or a combination of pepsin and pancreatin and the hydrolysates were fractionated by membrane ultrafiltration [391]. The chicken breast skin hydrolysates had significantly higher DPPH scavenging activity than the chicken thigh skin hydrolysates, but both had a significantly lower scavenging activity against DPPH radicals than reduced glutathione [391]. In another paper, chicken skin gelatin was hydrolyzed by Pronase E, Alcalase or collagenase and the hydrolysates submitted to ultrafiltration [392]. The hydroxyl radical activity, superoxide anion radical activity and Fe^{2+} chelating activity were higher than those of the commercial antioxidants BHT, trolox or ascorbic acid [392].

In order to improve the processing of porcine waste, porcine skin was hydrolyzed using Protamex, Bromeline, Neutrase, Alcalase, Flavorzym or papain [393]. It was found that hydrolysates obtained by Alcalase exhibited the highest degree of hydrolysis and

showed the highest antioxidant and collagenase inhibition activities [393]. Another research effort showed that the hydrolysates obtained from porcine blood by hydrolysis catalyzed by papain, trypsin or Alcalase, showed potent antioxidant and antimicrobial activities (one example of mixed bioactivities) [394].

3.2.8. Combined use of Alcalase with other proteases

The use of Alcalase in combination with other proteases may produce some synergistic effect [155]. In fact, it has been recently discussed that the use of several enzymes may have advantages in most reactions, even more so in reactions where multi-functional substrates are utilized, the so-called combi-enzymes [395]. In other cases, protein hydrolysis is carried out in several hydrolysis stages involving different enzymes. Examples of such researches are presented below.

First some examples of using the combi-protease concepts will be presented. For example, hydrolysate from yak bone obtained by hydrolysis with Alcalase plus Flavourzyme at 50°C for 4 h showed the strongest antioxidant activity in a H_2O_2 system and chelating activity to Cu^{2+} [396]. Other research reports showed that Alcalase + pepsin and Alcalase + trypsin were employed to prepare antioxidant peptides from oat protein, giving better results than the use of the individual enzymes [397]. In another paper, possible synergistic effects of combined action of proteases in antioxidant peptides production from soy protein isolate were evaluated [398]. In terms of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, the hydrolysates obtained with Flavourzyme combined with Alcalase showed the highest antioxidant activity, while the hydrolysates obtained using the ternary mixtures of Flavourzyme, Alcalase and YeastMax A showed the highest inhibition of linoleic acid autoxidation [398]. Hydrolysates from white bean protein concentrate were

obtained using a mixture of Alcalase and Flavourzyme [399]. The antioxidant activities of the hydrolysates increased from 45% to 70% after enzymatic hydrolysis and that the use of the binary enzyme mixture had a significant synergistic effect and resulted in maximum antioxidant activity of the protein hydrolysates in terms of DPPH-radical scavenging and reducing power assay [399]. Similar results were obtained in the hydrolysis of black bean protein where a mixture of Alcalase and Flavourzyme produced protein hydrolysate with the highest antioxidant activity [400]. Three novel antioxidant peptides were purified from corn gluten meal hydrolysate produced by Alcalase + Flavourzyme hydrolysis [401], and among the three peptides, Cys-Ser-Gln-Ala-Pro-Leu-Ala exhibited excellent scavenging capacities for DPPH radical and superoxide anion radical, with IC50 values of 0.116 and 0.39 mg/ml, respectively [401]. Wheat gluten was hydrolyzed through two treatments, single enzyme (Alcalase) and double enzyme (Alcalase-Flavor) [402]. The results showed that the hydrolysates produced by both enzymes had better solubility, reducing power, DPPH, superoxide anion and hydroxyl radical scavenging activity than single enzymatic hydrolysates [402]. In another research effort, antioxidant hydrolysates were produced from protein concentrate obtained from defatted flour of *Salvia hispanica* seeds hydrolyzed with Alcalase–Flavourzyme for up to 240 min [403]. In another paper, seven novel antioxidant peptides were obtained from sesame (*Sesamum indicum L.*) protein hydrolysate prepared by the hydrolysis with Alcalase and trypsin [404]. Among them, SYPTECRMR with DPPH and ABTS IC50 Values of 0.105 mg/mL and 0.004 mg/mL respectively, exhibited the highest antioxidant activity among the seven sesame peptides [404]. Later, anchovy (*Engraulis japonicus*) protein hydrolysates with a 1, 1-diphenyl-2-picryhydrazyl scavenging activity of 84.7% were obtained by hydrolysis using Protamex:Flavourzyme:Alcalase in a ratio of 1.1:1.0:0.9 under optimal conditions (total

protease concentration of 3.27%, pH 7.5, 55.4°C and 2.7 h) [405]. Eight antioxidant peptides were purified from hairtail (*Trichiurus japonicas*) muscle protein hydrolysate prepared by hydrolysis catalyzed by Papain + Alcalase [406]. In another research, a mixture of Alcalase, Brauzyn and Protamex was utilized to produce spent brewer yeast protein hydrolysates yeast with improved physicochemical and antioxidant properties [407]. In a further study, spent brewer yeast cell wall was ruptured with enzymatic hydrolysis catalyzed by Protamex, Brauzyn, Alcalase and Flavourzyme, and this was compared to the results obtained using conventional methods (autolysis and mechanical rupture) [408]. It was found that yeast compounds were more efficiently released after sequential enzymatic hydrolysis using Brauzyn and Alcalase, resulting in maximum solid recovery and an increase of 63% in antioxidant properties [408]. Shu *et al.* employed Plackett-Burman design to determine the significant factors that affect the preparation of antioxidant peptides by hydrolysis of goat milk casein with mixtures of protease, which were temperature, enzyme/substrate ratio and the ratio of compound protease [409]. It was found that the hydrolysis conditions that led the highest antioxidative activity of the produced peptides were 55°C, pH 7.5, substrate concentration of 3.0%, an enzyme/substrate of 4.0%, a ratio of Alcalase/papain of 1/3 and a reaction time of 180 min [409]. Later, the optimization of hydrolysis condition of goat milk casein using mixtures of Alcalase/papain was optimized *via* response surface methodology [410]. The optimal reaction conditions were 61°C, enzyme/substrate ratio of 5.6%, and a combi-protease papain: Alcalase of 1.8. This led a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity increased 1.17 folds compared to un-optimized conditions [410].

In other cases, the studies were performed using a sequential strategy. As they did not compare both, it is hard to see the advantages of these two-step protocols compared to the use of combiproteases. For example, eggshell membrane hydrolysate was prepared by sequential treatment with Alcalase and Protease S and the product was fractioned by ultrafiltration [411]. The obtained fractions showed scavenging activity against DPPH and hydroxyl radicals as well as Fe^{2+} chelating activity [411]. In the hydrolysis of chickpea (*Cicer arietinum L*.) protein it was showed that the hydrolysis treatment of 60 min with Alcalase followed by 30 min with Flavourzyme produced hydrolysates with the highest antioxidant activity and cholesterol micellar solubility inhibition (50%) [412]. In addition, a peptide (RQSHFANAQP) with high antioxidant activity was isolated from Chickpea (*Cicer arietinum L*.) albumin hydrolysate obtained by sequential hydrolysis with Alcalase and Flavorzyme, and fractionation using size exclusion chromatography [413]. Heat stable rice bran protein was hydrolyzed using Alcalase (1.8 h) followed by hydrolysis with Protamex (2 h) producing hydrolysates with high antioxidant activity [414]. In another research, protein enzymatic hydrolysates from a byproduct of chia (*Salvia hispanica L*.) oil extraction were obtained using Alcalase and Flavourzyme separately or in a sequential system [415]. Results revealed that the increase in the degree of hydrolysis (37.16 %) during the digestion with the sequential system Alcalase-Flavourzyme in 90 min showed higher ABTS antioxidant activity (12.56 mmol L-1 mg-1 protein) and higher DPPH radical sweep (77.47 %), compared to the individual enzymatic treatments [415]. Nile tilapia (*Oreochromis niloticus*) protein hydrolysates were prepared by one- and two-step hydrolysis using different commercial proteases [416], finding that the use of Alcalase in combination with papain rendered the hydrolysate with the best antioxidant properties and the most reduced bitterness, which could be used as the functional food supplement [416].

Collagen from the skin of yellowfin tuna obtained using papain was further hydrolyzed with Alcalase, and the hydrolysates obtained showed high antioxidant and antiglycation activities [417]. In addition, six antioxidant peptides were isolated (by ultrafiltration and chromatography methods) from protein hydrolysate obtained from blood cockle (*Tegillarca granosa*) treated with Alcalase for 1.5 h followed by a Neutrase treatment for 1.5 h [418]. Also, hydrolysates with high radical scavenging activity, reducing power, and lipid peroxidation inhibition capability from Antarctic krill (*Euphausia superba*) were prepared by sequential enzyme hydrolysis process using Alcalase and Flavourzyme under optimal conditions (pH 6.0, 2.5 h, 25°C, and solid–liquid ratio of 1:20) [419].

3.3. Production of peptides with angiotensin I–converting enzyme inhibitory activity

High blood pressure, better known as hypertension, is a major risk factor for cardiovascular diseases. It is related with stroke, myocardial infarction, heart failure and renal disease, which causes the premature death of about 9.4 million people every year [420, 421]. In this context, the dipeptidyl carboxypeptidase angiotensin I–converting enzyme (EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure and in the cardiovascular function [422], because it converts, by removing dipeptide from the C terminus, the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II, which has a tendency to increase blood pressure [423]. For this reason, many drugs intended to treat hypertension and related diseases rely on angiotensin I–converting enzyme inhibition; among them, the two most popular classes of pharmacological treatments are angiotensin receptor blockers, which block the type 1 receptor of angiotensin II, and angiotensin-converting enzyme inhibitors [424], which inhibit angiotensin-converting enzyme activity reducing the conversion of

angiotensin I to angiotensin II and the vasoconstricting activity of angiotensin II [420]. Synthetic angiotensin-converting enzyme inhibitors like captopril, enalapril, and lisinopril, have been shown to be relatively safe in the short term; however, their use has been associated with serious side effects [171] like the accumulation of substance P, which is expressed in lung cancer tissue and has been related with angiogenesis and tumor proliferation, and with a bradykinin accumulation in the lung, which has been reported to promote growth of lung cancer [425].

Therefore, there is a growing interest in finding natural angiotensin I-converting enzyme inhibitors to overcome the disadvantages of synthetic drugs. In this regard, peptides with angiotensin I-converting enzyme inhibitory activity have gained great popularity [426]. These bioactive peptides have been obtained by enzymatic hydrolysis mainly from seafood proteins such as bigeye tuna dark muscle, yellowfin sole, freshwater fish, seaweed pipefish, oyster, algae, sea cucumber, etc. [426], but also from other protein sources like coconut cake [427], sesame meal [428], *Phaseolus lunatus* [429] and walnut (*Juglans regia* L.) [430]. Among the most widely used enzymes to obtain peptides with angiotensin I-converting enzyme inhibitory activity is Alcalase.

3.3.1. Hydrolysis of vegetable proteins

3.3.1.1. Use of stand-alone Alcalase

Alcalase was used both to hydrolyze soybean proteins, and to catalyze the plastein reaction to modify the obtained soybean protein hydrolysates [431]. They showed an angiotensin I-converting enzyme inhibitory activity with an IC50 value that ranged from 0.64 to 1.11 mg/mL [431]. On the other hand, Li *et al*., showed that the hydrolysis of

soybean protein isolate catalyzed by Alcalase released antihypertensive peptides [432]. However, the simulated *in vitro* digestion of these peptides reduced the angiotensin I-converting enzyme inhibitory activities [432]. Zhang *et al*. obtained a potent antihypertensive tetrapeptide (Phe-Gly-Ser-Phe) from vinegar-soaked black soybean using Alcalase, which exhibited high *in vitro* angiotensin I-converting enzyme inhibitory activity (IC50 = 117.11 μM) and *in vivo* hypotensive effect in spontaneously hypertensive rats [433]. Rapeseed protein has also been hydrolyzed by Alcalase [434]. The obtained hydrolysate presented a degree of hydrolysis of ~11% after 4h digestion with Alcalase. This hydrolysate was fractioned and three peptides were purified. Among them, LY (IC50=0.11mM) was the most potent against angiotensin I-converting enzyme activity, and showed to be an effective hypotensive agent [434]. In another paper, the effect of Alcalase rapeseed hydrolysate on blood pressure was measured *in vivo* in Goldblatt rat model of hypertension finding a maximum difference in mean arterial pressure of approximately −50 mmHg by RP in comparison to vehicle treated rats [435], while in another study, Alcalase rapeseed protein hydrolysate inhibited angiotensin I-converting enzyme and renal activities in a dose-dependent manner [436].

Dadzie *et al.* optimized the Alcalase hydrolysis of vital wheat gluten by response surface methodology [436]. The optimized conditions were a substrate concentration of 5.04%, an enzyme-substrate ratio of 5.94%, and 30.79 min of reaction time. This hydrolysate presented a 78.93% ± 1.07 of angiotensin I-converting enzyme inhibitory activity [437]. On the other hand, *He et al*. used Alcalase for the establishment of an efficient enzymatic membrane reactor for the preparation of angiotensin I-converting enzyme inhibitory peptides from wheat germ protein isolates [438]. It was found that, in

comparison with the traditional enzymatic hydrolysis method, the conversion rate of protein increased by 36.17% and the IC50 of the produced hydrolysate was reduced by 30.6% [438]. Ramírez-Torres *et al*. reported the Alcalase hydrolysis optimization of the amaranth protein (optimal conditions were pH 7.01, enzyme concentration of 0.04 mU/mg, 52 °C and 6.16 h) [439]. The optimized hydrolysate showed a 93.5% of angiotensin I-converting enzyme inhibition, at a hydrolysis degree of 74.77%, and was bioavailable in mice from 5 to 60 min. Its hypotensive effect started after 4 h in spontaneously hypertensive rats [439]. Valdez-Meza *et al*. evaluated the antihypertensive properties of pasta enriched with an amaranth protein hydrolysate produced by Alcalase hydrolysis [440]. They found that the amaranth hydrolysates affected negatively the overall acceptability and, to a lesser extent, the pasta taste. However, under physiological conditions, it was possible to appreciate the antihypertensive properties of the supplemented pasta [440]. In another work, cookies prepared with Alcalase-generated amaranth hydrolysate reduced the blood pressure in spontaneously hypertensive rats [441].

Alcalase was also used to hydrolyze sweet sorghum grain protein [442]. The hydrolysate obtained with a degree of hydrolysis of 19% exhibited the strongest angiotensin-converting enzyme inhibitory activity [442]. The hydrolysis at 56 °C and pH 8.0 using an Alcalase dosage of 5200 U/g [443], produced an optimized sweet sorghum grain protein hydrolysate, that contained 24.3% 1–5 kDa (IC50=0.305 mg/ml) and 15.2% <1 kDa (IC50=0.116 mg/ml) peptide fractions having potent *in vitro* angiotensin I-converting enzyme inhibitory activities [443].

African yam bean seed proteins hydrolysates with angiotensin I-converting enzyme inhibitory activity were produced by the hydrolysis with Alcalase [444]. In another paper,

Alcalase hydrolysates of common bean protein showed remarkable antihypertensive effect over spontaneously hypertensive rats, similar to Captopril treatment [445]. In another very interesting work, beans damaged by anthracnose disease were hydrolyzed with Alcalase [446], and the obtained hydrolysates had angiotensin I-converting enzyme inhibitory activity (IC50 0.019 mg protein/mL) very similar to those from control beans, suggesting that preparation of hydrolysates from this protein source, a wasted material, would allow their revalorization [446]. Segura-Campos *et al*. produced Alcalase hydrolysates from defatted *Jatropha curcas* kernel meal and the protein hydrolysate with a 21.35% degree of hydrolysis produced 34.87% angiotensin I-converting enzyme inhibition, and the purified fraction with the highest angiotensin I-converting enzyme inhibitory activity had an IC50 value of 4.78 g/mL [447]. In another attempt, Xu *et al.* hydrolyzed soluble leaf protein from cauliflower processing by-products, using Alcalase, and the hydrolysate showed a potent angiotensin I-converting enzyme inhibitory activity *in vitro*, with an IC50 value of 138.545 μg/mL [448]. Lim *et al*. optimized by responsive surface methodology the Alcalase hydrolysis conditions of *Camellia japonica* protein [449]. The optimal conditions were 50.98°C, enzyme/substrate ratio of 2.85%, and pH of 7.12 to obtain hydrolysates with the highest angiotensin I-converting enzyme inhibitory activity. In an animal feeding study with spontaneously hypertensive rats, the authors found that even though systolic blood pressure was not statistically different, the high dose of *C. japonica* hydrolysate lowered diastolic blood pressure at the 5th week [449]. In another study, two peptides were obtained from horse gram flour by hydrolysis with Alcalase [450]. These peptides, TVGMTAKF and QLLLQQ, exhibited high angiotensin I-converting enzyme inhibitory activity with IC50 values of 30.3 μM and 75.0 μM, respectively [450].

Alcalase hydrolysis of pea protein produced angiotensin I-converting enzyme inhibitory dipeptides with IC50 values <25 mM [451], while the peptides obtained from chickpea accession BDN-9-3 by 1 h of Alcalase hydrolysis had an angiotensin I-converting enzyme activity with a IC50 value of 22.43 mg/ml [452]. On the other hand, lightly roasted cowpea flour was hydrolyzed with Alcalase for 6 h [453]. The resulting hydrolysate, with an angiotensin I-converting enzyme inhibitory IC50 value of 123.6 μg/ml, was subjected to different purification steps, and a peptide with an IC50 value of 22 μg/mL was obtained [453].

3.3.1.2. Comparison of Alcalase with other proteases

Several proteases have been explored in the angiotensin I-converting enzyme inhibitory peptide production from vegetable proteins. For example, Neutrase, Alcalase, Flavourzyme, Proleather, Protamex and papain were employed to hydrolyze apricot (*Prunus armeniaca* L.) kernel proteins [454]. Alcalase was selected for further study on the enzymatic preparation of angiotensin I-converting enzyme inhibitory peptides, finding that after 60 min of hydrolysis, the highest angiotensin I-converting enzyme inhibition was 82 ± 0.14% [454]. Soybean protein isolate was hydrolyzed by papain, Multifect Neutral, Neutrase, GC 106, Alcalase, Flavourzyme, and Protamex, at different enzyme and protein suspension concentrations, and at different reaction time [455]. Alcalase produced the best results under optimum hydrolysis conditions (1% enzyme concentration, 5% suspension concentration for 4 h) generating a hydrolysate with a IC50 value for angiotensin I-converting enzyme inhibitory activity of 79.94 μg/mL [455]. In another study, bromelain, Flavourzyme, papain, and Alcalase were utilized to hydrolyze this product, and Alcalase

generated the hydrolysate with the highest angiotensin I-converting enzyme inhibitory activity (IC50: 0.14 mg/mL at 6 h hydrolysis time) [456].

Phaseolus lunatus protein concentrates of flour from germinated and non-germinated seeds were hydrolyzed with Alcalase or pepsin-pancreatin and their hydrolysates were fractioned [429]. All obtained peptide fractions had angiotensin I-converting enzyme inhibitory activity in a range of 0.9 to 3.8 μg/mL [429]. It has also been reported that with a controlled protein hydrolysis using Alcalase, Flavourzyme or pepsin-pancreatin, it is possible to obtain angiotensin I-converting enzyme inhibitory and antioxidant peptides from *Vigna unguiculata* proteins [457]. In another paper, hydrolysates with angiotensin I-converting enzyme inhibitory activity were prepared from blue lupin (*Lupinus angustifolius*) protein isolate using Alcalase or Flavourzyme [458]. Alcalase hydrolysate showed the highest angiotensin I-converting enzyme inhibitory activities with IC50 values ranging from 0.10 to 0.21 mg/ml [458].

Angiotensin I-converting enzyme inhibitory activity of hydrolysates produced by Alcalase or Flavourzyme hydrolysis of protein isolate from pumpkin oil cake has also been investigated [459]. The highest activity was determined in the Alcalase hydrolysate after 60 min of reaction [459]. In another study, angiotensin I-converting enzyme inhibitory peptides with IC50 values ranging from 0.101 to 37.33 μg mL^{-1} were prepared from chickpea protein hydrolysates (fresh and hard-to-cook grains) using papain, pancreatin or Alcalase [460]. In another research, rapeseed protein hydrolysates were obtained by digestion with Alcalase and other proteases [461]. Alcalase, Proteinase K and thermolysin hydrolysates generated the highest *in vitro* inhibition of angiotensin I-converting enzyme. However, oral administration (100 mg/kg body weight) of Alcalase hydrolysate to

spontaneously hypertensive rats was the most effective treatment in blood pressure reduction [461]. On the other hand, angiotensin I-converting enzyme inhibitory activity of protein hydrolysates prepared by Alcalase hydrolysis of industrial defatted rapeseed [462], displayed the highest angiotensin I-converting enzyme inhibitory activity (IC50 value of 0.02 mg/ml) and exhibited good stability in an *in vitro* digestion model using human gastric and duodenal fluids, when compared to the results obtained using other proteases [462]. Another research showed the hydrolysis of canola protein isolate catalyzed by trypsin, chymotrypsin, pancreatin, pepsin and Alcalase [463]. Alcalase hydrolysate presented the highest *in vitro* inhibition of angiotensin I-converting enzyme activity, and showed antihypertensive effects, giving the fastest and the highest decrease in systolic blood pressure in spontaneously hypertensive rats among the produced hydrolysates [463]. Marrufo *et al*. used Alcalase or a sequential pepsin-pancreatin enzymatic system to hydrolyze defatted protein isolate of seeds from *Jatropha curcas* L [464]. Alcalase hydrolysate showed an angiotensin I-converting enzyme inhibitory effect with IC50 value of 2.8 μg/mL, while the IC50 value for the hydrolysate obtained by the pepsin-pancreatin system was 7.0 μg/mL [464]. In another research attempt, *Jatropha curcas L*. protein hydrolysates were produced by treatment of a non-toxic genotype with Alcalase as well as pepsin and pancreatin [465]. It was found that more efficient peptides in angiotensin I-converting enzyme inhibitory activity were produced in the Alcalase hydrolysates [465]. Another research used sesame meal, that was treated with pepsin, papain, Neutrase and Alcalase [428]. Alcalase generated the protein hydrolysate with the highest angiotensin I-converting enzyme inhibitory activity corresponding to an IC50 value of 0.6 mg/mL [428]. In another study, hydrolysates of wild almond proteins were prepared using chymotrypsin, trypsin, pepsin, Flavourzyme and Alcalase [466]. Alcalase, again, generated the

hydrolysates with the highest angiotensin I-converting enzyme inhibiting activity (IC50 = 0.8 mg/mL), and three peptides showing the highest angiotensin I-converting enzyme inhibitory activities were identified [466]. Malomo *et al*. produced antihypertensive hydrolysates of hemp seed proteins by hydrolysis with 2% or 4% pepsin, 1% or 2% Alcalase, 2% papain, or 2% pepsin + pancreatin [467]. The hydrolysates of hemp seed proteins obtained with 1% Alcalase were the most effective systolic blood pressure-reducing agents (32.5 ± 0.7 mm Hg after 4 h of ingestion) [467]. In another study, mungbean vicilin protein was enzymatically hydrolyzed by Alcalase and trypsin under optimal conditions [468]. The Alcalase hydrolysate exhibited the highest angiotensin I-converting enzyme inhibitory activity with IC50 value of 0.32 mg protein/mL [468]. Later, sweet potato protein was hydrolyzed by pepsin, papain and Alcalase under high hydrostatic pressure (100–300 MPa) [469]. It was found that molecular weight peptide fractions <3 kDa from sweet potato protein prepared with Alcalase under 100 MPa, showed the highest angiotensin I-converting enzyme inhibitory activity with a IC50 value 32.24 μg mL^{-1} [469]. Xu *et al*. evaluated pancreatin, pepsin and Alcalase for the hydrolysis of cauliflower processing by-products protein [470], and later Arise *et al*. compared trypsin, Alcalase, and pepsin in the hydrolysis of bambara protein [471]. In both cases, the peptides produced by Alcalase showed the highest inhibitory activity against angiotensin I-converting enzyme [470, 471]. In another paper, Dispase, trypsin, Alcalase, and Flavourzyme were used to hydrolyze a protein isolate extracted from *Ginkgo biloba* seeds, obtaining peptides with angiotensin I-converting enzyme inhibitory activity [472].

3.3.2. Hydrolysis of fish and seafood proteins

3.3.2.1. Use of stand-alone Alcalase

There are many examples of using Alcalase to hydrolyze fish and seafood proteins to get hydrolysates and peptides with angiotensin I-converting enzyme inhibitory activity. For example, the peptides al-Trp-Asp-Pro-Pro-Lys-Phe-Asp, Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe and Phe-Asn-Val-Pro-Leu-Tyr-Glu [473], with IC50 values against angiotensin I-converting enzyme activity of 9.10 μM, 10.77 μM and 7.72 μM, respectively, were isolated from Alcalase hydrolysate from salmon byproduct proteins [473]. Similarly, seven peptides were isolated from protein hydrolysate (5% degree of hydrolysis) of defatted skipjack roe (*Katsuwonus pelamis*) produced by Alcalase digestion [474]. The peptide MLVFAV peptide exhibited the highest angiotensin I-converting enzyme inhibitory activity with an IC50 value of 3.07μM [474]. In another study, collagen extracted from jellyfish (*Rhopilema esculentum*) was hydrolyzed with Alcalase at optimal hydrolyzing conditions (52. 7 °C, pH of 8.6 and enzyme-to-substrate ratio of 3. 46%) [475], producing a hydrolysate with an angiotensin I-converting enzyme inhibitory activity of 81.7% [475]. Amado *et al*. reported the purification and identification of angiotensin I-converting enzyme inhibitory peptides with IC50 values ranging from 1.92 to 8.83 μg mL^{-1}, obtained by 8 h of Alcalase hydrolysis of a protein concentrate recovered from a cuttlefish industrial manufacturing effluent [476]. Similarly, two potential angiotensin I-converting enzyme inhibitory peptides with molecular weight of 959.46 and 1,141.29 Da, were obtained from tuna cooking juice by Alcalase hydrolysis in a continuous enzymatic membrane reactor coupling with 1 kDa MWCO membrane [477]. In another paper, barbel (*Barbus callensis*) muscle protein was hydrolyzed with Alcalase producing a hydrolysate with an angiotensin I-converting enzyme inhibitory activity with an IC50 of 0.92 mg/mL [478]. Mahmoodani *et al*. used Alcalase hydrolysis to obtain angiotensin I-converting enzyme inhibitory peptides

from skin and bone gelatins of pangasius catfish (*Pangasius sutchi*), which showed an IC50 value of 3.2 μg/ml and 1.3 μg/ml, respectively [479].

Two angiotensin I-converting enzyme inhibitory peptides, identified as VKP and VKCFR, with IC50 values of 1.3 μM and 34.5 μM, respectively, from Jellyfish (*Rhopilema esculentum*) protein [480], and three peptides, EVSQGRP, CRQNTLGHNTQTSIAQ and VSRHFASYAN, with IC50 values of 0.05, 0.08 and 0.21 mM, respectively, from sea cucumber (*Stichopus horrens*) protein were obtained by Alcalase hydrolysis [481]. The sea cucumber hydrolysate was found to *in vivo* stabilize the blood pressure in normotensive rats [481]. Rasli and Sarbon optimized the Alcalase hydrolysis conditions for protein hydrolysate production from shortfin scad (*Decapterus Macrosoma*) skin gelatin [482]. The optimum hydrolysis conditions were 60°C , pH 9, 2.92% of enzyme/substrate concentration and 114.56 min, with an experimental yield of shortfin scad skin gelatin hydrolysis of 90.05%, degree of hydrolysis of 90.48%, This hydrolysate exhibited an experimental angiotensin I-converting enzyme inhibitory activity of 79.61% [482]. In another research, 1 h of Alcalase hydrolysis of tilapia (*Oreochromis niloticus*) processing by-product and tilapia muscle, produced a low molecular weight peptide fraction with a very high angiotensin I-converting enzyme inhibitory activity [483].

3.3.2.2. Comparison of Alcalase with other proteases

As performed in other cases, Alcalase has been seen compared to other proteases in the hydrolysis of fish and seafood proteins to produce hydrolysates or peptides with angiotensin I-converting enzyme inhibitory activity. Such is the case of the hydrolysis of seaweed pipefish muscle proteins [484], where Alcalase hydrolysate exhibited the highest angiotensin I-converting enzyme inhibitory activity, compared to the hydrolysates produced

with Pronase, pepsin, Neutrase, papain or trypsin [484]. Similar results have been obtained in the hydrolysis of gelatin from giant squid (*Dosidicus gigas*) [485], where the Alcalase hydrolysate was the most potent angiotensin I-converting enzyme inhibitor (IC50=0.34mg/mL) compared to the hydrolysates produced with NS37005, Savinase, Protamex, Neutrase, trypsin, and Esperase [485]. Also, in the hydrolysis of mussel (*Mytilus edulis*) protein, Alcalase catalyzed the hydrolysis most efficiently [486], with the highest protein recovery and the strongest angiotensin I-converting enzyme inhibitory activity, among six different proteases [486], and in the hydrolysis of *Sepiolopus horrens* flesh [487], Alcalase hydrolysate showed the highest degree of hydrolysis value (39.8%) and the highest angiotensin I-converting enzyme inhibitory activity, with an IC50 value of 0.41 mg/mL, compared with trypsin, papain, bromelain, Flavourzyme, or Protamex hydrolysates [487]. In another study, Nile tilapia (*Oreochromis niloticus*) gelatin was hydrolyzed using Pronase E, pepsin, Alcalase and trypsin [488]. The Alcalase hydrolysate exhibited the highest angiotensin I-converting enzyme inhibitory activity, and the peptide DPALATEPDPMPF exhibits a potent angiotensin I-converting enzyme inhibitory activity [488]. Flavourzyme, Neutrase, Alcalase and Protamex were used to hydrolyze skin gelatin of skate (*Okamejei kenojei*) [489], and it was found that Alcalase hydrolysate exhibited the highest angiotensin I-converting enzyme inhibitory activity [489]. Among various commercial enzymes, Alcalase was selected to hydrolyze snakehead fish sarcoplasmic protein due to its better performance [426]. Two angiotensin I-converting enzyme inhibitory peptides, with IC50 values of 1.3 and 2.8μM, respectively, were isolated from the Alcalase hydrolysate, these peptides showed no cytotoxicity effects on human embryonic fibroblast cell line and human hepatocarcinoma cell line [426]. In addition, among many other different proteases, Alcalase produced peptides with higher angiotensin

I-converting enzyme inhibitory activity from the shrimp shell waste [490]. The optimal Alcalase hydrolysis conditions were pH 9.5, 60 °C, 25 g L^{-1} substrate and 4000 U g^{-1} of enzyme [490].

Thornback ray gelatin hydrolysates were prepared by hydrolysis with Alcalase and Neutrase, and the proteases from *Bacillus subtilis* A26 or from *Raja clavate* [491]. In this study, gelatin hydrolysate treated with Alcalase and A26 exhibited the highest angiotensin I-converting enzyme activity with 82 ± 0.49% and 85 ± 0.65, respectively, at 5 mg/ml [491]. In another work, Alcalase, papain, bromelain, Flavourzyme, pepsin, and trypsin were used to produce angiotensin I-converting enzyme inhibitory hydrolysates from sea cucumber (*Actinopyga lecanora*) [492]. Alcalase hydrolysate presented the highest angiotensin I-converting enzyme inhibitory activity (69.8%) after 8 h of hydrolysis [492].

Collagenase, Proteinase K, Alcalase, and/or trypsin at their optimum conditions were used for the hydrolysis of grass carp (*Ctenopharyngodon idella*) skin pieces [493]. Alcalase and collagenase released peptides with angiotensin I-converting enzyme inhibitory activity [493]. In another work, angiotensin I-converting enzyme inhibitory and anticoagulant peptides from tuna cooking juice were prepared by enzymatic hydrolysis with Flavourzyme, pancreatin, Alcalase and pepsin [494]. The Alcalase hydrolysate after a hydrolysis time of 240 or 120 min showed the highest angiotensin I-converting enzyme inhibitory activity (96.9 ± 0.54%) [494].

Flavourzyme and Alcalase were employed in the hydrolysis of protein-rich flour from mojarra of Nile tilapia (*Oreochromis niloticus*) skeleton for the preparation of protein hydrolysates with angiotensin I-converting enzyme inhibitory activity [495]. Both obtained

hydrolysates showed greater angiotensin I-converting enzyme inhibitory activity with IC50 values of 0.344 and 0.238 mg/mL, respectively [495].

Dewi *et al*. reported the hydrolysis of three species of under-utilized sea cucumbers from Lampung and Gorontalo provinces, using Alcalase, bromelain, or the combination of both enzymes, at hydrolysis conditions of pH 7, 45 °C, 24 h and enzyme/substrate ratio of 1% [496]. Results revealed that the Alcalase hydrolysates of *H. atra* contained the most active angiotensin I-converting enzyme inhibition activity with an IC50 value of 0.32 mg/mL [496].

In addition, angiotensin I-converting enzyme inhibitory peptides from coastal trashes of squilla muscle (*Harpiosquilla raphidea*) were prepared by enzymatic hydrolysis using thermolysin, trypsin and Alcalase [497]. The hydrolysates produced after 5 h of hydrolysis with Alcalase and 6 h with thermolysin had the highest angiotensin I-converting enzyme inhibition activity (64.8 ± 0.3% and 68.4 ± 1.0%, respectively) [497].

3.3.3. Hydrolysis of whey and casein proteins

3.3.3.1. Use of stand-alone Alcalase

Alcalase has been frequently used for the hydrolysis of casein protein in order to obtain peptides with angiotensin I-converting enzyme inhibitory activity. For instance, it was reported that a casein hydrolysate prepared by hydrolysis of casein with Alcalase during 6 h had an *in vitro* angiotensin I-converting enzyme inhibitory activity with an IC50 value of 47.1 μg mL^{-1} [498], while in another paper, the hydrolysate showed an IC50 of 760 μg mL^{-1} [499].

Several studies report the modification of the Alcalase hydrolysates of casein, through the plastein reaction in order to improve angiotensin I-converting enzyme inhibition activity. About that, Alcalase casein hydrolysate with a degree of hydrolysis of 13.5% showed an IC50 value of 45.2 μg mL-1 for *in vitro* angiotensin I-converting enzyme inhibition activity [500], which was improved by Neutrase-catalyzed plastein reaction obtaining IC50 values ranging from 15.6 to 20.0 μg/mL [500]. Similarly, a casein hydrolysate with a degree of hydrolysis of 10.9% prepared with Alcalase [501], had *in vitro* angiotensin I-converting enzyme inhibition with an IC50 value of 52.6 μg/mL, which after modification by Alcalase-catalyzed plastein reaction, resulted in an IC50 value of 13.0 μg/mL [501].

Zhang and Zhao studied the hydrolysis of casein with Alcalase obtaining hydrolysates with *in vitro* angiotensin I-converting enzyme inhibitory activity of 44.4% [502]. The hydrolysates were later modified by Alcalase-catalyzed plastein reaction in an ethanol-water medium finding that most of the treated hydrolysates enhanced their angiotensin I-converting enzyme inhibition activities compared to the initial casein hydrolysate, mainly at 4 h of reaction time [502]. The same authors reported the optimization of the Alcalase-catalyzed plastein reaction in ethanol-water medium to improve the *in vitro* angiotensin I-converting enzyme inhibitory activity (44.4%) of the Alcalase casein hydrolysate [503]. The optimized conditions were Alcalase addition of 8.36 kU/g peptides, ethanol of 56.8% (v/v), substrate concentration of 56.8% (w/v), and 37.5°C, which led a casein hydrolysate with an angiotensin I-converting enzyme inhibitory activity of 62.5% [503]. In another study, casein was digested with Alcalase, and the obtained hydrolysate presented an *in vitro* angiotensin I-converting enzyme inhibitory activity of

48.2% [504]. When this product was modified by plastein reaction in propanol-water medium with addition of tyrosine or phenylalanine, after 1 h of reaction, produced modified hydrolysates with an inhibitory activity of 61.6-68.5% [504].

3.3.3.2. Comparison of Alcalase with other proteases

Hydrolysates from whey protein concentrate were generated using Flavourzyme, Alcalase or Neutrase, and they presented inhibition angiotensin I-converting enzyme activities of 51.52 %, 73.22 % and 71.14 %, respectively [505]. Alcalase and Neutrase hydrolysates were used to incubate human umbilical vein endothelial cells for 48 h, and this resulted in a beneficial differential expression of genes relevant to blood pressure control [505]. In another study, the angiotensin I-converting enzyme inhibitory effect of yoghurt beverage fortified with different whey protein hydrolysates was investigated [506]. To this goal, whey protein was hydrolyzed using Protamex, Alcalase and trypsin and the obtained hydrolysates were added to yoghurt beverage at concentrations of 1.25, 2.5, and 5 mg/mL. It was found that yoghurt beverage fortified with 2.5 mg/mL and 5 mg/ mL of hydrolysates had 61-69% and 74% of angiotensin I-converting enzyme inhibitory activity, respectively, with no significant differences between the Alcalase or Protamex hydrolysates [506]. In addition, bromelain, Alcalase and papain were used to hydrolyze camel milk protein [507]. Papain and Alcalase hydrolysates presented the highest angiotensin I-converting enzyme inhibitory activity [507].

3.3.4. Hydrolysis of proteins from other sources

3.3.4.1. Use of stand-alone Alcalase

There are many other sources of proteins that have been explored to produce peptides with angiotensin I-converting enzyme inhibitory activities by hydrolysis with Alcalase.

For example, Alcalase was used to hydrolyze silk fibroin [508], and the results showed that the obtained hydrolysate with a hydrolysis degree of 17% exhibited the highest angiotensin I-converting enzyme inhibitory activity, and significantly lowered blood pressure of spontaneously hypertensive rats after chronic oral administration [508]. Lu *et al*. reported an angiotensin I-converting enzyme inhibitory peptide (Ile-Gln-Pro) with an IC50 value of 5.77 ± 0.09 μM, which was produced by Alcalase digestion of *Spirulina platensis* [509]. This peptide was resistant to *in vitro* digestion by gastrointestinal proteases and significant decreased the systolic and diastolic blood pressure in spontaneously hypertensive rats after 4, 6, and 8 h of its oral administration [509].

Alcalase hydrolysis of chicken blood meal has also been explored [510]. In this study, the results showed that peptides with the highest angiotensin I-converting enzyme inhibition activities were produced after five hours of hydrolysis, using 10% Alcalase enzyme [510]. In another research, hydrolysates from bovine plasma were obtained by Alcalase at different degrees of hydrolysis [511]. The highest angiotensin I-converting enzyme inhibition activity was obtained with a hydrolysis degree of 6.7%. After fractioning the hydrolysate, the most active fraction presented an IC50 value of 0.18 mg/mL, which remained constant after submitting it to *in vitro* digestion conditions [511]. In another work, a natural seasoning with antihypertensive effect was developed using beef hydrolysate produced by the hydrolysis with Alcalase for 4 h [512].

Insects are also used as feedstock. *Tenebrio molitor* (L.) larva was subjected to hydrolysis with Alcalase [513]. The hydrolysate with a degree of hydrolysis of 20% presented the highest angiotensin I-converting enzyme inhibition activity with an IC50 value of 0. 39 mg/mL, and after fractionation, the smallest peptides were the most active ones, increasing this value up to 0.23 mg/mL. Its multiple dose oral administration to spontaneously hypertensive rats led to a significant decrease in blood pressure. A novel peptide (Tyr-Ala-Asn) was purified and presented an IC50 value of 0.017 mg/mL [513]. In another example, ultrasound treated silkworm pupa (*Bombyx mori*) protein was hydrolyzed using Alcalase, and the hydrolysate with the highest angiotensin I-converting enzyme inhibitory activity was subjected to several purification steps which led to the identification of a novel peptide (Lys-His-Val) with IC50 value of 12.82 μM [514]. This peptide was stable against the gastrointestinal proteases [514]. In another paper, Alcalase was also used for the preparation of peptides with angiotensin I-converting enzyme inhibitory activity from *Enteromorpha clathrata* protein [515].

Alcalase has also been used to obtain potential angiotensin I-converting enzyme inhibitory peptide from egg white protein. For example, a peptide with a sequence of Arg-Val-Pro-Ser-Leu and remarkable angiotensin I-converting enzyme inhibitory activity (IC50 value of 20 μM) [516], and another one, identified as QIGLF which exhibited an angiotensin I-converting enzyme inhibitory activity with an IC50 value of 75 μM and resistance to digestion by proteases of the gastrointestinal tract [517], were produced by Alcalase hydrolysis of egg white protein.

3.3.4.2. Comparison of Alcalase with other proteases

Alcalase was selected among seven commercial enzymes due to its most effective activity in the hydrolysis of *Porphyra yezoensis* proteins [518]. Under optimum Alcalase hydrolysis conditions (1.5% substrate, 5% enzyme, pH 9.0, 50 °C and 60 min), an antihypertensive peptide with a high angiotensin I-converting enzyme inhibition activity of 55.0% and a low IC50 value of 1.6 g/l was produced [518].

Protein by-products produced from the oil extraction in the biodiesel production from *Nannochloropsis oculata* were hydrolyzed using PTN, Flavourzyme, Neutrase, Alcalase or Protamex [519]. The hydrolysate produced by Alcalase showed the highest angiotensin I-converting enzyme inhibitory activity with and IC50 value of 0.126 mg ml^{-1} [519]. Similarly, trypsin, chymotrypsin, pepsin, Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase and papain were evaluated in the hydrolysis of *Chlorella ellipsoidea* proteins [520]. Among the tested enzymes, a potent angiotensin I-converting enzyme inhibitory peptide with IC50 value of 128.4 μM, was isolated from a hydrolysate produced by the hydrolysis with Alcalase [520].

Other studies report the hydrolysis of chicken skin protein from the thigh and breast muscles using Alcalase or a combination of pepsin/pancreatin [521]. The produced protein hydrolysates were fractionated by ultrafiltration membranes, and then were administrated to spontaneously hypertensive rats which reduced their systolic blood pressure [522]. Also, the production of chicken skin gelatin hydrolysates and peptides with angiotensin I-converting enzyme inhibitory activity using Pronase E, Alcalase and collagenase was reported [523]. They showed antihypertensive effect of some purified peptides by oral administration to spontaneously hypertensive rats [523].

Mudgil *et al*. studied the effect of different proteolytic enzymes (Alcalase and Protease), hydrolysis time and enzyme: substrate ratio on the bioactive properties of novel camel skin gelatin hydrolysates [524]. In general, no significant effect of the enzyme: substrate ratio and time of hydrolysis on the production of bioactive peptides was observed, while both enzymes, Alcalase and Protease, individually or in combination produced camel skin gelatin hydrolysates with highly potent antihypertensive activity [524].

Alcalase and papain were used to hydrolyze bovine collagen from connective tissue, a by- product in the meat processing industry [525]. The two most potent angiotensin I-converting enzyme inhibitory collagen hydrolysates with IC50 values of 0.17 and 0.35 mg mL^{-1} were obtained using Alcalase-catalyzed and papain-catalyzed hydrolysates, respectively. After fractionation, these values increased up to IC50 values of 3.95 and 7.29 μg mL^{-1}, respectively [525]. In another paper, edible bird nest protein was hydrolyzed by Alcalase or papain [526]. The results showed that 60 min of hydrolysis using Alcalase produced a protein hydrolysate with the highest angiotensin I-converting enzyme inhibitory activity with an IC50 value of 0.02 mg protein/ml [526]. Another contribution shows that *Achatina fulica* snail foot muscle protein was hydrolyzed with trypsin, papain or Alcalase [527]. It was found that Alcalase produced the hydrolysate with the highest degree of hydrolysis and a strong angiotensin I-converting enzyme inhibitory activity *in vitro* (IC50 value of 0.024 mg/mL) [527].

In another study, hydrolysates of egg protein were produced with pancreatin, pepsin, thermolysin or Alcalase [528]. After their fractionation by ultrafiltration and cation exchange chromatography, it was found that the hydrolysates produced with thermolysin or Alcalase showed the highest angiotensin I-converting enzyme inhibitory activity [528].

3.3.5. Combined use of Alcalase with other proteases

The production of angiotensin I-converting enzyme inhibitory peptides from whey protein isolate by hydrolysis using different proteases or combi-proteases [395] has been investigated.

There are some examples on the use of combi-proteases. For example, Chen *et al*., reported the optimization by response surface methodology and application of an Alcalase-trypsin enzymatic blend in the hydrolysis of goat milk casein [529]. Under optimal conditions (pH 8.4, enzymes ratio 1:1 and enzyme to substrate ratio 8.5%), the angiotensin I-converting enzyme inhibitory activity of the obtained hydrolysates was 91.99% [529]. In another paper, a blend of Alcalase and Protease was used for the hydrolysis of bovine milk to produce novel angiotensin I-converting enzyme inhibitory peptides [530]. In this study, the optimized hydrolysis conditions were determined to be pH 9.01, 61.81 °C and 6.5% ratio of enzyme to substrate. This led to hydrolysates with the highest angiotensin I-converting enzyme inhibitory activity (85.02%). Further fractioning gave a fraction with an angiotensin I-converting enzyme inhibitory activity as high as 92.7% [530]. In another work, Alcalase, Flavourzyme and thermolysin were used to produce protein hydrolysates from date seed flour [531]. Results showed that among all treatments, hydrolysates prepared using a combination of Alcalase and thermolysin exhibited the highest angiotensin I-converting enzyme inhibitory activity with an IC50 value of 0.53 mg/mL [531].

However, in this instance, we have been able to find more examples of the sequential use of several proteases. Such is the case of the study carried out by Wang *et al*., who studied the hydrolysis of whey protein isolate using Neutrase, Alcalase or trypsin and also their use in a sequential way [532]. The authors used two different hydrolysis

conditions, pH-controlled and not-controlled pH, where the pH will decrease during the reactions. After 3 h of incubation of the proteins with Alcalase plus 2 h with Neutrase without pH control, they produced a hydrolysate with the highest angiotensin I-converting enzyme inhibitory activity (54.30%) [532].

Rui *et al*. investigated angiotensin I-converting enzyme inhibitory activity of protein hydrolysates derived from protein isolates of three *Phaseolus vulgaris* varieties (navy, black and small red bean) produced by hydrolysis using sequential digestion of Alcalase/Flavourzyme or Alcalase/papain [533]. Results showed that Alcalase/papain hydrolysates for all investigated *Phaseolus vulgaris* varieties presented higher angiotensin I-converting enzyme inhibitory activity with IC50 values in a range of 68 ± 5 μg protein/mL to 83 ± 13 μg protein/mL than the other hydrolysates [533]. An angiotensin I-converting enzyme inhibitory octapeptide (PVNNPQIH) with an IC50 value of 206.7 ± 3.9 μM, was purified from small red bean (*Phaseolus vulgaris*) protein hydrolysate produced by sequential digestion catalyzed by Alcalase and papain followed by *in vitro* gastrointestinal simulation [534]. In another research, Alcalase was used in a sequential digestion of palm kernel expeller glutelin-2 with Flavourzyme, pepsin and trypsin [535]. The proteins were pretreated under high pressure. The obtained protein hydrolysates presented high angiotensin I-converting enzyme inhibitory activity (80.24 %) [535].

Alcalase-Flavourzyme sequential system was employed to hydrolyze a protein-rich fraction from chia (*Salvia hispanica* L.) seed, and the hydrolysate obtained had 58.46% angiotensin I-converting enzyme inhibitory activity [536]. In another research, the sequential hydrolysis of high pressure pretreated coconut cake globulin by Alcalase, Flavourzyme, pepsin and trypsin [427], produced a hydrolysate with an angiotensin I-

converting enzyme inhibitory activity of 52.16%, which markedly reduced the systolic blood pressure of spontaneously hypertensive rats after single and chronic oral administration [427].

In the same way, Alcalase-Protamex sequential process was used to hydrolyze almond protein, and the hydrolysates were purified in order to identify the most active peptides [537]. Two angiotensin I-converting enzyme inhibitory peptides with the IC50 values of 67.52 ± 0.05 and 43.18 ± 0.07 μg mL^{-1} were purified and the results showed that these peptides significantly regulated the release of nitric oxide and endothelin in human umbilical vein endothelial cells [537]. Zheng *et al*. employed Alcalase-trypsin sequential system to hydrolyze quinoa bran albumin [538]. The hydrolysates obtained had angiotensin I-converting enzyme inhibitory activity with IC50 of 38.16 μM and significant antihypertensive effect in spontaneously hypertensive rats [538].

Enzymatic sequential system has also been employed in the hydrolysis of seafood and fish proteins. For instance, Gu *et al*. reported the use of Alcalase-papain sequential system in the production of peptides with angiotensin I-converting enzyme inhibitory activity from collagen of Atlantic salmon (*Salmo salar* L.) skin [539]. Among the peptides produced, two dipeptides identified as Ala-Pro and Val-Arg presented the highest angiotensin I-converting enzyme inhibitory activities with an IC50 of 0.060 mg/ml for Ala-Pro and IC50 of 0.332 mg/ml for Val-Arg [539]. Similarly, proteins from abalone (*Haliotis discus hannai*) gonads were hydrolyzed by Alcalase followed by papain treatment [540]. The hydrolysate was fractionated and a peptide was isolated which showed a angiotensin I-converting enzyme inhibitory activity of 0.44 mg/mL [540]. Later, the Alcalase-papain sequential digestion of abalone gonads led to the production of a tripeptide which had an

angiotensin I-converting enzyme inhibitory activity with IC50 value of 106.24 μg/mL [541]. This activity remained after gastrointestinal digestion [541].

In another study, Alcalase/Protease produced peptides with angiotensin I-converting enzyme inhibitory activity from skate (*Okamejei kenojei*) skin gelatin, which were able to reduce the systolic blood pressure in spontaneously hypertensive rats [542]. Also, pepsin-pancreatin and Alcalase-Flavorzyme sequential systems were used to prepare hydrolysates with angiotensin I-converting enzyme inhibitory capacity from sea cucumber (*Isostichopus badionotus*) [543]. It was found that the Alcalase-Flavourzyme system produced hydrolysates with the highest degree of hydrolyses and angiotensin I-converting enzyme inhibitory action (86%) [543].

3.4. Production of metal binding peptides

It is extensively known that nutritional disorders often come from a deficit in the intestinal absorption of metals which are essential for the organism [544, 545]. To prevent it, many researchers have been trying to improve the chelating activity of functional foods increasing the bioavailability of these metals [546]. In this context, Alcalase presents itself as an excellent alternative, and it has proven its efficacy in several studies.

Among the essential trace elements that humans need, iron is the most important one, and its deficiency causes many diseases [547, 548]. Typically, foods derived from animals are a better source of iron since it is more easily absorbed than from foods derived from vegetables [549]. There are plant factors such as polyphenols, phytate and soy protein that inhibit the non-heme iron absorption, while ascorbic acid and some components of

animal tissues enhance it. It has been suggested that some peptides, released during the protein digestion, may help iron absorption [550].

Zinc is also a trace element of great importance for the organism as it has a key role in the activation of hundreds of enzymes and gene expression [551-553]. Zinc also participates in the innate immunity helping the normal function of neutrophil and natural killer cells [554, 555]. It is used in the treatment against several diseases like atherosclerosis or immunologic disorders [556]. Calcium is another essential mineral nutrient, involved in many basic biological processes such as nerve conduction, mitosis, muscle contraction, blood coagulation and, of course, it is indispensable as the structural support of the skeleton [557]. Its deficit can provoke serious systemic illnesses like osteoporosis [558]. Looking for alternatives to improve the calcium intake, several experiments have been carried out in order to obtain functional foods rich in this element. Proteolysis is again, a good choice to obtain hydrolysates with high calcium-binding ability. Next, we will present some papers showing the preparation of peptides with capability to bind zinc, iron or calcium and that way, facilitate their absorption and bioavailability.

3.4.1. Hydrolysis of vegetable proteins

The use of Alcalase employed in the hydrolysis of vegetable proteins will be presented in the next paragraphs, in some instances comparing its performance with that of other proteases.

In one of them, Alcalase or Flavourzyme were used to hydrolyze proteins from sunflower (*Helianthus annuus* L.) seeds and obtain iron-binding peptides [559]. The most

interesting peptide fraction to produce iron supplements was the one having a molecular weight below 3 kDa [559]. Some other studies have used Alcalase in order to obtain hydrolysates with increased zinc-binding ability. In one of these studies, four peptides were isolated from rapeseed Alcalase hydrolysate, and among them, Asn-Ser-Met showed an especially high zinc-chelating activity (better than the one of reduced glutathione) [556]. Other examples are the use of Alcalase to produce zinc-chelating peptides from mung bean [560] and rapeseed meal [561]. In other instance, wheat germ protein hydrolysates obtained through Alcalase hydrolysis were found to present the capability to bind calcium [558]. This capacity was dependent on various factors like the degree of hydrolysis, amino acid composition and molecular mass distribution of different hydrolysates. The calcium-binding peptides was mainly composed by Glu, Arg, Asp and Gly, and the level of Ca^{2+} bound was related to the hydrophobic amino acid content in the wheat germ protein hydrolysates [558].

In some studies, the results are not focused on one individual metal, but on a general capacity of the hydrolysate to chelate metals. In one of these studies, Alcalase was used to hydrolyze wheat germ protein [562], and the hydrolysates prepared under optimal conditions (200 min) had the highest degree of hydrolysis (15.61 ± 0.09%) and metal chelating ability (69.62 ± 0.96%), being this result better than using other proteases like Flavourzyme or papain [562]. Similarly, wheat germ protein was also hydrolyzed by papain, Flavourzyme and Alcalase [563]. The hydrolysate with the highest metal-binding ability (69.62 ± 0.96%) was obtained when Alcalase was used [563].

It has been reported that, when Alcalase is used to hydrolyze soy protein, the hydrolysate is rich in calcium-chelating activity, but if the reaction conditions are optimized

using different media (water, ethanol-water, methanol-water), the calcium-chelating activities could be improved [564]. In another paper, casein and soybean proteins were hydrolyzed with Alcalase and trypsin [565]. Proteolytic hydrolysis enhanced the bioaccessibility of iron and zinc in proportion to the degree of hydrolysis. Alcalase hydrolysis showed a comparatively higher metal chelating activity with both proteins [565].

3.4.2. Hydrolysis of animal proteins

Some studies where Alcalase was employed in the hydrolysis of animal proteins will be presented. In one of these studies, it was reported that sea cucumber (*Stichopus japonicus*) ovum hydrolysates obtained with Alcalase at a hydrolysis degree of 25.9% possessed a very high iron binding capacity (92.1%) [566]. In another work, Alcalase was utilized to obtain zinc-chelating peptides from sea cucumber with a zinc-chelating ability of a 33.31%, and the zinc mainly bonded to carboxylic and amide groups [567]. Alcalase can also be used to generate good iron-binding peptides from heated colostral whey [568]. And as another instance, whey protein was hydrolyzed by Alcalase, and the hydrolysate exhibited a high calcium rich chelate capability [569]. A different work showed that β-lactoglobulin hydrolysates obtained with Alcalase after 6 h of hydrolysis possessed the highest iron-binding capacity among the hydrolysates produced in the several assayed conditions [570].

There are many studies where not only Alcalase, but other proteases were also used to obtain the hydrolysate with iron-chelating properties. However, in most of them, Alcalase was reported to produce the peptides with the highest iron-chelating ability, as it can be seen in some experiments made with scad (*Decapterus maruadsi*) processing by-products [549, 571], buffalo αS-casein [572] and marine mackerel processing byproducts

[573]. In another instance, among the hydrolysates obtained using several proteases (trypsin, pepsin, Flavourzyme, Alcalase, and papain), the yak casein hydrolysate obtained with Alcalase presented the highest Zn^{2+}-binding capacity [574]. It is remarkable that although compared with native yak casein, the Zn^{2+}-binding activity of yak casein hydrolysate was significantly lower, its solubility was markedly higher under intestinal basic pH ranges, which indicates a better bioavailability [574]. In another paper, four different proteases including Alcalase were used to obtain calcium-binding peptides from tilapia (*Oreochromis niloticus*) protein [575]. Alcalase produced the hydrolysate with the highest calcium-binding capacity (65 mg/g protein) at 27.7 % degree of hydrolysis [575]. Another example is a study where calcium binding peptides were isolated from bovine serum proteins hydrolysates using Flavourzyme, Protamex and Alcalase [576]. From the peptide fraction below 3 kDa of the Alcalase hydrolysate, a peptide (Asp-Asn-Leu-Pro-Asn-Pro-Glu-Asp-Arg-Lys-Asn-Tyr-Glu) with the highest calcium binding capacity was obtained [576].

There are also reports of works looking for the improvement of the chelating ability of the proteins of other elements. In this way, Alcalase was used to hydrolyze chicken sternal cartilage to obtain several peptides with protective effect in a cadmium-induced osteoporosis model [577]. Another example is the study where Alcalase and other proteases were used to obtain an Mg^{2+}-binding hydrolysate from casein [578]. The hydrolysate that showed the highest Mg^{2+}-chelation efficiency (96.1%) was obtained using Alcalase at an enzyme substrate ratio of 30%. After the hydrolysate was fractioned, the smallest fraction exhibited 100% Mg^{2+} solubilization and 39.5% of bioavailability [578]. In a slightly different turn a study proved that Alcalase could be employed to hydrolyze casein in order

to obtain casein phosphopeptides which can be used for enhancing the bioaccessibility of iron and zinc in pure iron solutions or even in high phytate foods [579].

Some studies where the protein source is neither animal nor vegetal are also presented. For instance, in one study, *Spirulina*, a cyanobacteria, was hydrolyzed using Alcalase and Flavourzyme to finally obtain iron-chelating peptides in the peptide faction below 3 kDa [580]. In another paper, Alcalase was used to hydrolyze a fungus (*Grifola frondosa)* protein [581]. The hydrolysate was filtered through 5 and 1 kDa nominal cut-off ultrafiltration membranes, two fractions with chelating activity were obtained, and named GFP-1 and GFP-2 respectively. GFP-2 had the highest Fe (II) chelating activity and both fractions kept this activity even after *in vitro* gastrointestinal digestion [581].

3.4.3. Combined use of Alcalase with other proteases

There are some examples of use of combi-proteases [395] to produce chelating peptides. In one study, ovomucoid was hydrolyzed by different proteases (pepsin, α-chymotrypsin, papain, and Alcalase) alone or in combinations [582]. Among the different treatments, the hydrolysate after hydrolysis of Alcalase plus papain showed the highest iron-chelating and antioxidant activities [582]. In another paper, Alcalase and Neutrase were used in combination for the hydrolysis of pig bone collagen to obtain peptides with a high calcium binding ability [583]. Defatted rice bran protein was treated with Alcalase, Flavourzyme or a combination of both, in order to obtain a hydrolysate rich in iron binding peptides [584]. The iron bioavailability was also studied using an *in vitro* digestion and absorption model (Caco-2 cells). The best results were obtained with a combined hydrolysis catalyzed by Alcalase and Flavourzyme [584].

3.5. Production of peptides with antidiabetic potential activity

Diabetes mellitus is a chronic metabolic disease which represents a worldwide health problem with strong socioeconomic and health impacts [585]. Among the three existing types of diabetes (type 1 and type 2 diabetes mellitus, and gestational diabetes), type 2 diabetes mellitus is the most common type, comprising 90% of the world diabetic population, and the number of people suffering from type 2 diabetes is expected to reach 439 million by 2030 [586, 587]. Diabetes can cause many complications, which include diabetic ketoacidosis, nonketotic hyperosmolar coma and hyperglycemia. Hyperglycemia, which is caused by the disability or lack of insulin production by pancreatic β-cells, reduced sensitivity of the tissue to insulin, or both [588], is an early abnormality that signals the presence of type 2 diabetes mellitus and it is an important risk factor for the development of diabetes mellitus-derived complications such as microangiopathy, retinal damage, neuropathy, chronic renal failure and cardiovascular disease. For these reasons, a good management of hyperglycemia is critical to prevent or delay the manifestation and complications of type 2 diabetes mellitus [589]. One therapeutic approach to control type 2 diabetes is the use of synthetic medicines like acarbose and voglibose, which suppress the absorption of glucose by the inhibition of carbohydrate-hydrolyzing enzymes [590] such as α-glucosidase [591], which catalyzes the cleavage of glucose from disaccharides, or α-amylase, which acts on long-chain carbohydrates [592, 593]. However, the cost of these drugs is high, and they are associated with gastrointestinal side effects like diarrhea (14% of patients) and flatulence (78% of patients) [594]. The inhibition of dipeptidyl peptidase-IV activity is another mechanism for type 2 diabetes mellitus control. Dipeptidyl peptidase-IV is responsible for the rapid degradation of both glucagon-like peptide 1 and glucose-

dependent insulinotropic polypeptide [595], two insulinotropic incretin hormones that enhance glucose-dependent insulin secretion from pancreatic cells and regulate postprandial blood glucose levels [596-599]. Many synthetic dipeptidyl peptidase-IV inhibitors are used, including vildagliptin, linagliptin, saxagliptin, and sitagliptin [600]; however, these drugs, which are collectively known as gliptins, provide inadequate glycemic control and are associated with frequent side-effects such as hypoglycemia, weight gain, cardiovascular problems, headaches and urinary and upper respiratory tract infections [601].

Due to the previously mentioned synthetic drugs disadvantages, there is currently a growing global demand for the search of natural therapeutic agents with reduced or no side-effects to control, prevent and treat this disease. In this sense, recent approaches for the management of type 2 diabetes mellitus have focused on nutritional interventions using food-derived components like phenols, flavonoids, protein and peptides, which exhibit antidiabetic activity [602]. In fact, it has been established that some proteins, protein hydrolysates, peptides and amino acids can beneficially regulate blood glucose levels [601]. In this context, the antidiabetic activity of food protein hydrolysates and their peptides from milk proteins and hemp, pea, rice, soy and macroalgae proteins, has been demonstrated [598]. These peptides can be successfully obtained by enzymatic hydrolysis of different source proteins, using proteases. Among them, Alcalase has been used to produce these hydrolysates. Next, we will comment some examples.

3.5.1. Use of stand-alone Alcalase

Several proteins from vegetable sources have been evaluated as feedstocks to produce peptides with antidiabetic activity by Alcalase hydrolysis, such is the case of the

study carried out by De Souza *et al*., who studied the impact of germination of cowpea (*Vigna unguiculata*) combined with Alcalase hydrolysis of the protein extract from this source, on the generation of bioactive peptides with dipeptidyl peptidase IV inhibition activity [603]. These authors found that the hydrolysates produced from non-germinated seeds after 1h of Alcalase hydrolysis exerted the highest dipeptidyl peptidase IV inhibition after *in vitro* simulated gastrointestinal digestion [603]. Later, De Souza *et al*. evaluated the effect of germination time and Alcalase hydrolysis of common bean proteins in the generation of bioactive peptides with potential to reduce parameters related to the risk of developing type 2 diabetes mellitus *in vitro* [604]. Computational modeling showed that the peptide RGPLVNPDPKPFL obtained after 48h seed germination and 1h Alcalase hydrolysis was able to strongly inhibit dipeptidyl peptidase-IV by interacting with the S1, S2, and S3 pockets of its active site [604]. Later, Connolly *et al.* reported the *in vitro* dipeptidyl peptidase-IV inhibitory activity of a hydrolysate obtained by hydrolysis of brewers spent grain protein-enriched isolate catalyzed by Alcalase [598], which after 240-min digestion generated a hydrolysate presenting a dipeptidyl peptidase-IV inhibitory concentration (IC50) value of 3.57 ± 0.19 mg mL^{-1} (half of the values of the initial protein) [598]. It has also been reported that Alcalase-generated potato protein hydrolysate is a potential bioactive peptide against diabetes mellitus in animal models [605]. Asokan *et al*. investigated the efficiency of the peptide DIKTNKPVIF purified from the previous hydrolysate against diabetes mellitus [606]. This peptide effectively regulated blood glucose level and also controlled plasma total glycerol, total cholesterol, insulin, and hemoglobin A1c levels in animals with diabetes mellitus. Furthermore, treatment with this peptide also ameliorated diabetes mellitus-associated damages in the pancreatic islets and in the liver, heart, and kidney tissues [606].

Another source of protein that has been studied to produce antidiabetic peptides is white egg protein. Yu *et al.* identified potential antidiabetic peptides obtained from egg white protein hydrolyzed by Alcalase [594]. Among the eight peptides evaluated, the peptide RVPSLM produced α-glucosidase inhibition with an IC50 value of 23.07 μmol L^{-1}. However, it did not exhibit a detectable inhibitory efficiency on the α-amylase activity [594].

3.5.2. Comparison of Alcalase with other proteases

Alcalase has also been compared in its ability to produce antidiabetic peptides with other proteases, mainly using proteins from plant sources. Proteins from de-hulled hard-to-cook beans (Pinto Durango and Negro 8025 beans) have been hydrolyzed with either Alcalase or bromelain [607]. After 120 min of reaction, the hydrolysates were separated into five peptide fractions by ultrafiltration. It was found that the < 1. kDa pinto Durango-bromelain fraction was the best inhibitor of α-amylase (49.9. ± 1.4%); however, the < 1. kDa pinto Durango-Alcalase fraction inhibited both, α-glucosidase (76.4. ± 0.5%), and dipeptidyl peptidase-IV (55.3 ± 1.6%). In general, hydrolysates from de-hulled hard-to-cook beans inhibited enzymes related to diabetes management, being the smallest peptides (< 1 kDa) the most powerful [607]. Peptides released from oat, buckwheat, and highland barley proteins by Alcalase hydrolysis or gastrointestinal and tryptic digestion, were studied in terms of their *in vitro* inhibitory effects on dipeptidyl peptidase IV [608]. All obtained hydrolysates exhibited dipeptidyl peptidase IV inhibitory activities, with IC50 values ranging from 0.13 mg/mL (oat glutelin after Alcalase digestion) to 8.15 mg/mL (highland barley albumin after tryptic digestion). In this study, Alcalase was more efficient than trypsin in the production of peptides that were good inhibitors of dipeptidyl peptidase IV

[608]. In another paper, Mojica and De Mejia optimized the antidiabetic peptides production from black bean (*Phaseolus vulgaris* L.) protein isolate, using eight commercial proteases [609]. It was found that the highest antidiabetic effect of the hydrolysate was obtained using Alcalase, with a hydrolysis time of 2 h and an enzyme/substrate ratio of 1/20. The detected inhibition values for dipeptidyl peptidase IV, α-amylase and α-glucosidase were 96.7%, 53.4% and 66.1% , respectively [609].

In another interesting study, Alcalase, Neutrase, Flavourzyme and Protamax were used to obtain rice bran protein hydrolysates [610]. Alcalase and Protamax produced hydrolysates that generally had the highest antidiabetic activities. The α-amylase and α-glucosidase inhibitory activities these hydrolysates were similar to those of the commercial antidiabetic drug acarbose [610]. Another work reports the hydrolysis of pea protein concentrate with chymotrypsin, pepsin, Alcalase or trypsin [611]. Alcalase was the enzyme that produced hydrolysate with the highest production of di- and tripeptides and the higher inhibition activity versus α-amylase than versus α-glucosidase [611].

3.5.3 Combined use of Alcalase with other proteases

Regarding the production of peptides with antidiabetic activity using Alcalase, there are several studies where this enzyme is used in combination with other proteases, either through co-hydrolysis or in sequential systems.

The use of combi-proteases [395] has many different examples. In this context, some vegetable proteins have been evaluated. For instance, Alcalase and bromelain were used to produce peptides from pinto Durango and black 8025 beans proteins [612]. The hydrolysates effect on insulin secretion from pancreatic β-cells and glucose uptake from

insulin-resistant adipocytes was studied [612]. Hydrolysates and peptide fractions increased glucose-stimulated insulin secretion from rat insulinoma INS-1E cells, reduced the expression of proteins like dipeptidyl peptidase IV and receptor for advanced glycation end products, and significantly reduced oxygen species (up to 70%). Besides, peptides inhibited lipid accumulation in mature adipocytes 3T3-L1 and increased glucose uptake (67%) enhancing insulin signaling and reducing the phosphatase and tensin homologue activation [612]. *In vitro* hypoglycemic activity of four kinds of dark tea (*Camellia sinensis* L.) proteins and their hydrolysates were investigated by Su *et al.* Alcalase and trypsin were used to hydrolyze four water-extracted dark tea proteins [613]. Their results showed that most of the dark tea proteins and hydrolysates significantly inhibited α-glucosidase and dipeptidyl peptidase, with a half maximal inhibitory concentration values in the range of 0.0103 -1.3114 mg/mL and 0.1000 -1.3364 mg/mL, respectively [613].

On the other hand, Nuñez-Aragón *et al.* evaluated the antihyperglycemic activity and inhibition of α-glucosidase, and intestinal glucose absorption, and acute toxicity of total hydrolysates and <1 kDa fractions from *Phaseolus lunatus L., Phaseolus vulgaris L., and Mucuna pruriens* (L.) DC. obtained by hydrolysis with Alcalase-Flavourzyme or pepsin-pancreatin enzymatic systems [614]. *In vitro*, total hydrolysates and fractions, particularly from *M. pruriens*, inhibited carbohydrate intestinal absorption and α-glucosidase activity, and *in vivo*, three out of six total hydrolysates and four of six <1 kDa fractions suppressed starch-induced postprandial hyperglycemia. In addition, none of the hydrolysates and fractions tested showed any signs of toxicity (median lethal dose >5000 mg kg−1) [614].

Napin extracted from rapeseed was hydrolyzed by several commercial enzymes to produce hydrolysates with dipeptidyl peptidase-IV inhibitory activity [615]. Among the

evaluated enzymes, a two-enzyme-combination approach with Alcalase and trypsin was selected due to the favorable dipeptidyl peptidase-IV inhibitory activity (IC 50 = 0.68 mg/mL) of the napin hydrolysate [615].

Also, examples of sequential hydrolysis by several proteases may be found. Castañeda-Pérez *et al.* investigated the antidiabetic potential of cowpea (*Vigna unguiculata* L.) protein hydrolysates and ultra-filtered peptide fractions produced by sequential hydrolysis with Alcalase-Flavourzyme [616]. The peptide fraction greater than 10 kDa showed the highest α-amylase inhibitory activity with an IC 50 value of 31.58 mg protein/ml, and the highest inhibitory activity of α-glucosidase with an IC 50 value of 40.17 mg protein/mL. However, protein hydrolysates showed the highest inhibitory activity of dipeptidyl peptidase-IV with an IC 50 value of 139.04 mg protein/mL. Moreover, protein hydrolysates and ultra-filtered peptide fractions with higher inhibitory activity of α-amylase, α-glucosidase, and dipeptidyl peptidase-IV did not show *in vitro* cytotoxicity in Vero cells [616].

In addition to vegetable proteins, some proteins from fish have also been studied using combi-proteases. Harnedy *et al*. demonstrated that the blue whiting protein hydrolysate generated using Alcalase and Flavourzyme had significant metabolic effects relevant to glucose control *in vivo*, by inhibition of dipeptidyl peptidase-IV and mediation of insulin and glucagon-like peptide-1 release from BRIN-BD11 and GLUTag cells, respectively [617]. In another attempt, these authors reported the production of salmon co-product hydrolysates with promising *in vitro* antidiabetic activity [618]. They found that gelatin and trimmings hydrolysates generated by hydrolysis with Alcalase and Flavourzyme exhibited high insulin and glucagon-like peptide-1 secretory activity stimulation from

pancreatic BRIN-BD11 and enteroendocrine GLUTag cells, respectively, and potent dipeptidyl peptidase-IV inhibitory activity [618]. Also, Alcalase and Flavourzyme were used to obtain boarfish (*Capros aper*) protein hydrolysate, in order to investigate their antidiabetic actions in cultured cells and mice [619]. They found that boarfish protein hydrolysate caused a dose-dependent increase in insulin secretion from BRIN-BD11 cells. Moreover, it mediated an increase in plasma insulin levels and a consequent reduction in blood glucose concentration after oral glucose tolerant test in mice. This way, boarfish protein hydrolysate showed potent antidiabetic actions in cells and improved glucose tolerance in mice [619].

In a more recent investigation, twenty-two novel dipeptidyl peptidase-IV inhibitor peptides and fifteen novel insulinotropic peptides were identified in a boarfish protein hydrolysate generated at semi-pilot scale using Alcalase and Flavourzyme [601]. Among them, the most potent dipeptidyl peptidase-IV inhibitory peptide had a dipeptidyl peptidase-IV IC50 value of 21.72 ± 1.08 µM in a conventional *in vitro* assay and 44.26 ± 0.65 µM in an *in situ* cell-based (CaCo-2) dipeptidyl peptidase-IV inhibition assay. This peptide stimulated insulin secretory activity from pancreatic BRIN-BD11 cells grown in culture [601].

According to the reviewed literature, antidiabetic hydrolysates and peptides obtained by protein hydrolysis with Alcalase of protein from egg, fish by-products, legumes, etc. have emerged as a new alternative to treat hyperglycemia and have the potential to be developed into a dietary or nutraceutical supplement for the management of type 2 diabetes mellitus and its complications.

3.6. Production of peptides with anti-inflammatory activity

Inflammation is an essential, complex and highly regulated physiological adaptive response of the body to cell damage and tissue vascularization, that enables patient survival during infection or injury and maintains tissue homeostasis under different noxious conditions [620]. This response is part of the host defense mechanism against inflammatory inducers like chemical and noxious mechanical agents, microbial infections, and conditions such as infection and tissue injury [621, 622]. During the early phases of inflammatory response, tissue-resident cells (inflammatory sensors) detect the inflammatory stimulus and release soluble inflammatory mediators, including cytokines, vasoactive amines, free radicals, chemokines and eicosanoids [620, 621]. It is important to mention that, although a typical inflammatory response consists of four components (inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators), each component comes in multiple forms and their combinations function in distinct inflammatory pathways which depend on the nature of the inflammatory trigger. Thus, for example, bacterial pathogens are detected by receptors of the innate immune system, such as Toll-like receptors, which are expressed on tissue-resident macrophages and induce the production of inflammatory cytokines (e.g., tumor necrosis factor α, interleukins-1, interleukins -6, interleukin-1β) and chemokines (e.g., chemokine C-C ligand 2 and C-X-C chemokine 8), nitric oxide as well as prostaglandin-E2 [620, 623]. Excessive and uncontrolled inflammation is harmful to all tissues, since it may cause many acute and chronic human diseases including obesity, atherosclerosis, type 2 diabetes, cancer and neurodegenerative diseases [620, 624]. For example, dysregulated activation of some inflammatory enzymes such as cyclooxygenase-2, generating prostaglandin-E2 from arachidonic acid, and inducible nitric oxide synthase, which catalyzes the reaction that oxidizes L-arginine to nitric oxide and citruline, play

important roles in the progression of oncogenesis [625]. Therefore, suppressing the overproduction of inflammatory mediators and the control of the abnormal up-regulations of the inflammatory enzymes (that promote excessive inflammation) is important for the treatment and prevention of inflammation and to reduce the risk of inflammation-derived diseases [626]. For this reason, some synthetic drugs have been employed to regulate the response of the immune system. Unfortunately, the prolonged use of these chemical anti-inflammatory drugs may result in cardiovascular, renal or gastrointestinal damage. Therefore, there is a growing interest on the use of non-toxic natural compounds to reach this goal [627, 628]. In this regard, the anti-inflammatory activity of many plant and animal derived food proteins and protein hydrolysates has been demonstrated [629, 630].

In order to improve the bioactivity of food proteins, enzymatic hydrolysis has been applied to many food proteins to release bioactive peptides with desired functional properties [626]. Alcalase is one of the protesase used to produce protein hydrolysates with anti-inflammatory activity [631].

3.6.1. Use of stand-alone Alcalase

Focusing on the use of Alcalase, Oseguera-Toledo *et al*. demonstrated that Alcalase hydrolysates of pinto Durango and Negro beans inhibit cyclooxygenase-2 expression, prostaglandin E2 production, inducible nitric oxide synthase expression and nitric oxide production [632]. For this reason, these hydrolysates from common beans can be used to treat inflammatory associated diseases [632]. In another paper, an anti-inflammatory peptide was identified in lupine protein hydrolysates obtained by Alcalase hydrolysis [633]. This peptide, with a sequence of Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg, was synthesized and its anti-inflammatory activity was tested. It was found that the peptide may help prevent

chronic inflammation due to a significant reduction of the expression of tumor necrosis factors, interleukin-1β, and C-C motif chemokine ligand 2, and the induction of the anti-inflammatory cytokine interleukin-10 expression, together with a decrease of nitric oxide production [633]. Lee *et al*. obtained velvet antler Alcalase hydrolysate and assessed their anti-inflammatory effects in zebrafish as well as *in vitro*, using different cell lines [634]. They found that the Alcalase hydrolysate inhibited the production of nitric oxide by lipopolysaccharide-induced cells in a dose-dependent manner and also reduced the expression of inflammatory mediators such as nitric oxide synthase and cyclooxygenase-2. In addition, the analysis of anti-inflammatory effects of velvet antler Alcalase hydrolysate using lipopolysaccharide-stimulated zebrafish showed that this hydrolysate significantly inhibited the extent of lipopolysaccharide-stimulated cell death and generation of nitric oxide and reactive oxygen species in zebrafish [634]. These authors emphasize that velvet antler Alcalase hydrolysate could be used as a natural and strong anti-inflammatory, and that enzymatic hydrolysis of velvet antler may be an effective process to produce antler derivatives that can be used in the preparation of health foods and nutraceutical products [634]. In another study, it was demonstrated that the low-molecular weight fractions prepared from ovomucin Alcalase hydrolysate may have potential applications for the maintenance of dermal health and treatment of skin diseases [635], due to the their anti-inflammatory activity regulated through the inhibition of tumor necrosis factor-mediated nuclear factor κ-light-chain-enhancer of activated B cells activity [635] .

Alcalase has also been used in the hydrolysis of whey protein to produce, isolate and characterize anti-inflammatory peptides. In one study, eight peptides, including 2 new peptides (DYKKY and DQWL) were identified [636]. DQWL showed the strongest

inhibitory ability on cyclooxygenase-2, interleukin-1β, and tumor necrosis factor-α mRNA expression and production of interleukin-1β and tumor necrosis factor-α proteins [636].

3.6.2. Comparison of Alcalase with other proteases

Ruditapes philippinarum protein extract was hydrolyzed using eight proteases, being Alcalase among them [637]. It was found that the Alcalase-produced hydrolysate exhibited the highest nitric oxide production inhibitory activity, and one of the produced peptides displayed potent anti-inflammatory activity through inhibition of the lipopolysaccharides-induced nitric oxide production in RAW264.7 cells [637]. In another research, tuna cooking juice was hydrolyzed by three commercial enzymes (Flavourzyme, Orientase and Alcalase) [629]. Among the evaluated enzymes, Alcalase hydrolysate exhibited the most potent anti-inflammatory capability, and its peptide fraction with molecular weight ranging from 204 to 1672.9 Da possessed the greatest activity [629]. O'Sullivan *et al*. reported the production of hydrolysates from bovine lung tissue using pepsin, papain or Alcalase, and they assessed the anti-inflammatory activity of these hydrolysates in RAW264.7 macrophages and Jurkat T cells [638]. They found that the cell treatment with the Alcalase hydrolysate significantly decreased the production of the pro-inflammatory cytokines interleukin-6 and interleukin-1β in a dose dependent manner in RAW264.7 cells, and the nitric oxide production; therefore, the authors concluded that the Alcalase hydrolysis of bovine lung may have potential as an anti-inflammatory agent [638]. Finally, Meram and Wu, evaluated the anti-inflammatory effects of egg yolk livetins (α, β, and γ-livetin) fraction and its hydrolysate, prepared by hydrolysis with Alcalase or pepsin, on lipopolysaccharide-induced RAW 264.7 macrophages as an *in vitro* model [626]. They found that the treatment with livetins and peptides from its hydrolysate significantly

reduced the inflammatory response by the inhibition of production of nitric oxide, pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6 and interleukin-1β, and the expression of inducible nitric oxide synthase. In addition, Alcalase hydrolysate showed more effects in inhibiting prostaglandin-E2 production as well as expression of cyclooxygenase-2 [626].

3.6.3. Combined use of Alcalase with other proteases

Regarding the use of Alcalase in combination with other proteases, there is just one example in the analyzed time frame. Alcalase and Izyme AL were used to hydrolyze lupine protein isolate to obtain protein hydrolysates with potential anti-inflammatory capacities through their *in vitro* inhibition capabilities of phospholipase A2, cyclooxygenase 2, thrombin, and transglutaminase, which are all enzymes that are involved in the inflammatory process [639]. The protein hydrolysates prepared after 15 min of hydrolysis with Alcalase and lupine protein hydrolysates obtained after 60 min of hydrolysis with Izyme followed by 15 min of hydrolysis with Alcalase, exhibited the best inhibitory activities [639].

Evidently, Alcalase hydrolysis of different proteins is an excellent tool for producing anti-inflammatory peptides which have potential to be used in the preparation of health foods and nutraceutical and pharmaceutical products that promote and protect global health, against acute or chronic diseases derived from the inflammatory response.

3.7. Production of peptides with antimicrobial activity

One of the main concerns of the food industry is ensuring the safety and shelf life of foods which are threatened by the incidence of pathogenic and spoilage bacteria that can

contaminate food [640-642]. In order to avoid the growth of such bacteria, natural and synthetic antibacterial agents have been used; however, due to the possible negative impact of such chemicals on human health and the environment, the use of synthetic agents is restricted [643, 644]. Such problems have led to the search and identification of safe and potential natural biomolecules that avoid toxic effects. In this sense, bioactive peptides have gained attention as an alternative to conventional antibiotics [645], being of great relevance in the pharmaceutical and food industries due to their high specificity and low toxicity [646]. For this reason, there is a growing interest in the utilization of these bioactive peptides as food grade bio-preservatives or as health-promoting food supplements in the food industry [647]. Food proteins are an important source of such bioactive peptides, but they can be obtained from different protein sources, including milk, eggs, fish, wheat, bacteria, insects, plants, vertebrates, etc. [648, 649]. Among the strategies used to improve the antimicrobial activity of proteins, enzymatic hydrolysis using microbial, plant or digestive proteolytic enzyme has been widely reported, and Alcalase has been used extensively to prepare soluble protein hydrolysates and peptides with antibacterial activities from different protein sources [643].

3.7.1. Use of stand-alone Alcalase

Vegetable proteins have been frequently used for this aforementioned goal. Tan *et al*. used Alcalase to obtain peptides from palm kernel expeller with antimicrobial activity against spore-forming and non-spore-forming bacteria [650]. These authors found that, according to the minimum inhibitory concentration, a degree of hydrolysis of 70% of palm kernel expeller peptide effectively inhibited the growth of spore-forming and non-spore-forming Gram-positive bacteria (*B. cereus, B. coagulans*, *B. megaterium, B. pumilus*, *B.*

stearothermophillus, B. subtilis, B. thuringiensis, Cl. perfringens, Lisinibacillus sphaericus and *L. monocytogenes*). Because of that, these peptides obtained from palm kernel expeller could be used as additives in food preservation and developed as antibacterial agents in the pharmaceutical industry [650]. Later, Alcalase was used to obtain an antimicrobial hydrolysate from palm kernel cake-derived protein [651]. The hydrolysate was purified by gel filtration chromatography, and one purified fraction bearing 14.63 ± 0.70% (w/w) protein, a molecular mass of 2.4 kDa, low hemolytic activity (~50% hemolysis of human erythrocytes at concentration of 1000 μg/ml) and a major component of lauric acid derivative was found. The purified compound was suitable for its use as an antimicrobial agent with potent antibacterial activity, particularly against *Bacillus* species [651]. Song *et al*. reported the fractionation and identification of antibacterial peptides from cottonseed protein hydrolysates obtained using Alcalase [649]. In this study, nine novel peptides encoded in cottonseed proteins were identified and three peptides (KDFPGRR, LGLRSGIILCNV, and DENFRKF) with antibacterial activities of 77.7%, 69.3%, and 45.0% at 1.0 mg/mL, respectively, were chemically synthesized [649]. This result suggest that hydrolysate of cottonseed protein could be used as a potential source of antibacterial peptides that could be applied to food systems and the feed industry.

Other proteins have also been employed for this goal. For example, peptides with antibacterial activity against Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus luteus* and *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter sp*.) bacteria, were obtained from barbel (*Barbus callensis*) muscle protein hydrolysates obtained by treatment with Alcalase (degree of hydrolysis=6.6%)

[652]. Peptides were fractionated by size exclusion chromatography and purified by reverse-phase high performance liquid chromatography. The most active peptide fraction contained three peptides (Ala-Ala-Ala-Leu, Ala-Ala-Gly-Gly-Val and Ala-Ala-Val-Lys-Met). According to the authors, the antibacterial peptides derived from barbel protein hydrolysates could be useful as preservatives for the storage and distribution of meat-based products [652]. Alcalase has also been used in the hydrolysis of goat whey to release peptides possessing potent antimicrobial activity [643]. The produced peptides exhibited bactericidal activity against *S. typhimurium*, *E. coli* and *B. cereus* and bacteriostatic activity against *S. aureus*, significantly higher than the antibacterial activity of the non-hydrolyzed goat whey, which shows that the hydrolysis of goat whey by Alcalase is an easy tool to enhance its antibacterial activity [643].

3.7.2. Comparison of Alcalase with other proteases

In the field of the production of peptides with antimicrobial activity by enzymatic hydrolysis, there are studies where the comparison of various proteases is reported. For instance, Kumar *et al*. used Alcalase and other enzymes to produce camel milk casein hydrolysates [647]. These authors observed that Alcalase and α-chymotrypsin produced the peptides with the highest antimicrobial activity [647]. In other papers, the utilization of blood from the meat industry was the raw material to produce antimicrobial peptides. This permitted to prevent the loss of valuable by-products and reducing environmental pollution. For example, Verma *et al*. investigated the production of protein hydrolysates from porcine blood by enzymatic hydrolysis using trypsin, Alcalase or papain [648]. The results showed that the hydrolysate antimicrobial efficacy was higher for whole porcine blood hydrolysate than for their respective fractions, and that among the tested enzymes trypsin and Alcalase

could produce peptides with comparatively higher antimicrobial activity than papain for all tested microbes [648]. That way, these porcine blood hydrolysates can be a potential source of natural preservatives for shelf-life extension of meat and meat products and can further be exploited by nutraceutical and pharmaceutical industries for their antioxidant and antimicrobial properties [648].

In another study, Protamex or Alcalase were used to produce protein hydrolysates of byproducts of industrial processing of stripped weakfish (*Cynoscion guatucupa*) [653]. It was found that the highest antimicrobial activity against *Escherichia coli* O157:H7 (5.50 ± 0.17 mm) was exhibited for Alcalase hydrolysates with a degree of hydrolysis of 5% [653].

3.7.3. Combined use of Alcalase with other proteases

In the production of antimicrobial peptides, Alcalase has also been utilized together with other proteases, looking for a synergistic effect that allows obtaining peptides with superior antimicrobial activity. In the combi-protease concept [395], the use of a mixture of Flavourzyme and Alcalase to hydrolyze sunflower protein has been reported [654]. The results revealed that the obtained hydrolysates inhibited five microbial strains (*E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Salmonella typhimurium*) [654].

Other authors use a protein sequential hydrolysis using different proteases. For example Coelho *et al*. reported the use of Flavourzyme, Alcalase and sequential Alcalase-Flavourzyme to produce hydrolysates from chia protein, protein-rich fraction and chia protein concentrates, to generate chia protein-based antibacterial hydrolysates/peptides [655]. For Alcalase, the hydrolysates obtained showed antibacterial activity in the majority

of the samples, but the antibacterial effects of the hydrolysates produced by Flavourzyme and mainly by sequential Alcalase-Flavourzyme system were better than those [655].

In conclusion, hydrolysates and peptides obtained from different protein sources by enzymatic hydrolysis using Alcalase, have a great potential to be used as a natural antimicrobial agent in food systems to avoid the food deterioration and improve their safety, with no negative impacts for human health or the environment.

3.8. Production of peptides with functional, sensory and nutritional properties in food products

It is very common to employ proteases in industry to convert by-products and different kinds of residues from food industry into valuable products. Since Alcalase is a very efficient tool to hydrolyze proteins and produce small peptides, it is very straightforward to find studies where it is used to obtain hydrolysates with functional, sensory and nutritional properties.

3.8.1 Hydrolysis of vegetable proteins

3.8.1.1 Use of stand-alone Alcalase

In the literature there are many examples showing the use of Alcalase to hydrolyze proteins from a vegetal origin for this objective. For example, Alcalase was used to hydrolyze soy β-conglycinin-rich (7S-rich) fractions [656]. Functional properties such as solubility, droplet size distribution of emulsion and heat-induced gelling properties of the protein and its hydrolysate were studied [656]. Later, different Alcalase concentrations and pH values were employed to hydrolyze soy protein isolate [657]. Solubility, functional

properties, Angiotensin I-converting enzyme inhibitory and DPPH scavenging activities of the resulting hydrolysates were investigated [657].

Concerning rice residues, the functional properties of defatted rice bran protein hydrolyzed by Alcalase were studied, showing that the treatment improved the quality of the protein [658]. It was also studied how the aroma characteristics of rice bran protein concentrate hydrolysates obtained by Alcalase hydrolysis were improved by spray drying and sugar addition. [659]. In another research, it was observed that the hydrolysate obtained after rice protein hydrolysis by Alcalase Peptides had the maximum emulsibility (48.80 mL/g) and emulsion stability (43.01 min) at pH 3.0 and pH 5.0. [660].

There are also studies where potato proteins were subjected to Alcalase hydrolysis. In one of them, it was shown how profitable preparations of well-balanced amino acid composition and positive functional properties could be obtained by a 2 h hydrolysis of fodder potato protein concentrates by Alcalase [661]. The resulting product was proposed as suitable for preparations characterized by high nutritive value and functional properties [661]. Years later, potato protein hydrolysates prepared by Alcalase hydrolysis were determined to be suitable as a functional food component in the food industry [662].

Alcalase has also been employed to hydrolyze sunflower proteins. In one of the studies, sunflower 11S globulin was hydrolyzed by this protease showing that the hydrolysate functional (solubility, emulsifying properties, foaming properties, oil binding capacity, and surface hydrophobicity) properties of the hydrolysates could be altered by varying the hydrolysis time. [663]. In another paper, sunflower protein isolates, extracted from defatted sunflower flour, were hydrolyzed by Alcalase at different degrees of

hydrolysis, showing changes on the structural and interfacial properties of the hydrolysates [664].

Wheat gluten, due to its difficult solubilization and bitter taste, has a limited application [665]. One of the strategies to solve this problem is its deamidation followed by enzymatic hydrolysis. The best functional properties were found when Alcalase was employed after the treatment with citric acid, showing a great potential as a modified wheat gluten product [665]. In a different study, the synergistic effect of wheat gluten proteins hydrolysis catalyzed by Alcalase together with a heat treatment was investigated, improving the quality of the protein sensorial properties [666].

There are many other examples where Alcalase has been used to hydrolyze vegetal proteins. For instance, the hydrolysate obtained hydrolyzing mentarang (*Pholas orientalis*) protein [667], possessed a high amount of essential amino acids and their foaming properties decreased significantly with increasing foaming time, making mentarang hydrolysate suitable for application as a natural additive in food [667]. In a parallel study, the effects of the degree of hydrolysis were studied on the pine nut protein isolates and its enzymatic hydrolysates after digestion with Alcalase [668]. The control of the degree of hydrolysis could be an effective strategy to modify specific functional and bioactive properties of the protein hydrolysate [668]. The use of Alcalase to hydrolyze sesame cake protein at 50 ºC and pH 8.5 produced hydrolysates where water-holding capacity, oil-holding capacity, foam capacity and stability, emulsifying activity and stability were improved with respect to the non-hydrolyzed protein [669]. These results make sesame cake protein hydrolysate a useful additive in several foods [669]. In another paper, Alcalase was used to hydrolyze chickpea protein hydrolysate, improving the physicochemical,

interfacial tension and surface characteristics of the protein isolate [670]. In another paper, palm kernel expeller protein was subjected to limited hydrolysis using Alcalase and this improved its nutritional value, physicochemical and functional properties [671]. In another research, a limited proteolytic hydrolysis was performed on coconut (*Cocos nucifera L.*) protein using Alcalase [672]. The resulting hydrolysates improved the stability and rheological properties of oil-in-water emulsions, [672]. In another work, protein from *Corylus mandshurica* kernel meal was extracted using Alcalase as a protein hydrolysate solution [673]. The hydrolysate presented suitable values of amino acid nutritional composition [673]. Another study shows that fava bean protein isolate hydrolyzed by Alcalase presented show positive effects as emulsion stabilizing agent, depending on the hydrolysis [674]. In a last paper, horse gram flour proteins were hydrolyzed by Alcalase, improving its functional properties [675].

3.8.1.2. Comparison of Alcalase with other proteases

Studies where the hydrolysis of Alcalase is compared to the use of proteases to produce peptides with functional, sensory and nutritional properties are very common. For example, pumpkin (*Cucurbita moschata*) oil processing by-products were hydrolyzed by Alcalase, Protamex, Flavourzyme or Neutrase [676]. The physicochemical characteristics of the obtained hydrolysates were studied, but each enzyme was the most suitable for a determined characteristic, improving its role as protein fortification and a potential food ingredient [676]. In another study, using the same enzymes and proteins, Alcalase was the protease giving the pumpkin protein hydrolysates with the best improved nutritional quality [677]. Later, a different species of pumpkin (*Cucurbita pepo*) seed protein isolate was hydrolyzed by pepsin or Alcalase [678]. The solubility of both hydrolysates was higher

than the solubility of the initial protein, mainly at pH near the isoelectric point. Both hydrolysates, successfully stabilized oil emulsions at all the pH and ionic strengths analyze, while the original protein failed at pH 5.0 [678]. In one instance various proteases (Protamex, Alcalase or Flavourzyme) were tested on the production of wheat gluten hydrolysates [679]. Alcalase hydrolysate presented taste-enhancing properties in a concentration-dependent manner [679]. In another research, Flavorzyme, Pepsase or Alcalase were employed to hydrolyze wheat gluten [680]. The 26 h Alcalase hydrolysate presented the best effect for promoting yoghurt fermentation [680]. In another work, four enzymes (papain, bromelain, Alcalase or Neutrase,) were able to hydrolyze proteins from rice residue [681]. The induction time was longer when using Alcalase, and its hydrolysate had the best emulsifying activity as well [681]. In another study, defatted peanut flour protein was hydrolyzed by papain, Protamex and Alcalase [682]. The protease pretreatment was a highly effective way to extract peanut protein concentrate with good functional properties from defatted peanut flour. An increase of nitrogen solubility index was reported after hydrolysis. The yield was also significantly increased together with some other sensorial features [682]. The Alcalase or pepsin treatment hydrolysis of black bean (*Phaseolus vulgaris L.*) protein by 120 min were prepared [683]. Pepsin permitted to reach higher degrees of hydrolysis. However, the Alcalase-treated bean protein hydrolysates presented higher surface hydrophobicity, higher emulsion stability during 30-days than those obtained from pepsin digestion. The Alcalase protein hydrolysates were adequate protein additives in the diet as bioactive and nutritional foods [683].

3.8.2. Hydrolysis of fish proteins

3.8.2.1. Use of stand-alone Alcalase

Regarding the hydrolysis of proteins from animal sources to produce this kind of peptides, fish by-products hydrolysis is the most employed source, with an intense research done in the last years. Protein hydrolysates with different degrees of hydrolysis were obtained by Alcalase hydrolysis of blue whiting (*Micromesistius poutassou*) proteins [684]. Solubility, emulsion capacity, chemical composition and oil-binding capacity were altered with different degrees of hydrolysis, while water-holding capacity, color and emulsion stability did not significantly change. Protein solubility increased from 10% to 70% when the degree of hydrolysis increased [684]. In another research, defatted roe protein concentrates of *Catla catla* were hydrolyzed using 1% Alcalase at pH 8.5-9.0 and 50-55 °C [685]. The solubility of the hydrolysates was 70.5-95% over pH values from 2 to 12). Oil absorption capacity, emulsifying capacity and foaming capacity were found to be protein content dependent. This could be linked to simple peptides by SDS-PAGE [685]. Cobia (*Rachycentron canadum*) was also hydrolyzed by Alcalase at different degrees of hydrolysis. The highest hydrolysis degree (96%) presented showed desired essential amino acid profile for human requirement, except for methionine and isoleucine [686]. The color, emulsifying capacity and foaming properties were adequate for utilization. However, peptide solubility, oil-holding capacity, water-holding capacity remaining almost unaltered. The authors suggested that this protein hydrolysate is a potential foaming agent and additive for food industry [686]. Later, protein hydrolysates from skipjack (*Katsuwonous pelamis*) roe using Alcalase were obtained with different degrees of hydrolysis [687]. The hydrolysis increased the protein solubility [687]. Some years later, a protein hydrolysate from the same source was obtained through Alcalase hydrolysis [688]. The high amount of essential amino acids found in this hydrolysate made it a good candidate to be used as diet nutrients, food additives and even pharmaceutical agents [688].

Rainbow trout (*Onchorhynchus mykiss*) seems to be a recurring substrate for hydrolysis which is frequently utilized for this goal using Alcalase. For example, functional properties of hydrolysates obtained by the hydrolysis of rainbow trout viscera using Alcalase were compared to those obtained from poultry by-products protein [689]. Foaming properties, emulsifying stability, emulsifying activity, water holding capacity and color of the trout viscera protein hydrolysate was higher than those obtained using poultry by-products protein hydrolysate while oil absorption capacity was not significantly altered [689]. Methionine and histidine in both protein hydrolysates were the limiting amino acids and trout viscera protein hydrolysate had more hydrophobic residue. The amino acid composition also different, and could be related to the different pH solubility of both hydrolysates [689]. In a later study, the use of enzymatic hydrolysis using Alcalase coupled to microwave heating to hydrolyze rainbow trout by-products was used to improve the functional and antioxidant properties of the produced hydrolysates [690]. The use of Alcalase after chemical pretreatment of rainbow trout processing by-products produced hydrolysates that were successfully employed as an additive in frozen fish mince [691]. Alcalase hydrolysis and subsequent treatment by centrifugation and spray drying were employed to obtain silver catfish (*Pangasius* sp.) frame hydrolysate powder that possesses good solubility, good foaming properties and light color profile [692]. The hydrolysates were also rich in glutamate and lysine which grants it with a high potential as food additive [692]. The hydrolysis conditions to obtain eel (*Monopterus sp.*) protein hydrolysate using Alcalase were optimized using response surface methodology, and an experimental protein hydrolysis degree of 15.01% (that was lower than the predicted values) was obtained [693]. The nitrogen solubility index was 85% and the emulsion stability index decreased with the increase in the hydrolysate concentration while the foam expansion increased. High

solubility and the ability of hydrolysate to emulsify and form foam show its potential for use as a natural binding and emulsifying agent [693]. Recently, Asian swamp eel protein hydrolysates were prepared using Alcalase [694]. The hydrolysate showed the presence of aromatic groups, hydrophobic and hydrophilic amino acids. There were no significant differences of the hydrolysate solubilities at different pH values. The emulsifying and foaming properties of the hydrolysate depended on the pH, while water holding capacity depended on the protein concentrations. There were no significant differences in the oil binding capacities of the hydrolysate at different concentrations [694]. Alcalase hydrolysis of fish protein from seabass (*Dicentrarchus labrax*) by-products gave a hydrolysate that was added to whiting (*Merlangius merlangus*) mince as texture softening effect [695]. In a different study, Alcalase was employed in the pretreatment of the scales of a different species of seabass (*Lates calcarifer*) and grey mullet (*Mugil cephalus*) for the production with yields of gelatin 14.1–15.2% that presented high protein content (88.6–90.0%) with ash (1.43–1.55%) and no fat [696]. The gelatin was identified as type A due to its pH value. The viscosities of gelatin were found to be 6.97 cP for seabass and 8.73 cP for grey mullet hydrolysates. Both gelatins contained α-chain and β-chain as the major components. Gelatin from seabass and grey mullet scales could be used as a potential replacement for mammalian gelatin [696]. Another work studied the flavor properties of the Maillard reaction products obtained from the hydrolysis by Alcalase of *Collichthys niveatus* protein [697]. A total of 80 volatile compounds were separated and identified [697]. Shortfin scad (*Decapterus macrosoma*) protein hydrolysates were prepared using Alcalase [698]. They have high protein content and concentration, lower molecular weight, high solubility, and high percentage of essential amino acids which fulfil adult human requirements [698]. The next year the hydrolysate from skin of shortfin scad was produced using Alcalase in order

to prepare gelatin hydrolysate [699]. The yield of hydrolysate was 51.01%, the moisture (13.82%), protein (90.05%), fat (1.95%), and ash 12.48%, contents were adequate for its use [699].

In one additional example, the physical and oxidative stabilities of cod liver oil-in-water emulsions were fortified by the protein hydrolysate of discarded common carp (*Cyprinus carpio*) roe [700]. Fish skin gelatin rich in α-chain was obtained through Alcalase digestion and this product can be used in food, pharmaceutical and biological industries [701]. Yellowstripe scad fish (*Selaroides leptolepis*) protein hydrolysate was produced by hydrolysis with Alcalase and processed by spray or freeze drying [702]. The water holding capacity of freeze-dried protein hydrolysates was higher than spray-dried hydrolysates in [702]. Shark (spiny dogfish) skin gelatin obtained by Alcalase hydrolysis was rich in high molecular weight polypeptide chains [703]. Optimized gelatin presented 7.9% of hydroxyproline, 10% of proline and 31.6% of glycine. This gelatin had a strong ability to form films from solutions with even only 0.5% gelatin concentrations. Microstructure of 3% gelatin displayed a smooth and compact film network [703]. Alcalase was employed to hydrolyze Chinese sturgeon (*Acipenser sinensis*) [704]. The protein hydrolysates could be useful in many applications of the food industry because of its functional and antioxidant properties [704].

3.8.2.2. Comparison of Alcalase with other proteases

Again, there are many studies that compare the performance of various proteases in the hydrolysis of fish by-products and the quality of the obtained products. For instance, the functional properties and the amino acid profile of bluewing sea robin (*Prionotus punctatus*) hydrolysates obtained by digestion with Flavourzyme and Alcalase were

evaluated [705]. Both showed a good essential amino acid composition [705]. Functional properties of Nile tilapia (*Oreochromis niloticus*) hydrolysates obtained with Alcalase, Neutrase and Flavourzyme were analyzed [706]. Essential amino acids were over the recommended amounts by the Food and Agricultural Organization/World Health Organization for humans. Low molecular weight peptides were abundant in hot water dip hydrolysates (328- 1876 Da). The hot water dip concentrates were mainly composed of high molecular weight peptides (214-19,576 Da). The solubilities were higher than 80% at pH 12.0 [706]. Emulsifying capacity of 21.40 and 20.40 mL, hydrophobicities of 168.01 and 200.28, bulk density of 0.53 and 0.36 mL g^{1}, oil absorption capacity ranged between 2.23 and 3.36 g mL^{-1}, and water-binding capacity was in the range of 1.77 and 2.43 mL g^{-1} respectively for hot water dip hydrolysates and hot water dip concentrates. Foam capacity and foam stability ranged from 124.53 to 37.25 mL g^{-1} for hot water dip hydrolysates and from 80.3 to 45.57 mL g^{-1} for hot water dip concentrates. The hydrolysate was more easily digestible than the concentrate [706]. Another study shows the differences in functional properties of hydrolysates from *Cirrhinus mrigala* egg, obtained by hydrolysis using papain and Alcalase [707]. The degree of hydrolysis was 62% for Alcalase and 17.1% for papain, after 90 min digestion. The hydrolysate produced by Alcalase presented higher protein content (85% *versus* 70%). The hydrolysates showed an increased solubility from pH 2 to pH 12. The hydrolysates exhibited high fat absorption capacity (0.9 and 1.0 g/g sample), foam capacity (70% and 25%) and emulsifying capacity (4.25 and 5.98 ml/g hydrolysate), respectively for Alcalase and papain protein hydrolysates [707]. Fish protein hydrolysates were prepared from fish by-product using Flavourzyme or Alcalase [708]. The Alcalase hydrolysate showed an overall better performance [708]. In another paper, cuttlefish (*Sepia officinalis*) muscle proteins were hydrolyzed by Alcalase and *Bacillus licheniformis* NH1

proteases [709]. A nitrogen recovery of 63% was obtained after a hydrolysis degree of 12.5%, using Alcalase. This hydrolysate presented a water holding capacity and a fat absorption capacity lower than the hydrolysate produced using NH1 proteases. The interfacial (emulsion stability index, emulsion activity index) and the surface (foaming stability and capacity) properties decreased when the degree of hydrolysis increased [709]. Rainbow trout roe protein hydrolysates were obtained *via* pepsin or Alcalase hydrolysis presented essential amino acids in a very interesting proportion [710]. In another paper, hydrolysates from the livers of *Oncorhynchus keta* and *Oncorhynchus gorbuscha* were produced using different proteases and Alcalase was the most efficient one [711]. The adequate amounts of essential amino acids, the balanced amino acid composition and the presence of some possible bio-active peptides, make the Alcalase liver protein hydrolysate a good alternative in functional food applications and as a source of novel products [711]. Atlantic salmon (*Salmo salar*) protein hydrolysates were obtained employing the endopeptidases Protex, Promod or Alcalase at three degrees of hydrolysis [712]. Alcalase was the enzyme producing more bitter peptides [712]. Marugoto E, Alcalase, Flavourzyme and Protamex were used to hydrolyze anchovy fine powder at 300 MPa and ambient pressure [713]. The high pressure gave hydrolysates with higher contents of total soluble solids, total water-soluble nitrogen and trichloroacetic acid-soluble nitrogen [713]. Brewer's spent yeast proteases, Neutrase and Alcalase [714] were used to hydrolyze muscle and viscera proteins from canned sardine by-products in order to obtain products with biological and functional properties. All the treatments produced improved biological and functional properties [714].

3.8.3. Hydrolysis of proteins from different sources

3.8.3.1. Use of stand-alone Alcalase

Apart from fish, there are many other animal sources that have been utilized to produce hydrolysates with different food functional properties using Alcalase. For example, Alcalase was employed to hydrolyze green mussel (*Perna viridis*) at pH 7 or 9 (where the hydrolysis degree was higher) [715]. The bitterness of both hydrolysates did not exceed that of the standard caffeine solutions. The authors concluded that further work must be performed to produce a green mussel hydrolysate with sensorial properties suitable for use in food products [715]. In another work, tropical banded cricket (*Gryllodes sigillatus*) was hydrolyzed by Alcalase at different concentrations, improving the protein solubility, foaming properties and emulsion capabilities [716]. Later, Alcalase was employed to hydrolyze buffalo whey. The hydrolysate was applied to apple, and it prevented apple browning [717]. Sheep plasma has also been hydrolyzed by Alcalase, the hydrolysate improved the color stability in mutton patties [718]. Fish and bovine gelatins and caseinate were hydrolyzed using Alcalase, and the hydrolysates were added to skimmed bovine milk that was then fermented to produce yoghurt [719]. Both gelatin hydrolysates lowered the titratable acidity but increased the pH values, delaying yoghurt fermentation, while the caseinate hydrolysate showed the opposite effect. The two gelatin hydrolysates worsened the quality features of the yoghurt: lower viscous moduli apparent viscosity, elastic, hardness, and adhesiveness, smaller hysteresis loop areas and higher syneresis extent, while the caseinate hydrolysate improved these quality attributes. Bovine gelatin hydrolysate always presented a higher negative effect than fish gelatin hydrolysate on yogurt texture and acidification [719]. Recently, Alcalase was employed to obtain hydrolysates from non-penaeid shrimp (*Acetes indicus*) that presented 56% essential amino acids [720]. The spray-

dried protein hydrolysates solubility was 90.20% at pH 2 and 96.92% at pH 12. The emulsifying features of the hydrolysate depended on the protein concentration and the highest emulsifying capacity (26.67%) and emulsion stability (23.33%) were obtained at a concentration of 20 mg mL^{-1}. At a concentration of 20 mg $mL^{-1,}$ the lowest and the highest foaming capacity were appreciated at pH 10 and pH 6. The water holding capacity of protein hydrolysate increased with its concentration [720].

3.8.3.2. Comparison of Alcalase with other proteases

There are many examples where Alcalase was compared to proteases in this goal. Some of them use whey proteins as the protein source to be hydrolyzed. For example, whey proteins were hydrolyzed by Flavourzyme, Neutrase, Protamax and Alcalase and spray-dried [721]. Samples treated with Alcalase for 3 hours produced various bioactive peptides identified by offline-electrospray-ionization mass spectrometry measurements and offline-matrix-assisted laser desorption/ionization mass spectrometry [721]. In a later study, pepsin, Protease M, trypsin, Protease S and Alcalase were employed to obtain whey protein hydrolysates [722]. Although depending on the used protease, the features of the final product varied greatly, in all cases, an increase in the hydrolysis time increased the degree of hydrolysis, bulk density, foaming capacity and solubility. It was shown that the hydrolysates improved the characteristics of several food products [722]. Beta-lactoglobulin (β-Lg) is the major whey protein of cow milk and determines the technofunctional properties of products like whey protein concentrates and isolates, which are available in large quantities in an industrial scale. β-lactoglobulin obtained from whey protein isolate was hydrolyzed by the hydrolysis with Alcalase, pepsin or trypsin [723]. A limited pepsin hydrolysis led to both, superior foam stability and increased overrun, while

foam drainage decreased by more than 50% compared to foams produced by trypsin and Alcalase treated hydrolysates. The authors suggested that only denatured molecules are hydrolyzed, and this permits synergistic effects of the produced peptides and the strong surface activity of the protein [723]. Trypsin, Protamex and Alcalase were used to hydrolyze collagen from the jellyfish *Chrysaora* sp. [724]. Although Alcalase produced the highest degree of hydrolysis, a high water holding capacity, oil absorption capacity, water binding, and water absorption was obtained by all hydrolysates together with a good emulsifying and moderate foaming properties [724]. Flavourzyme and Alcalase were employed to hydrolyze mud clam (*Polymesoda erosa*) protein [725]. Alcalase hydrolysate contains smaller peptides than Flavourzyme hydrolysate. Eighteen, six and seven volatile compounds were identified in the flesh, Flavourzyme hydrolysate and Alcalase hydrolysate, respectively. Bitterness was higher in Alcalase hydrolysate than in Flavourzyme hydrolysate. Quantitative descriptive analysis revealed that Flavourzyme hydrolysate was the least bitter but caused more umami taste compared to Alcalase hydrolysate [725]. Goat viscera protein hydrolysates obtained by hydrolysis using Brauzyn and Alcalase showed maximum solubility values for the samples with a higher degree of hydrolysis while oil retention capacity showed higher values for the hydrolysates with lower degree of hydrolysis [726]. Emulsifying properties and emulsion stabilities of the different hydrolysates did not change. The authors conclude that the protein hydrolysates of goat viscera are outstanding sources of nutrients and may be useful in the food industry [726]. Egg yolk proteins were hydrolyzed with Neutrase, Flavourzyme and Alcalase; Alcalase was the protease with the highest hydrolysis efficiency and its hydrolysates could be an excellent emulsifying agent [727]. In another study, among five enzymes used proteases, Alcalase resulted to be the optimal enzyme to hydrolyze the offal of octopus and abalone

[728], although even better results could be obtained if used together with Flavourzyme, producing a very interesting food condiment [728]. Flavourzyme, Neutrase, trypsin, Protamex and Alcalase were used in the recovery of fat and protein hydrolysates from chicken skin [729]. The highest (49.19%) degree of protein hydrolysis was achieved using Alcalase, but Flavourzyme hydrolysates presented the highest emulsifying activity index, oil-holding capacity and water-holding capacity. The highest foaming capacity was observed in the trypsin, Protamex or Alcalase hydrolysates. Hydrolysis using Protamex or trypsin provided in the highest fat yield [729]. Papain and Alcalase were employed to hydrolyze golden apple snail (*Pomacea canaliculata*) protein [730]. The Alcalase hydrolysate showed higher yields (12.61%) and hydrolysis degree (88.18%) than that obtained with papain. Alcalase hydrolysate presented higher foaming stability, solubility, emulsifying activity and stability index, while differences in fat binding, foaming, water holding capacities or protein concentration were scarce. This was correlated to structural differences between both produced hydrolysates [730]. Later, these enzymes were compared in the hydrolysis of squid (*Loligo formosana*) ovary [731]. One of the Alcalase hydrolysates presented the highest foaming capacity showing high solubility and surface hydrophobicity. If a pre-heating at 60 °C was performed, the hydrolysate showed the highest foaming capacity and had the lowest liquid drainage, also microstructure and viscoelastic features of foam were much improved [731]. In another paper, Neutrase, trypsin and Alcalase were employed to hydrolyze egg white protein, giving an hydrolysate that could be used as stabilizer for emulsions [732].

3.8.4. Combined use of Alcalase with other proteases

Alcalase and Flavourzyme were simultaneously used to hydrolyze the protein of little hairtail (*Trichiurus haumela*) [733]. The reaction conditions were optimized attending to hydrolysis time, temperature, pH, enzymes/substrate ratio, and Alcalase/Flavourzyme ratio. The optimal hydrolysate possesses high nutritional value and could be used as a nutritious supplement in various food products [733]. Alcalase and Flavourzyme have been employed both separately and simultaneously to hydrolyze chickpea protein isolate [734]. The degree of hydrolysis was higher when both enzymes were used together. The results of this study revealed that the hydrolysis enhanced the functional properties and antioxidant activity of chickpea protein [734].

Later a sequential enzymatic hydrolysis using Alcalase and Flavourzyme was proposed to hydrolyze hard-to-cook bean (*Phaseolus vulgaris L.*) protein [735]. Once the hydrolysate was prepared, it was added to durum wheat semolina pasta at different concentrations. The 10% hydrolysate was the best concentration in terms of nutritional parameters and sensory scores [735], later this product was remarked as a functional food [736]. Corolase, papain, Alcalase and Neutrase were individually used or in a two-step process to hydrolyze lupin (*Lupinus angustifolius* cultivar Boregine) protein isolates [737]. Combinations of Alcalase and papain were most effective in the degradation of polypeptides in *L. angustifolius*, although all hydrolysates increased the foam activity, emulsifying capacity and protein solubility. The combination of Alcalase and papain increased the bitterness while the fragrance characteristics of the hydrolysates were very similar to untreated protein. The protein hydrolysis greatly reduced the major IgE-reactive polypeptides [737].

3.9. Production of peptides with other bioactivities

This section attempts to summarize, with some examples published since 2010, the huge range of possible bioactivities that the peptides produced by Alcalase (individually or in combination with other proteases) via the hydrolysis of different proteins in order to obtain products with a higher value, mainly in the health industry.

In many instances, Alcalase was individually used, in some instances comparing its performance with that of other proteases. For example, Alcalase hydrolysates of peanut proteins were found to have the best *in vitro* antithrombotic activities among the hydrolysates produced by various proteases [738]. Under optimal conditions (pH 8.5, 50 °C, 50 mg/ml of peanut protein and an enzyme concentration of 5000 IU/g of peanut protein), the antithrombotic activities were increased to 26% after 2 h of reaction [738]. In another instance, the peptide WA3-1 was obtained from *Whitmania pigra* protein by hydrolysis with Alcalase [739]. It had a high anticoagulant activity and significantly prolonged the plasma clotting time on activated partial thromboplastin time, prothrombin time and thrombin time [739]. Alcalase was also proposed to be the best catalyst to hydrolyze egg white powder derived anticoagulant peptides [740]. After purification, the anticoagulant activity of the selected fraction determined by micro plate reader was 84.74% [740]. The optimization of this work showed that the anticoagulant activity was optimum with a substrate concentration of 1% and pH 7, and that low temperatures produce hydrolysates with higher anticoagulant activity [741]. Protein from the scorpion *Buthus martensii Karsch* was enzymatically hydrolyzed to obtain a bioactive hydrolysate [742]. Alcalase was considered the best enzyme for this hydrolysis and the highest anticoagulant activity was achieved at a degree of hydrolysis of 18 % [742].

To get peptides with this kind of bioactivities, also some examples of combined use of different proteins may be found. For example, a sequential hydrolysis of amaranth protein using Alcalase at pH 10 and 37 °C, followed by a trypsin hydrolysis at pH 8 and 37 °C, were carried out to obtain a hydrolysate with antithrombotic activity [743]. In this case, the glutelin fraction exhibited an antithrombotic activity significantly superior to the other fractions [743].

Alcalase hydrolysates have also been used in cell proliferation. In one of these studies Alcalase was employed to obtain a *Thunnus orientalis* bone-based collagen hydrolysate [744]. A stimulated proliferation and enhanced osteogenic differentiation of MC3T3-E1 cell were observed even at extremely low hydrolysate concentrations (2μg/mL). It also upregulated mRNA levels of osteogenic markers, like runt-related transcription factor 2, osteopontin, alkaline phosphatase and osteocalcin [744]. In another instance, Neutrase, Protamex, Kojizyme, Flavourzyme or Alcalase were used to hydrolyze *Hippocampus abdominalis* and the effects of the hydrolysates on skeletal muscle growth in C2C12 myoblasts and zebrafish were investigated [745]. The highest proliferation was observed when the Alcalase hydrolysate was used and it significantly increased creatine kinase activity and glycogen levels in the cells. It also down-regulated the myostatin–Smad pathway and up-regulated the IGF-1-Akt pathway. When this hydrolysate was applied to the zebrafish model, the endurance against water flow and slope without training performance were enhanced [745].

Collagen is related to proliferation and differentiation of the skin fibroblasts and it is the main component of extracellular matrix [746]. To reinforce this, red deer (*Cervus elaphus*) antler collagen peptides with the capacity of promoting proliferation of human

skin fibroblasts were obtained via hydrolysis using Alcalase and, after, trypsin in the same reaction under optimal conditions [746].

Some of the Alcalase hydrolysates presented immunomodulatory properties. For example, two selenium-enriched rice protein hydrolysates were obtained through Alcalase hydrolysis [747]. Two peptides (SeMDPGQQ and TSeMMM) were characterized as novel selenium-containing peptide sequences. TSeMMM, presented a stronger immunomodulatory activity, and exhibited potential in the field of functional food additives to improve human health [747]. Ultrafiltered fractions of simulated gastrointestinal digestion of milk products supplemented with brewery spent grain protein hydrolysates obtained by Alcalase hydrolysis, confers anti-inflammatory effects in Concanavalin-A (ConA)-stimulated Jurcat T cells [748]. The hydrolysates caused a reduction in interleukin-6 (IL-6) production in Jurkat T cells and the IL-2 and interferon-γ was not affected. The production of IL-6, IL-1β and tumor necrosis factor-α production in lipopolysaccharide-stimulated RAW 264.7 cells was not significantly altered [748]. Mung bean protein hydrolysate was obtained by hydrolysis with different proteases (Flavourzyme, Neutrase, trypsin and Alcalase) and it was employed to study the immunomodulatory activity in lipopolysaccharide-induced RAW 264.7 cells [749]. The 3-h Alcalase hydrolysate had a suppressing activity of pro-inflammatory mediators, depending on the dose [749]. Another instance shows how trypsin, pepsin, papain, Neutrase or Alcalase were used to hydrolyze defatted wheat germ globulin [750]. When the Alcalase hydrolysate was employed, the highest immunomodulatory activity with respect to lymphocyte proliferation, secretion of pro-inflammatory cytokines and phagocytosis of neutral red was obtained [750]. In another paper, an immunomodulatory peptide was obtained using Alcalase to hydrolyze silkworm

(*Bombyx mori*) pupa protein [751]. Splenocyte proliferation could be upgraded from 87.35% to 248.4% after induction by Concanavalin A, in the presence of 100 μg/ml of purified peptide [751]. In another research, a novel 441.06 Da immunomodulatory peptide was produced and isolated from ultrasound-pretreated silkworm (*Bombyx mori*) pupa protein after hydrolysis using Alcalase [752]. Splenic lymphocyte proliferation assay was used to test its pro-proliferative activity, and it was found that with 100 μg/mL of the purified peptide the splenocyte proliferation rate was 91.1% [752]. In another work, the hydrolysates of ovalbumin, lysozyme and whole egg white produced by Alcalase hydrolysis were used to test the effects of the peptides produced on antibody production, cytokine secretion, oxidative stress and proliferation of murine spleen and mesenteric lymph node cells [753]. All of them were stimulated with T-(concanavalin A-ConA) or B-cell mitogens (lipopolysaccharide-LPS). It was shown that ConA-stimulated lymphocyte proliferation was reduced and secretion of the Th1 cytokine TNF-α decreased [753]. In a different study, the anti-allergic capacity of hydrolysates of ovalbumin lysozyme and ovomucoid from egg white obtained by Alcalase treatment was evaluated [754]. The peptides present in the hydrolysates were identified and they produced the downregulation of the production of Th2-biased cytokines. Secretion of IgE to the culture media of Th2-skewed peripheral blood mononuclear cells was also reported. In peripheral blood leukocytes, the oxidative stress was significantly neutralized [754].

Another bioactivity that the peptides presented in protein hydrolysates may exhibit may be in the control of hyperuricemia. The imbalance between uric acid/urate production and excretion results in hyperuricemia, with an excess of xanthine oxidase activity causing gout, kidney stones, and sometimes even renal and cardiovascular diseases. The inhibition

of xanthine oxidase could reduce both vascular oxidative stress and circulating uric acid, since xanthine oxidase inhibitors can block the biosynthesis of uric acid from purines [755]. The tuna flesh hydrolysate obtained by Alcalase hydrolysis was analyzed for this goal, finding that peptides having Phe-His in the sequence possess the highest xanthine oxidase inhibitory activity in potassium oxonate-induced hyper-uricemic rats [755]. In another work, the water extract of shark cartilage was hydrolyzed by Alcalase [756]. Using an animal model, anti-hyperuricemic activity of the Alcalase hydrolysate was detected. Two peptides (Tyr-Leu-Asp-Asn-Tyr and Ser-Pro-Pro-Tyr-Trp-Pro-Tyr) lowered the serum uric acid level when used at 5 mg/kg of body weight via intravenous injection, and another peptide (Tyr-Leu-Asp-Asn-Tyr) showed anti-hyperuricemic activity when orally administrated [756].

Bioactive peptides derived from food and many other sources offer an interesting alternative to fight against alcoholic liver disease, with the aim of controlling alcohol concentration and also certain alcohol degradation metabolites such as acetaldehyde and reactive oxygen species. Among the peptides bioactivities that could be of interest in this issue, some of them may be remarkable, for example antioxidant, antihypertensive, anti-diabetic, anti-inflammatory, antimicrobial, mineral binding, hepato-protective effect, etc. [757]. Corn hydrolysates, mainly the fractions with low molecular mass, have been reported to possess many of these bioactive functions. In this context, papain, neutral protease and Alcalase were employed to obtain the hydrolysate of corn gluten meal and study its effect on anti-inebriation treatment [758]. Two bioactive peptides were obtained and the mixture of them helped prevent acute alcohol intoxication in the liver by accelerating the alcohol metabolism and reducing the oxidative damage as well [758]. In

another study, the objective was to obtain low molecular mass peptides from corn hydrolyzed with Alcalase to facilitate alcohol metabolism by activating hepatic alcohol dehydrogenase [759]. The highest activity to activate alcohol dehydrogenase *in vitro* was exhibited by the fraction below 1000 Da [759]. Chicken hydrolysates obtained using Alcalase have been described to possess peptides that stabilize alcohol dehydrogenase [757]. In this study, 21 peptides were potentially active and three could stabilize alcohol dehydrogenase in a dose-dependent manner (DPQYPPGPPAF, IPC, and APGH) [757]. In another study, peptides from seahorse (*Hippocampus abdominalis*) hydrolysate produced by Alcalase hydrolysis [760], was found to protect Huh7 cells from ethanol toxicity and increase the viability of Chang cells, suggesting that the hydrolysate from seahorse could have a hepatoprotective effect [760]. It has also been reported how the Alcalase-treated silk protein hydrolysate has a beneficial effect in rats [761]. No cytotoxicity on hepatic tissues and blood biochemistry was observed by Alcalase-treated silk protein hydrolysate and some indicator values of liver function like aspartate aminotransferase and alanine aminotransferase were alleviated in a dose dependent manner [761]. Alcalase and Neutral were sequentially used to hydrolyze wheat germ proteins [762], and the hydrolysates obtained showed that they can facilitate alcohol metabolism by activation of the alcohol dehydrogenase enzyme with an activation rate of 68.37% [762]. Alcalase and Flavourzyme were also used sequentially to hydrolyze *Schizochytrium sp.* [763], and the hydrolysate produced could effectively modulate alcohol metabolism related enzymes levels and activities in mice using the model of alcohol-induced liver injury [763].

It is possible to find other studies where Alcalase-hydrolysates have been used as hepatoprotective agents but not related to alcohol metabolism. For instance, high-fat diets

can induce nonalcoholic fatty liver disease, especially hard to treat in elderly subjects. Alcalase was employed to obtain a peptide (DIKTNKPVIF) from potato protein [764]. The potato hydrolysate was orally administered and the purified peptide was intraperitoneally injected, finding that these treatments alleviate pro-inflammatory reaction associated with hepatosteatosis development in elderly subjects through activation of AMPK [764]. Another study shows the production of potato protein hydrolysate through Alcalase hydrolysis [765]. This was used to treat high-fat diets fed aging rats, which presented an increased body weight. This treatment attenuated the high-fat diets induced hepatic fat accumulation. Hepatic apoptosis- and fibrosis-related proteins induced by high-fat diets were also suppressed [765]. Alcalase and Termamyl SC were used to obtain the soluble rice protein from rice-derived by-products, specifically from rice syrup meal [766]. The effect of peptides obtained was studied *in vitro* (rat primary hepatocytes) and *in vivo* (mice). Results showed that the viability of rat primary hepatocytes was not affected, and tert-butyl hydroperoxide induced cytotoxicity was ameliorated. The peptides also reduced the activities of hepatocyte alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase in a dose-dependent manner. The reduction of these parameters was also observed *in vivo* [766]. In another study, Alcalase was used to produce low molecular weight corn peptides [767]. This study concluded that the hydrolysate obtained with Alcalase, applied at the dose of 200 mg/kgbw showed a significant protective effect to alleviate carbon tetrachloride-induced hepatocellular injury [767].

In addition, it has been described that the peptide IF obtained from the hydrolysis of potato protein using Alcalase has promising therapeutic effects on renal damage related to the hypertension [768]. Kidney sections of hypertensive rats treated with the IF peptide

showed restoration of the glomerulus and Bowman´s capsule. Also the expression levels Nrf2-mediated antioxidants were increased in these rats and the amount of apoptotic cells in the groups treated with the peptide IF was reduced [768].

In another study enzymatic hydrolysates obtained from seahorse (*Hippocampus abdominalis*) by hydrolysis with Alcalase have been studied [769]. This is relevant since oxidative stress-mediated endothelial dysfunction and LDL oxidation play an important role in the pathogenesis of atherosclerosis. These hydrolysates showed high antioxidant activities in DPPH, ABTS+ and ORAC assays. They also ameliorated H_2O_2-mediated injury through the restoration of antioxidant enzyme activities and glutathione in human umbilical vein endothelial cell [769].

There are many unexplored protein sources of bioactive peptides encrypted in their protein sequence that could be released by enzymatic hydrolysis, an some of them may present anti-cancer activities [770]. To reach this goal, Alcalase hydrolysates from soybean meal were investigated [771]. For this study, two high oleic acid soybean lines and one high protein soybean line were used. The hydrolysates bioactivity was tested against colon, liver and lung cancer cell lines, obtaining growth inhibition rates of 73% against colon cancer, 70% against liver cancer cells and 68% against lung cancer cells using peptides from the two high oleic acid soybean lines [771]. In another work, two maize lines (Asgrow-773 and CML-502) were used to produce hydrolysates by Alcalase treatment [770]. The derived peptides were used *in vitro* model of human liver cancer with HepG2 cells. Anti-proliferative effects from both maize lines on the HepG2 cells were found, related to the induction of apoptosis due to a decrease of the expression of anti-apoptotic factors [770]. Sweet potato proteins were hydrolyzed using six different proteases, and

among them, Alcalase hydrolysate was the one with the best results in terms of anti-proliferative effects [772]. The peptides were tested in HT-colon cancer cells after being previously separated into four fractions. The highest anti-proliferative effect (43.87% at 100 μg/mL) was found in the fraction < 3 kDa [772]. Other less common protein sources have been used to this goal. For example, *Dendrobium catenatum* Lindley protein was subjected to hydrolysis using trypsin or Alcalase [773]. The hydrolysates were separated into nine fractions by gel filtration chromatography. The fraction called A3 had the best anti-proliferative activity in vitro against HepG-2 (73.38%), SGC-7901 (78.91%) and MCF-7 (86.8%) cancer cells and O_2 normal liver cells (5.52%) at a dose of 500 μg/mL [773]. Silkworm pupae (*Bombyx mori*) protein was subjected to Alcalase hydrolysis, and the hydrolysates were tested in human gastric cancer cells (SGC-7901) showing a specific inhibition of the cell proliferation and reducing some abnormal morphologic features in a dose and time dependent manner [774]. Thus, the authors concluded that silkworm pupae protein hydrolysates can, through an intrinsic apoptosis pathway, ROS (Reactive Oxygen Species) accumulation and cell cycle arrest, specifically suppress growth of SGC-7901 cells [774]. In another research, a sequential hydrolysis catalyzed using Alcalase and papain was employed to produce hydrolysates form *Arthrospira platensis* protein [775]. The hydrolysates were separated by gel filtration chromatography. From the fraction with the strongest antitumor effects on MCF-7 and HepG-2 cells, three peptides were isolated and identified (AGGASLLLLR, LAGHVGVR, and KFLVLCLR). Together they possess a strong antitumor activity but low cytotoxicity on normal cells [775].

Oxidative stress is another point where bioactive peptides may have some positive incidence [776]. It may be described as an imbalance between the generation of oxidants

and their elimination systems. Damages caused by non-physiological high oxidative stress may lead to a wide range of phenotypic changes, including altered gene expression, arrested cell proliferation and cell growth, and cellular senescence. To suppress this negative action in the organism, the antioxidants act as scavengers of oxidants to maintain the biological redox steady states [776]. Casein hydrolysates were produced by enzymatic hydrolysis using Alcalase, finding that they elevated catalase activity, increased cell viability, and decreased superoxide dismutase activity in HepG2 cells [777]. On another approach, ultrasound-pretreated porcine cerebral hydrolysate was produced using Alcalase [778]. This produced a hydrolysate where 11 peptides were identified. These peptides were administered to developing mice. Pb^{2+}-induced spontaneous locomotor activity, latencies to reach the platform and the time in target quadrant were decreased by the hydrolysate. Accumulation of lead in the blood and brain of Pb^{2+}-exposed developing mice was also reduced by this treatment [778]. In a sequential reaction, Alcalase followed by Flavourzyme hydrolysis were carried out using bovine colostrum whey protein as substrate [779]. The produced hydrolysate was later fractioned by ultrafiltration (10 kDa cutoff membrane). The hydrolysate showed high inhibitory activities of oxidative damage of deoxyribose and it also presented an inhibitory effect on the breakdown of supercoiled DNA into open circular DNA and linear DNA [779].

On a global scale, obesity has reached epidemic proportions and is a major problem to human health and an economic burden of chronic disease and disability [780]. There are some chemical drugs that are typically used to treat these kinds of diseases. Nonetheless, hey produce some adverse side effects, including increased blood pressure, dry mouth, constipation, headache and insomnia. That way, it is necessary to find new sources of

compounds that can have potential positive effects. The use of natural products with anti-obesity properties may be an excellent alternative to prevent the negative effects mentioned previously, and in this area bioactive peptides may have also some relevance [780]. For example, hypocholesterolemic peptides were obtained from isolated chickpea protein after Alcalase hydrolysis [781]. Under optimal hydrolysis conditions, the inhibiting rate of cholesterol production of chickpea peptides is 71.55% *in vitro*. *In vivo* assays using Wistar rats, the concentration of cholesterol could be decreased by 22.33% with chickpea peptides at 100 mg/kg bw [781]. In another study, Alcalase was employed to hydrolyze potato protein [782]. After fractioning, the fraction of 10 kDa enhanced lipolysis-stimulating activity in 3T3-L1 adipocytes these cells since the relative triglyceride residue significantly decreased from 88.4 to 83.8% at the 800 ppm level [782]. In another research, aging rats with a high fat diet induced hyperlipidemia were treated with Alcalase potato protein hydrolysates and probucol in order to evaluate serum lipid profiles and heart protective effects [783]. Serum triacylglycerol, total cholesterol, and LDL levels were reduced after hydrolysate treatments and they could also reduce serum lipids without affecting HDL expression. This reduction in serum lipids together with the enhancement of the activation of the compensatory IGF1R-PI3K-Akt survival pathway could explain the heart protective effect of the hydrolysate in aging rats with hyperlipidemia [783]. Following this research line, hamsters who were fed with a high fat diet, which caused them significant deterioration in their heart function, where treated with hydrolysates from the Alcalase potato protein hydrolysates for fifty days at different concentrations (15, 45 and 75 mg/kg/day) [605]. After the treatment, in all cases, after the initial increase of apoptosis positive cells and the expression of protein markers of apoptosis in the hamster fed with high fat diet, their cardiac ejection fraction percentage and fraction shortening percentage

became similar to those of the control group. It is suggested that these effects might be mediated by SIRT1 pathway indicating a restoration from the metabolic disorders induced by high fat diet [605]. Four proteases including trypsin, papain, Neutrase and Alcalase were used to obtain rice bran protein hydrolysates [784]. Later, they were fractionated by hydrophobicity using styrene/divinylbeneze resins. The highest micellar cholesterol inhibition ability was observed using the Alcalase hydrolysate, which suggests that it may have hypo-cholesterolaemic properties [784]. In another paper, canola protein isolate and its enzymatic hydrolysates were used to study the ability to inhibit adipogenic differentiation of C3H10T1/2 murine mesenchymal stem cells *in vitro* [780]. While cell viability was not affected by the treatment, the protein and its hydrolysate contain bioactive components which modulated in vitro adipocyte differentiation. However, the Alcalase hydrolysate was found to produce a higher reduction in anti-adipogenic differentiation [780]. One example of a sequential hydrolysis is the use of a first sunflower protein hydrolysis catalyzed by Alcalase (1 h) followed by 2 h of hydrolysis with Flavourzyme [785]. Rats were injected with a high dose of this hydrolysate and no signs of lethality or acute toxicity were showed. However, the administration of sunflower protein hydrolysate produced a significant decrease in both serum total cholesterol (18.55%) and triglyceride (29.70%) levels in induced hyper-lipidemic rats [785].

Alcalase treatment may be used to transform industrial protein residues into bioactive peptide, in some instances with a high value, solving the contamination produced by the disposal of these residues in Nature. For example, rice residues were subjected to Alcalase hydrolysis to obtain a hydrolysate with high protein content [786]. The <1000 kDa fraction was able to prolong significantly swimming fatigue time and blood sugar levels in

mice compared to saline and hydrolysate fractions of higher molecular weight. The blood lactate content was also significantly reduced. The HPLC separation permitted to obtain the peptide that was the main responsible for these activities, and its sequence was Gln-Ser-Pro-Glu-Ile [786]. Defatted rice bran was hydrolyzed with Alcalase to produce rice bran protein concentrate [787]. The degree of hydrolysis increased with time and it was at 50 min when the highest inhibitory efficiency on soybean lipoxygenase activity was found (66%). It behaves as a competitive inhibitor [787]. Another interesting study presents the Alcalase hydrolysis of soybean β-conglycinin [788]. The hydrolysate exhibited the effect of the *in vitro* inhibition of pathogen adhesion or translocation to intestinal cells. Mice treated with dextran sulfate sodium-induced intestinal mucosa injury were used to study the protective and reparative effects of β-conglycinin hydrolysate on intestinal mucosa injury. The results show how the histological injury in both, the protective and reparative experiments, was significantly reduced. The myeloperoxidase activity also decreased compared to the control group [788]. Sericin hydrolysate, extracted from silk cocoon shells by heat treatment and later hydrolyzed using Alcalase, presented inhibitory effects over polyphenol oxidase, avoiding the browning of fresh-cut products [789]. This hydrolysate was able to reduce polyphenol oxidase activity from apple extract by 95%, from eggplant extract by 79% and from bean sprouts and banana flower extracts by 70% [789].

Alcalase was found to be the best protease to produce a hydrolysate rich in 5-hydroxytryptophan from liquid egg white in a liquid egg white-water ratio of 1:1 [790]. When liquid egg white was administered at an equivalent dose to 6 mg/kg of 5-hydroxytryptophan to mice, the sleep duration significantly increased, while sleep latency time decreased in a similar way to the 5-hydroxytryptophan treatment. These results

suggest that liquid egg white could be employed as an alternative sleep-potentiating agent [790]. In another paper, Alcalase was used to prepare a gelatin hydrolysate from blue shark skin and bovine skin gelatin [791]. The hydrolysis times were 15 and 90 min for blue shark skin and 30 and 120 min for bovine skin gelatin, and the latter possessed higher amounts of low molecular weight peptides. Then, surimi was frozen at -25 °C for 135 days, adding or not adding the hydrolysates. The amino acid content and the suppression in freezing-induced denaturation of surimi samples treated with both hydrolysates was similar and they were more effective than the samples of shorter times of hydrolysis [791].

In another paper, the Alcalase hydrolysates of fresh and boiled Venus clams exhibited the strongest hyaluronidase inhibitory activity among the hydrolysates produced by five different proteases [792]. After fractioning, one of the fractions presented the highest hyaluronidase and elastase specific activities of 141.15 and 81.36% mL/mg, respectively. Thus, Alcalase hydrolysate of boiled Venus clams was suggested to be used as a cosmetics agent [792].

Sunflower defatted seed meal is an abundant by-product of biodiesel chain oil extraction [793]. In a two-step hydrolysis approach using Alcalase and Flavourzyme in a sequential manner, a high-quality hydrolysate was obtained [793]. This hydrolysate was interesting in terms of nutrient, amino acid, and peptide content as a potential biostimulant in agriculture. The sunflower hydrolysate presented auxin-like and interesting effects on plant root elongation, but no gibberellin-like activity, therefore this product may be considered as an effective biostimulant [793]. In another paper, hemp (*Cannabis sativa* L.) seeds were hydrolyzed using Alcalase and Flavourzyme in a sequential way to obtain a potentially bioactive hemp protein hydrolysate [794]. *In vitro* experiments permitted to

identify two bioactive hemp protein hydrolysates that down-regulated TNF-α, IL-1β, and IL-6 mRNA transcriptional levels. On the other hand, the gene expression of anti-inflammatory cytokine IL-10 was up-regulated. Therefore, hemp protein hydrolysates may improve the neuro-inflammatory and inflammatory states [794].

4. Conclusions

This review has outlined the impressive potential of Alcalase in the production of peptides with very different bioactivities. The enzyme, due to the high number of positions where it may hydrolyze a protein, has come out on top in most of the comparisons with other popular proteases as the best enzyme in the production of bioactive peptides (as it produces more peptides, and of a smaller size because its wider selectivity). However, not always Alcalase is the enzyme that produces the best results, because these smaller peptides are not always the most active ones. Although bioactive peptides may be obtained from any protein source, the use of high-end proteins for this goal seems inadequate. In this context, a special interest may be addressed towards its utilization as feedstocks of residues from different industries, such as vegetable oil, fish or poultry processing. As shown in this review, it is possible to obtain hydrolysates with good bioactivities from these residues. This way, a greener economy and the reduction of waste may be achieved, producing highly added valued products without a competition with the usage of the proteins as food.

The immobilization of this enzyme permits to increase its stability, mainly if applying appropriate immobilization protocols: suitable supports, adequate groups in the support, and appropriate immobilization protocols that can permit an intense multipoint covalent attachment. As a result, the range of conditions where it can be utilized may be expanded. In this context, it should be remarked that a problem in the use of immobilized

Alcalase is the requirement for a proper enzyme orientation. Only properly oriented enzyme molecules will be able to attach to substrates as large as proteins and the diffusional problems that a concentrate enzyme solutions may have to go inside the solid porous particle.

Even being Alcalase a very suitable enzyme to hydrolyze proteins from different sources, with a wide selectivity that permits it to produce very small peptides, in general the combined use of Alcalase with other proteases, to further decrease the size of the peptides, further improves the properties of the obtained hydrolysates, as the number of peptides increase and their average size decrease. These better results are due to the combination of the different proteases regioselectivity, which permits to increases the number of broken bonds on the polypeptide chain of the substrate protein. These advantages of the coupled use of enzymes with the same bioactivity has been recently reviewed for the case of the full modification of oils and fats by lipases [395], and in this instance the advantages follow very similar pathways: this combination of enzymes not only combines the specificity and selectivity of several enzymes, but also the change of the medium conditions (e.g., a decrease in the pH) or some inhibitions by the products may differently affect the different enzymes. That way, the authors of this review foresee a greater development in the use of combination of several proteases (or combi-proteases) in this kind of processes in the future.

Regarding the use of immobilized proteases, the use of a combi-protease can increase the number of papers where proteases are co-immobilized to take full advantages of the kinetic improvements in the processes raised by the coimmobilization and the combination of different enzyme selectivities. In this regard, it must be remarked that

proteases (and enzymes in general) co-immobilization may have serious problems (e.g., immobilization using the same support surface and immobilization protocol, the lifetime of the co-immobilized protease biocatalysts will be marked by that of the least stable enzyme, if one enzyme is less stable it becomes difficult to keep the relation between the activities of all involved enzymes throughout the life of the biocatalysts) [155]. In the case of proteases to be used in proteins hydrolysis, an additional problem to consider in co-immobilization is the requirement of having a correct orientation of all involved enzymes. That is, co-immobilization must be employed only if the presence of both enzymes outweighs the advantages of the combined use of the different proteases, for example if there are some synergic effects. Only after careful evaluations of the pros and cons co-immobilization may be recommended. Nevertheless, there are many efforts in the area of enzyme immobilization to solve some of the problems of enzyme co-immobilization, and perhaps they can open a massive use of co-immobilized enzymes in the future.

That way, in our opinion, the future use of Alcalase for the production of active bio-peptides should evolve toward the use of Alcalase co-immobilized with other proteases, and be extended mainly to the use of waste products. This will valorize these materials improving the economy of the global processes, and will avoid the waste dumping that may become a serious environmental problem.

Acknowledgments

We gratefully recognize the support from the Ministerio de Ciencia e Innovación from Spanish Government (project number CTQ2017-86170-R). The FPU fellowship (Ministerio de Educacion) for Mr. Morellon–Sterling and the fellowship for Mr. Siar from the Algerian Ministry of Higher Education and Scientific Research are also thanked. Dr.

Tacias-Pascacio thanks the financial support from “Programa para el Desarrollo Profesional Docente” (PRODEP) from Mexican Government.

References

[1] O.L. Tavano, A. Berenguer- Murcia, F. Secundo, R. Fernandez- Lafuente, Biotechnological applications of proteases in food technology, Compr. Rev. Food sci. Food Saf. 17 (2018) 412-436, https://doi.org/10.1111/1541-4337.12326.

[2] D. Bhandari, S. Rafiq, Y. Gat, P. Gat, R. Waghmare, V. Kumar, A review on bioactive peptides: physiological functions, bioavailability and safety, Int. J. Pept. Res. Ther. 26 (2020) 139-150, https://doi.org/10.1007/s10989-019-09823-5

[3] O.L. Tavano, S.I. da Silva Júnior, Food Proteins as a Tool in Human Longevity: A Mini-Review, Nov. Tech. Nutri. Food. Sci. 4 (2019) 347-349, https://doi.org/10.31031/NTNF.2019.04.000590.

[4] X. Sun, C. Acquah, R.E. Aluko, C.C. Udenigwe, Considering food matrix and gastrointestinal effects in enhancing bioactive peptide absorption and bioavailability, J. Func. Foods. 64 (2020) 103680. https://doi.org/10.1016/j.jff.2019.103680.

[5] E.C. Webb, Enzyme nomenclature: a personal retrospective, FASEB J. 7 (1993) 1192-1194, https://doi.org/10.1096/fasebj.7.12.8375619.

[6] J.K. McDonald, An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification, Histochem J. 17 (1985) 773-85, https://doi.org/10.1007/BF01003313.

[7] O.L. Tavano, Protein hydrolysis using proteases: an important tool for food biotechnology, J. Mol. Catal. B Enzym. 90 (2013) 1-11, https://doi.org/10.1016/j.molcatb.2013.01.011.

[8] N.D. Rawlings, M. Waller, A.J. Barrett, A. Bateman, MEROPS: the database of proteolytic enzymes, their substrates and inhibitors, Nucleic Acids Res. 42 (2014) D503-D509, https://doi.org/10.1093/nar/gkt953.

[9] L. Polgár, The catalytic triad of serine peptidases, Cell. Mol. Life Sci. 62 (2005) 2161-2172, https://doi.org/10.1007/s00018-005-5160-x.

[10] R.J. Delange, E.L. Smith, Amino acid composition; isolation and composition of peptides from the tryptic hydrolysate, J. Biol. Chem. 243 (1968) 2134-2142.

[11] A.V. Güntelberg, M. Ottesen, Preparation of Crystals containing the Plakalbumin-forming Enzyme from Bacillus subtilis, Nature. 170 (1952) 802-802, https://doi.org/10.1038/170802a0.

[12] E.L. Smith, The complete sequence: Comparison with subtilisin BPN'; evolutionary relationshop, J. Biol. Chem. 243 (1968) 2184-2191.

[13] C.G. Kumar, H. Takagi, Microbial alkaline proteases: from a bioindustrial viewpoint, Biotechnol. Adv. 17 (1999) 561-594, https://doi.org/10.1016/s0734-9750(99)00027-0.

[14] A. Sumantha, C. Larroche, A. Pandey, Microbiology and industrial biotechnology of food-grade proteases: a perspective, Food Technol. Biotechnol. 44 (2006) 211.

[15] S. Singh, B.K. Bajaj, Potential application spectrum of microbial proteases for clean and green industrial production, Energ. Ecol. Environ. 2 (2017) 370-386, https://doi.org/10.1007/s40974-017-0076-5.

[16] J. Kim, M. Kwon, S. Kim, Biological degumming of silk fabrics with proteolytic enzymes, J. Nat. Fibers. 13 (2016) 629-639, https://doi.org/10.1080/15440478.2015.1093578.

[17] M. Kanelli, S. Vasilakos, S. Ladas, E. Symianakis, P. Christakopoulos, E. Topakas, Surface modification of polyamide 6.6 fibers by enzymatic hydrolysis, Process Biochem. 59 (2017) 97-103, https://doi.org/10.1016/j.procbio.2016.06.022.

[18] M.B. Hale, Making fish protein concentrates by enzymatic hydrolysis: a status report on research and some processes and products studied by NMFS, Seattle, Washington (1972).

[19] McDonald, J. K., An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification. Histochemical J. 17(1985) 773-785, https://doi.org/10.1007/BF01003313

[20] N. Ahmadifard, J.H. Mureta, A. Abedian-Kenari, A. Motamedzadegan, H. Jamali, Comparison the effect of three commercial enzymes for enzymatic hydrolysis of two substrates (rice bran protein concentrate and soy-been protein) with SDS-PAGE, J Food Sci. Technol. 53 (2016) 1279-84, https://doi.org/10.1007/s13197-015-2087-6.

[21] E. Kula, E. Kocadag Kocazorbaz, H. Moulahoum, S. Alpat, F. Zihnioglu, Extraction and characterization of novel multifunctional peptides from *Trachinus Draco* (greater weever) myofibrillar proteins with ACE/DPP4 inhibitory, antioxidant, and metal chelating activities, J. Food Biochem. 44 (2020) e13179, https://doi.org/10.1111/jfbc.13179.

[22] A. Clemente, Enzymatic protein hydrolysates in human nutrition, Trends Food Sci. Technol. 11 (2000) 254-262, https://doi.org/10.1016/S0924-2244(01)00007-3.

[23] Y. Hou, Z. Wu, Z. Dai, G. Wang, G. Wu, Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance, J. Animal Sci. Biotechnol. 8 (2017) 24, https://doi.org/10.1186/s40104-017-0153-9.

[24] C.C. Udenigwe, R.E. Aluko, Food protein-derived bioactive peptides: production, processing, and potential health benefits, J. Food Sci. 77 (2012) R11-24, https://doi.org/10.1111/j.1750-3841.2011.02455.x.

[25] Hassan, Y. I., Trofimova, D., Samuleev, P., Miah, M. F., Zhou, T., Omics Approaches in Enzyme Discovery and Engineering. In: D. Barh, V. Azebedo (Eds.), Omics Technologies and Bio-Engineering, Academic Press, London, UK, pp. 297-322, https://doi.org/10.1016/B978-0-12-815870-8.00016-4.

[26] Novozyme, Proteases for biocatalysis. https://www.novozymes.com/es/advance-your-business/pharma (accessed 11 September 2020).

[27] Chen, S. T., Chen, S. Y., Wang, K. T., Kinetically controlled peptide bond formation in anhydrous alcohol catalyzed by the industrial protease Alcalase, J. Org. Chem. 57 (1992) 6960-6965, https://doi.org/10.1021/jo00051a052.

[28] Q. Xu, H. Hong, J. Wu, X. Yan, Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review, Trends Food Sci. Technol. 86 (2019) 399-411, https://doi.org/10.1016/j.tifs.2019.02.050.

[29] W.M. Miner-Williams, B.R. Stevens, P.J. Moughan, Are intact peptides absorbed from the healthy gut in the adult human?, Nutr. Res. Rev. 27 (2014) 308-329, https://doi.org/10.1017/S0954422414000225.

[30] I.D. Nwachukwu, R.E. Aluko, Structural and functional properties of food protein-derived antioxidant peptides, J. Food Biochem. 43 (1) (2019) e12761, https://doi.org/10.1111/jfbc.12761.

[31] S.Y. Lee, S.J. Hur, Antihypertensive peptides from animal products, marine organisms, and plants, Food Chem. 228 (2017) 506-517, https://doi.org/10.1016/j.foodchem.2017.02.039.

[32] L. Yi, M.A.J.S. Van Boekel, S. Boeren, C.M.M. Lakemond, Protein identification and *in vitro* digestion of fractions from *Tenebrio molitor*, Eur. Food Res. Technol. 242 (2016) 1285-1297, https://doi.org/10.1007/s00217-015-2637-6.

[33] M. Salami, R. Yousefi, M.R. Ehsani, M. Dalgalarrondo, J.-M. Chobert, T. Haertlé, S.H. Razavi, A.A. Saboury, A. Niasari-Naslaji, A.A. Moosavi-Movahedi, Kinetic characterization of hydrolysis of camel and bovine milk proteins by pancreatic enzymes, Int. Dairy J. 18 (2008) 1097-1102, https://doi.org/10.1016/j.idairyj.2008.06.003.

[34] G. Mandalari, N.M. Bisignano, C. Bisignano, R.B. Lo Curto, F. Mulholland, M. Su, M. Venkatachalam, J.M. Robinham, L.N. Willison, K. Lapsley, K.H. Roux, S.K. Sathe, Effect of food matrix and processing on release of almond protein during simulated digestion, LWT-Food Sci. Technol. 59 (2014) 439-447, https://doi.org/10.1016/j.lwt.2014.05.005.

[35] S. Benedé, I. López-Expósito, E. Molina, R. López-Fandiño, Egg proteins as allergens and the effects of the food matrix and processing, Food Funct. 6 (2015) 694-713, https://doi.org/10.1039/c4fo01104j.

[36] M. Zahir, V. Fogliano, E. Capuano, Food matrix and processing modulate *in vitro* protein digestibility in soybeans, Food Funct. 9 (2018) 6326-6336 https://doi.org/10.1039/C8FO01385C.

[37] M. Friedman, Nutritional value of proteins from different food sources. A review, J. Agric. Food Chem. 44 (1996) 6-29, https://doi.org/10.1021/jf9400167.

[38] M. Morifuji, M. Ishizaka, S. Baba, K. Fukuda, H. Matsumoto, J. Koga, M. Kanegae, M. Higuchi, Comparison of different sources and degrees of hydrolysis of dietary protein: effect on plasma amino acids, dipeptides, and insulin responses in human subjects, J. Agric. Food Chem. 58 (2010) 8788-97, https://doi.org/10.1021/jf101912n.

[39] E. Marconi, G. Panfili, L. Bruschi, V. Vivanti, L. Pizzoferrato, Comparative study on microwave and conventional methods for protein hydrolysis in food, Amino Acids. 8 (1995) 201-208, https://doi.org/10.1007/BF00806493.

[40] P. Borrajo, M. Pateiro, M. Gagaoua, D. Franco, W. Zhang, J.M. Lorenzo, Evaluation of the antioxidant and antimicrobial activities of porcine liver protein hydrolysates obtained using Alcalase, bromelain, and papain, Appl. Sci. 10 (2020) 2290, https://doi.org/10.3390/app10072290.

[41] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Industrial biocatalysis today and tomorrow, Nature. 409 (2001) 258-268, https://doi.org/10.1038/35051736.

[42] R.A. Sheldon, J.M. Woodley, Role of biocatalysis in sustainable chemistry, Chem. Rev. 118 (2018) 801-838, https://doi.org/10.1021/acs.chemrev.7b00203.

[43] J. M. Choi, S. S. Han, H. S. Kim, Industrial applications of enzyme biocatalysis: Current status and future aspects, Biotechnol. Adv. 33 (2015) 1443-1454, https://doi.org/10.1016/j.biotechadv.2015.02.014.

[44] A.J.J. Straathof, S. Panke, A. Schmid, The production of fine chemicals by biotransformations, Curr. Opin. Biotechnol. 13 (2002) 548-556, https://doi.org/10.1016/S0958-1669(02)00360-9.

[45] M.T. Reetz, Biocatalysis in organic chemistry and biotechnology: past, present, and future, J. Am. Chem. Soc. 135 (2013) 12480-12496, https://doi.org/10.1021/ja405051f.

[46] P. Jeandet, E. Sobarzo-Sánchez, A.S. Silva, C. Clément, S.F. Nabavi, M. Battino, M. Rasekhian, T. Belwal, S. Habtemariam, M. Koffas, Whole-cell biocatalytic, enzymatic and green chemistry methods for the production of resveratrol and its derivatives, Biotechnol. Adv. 39 (2019) 107461, https://doi.org/10.1016/j.biotechadv.2019.107461.

[47] V.S. Ferreira-Leitão, M.C. Cammarota, E.C. Gonçalves Aguieiras, V. de Sá, L. Ribeiro, R. Fernandez-Lafuente, D.M.G. Freire, The protagonism of biocatalysis in green chemistry and its environmental benefits, Catalysts 7 (2017) 9, https://doi.org/10.3390/catal7010009.

[48] Z. Chen, J. Liu, J. Tao, Biocatalysis for green chemistry and drug development, Prog. Chem. 19 (2007) 1919-1927.

[49] H.E. Schoemaker, D. Mink, M.G. Wubbolts, Dispelling the myths--biocatalysis in industrial synthesis, Science. 299 (2003) 1694-1697, https://doi.org/10.1126/science.1079237.

[50] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chem. Soc. Rev. 42 (2013) 6223-6235, https://doi.org/10.1039/c3cs60075k.

[51] R. DiCosimo, J. McAuliffe, A.J. Poulose, G. Bohlmann, Industrial use of immobilized enzymes, Chem. Soc. Rev. 42 (2013) 6437-6474, https://doi.org/10.1039/c3cs35506c.

[52] A. Liese, L. Hilterhaus, Evaluation of immobilized enzymes for industrial applications, Chem. Soc. Rev. 42 (2013) 6236-6249, https://doi.org/10.1039/c3cs35511j.

[53] V.M. Balcão, A.L. Paiva, F.X. Malcata, Bioreactors with immobilized lipases: state of the art, Enzyme Microb. Technol. 18 (1996) 392-416, https://doi.org/10.1016/0141-0229(95)00125-5.

[54] J.K. Poppe, R. Fernandez-Lafuente, R.C. Rodrigues, M.A.Z. Ayub, Enzymatic reactors for biodiesel synthesis: present status and future prospects, Biotechnol. Adv. 33 (2015) 511-525, https://doi.org/10.1016/j.biotechadv.2015.01.011.

[55] I. Eş, J.D.G. Vieira, A.C. Amaral, Principles, techniques, and applications of biocatalyst immobilization for industrial application, Appl. Microbiol. Biotechnol. 99 (2015) 2065-2082, https://doi.org/10.1007/s00253-015-6390-y.

[56] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451-1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.

[57] V.M. Balcão, M.M.D.C. Vila, Structural and functional stabilization of protein entities: state-of-the-art, Adv. Drug Deliv. Rev. 93 (2015) 25-41, https://doi.org/10.1016/j.addr.2014.10.005.

[58] P.V. Iyer, L. Ananthanarayan, Enzyme stability and stabilization—aqueous and non-aqueous environment, Process Biochem. 43 (2008) 1019-1032, https://doi.org/10.1016/j.procbio.2008.06.004.

[59] L. Betancor, M. Fuentes, G. Dellamora-Ortiz, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, C. Mateo, J.M. Guisán, R. Fernández-Lafuente, Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles, J. Mol. Catal. B Enzym. 22 (2005) 97-101, https://doi.org/10.1016/j.molcatb.2004.11.003.

[60] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, M. Fuentes, R. Fernández-Lafuente, J.M. Guisán, Prevention of interfacial inactivation of enzymes by coating the enzyme surface with dextran aldehyde, J. Biotechnol. 110 (2004) 201-207, https://doi.org/10.1016/j.jbiotec.2004.02.003.

[61] C. Garcia- Galan, Á. Berenguer- Murcia, R. Fernandez- Lafuente, R.C. Rodrigues, Potential of different enzyme immobilization strategies to improve enzyme performance, Adv. Synth. Catal. 353 (2011) 2885-2904, https://doi.org/10.1002/adsc.201100534.

[62] R.C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. Fernandez-Lafuente, Modifying enzyme activity and selectivity by immobilization, Chem. Soc. Rev. 42 (2013) 6290-307, https://doi.org/10.1039/c2cs35231a.

[63] L. Dal Magro, J.F. Kornecki, M.P. Klein, R.C. Rodrigues, R. Fernandez-Lafuente, Optimized immobilization of polygalacturonase from *Aspergillus niger* following different protocols: Improved stability and activity under drastic conditions, Int. J. Biol. Macromol. 138 (2019) 234-243, https://doi.org/10.1016/j.ijbiomac.2019.07.092.

[64] L. Dal Magro, J.F. Kornecki, M.P. Klein, R.C. Rodrigues, R. Fernandez-Lafuente, Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range, Enzyme Microb. Technol. 132 (2020) 109397, https://doi.org/10.1016/j.enzmictec.2019.109397.

[65] R. Fernandez-Lafuente, Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation, Enzyme Microb. Technol. 45 (2009) 405-418, https://doi.org/10.1016/j.enzmictec.2009.08.009.

[66] R. Fernández-Lafuente, O. Hernández-Jústiz, C. Mateo, M. Terreni, G. Fernández-Lorente, M.A. Moreno, J. Alonso, J.L. García-López, J.M. Guisan, Biotransformations catalyzed by multimeric enzymes: stabilization of tetrameric ampicillin acylase permits the optimization of ampicillin synthesis under dissociation conditions, Biomacromolecules. 2 (2001) 95-104, https://doi.org/10.1021/bm000072i.

[67] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts, Biotechnol. Adv. 33 (2015) 435-456, https://doi.org/10.1016/j.biotechadv.2015.03.006.

[68] E.A. Manoel, J.C.S. dos Santos, D.M.G. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme Microb. Technol. 71 (2015) 53-57, https://doi.org/10.1016/j.enzmictec.2015.02.001.

[69] R.C. Rodrigues, J.J. Virgen-Ortíz, J.C.S. dos Santos, Á. Berenguer-Murcia, A.R. Alcantara, O. Barbosa, C. Ortiz, R. Fernandez-Lafuente, Immobilization of lipases on

hydrophobic supports: immobilization mechanism, advantages, problems, and solutions, Biotechnol. Adv. 35 (2019) 746-770, https://doi.org/10.1016/j.biotechadv.2019.04.003.

[70] Z. Chen, L. Liu, R. Yang, Improved performance of immobilized lipase by interfacial activation on Fe_3O_4@PVBC nanoparticles, RSC Adv. 7 (2017) 35169-35174, https://doi.org/10.1039/C7RA05723G.

[71] I. Francolini, V. Taresco, A. Martinelli, A. Piozzi, Enhanced performance of *Candida rugosa* lipase immobilized onto alkyl chain modified-magnetic nanocomposites, Enzyme Microb. Technol. 132 (2020) 109439, https://doi.org/10.1016/j.enzmictec.2019.109439.

[72] J.C.S.d. Santos, O. Barbosa, C. Ortiz, A. Berenguer- Murcia, R.C. Rodrigues, R. Fernandez- Lafuente, Importance of the support properties for immobilization or purification of enzymes, ChemCatChem. 7 (2015) 2413-2432, https://doi.org/10.1002/cctc.201500310.

[73] M. Bilal, M. Asgher, H. Cheng, Y. Yan, H.M.N. Iqbal, Multi-point enzyme immobilization, surface chemistry, and novel platforms: a paradigm shift in biocatalyst design, Crit. Rev. Biotechnol. 39 (2019) 202-219, https://doi.org/10.1080/07388551.2018.1531822.

[74] M. Hoarau, S. Badieyan, E.N.G. Marsh, Immobilized enzymes: understanding enzyme–surface interactions at the molecular level, Org. Biomol. Chem. 15 (2017) 9539-9551, https://doi.org/10.1039/C7OB01880K.

[75] O. Barbosa, R. Torres, C. Ortiz, A.n. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Heterofunctional supports in enzyme immobilization: from traditional

immobilization protocols to opportunities in tuning enzyme properties, Biomacromolecules. 14 (2013) 2433-2462, https://doi.org/10.1021/bm400762h.

[76] A. Anwar, M. Saleemuddin, Alkaline proteases: a review, Bioresour. Technol. 64 (1998) 175-183, https://doi.org/10.1016/S0960-8524(97)00182-X.

[77] Z. Fang, Y.-C. Yong, J. Zhang, G. Du, J. Chen, Keratinolytic protease: a green biocatalyst for leather industry, Appl. Microbiol. Biotechnol. 101 (2017) 7771-7779, https://doi.org/10.1007/s00253-017-8484-1.

[78] A.M. Białkowska, K. Morawski, T. Florczak, Extremophilic proteases as novel and efficient tools in short peptide synthesis, J. Ind. Microbiol. Biotechnol. 44 (2017) 1325-1342, https://doi.org/10.1007/s10295-017-1961-9.

[79] M. Sharma, Y. Gat, S. Arya, V. Kumar, A. Panghal, A. Kumar, A review on microbial alkaline protease: An essential tool for various industrial approaches, Ind. Biotechnol. 15 (2019) 69-78, https://doi.org/10.1089/ind.2018.0032.

[80] M. Kuddus, P.W. Ramteke, Recent developments in production and biotechnological applications of cold-active microbial proteases, Crit. Rev. Microbiol. 38 (2012) 330-338, https://doi.org/10.3109/1040841X.2012.678477.

[81] S. Joshi, T. Satyanarayana, Biotechnology of cold-active proteases, Biology. 2 (2013) 755-783, https://doi.org/10.3390/biology2020755.

[82] Q. Husain, Nanocarriers immobilized proteases and their industrial applications: an overview, J. Nanosci. Nanotechnol. 18 (2018) 486-499, https://doi.org/10.1166/jnn.2018.15246.

[83] J. Vioque, A. Clemente, R. Sánchez- Vioque, J. Pedroche, F. Millán, Effect of Alcalase™ on olive pomace protein extraction, J. Amer. Oil Chem. Soc. 77 (2000) 181-185, https://doi.org/10.1007/s11746-000-0029-1.

[84] C. Esteve, M.L. Marina, M.C. García, Novel strategy for the revalorization of olive (*Olea europaea*) residues based on the extraction of bioactive peptides, Food Chem. 167 (2015) 272-280, https://doi.org/10.1016/j.foodchem.2014.06.090.

[85] E. H. Siar, H. Zaak, J.F. Kornecki, M.N. Zidoune, O. Barbosa, R. Fernandez-Lafuente, Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins, Process Biochem. 58 (2017) 98-104, https://doi.org/10.1016/j.procbio.2017.04.009.

[86] E. H. Siar, R. Morellon-Sterling, M.N. Zidoune, R. Fernandez-Lafuente, Amination of ficin extract to improve its immobilization on glyoxyl-agarose: Improved stability and activity versus casein, Int. J Biol. Macromol. 133 (2019) 412-419, https://doi.org/10.1016/j.ijbiomac.2019.04.123.

[87] N. Ranjbari, M. Pazhang, R. Fernandez-Lafuente, F. Shojaei, M. Satari, A. Homaei, Improved features of a highly stable protease from *Penaeus vannamei* by immobilization on glutaraldehyde activated graphene oxide nanosheets, Int. J. Biol. Macromol. 130 (2019) 564-572, https://doi.org/10.1016/j.ijbiomac.2019.02.163.

[88] S.A. Shirdel, K. Khajeh, S.M. Asghari, H.R. Karbalaei- Heidari, Enhancement of thermostability and resistance against autolysis in a zinc metalloprotease, Eng. Life Sci. 14 (2014) 229-234, https://doi.org/10.1002/elsc.201200218.

[89] X. Liang, Y. Bian, X.-F. Tang, G. Xiao, B. Tang, Enhancement of keratinolytic activity of a thermophilic subtilase by improving its autolysis resistance and thermostability under reducing conditions, Appl. Microbiol. Biotechnol. 87 (2010) 999-1006, https://doi.org/10.1007/s00253-010-2534-2.

[90] D. Russell, N.J. Oldham, B.G. Davis, Site-selective chemical protein glycosylation protects from autolysis and proteolytic degradation, Carbohydr. Res. 344 (2009) 1508-1514, https://doi.org/10.1016/j.carres.2009.06.033.

[91] S. Klomklao, H. Kishimura, S. Benjakul, B.K. Simpson, Autolysis and biochemical properties of endogenous proteinases in Japanese sandfish (*Arctoscopus japonicus*), Int. J. Food Sci. Technol. 44 (2009) 1344-1350, https://doi.org/10.1111/j.1365-2621.2009.01963.x.

[92] N. Ktari, N. Fakhfakh, R. Bani, H. Ben Khaled, M. Nasri, A. Bougatef, Effect of degree of hydrolysis and protease type on the antioxidant activity of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products, J. Aquat. Food Prod. Technol. 22 (2013) 436-448, https://doi.org/10.1080/10498850.2012.658961.

[93] H. Herpandi, N. Huda, A. Rosma, W.A. Wan Nadia, Degree of hydrolysis and free tryptophan content of skipjack tuna (*Katsuwonus pelamis*) protein hydrolysates produced with different type of industrial proteases, Int. Food Res. J. 19 (2012) 863-867.

[94] M.K. Nyo, L.T. Nguyen, Value-addition of defatted peanut cake by proteolysis: Effects of proteases and degree of hydrolysis on functional properties and antioxidant capacity of peptides, Waste Biomass Valor. 10 (2019) 1251-1259, https://doi.org/10.1007/s12649-017-0146-0.

[95] D. Konieczny, A.K. Stone, M.G. Nosworthy, J.D. House, D.R. Korber, M.T. Nickerson, T. Tanaka, Nutritional properties of pea protein- enriched flour treated with different proteases to varying degrees of hydrolysis, Cereal Chem. 97 (2020) 429-440, https://doi.org/10.1002/cche.10258.

[96] K. Venkatachalam, M. Nagarajan, Assessment of different proteases on degree of hydrolysis, functional properties and radical scavenging activities of salted duck egg white hydrolysate, J. Food Sci. Technol. 56 (2019) 3137-3144, https://doi.org/10.1007/s13197-019-03645-5.

[97] M. Cheryan, P.J. Van Wyk, N.F. Olson, T. Richardson, Continuous coagulation of milk using immobilized enzymes in a fluidized-bed reactor, Biotechnol. Bioeng. 17 (1975) 585-598, https://doi.org/10.1002/bit.260170410.

[98] A. Carlson, G.C. Hill, N.F. Olson, The coagulation of milk with immobilized enzymes: a critical review, Enzyme Microb. Technol. 8 (11) (1986) 642-650, https://doi.org/10.1016/0141-0229(86)90059-1.

[99] M. Esposito, P. Di Pierro, W. Dejonghe, L. Mariniello, R. Porta, Enzymatic milk clotting activity in artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus*) flowers. Immobilization of alpine thistle aspartic protease, Food Chem. 204 (2016) 115-121, https://doi.org/10.1016/j.foodchem.2016.02.060.

[100] E. H. Siar, R. Morellon-Sterling, M.N. Zidoune, R. Fernandez-Lafuente, Use of glyoxyl-agarose immobilized ficin extract in milk coagulation: Unexpected importance of the ficin loading on the biocatalysts, Int. J. Biol. Macromol. 144 (2020) 419-426, https://doi.org/10.1016/j.ijbiomac.2019.12.140.

[101] B.C.C. Pessela, R. Torres, M. Fuentes, C. Mateo, R. Fernandez-Lafuente, J.M. Guisán, Immobilization of Rennet from *Mucor miehei* via its sugar chain. Its use in milk coagulation, Biomacromolecules. 5 (2004) 2029-2033, https://doi.org/10.1021/bm049735c.

[102] R. Morellon-Sterling, H. El-Siar, O.L. Tavano, Á. Berenguer-Murcia, R. Fernández-Lafuente, Ficin: A protease extract with relevance in biotechnology and biocatalysis, Int. J. Biol. Macromol. 162 (2020) 394-404, https://doi.org/10.1016/j.ijbiomac.2020.06.144.

[103] C.M. Manohar, V. Prabhawathi, P.M. Sivakumar, M. Doble, Design of a papain immobilized antimicrobial food package with curcumin as a crosslinker, PloS One. 10 (2015), e0121665. https://doi.org/10.1371/journal.pone.0121665.

[104] P. Veluchamy, P.M. Sivakumar, M. Doble, Immobilization of subtilisin on polycaprolactam for antimicrobial food packaging applications, J. Agric. Food Chem. 59 (2011) 10869-10878, https://doi.org/10.1021/jf201124v.

[105] H. Eshamah, I. Han, H. Nam, J. Acton, P. Dawson, Antibacterial effects of natural tenderizing enzymes on different strains of *Escherichia coli* O157: H7 and *Listeria monocytogenes* on beef, Meat Sci. 96 (2014) 1494-1500, https://doi.org/10.1016/j.meatsci.2013.12.010.

[106] K. Atacan, M. Özacar, M. Özacar, Investigation of antibacterial properties of novel papain immobilized on tannic acid modified Ag/$CuFe_2O_4$ magnetic nanoparticles, Int. J. Biol. Macromol. 109 (2018) 720-731, https://doi.org/10.1016/j.ijbiomac.2017.12.066.

[107] C.M. Manohar, S.D. Kundgar, M. Doble, Betanin immobilized LDPE as antimicrobial food wrapper, LWT. 80 (2017) 131-135, https://doi.org/10.1016/j.lwt.2016.07.020.

[108] K. Hernandez, R. Fernandez-Lafuente, Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance, Enzyme Microb. Technol. 48 (2011) 107-122, https://doi.org/10.1016/j.enzmictec.2010.10.003.

[109] E. H. Siar, S. Arana-Peña, O. Barbosa, M. Zidoune, R. Fernandez-Lafuente, Immobilization/stabilization of ficin extract on glutaraldehyde-activated agarose beads. Variables that control the final stability and activity in protein hydrolyses, Catalysts. 8 (2018) 149, https://doi.org/10.3390/catal8040149.

[110] E.P. Cipolatti, A. Valerio, R.O. Henriques, D.E. Moritz, J.L. Ninow, D.M.G. Freire, E.A. Manoel, R. Fernandez-Lafuente, D. de Oliveira, Nanomaterials for biocatalyst immobilization–state of the art and future trends, RSC Adv. 6 (2016) 104675-104692, https://doi.org/10.1039/C6RA22047A.

[111] L.A. Lopes, P.K. Novelli, K. Fernandez-Lafuente, P.W. Tardioli, R.L.C. Giordano, Glyoxyl-activated agarose as support for covalently link Novo-Pro D: Biocatalysts performance in the hydrolysis of casein, Catalysts. 10 (2020) 466, https://doi.org/10.3390/catal10050466.

[112] R.M. Blanco, J.J. Calvete, J. Guisán, Immobilization-stabilization of enzymes; variables that control the intensity of the trypsin (amine)-agarose (aldehyde) multipoint attachment, Enzyme Microb. Technol. 11 (1989) 353-359, https://doi.org/10.1016/0141-0229(89)90019-7.

[113] J. Pedroche, M. del Mar Yust, C. Mateo, R. Fernández-Lafuente, J. Girón-Calle, M. Alaiz, J. Vioque, J.M. Guisán, F. Millán, Effect of the support and experimental conditions

in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: correlation between enzyme–support linkages and thermal stability, Enzyme Microb. Technol. 40 (2007) 1160-1166, https://doi.org/10.1016/j.enzmictec.2006.08.023.

[114] M. del Mar Yust, J. Pedroche, M. del Carmen Millán-Linares, J.M. Alcaide-Hidalgo, F. Millán, Improvement of functional properties of chickpea proteins by hydrolysis with immobilised Alcalase, Food Chem. 122 (2010) 1212-1217, https://doi.org/10.1016/j.foodchem.2010.03.121.

[115] P.W. Tardioli, R. Sousa Jr, R.C. Giordano, R.L.C. Giordano, Kinetic model of the hydrolysis of polypeptides catalyzed by Alcalase® immobilized on 10% glyoxyl-agarose, Enzyme Microb. Technol. 36 (2005) 555-564, https://doi.org/10.1016/j.enzmictec.2004.12.002.

[116] M.M. Resende, R. Sousa Jr, P.W. Tardioli, R.L.C. Giordano, R.C. Giordano, Enzymatic tailor- made proteolysis of whey in a vortex flow reactor, AIChE J. 51 (2005) 314-322, https://doi.org/10.1002/aic.10241.

[117] P.W. Tardioli, J. Pedroche, R.L.C. Giordano, R. Fernández- Lafuente, J.M. Guisan, Hydrolysis of proteins by immobilized- stabilized Alcalase- glyoxyl agarose, Biotechnol. Prog. 19 (2003) 352-360, https://doi.org/10.1021/bp025588n.

[118] T.B. Pessato, N.C. de Carvalho, O.L. Tavano, L.G.R. Fernandes, R.d.L. Zollner, F.M. Netto, Whey protein isolate hydrolysates obtained with free and immobilized Alcalase: Characterization and detection of residual allergens, Food Res. Int. 83 (2016) 112-120, https://doi.org/10.1016/j.foodres.2016.02.015.

[119] C. Bernal, F. Guzman, A. Illanes, L. Wilson, Selective and eco-friendly synthesis of lipoaminoacid-based surfactants for food, using immobilized lipase and protease biocatalysts, Food Chem. 239 (2018) 189-195, https://doi.org/10.1016/j.foodchem.2017.06.105.

[120] L.N. Corîci, A.E. Frissen, D.-J. van Zoelen, I.F. Eggen, F. Peter, C.M. Davidescu, C.G. Boeriu, Sol–gel immobilization of Alcalase from *Bacillus licheniformis* for application in the synthesis of C-terminal peptide amides, J. Mol. Catal. B Enzym. 73 (2011) 90-97, https://doi.org/10.1016/j.molcatb.2011.08.004.

[121] P. Vossenberg, H.H. Beeftink, M.A. Cohen Stuart, H. Tramper, Immobilization to prevent enzyme incompatibility with proteases, Biocatal. Biotransform. 29 (2011) 288-298, https://doi.org/10.3109/10242422.2011.631213.

[122] P. Vossenberg, H.H. Beeftink, T. Nuijens, P. Quaedflieg, M.A.C. Stuart, J. Tramper, Performance of Alcalase formulations in near dry organic media: Effect of enzyme hydration on dipeptide synthesis, J. Mol. Catal. B Enzym. 78 (2012) 24-31, https://doi.org/10.1016/j.molcatb.2012.01.022.

[123] V. Kasche, Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, peptides and other condensation products, Enzyme Microb. Technol. 8 (1986) 4-16, https://doi.org/10.1016/0141-0229(86)90003-7.

[124] R. Fernandez-Lafuente, C.M. Rosell, J.M. Guisan, The presence of methanol exerts a strong and complex modulation of the synthesis of different antibiotics by immobilized penicillin G acylase, Enzyme Microb. Technol. 23 (1998) 305-310, https://doi.org/10.1016/S0141-0229(98)00053-2.

[125] P. Vossenberg, H.H. Beeftink, M.A.C. Stuart, J. Tramper, Kinetics of Alcalase-catalyzed dipeptide synthesis in near-anhydrous organic media, J. Mol. Catal. B Enzym. 87 (2013) 113-120, https://doi.org/10.1016/j.molcatb.2012.11.002.

[126] P. Vossenberg, H.H. Beeftink, T. Nuijens, P. Quaedflieg, M.A.C. Stuart, J. Tramper, Dipeptide synthesis in near-anhydrous organic media: Long-term stability and reusability of immobilized Alcalase, J. Mol. Catal. B Enzym. 93 (2013) 23-27, https://doi.org/10.1016/j.molcatb.2013.03.014.

[127] P. Vossenberg, R. Beeftink, M.C. Stuart, H. Tramper, Effect of enzyme dehydration on Alcalase- catalyzed dipeptide synthesis in near- anhydrous organic media, Biotechnol. Prog. 29 (2013) 870-875, https://doi.org/10.1002/btpr.1737.

[128] L. Cao, F. van Rantwijk, R.A. Sheldon, Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase, Org. Lett. 2 (2000) 1361-1364, https://doi.org/10.1021/ol005593x.

[129] R. Schoevaart, M.W. Wolbers, M. Golubovic, M. Ottens, A.P.G. Kieboom, F. Van Rantwijk, L.A.M. Van der Wielen, R.A. Sheldon, Preparation, optimization, and structures of cross- linked enzyme aggregates (CLEAs), Biotechnol. Bioeng. 87 (2004) 754-762, https://doi.org/10.1002/bit.20184.

[130] P. Vossenberg, H.H. Beeftink, T. Nuijens, M.A.C. Stuart, J. Tramper, Selecting optimal conditions for Alcalase CLEA-OM for synthesis of dipeptides in organic media, J. Mol. Catal. B Enzym. 75 (2012) 43-49, https://doi.org/10.1016/j.molcatb.2011.11.008.

[131] M. López- Iglesias, E. Busto, V. Gotor, V. Gotor- Fernández, Use of protease from *Bacillus licheniformis* as promiscuous catalyst for organic synthesis: Applications in C-C

and C-N bond formation reactions, Adv. Synth. Catal. 353 (2011) 2345-2353, https://doi.org/10.1002/adsc.201100347.

[132] P. Ferraboschi, M. De Mieri, F. Galimberti, Chemo-enzymatic approach to the synthesis of the antithrombotic clopidogrel, Tetrahedron Asymmetry. 21 (2010) 2136-2141, https://doi.org/10.1016/j.tetasy.2010.06.040.

[133] S. Wang, C. Zhang, B. Qi, X. Sui, L. Jiang, Y. Li, Z. Wang, H. Feng, R. Wang, Q. Zhang, Immobilized Alcalase alkaline protease on the magnetic chitosan nanoparticles used for soy protein isolate hydrolysis, Eur. Food Res. Technol. 239 (2014) 1051-1059, https://doi.org/10.1007/s00217-014-2301-1.

[134] G. Tofani, A. Petri, O. Piccolo, Preparation of enantiomerically pure N-heterocyclic amino alcohols by enzymatic kinetic resolution, Tetrahedron Asymmetry. 26 (2015) 638-643, https://doi.org/10.1016/j.tetasy.2015.04.009.

[135] P. Falus, L. Cerioli, G. Bajnóczi, Z. Boros, D. Weiser, J. Nagy, D. Tessaro, S. Servi, L. Poppe, A continuous- flow cascade reactor system for subtilisin A- catalyzed dynamic kinetic resolution of N- *tert*- Butyloxycarbonylphenylalanine ethyl thioester with benzylamine, Adv. Synth. Catal. 358 (2016) 1608-1617, https://doi.org/10.1002/adsc.201500902.

[136] C.L. Fenoglio, N. Vierling, R.M. Manzo, R.J. Ceruti, G.A. Sihufe, E.J. Mammarella, Whey protein hydrolysis with free and immobilized Alcalase®: effects of operating parameters on the modulation of peptide profiles obtained, Am. J. Food. Technol. 11 (2016)152-158, https://doi.org/10.3923/ajft.2016.152.158.

[137] B. Wang, T. Meng, H. Ma, Y. Zhang, Y. Li, J. Jin, X. Ye, Mechanism study of dual-frequency ultrasound assisted enzymolysis on rapeseed protein by immobilized Alcalase, Ultrason. Sonochem. 32 (2016) 307-313, https://doi.org/10.1016/j.ultsonch.2016.03.023.

[138] T. Meng, H. Ma, W. Wang, X. Ye, Studies on the hydrolyzing casein by immobilized alkaline protease, Journal of Chinese Institute of Food Science and Technology, 16 (2016) 87-93, https://doi.org/10.16429/j.1009-7848.2016.08.013.

[139] W. Qu, R.M. Sehemu, T. Zhang, B. Song, L. Yang, X. Ren, H. Ma, Immobilized enzymolysis of corn gluten meal under triple-frequency ultrasound, Int. J. Food Eng. 14 (2018) 20170347, https://doi.org/10.1515/ijfe-2017-0347.

[140] M.G. Žuža, N.Z. Milašinović, M.M. Jovović, J.R. Jovanović, M.T.K. Krušić, B.M. Bugarski, Z.D. Knežević-Jugović, Design and characterization of Alcalase–chitosan conjugates as potential biocatalysts, Bioprocess Biosyst. Eng. 40 (2017) 1713-1723, https://doi.org/10.1007/s00449-017-1826-7.

[141] B. Özbek, Ş. Ünal, Preparation and characterization of polymer-coated mesoporous silica nanoparticles and their application in Subtilisin immobilization, Korean J. Chem. Eng. 34 (2017) 1992-2001, https://doi.org/10.1007/s11814-017-0045-x.

[142] T.A. Siswoyo, N.F. Matra, A.A. Safiera, A. Supriyadi, Synthesis of antioxidant peptides from Melinjo (*Gnetum gnemon*) seed protein isolated using sol-gel immobilized Alcalase, Inter. J. Adv. Sci. Eng. Inf. Tech 7 (2017) 2088-5334, http://dx.doi.org/10.18517/ijaseit.7.4.936.

[143] A. Yang, C. Long, J. Xia, P. Tong, Y. Cheng, Y. Wang, H. Chen, Enzymatic characterisation of the immobilised Alcalase to hydrolyse egg white protein for potential

allergenicity reduction, J. Sci. Food Agric. 97 (2017) 199-206, http://dx.doi.org/10.1002/jsfa.7712.

[144] C.-K. Wei, K. Thakur, D.-H. Liu, J.-G. Zhang, Z.-J. Wei, Enzymatic hydrolysis of flaxseed (*Linum usitatissimum* L.) protein and sensory characterization of Maillard reaction products, Food Chem. 263 (2018) 186-193, https://doi.org/10.1016/j.foodchem.2018.04.120.

[145] T. Sewczyk, M.H. Antink, M. Maas, S. Kroll, S. Beutel, Flow rate dependent continuous hydrolysis of protein isolates, AMB Express. 8 (2018) 1-9, https://doi.org/10.1186/s13568-018-0548-9.

[146] X. Wu, M. Hou, J. Ge, Metal–organic frameworks and inorganic nanoflowers: a type of emerging inorganic crystal nanocarrier for enzyme immobilization, Catal. Sci. Technol. 5 (2015) 5077-5085, https://doi.org/10.1039/C5CY01181G.

[147] C. Altinkaynak, S. Tavlasoglu, I. Ocsoy, A new generation approach in enzyme immobilization: Organic-inorganic hybrid nanoflowers with enhanced catalytic activity and stability, Enzyme Microb. Technol. 93 (2016) 105-112, https://doi.org/10.1016/j.enzmictec.2016.06.011.

[148] A.H. Memon, R. Ding, Q. Yuan, Y. Wei, H. Liang, Facile synthesis of Alcalase-inorganic hybrid nanoflowers used for soy protein isolate hydrolysis to improve its functional properties, Food Chem. 289 (2019) 568-574, https://doi.org/10.1016/j.foodchem.2019.03.096.

[149] F. Hussain, S. Arana-Peña, R. Morellon-Sterling, O. Barbosa, S. Ait Braham, S. Kamal, R. Fernandez-Lafuente, Further stabilization of Alcalase immobilized on glyoxyl

supports: Amination plus modification with glutaraldehyde, Molecules. 23 (2018) 3188, https://doi.org/10.3390/molecules23123188.

[150] N. Rueda, J.C.S. dos Santos, C. Ortiz, R. Torres, O. Barbosa, R.C. Rodrigues, Á. Berenguer- Murcia, R. Fernandez- Lafuente, Chemical modification in the design of immobilized enzyme biocatalysts: Drawbacks and opportunities, Chem. Rec. 16 (2016) 1436-1455, https://doi.org/10.1002/tcr.201600007.

[151] R.C. Rodrigues, O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. Fernandez-Lafuente, Amination of enzymes to improve biocatalyst performance: coupling genetic modification and physicochemical tools, RSC Adv. 4 (2014) 38350-38374, https://doi.org/10.1039/C4RA04625K.

[152] S. Ait Braham, F. Hussain, R. Morellon- Sterling, S. Kamal, J.F. Kornecki, O. Barbosa, D.E. Kati, R. Fernandez- Lafuente, Cooperativity of covalent attachment and ion exchange on Alcalase immobilization using glutaraldehyde chemistry: Enzyme stabilization and improved proteolytic activity, Biotechnol. Prog. 35 (2019) e2768, https://doi.org/10.1002/btpr.2768.

[153] A.I. Benítez-Mateos, M.L. Contente, S. Velasco-Lozano, F. Paradisi, F. López-Gallego, Self-sufficient flow-biocatalysis by coimmobilization of pyridoxal 5′-phosphate and ω-transaminases onto porous carriers, ACS Sustainable Chem. Eng. 6 (2018) 13151-13159, https://doi.org/10.1021/acssuschemeng.8b02672.

[154] S. Peirce, J.J. Virgen-Ortíz, V.G. Tacias-Pascacio, N. Rueda, R. Bartolome-Cabrero, L. Fernandez-Lopez, M.E. Russo, A. Marzocchella, R. Fernandez-Lafuente, Development of simple protocols to solve the problems of enzyme coimmobilization. Application to

coimmobilize a lipase and a β-galactosidase, RSC Adv. 6 (2016) 61707-61715, https://doi.org/10.1039/C6RA10906C.

[155] S. Arana-Peña, D. Carballares, R. Morellon-Sterlling, Á. Berenguer-Murcia, A.R. Alcántara, R.C. Rodrigues, R. Fernandez-Lafuente, Enzyme co-immobilization: always the biocatalyst designers' choice…or not?, Biotechnol. Adv. In press https://doi.org/10.1016/j.biotechadv.2020.107584.

[156] A. Clemente, J. Vioque, R. Sanchez-Vioque, J. Pedroche, F. Millán, Production of extensive chickpea (*Cicer arietinum* L.) protein hydrolysates with reduced antigenic activity, J. Agric. Food Chem. 47 (1999) 3776-3781, https://doi.org/10.1021/jf981315p.

[157] J. Pedroche, M.M. Yust, H. Lqari, C. Megías, J. Giron-Calle, M. Alaiz, J. Vioque, F. Millan, Obtaining of *Brassica carinata* protein hydrolysates enriched in bioactive peptides using immobilized digestive proteases, Food Res. Int. 40 (2007) 931-938, https://doi.org/10.1016/j.foodres.2007.04.001.

[158] Y. Wang, H. Chen, J. Wang, L. Xing, Preparation of active corn peptides from zein through double enzymes immobilized with calcium alginate–chitosan beads, Process Biochem. 49 (2014) 1682-1690, https://doi.org/10.1016/j.procbio.2014.07.002.

[159] Z. Chen, X. Wang, Y. Chen, Z. Xue, Q. Guo, Q. Ma, H. Chen, Preparation and characterization of a novel nanocomposite with double enzymes immobilized on magnetic Fe_3O_4-chitosan-sodium tripolyphosphate, Colloids Surf. B Biointerfaces. 169 (2018) 280-288, https://doi.org/10.1016/j.colsurfb.2018.04.066.

[160] C. Sutthiwanjampa, S.M. Kim, Antioxidant, Anti-tyrosinase and immunomodulatory activities of the enzymatic boiled *Venus Clam* hydrolysate, Chiang Mai Journal of Science. 45 (2018) 249-262.

[161] J. Xie, M. Du, M. Shen, T. Wu, L. Lin, Physico-chemical properties, antioxidant activities and angiotensin-I converting enzyme inhibitory of protein hydrolysates from Mung bean (*Vigna radiate*), Food Chem. 270 (2019) 243-250, https://doi.org/10.1016/j.foodchem.2018.07.103.

[162] J.G. dos Santos Aguilar, A.K.S. de Souza, R.J.S. de Castro, Enzymatic hydrolysis of chicken viscera to obtain added-value protein hydrolysates with antioxidant and antihypertensive Properties, Int. J. Pept. Res. Ther. 26 (2019) 717-725, https://doi.org/10.1007/s10989-019-09879-3.

[163] C. Lammi, G. Aiello, G. Boschin, A. Arnoldi, Multifunctional peptides for the prevention of cardiovascular disease: A new concept in the area of bioactive food-derived peptides, J. Func. Foods. 55 (2019) 135-145, https://doi.org/10.1016/j.jff.2019.02.016.

[164] Z. Karami, S.H. Peighambardoust, J. Hesari, B. Akbari-Adergani, D. Andreu, Antioxidant, anticancer and ACE-inhibitory activities of bioactive peptides from wheat germ protein hydrolysates, Food Biosci. 32 (2019) 100450, https://doi.org/10.1016/j.fbio.2019.100450.

[165] C.M. Montone, A.L. Capriotti, C. Cavaliere, G. La Barbera, S. Piovesana, R.Z. Chiozzi, A. Laganà, Characterization of antioxidant and angiotensin-converting enzyme inhibitory peptides derived from cauliflower by-products by multidimensional liquid

chromatography and bioinformatics, J. Func. Foods. 44 (2018) 40-47, https://doi.org/10.1016/j.jff.2018.02.022.

[166] Y. Zheng, Y. Li, G. Li, ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates: purification, identification and synthesis, RSC Adv. 9 (2019) 5925-5936, https://doi.org/10.1039/C8RA10269D.

[167] C.S. Yang, J.M. Landau, M.-T. Huang, H.L. Newmark, Inhibition of carcinogenesis by dietary polyphenolic compounds, Annu. Rev. Nutr. 21 (2001) 381-406, https://doi.org/10.1146/annurev.nutr.21.1.381.

[168] H.L. Jang, A.M. Liceaga, K.Y. Yoon, Purification, characterisation and stability of an antioxidant peptide derived from sandfish (*Arctoscopus japonicus*) protein hydrolysates, J. Func. Foods. 20 (2016) 433-442, https://doi.org/10.1016/j.jff.2015.11.020.

[169] A. Bougatef, N. Nedjar-Arroume, L. Manni, R. Ravallec, A. Barkia, D. Guillochon, M. Nasri, Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins, Food Chem. 118 (2010) 559-565, https://doi.org/10.1016/j.foodchem.2009.05.021.

[170] N. Ji, C. Sun, Y. Zhao, L. Xiong, Q. Sun, Purification and identification of antioxidant peptides from peanut protein isolate hydrolysates using UHR-Q-TOF mass spectrometer, Food Chem. 161 (2014) 148-154, https://doi.org/10.1016/j.foodchem.2014.04.010.

[171] S.-K. Kim, I. Wijesekara, Development and biological activities of marine-derived bioactive peptides: A review, J. Func. Foods. 2 (2010) 1-9, https://doi.org/10.1016/j.jff.2010.01.003.

[172] R. Kahl, H. Kappus, Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E, Z Lebensm Unters Forsch. 196 (1993) 329-338, https://doi.org/10.1007/BF01197931.

[173] A.G.P. Samaranayaka, E.C.Y. Li-Chan, Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications, J. Func. Foods. 3 (2011) 229-254, https://doi.org/10.1016/j.jff.2011.05.006.

[174] P. Puchalska, M.L. Marina, M.C. García, Isolation and identification of antioxidant peptides from commercial soybean-based infant formulas, Food Chem. 148 (2014) 147-154, https://doi.org/10.1016/j.foodchem.2013.10.030.

[175] S. Sakanaka, Y. Tachibana, N. Ishihara, J.K. Juneja, Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates, J. Agric. Food Chem. 53 (2005) 464-468, https://doi.org/10.1021/jf0487699.

[176] N.S. Sampath Kumar, R.A. Nazeer, R. Jaiganesh, Purification and identification of antioxidant peptides from the skin protein hydrolysate of two marine fishes, horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*), Amino Acids. 42 (2012) 1641-1649, https://doi.org/10.1007/s00726-011-0858-6.

[177] C. F. Chi, F. Y. Hu, B. Wang, X. J. Ren, S. G. Deng, C. W. Wu, Purification and characterization of three antioxidant peptides from protein hydrolyzate of croceine croaker (*Pseudosciaena crocea*) muscle, Food Chem. 168 (2015) 662-667, https://doi.org/10.1016/j.foodchem.2014.07.117.

[178] F. Hu, A. T. Ci, H. Wang, Y. Y. Zhang, J. G. Zhang, K. Thakur, Z.-J. Wei, Identification and hydrolysis kinetic of a novel antioxidant peptide from pecan meal using

Alcalase, Food Chem. 261 (2018) 301-310, https://doi.org/10.1016/j.foodchem.2018.04.025.

[179] S.Y. Kim, J.Y. Je, S.K. Kim, Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion, J. Nutr. Biochem. 18 (2007) 31-38, https://doi.org/10.1016/j.jnutbio.2006.02.006.

[180] R. Darmawan, N.A. Bringe, E.G. de Mejia, Antioxidant capacity of Alcalase hydrolysates and protein profiles of two conventional and seven low glycinin soybean cultivars, Plant Foods Hum. Nutr. 65 (2010) 233-240, https://doi.org/10.1007/s11130-010-0185-1.

[181] M.G. Vernaza, V.P. Dia, E.G. de Mejia, Y.K. Chang, Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours, Food Chem. 134 (2012) 2217-2225, https://doi.org/10.1016/j.foodchem.2012.04.037.

[182] S. Lin, J. Wang, P. Zhao, Y. Yang, H. Ye, Y. Yuan, J. Liu, G. Jones, Optimized antioxidant peptides fractions preparation and secondary structure analysis by MIR, Int. J. Biol. Macromol. 59 (2013) 151-157, https://doi.org/10.1016/j.ijbiomac.2013.04.008.

[183] X. H. Zhao, J. Z. Song, Evaluation of antioxidant properties *in vitro* of plastein-reaction-stressed soybean protein hydrolysate, Int. J. Food Prop. 17 (2014) 152-162, https://doi.org/10.1080/10942912.2011.617025.

[184] S.Y. Park, J.S. Lee, H.H. Baek, H.G. Lee, Purification and characterization of antioxidant peptides from soy protein hydrolysate, J. Food Biochem. 34 (2010) 120-132, https://doi.org/10.1111/j.1745-4514.2009.00313.x.

[185] Q. Zhang, X. Tong, B. Qi, Z. Wang, Y. Li, X. Sui, L. Jiang, Changes in antioxidant activity of Alcalase-hydrolyzed soybean hydrolysate under simulated gastrointestinal digestion and transepithelial transport, J. Func. Foods. 42 (2018) 298-305, https://doi.org/10.1016/j.jff.2018.01.017.

[186] Q. Zhang, X. Tong, X. Sui, Z. Wang, B. Qi, Y. Li, L. Jiang, Antioxidant activity and protective effects of Alcalase-hydrolyzed soybean hydrolysate in human intestinal epithelial Caco-2 cells, Food Res. Int. 111 (2018) 256-264., https://doi.org/10.1016/j.foodres.2018.05.046.

[187] Q. Zhang, X. Tong, Y. Li, H. Wang, Z. Wang, B. Qi, X. Sui, L. Jiang, Purification and characterization of antioxidant peptides from Alcalase-hydrolyzed soybean (*Glycine max* L.) hydrolysate and their cytoprotective effects in human intestinal Caco-2 cells, J. Agric. Food Chem. 67 (2019) 5772-5781, https://doi.org/10.1021/acs.jafc.9b01235.

[188] H.M. Li, X.I.N. Hu, P. Guo, Y. Fu, L.I. Xu, X.Z. Zhang, Antioxidant properties and possible mode of action of corn protein peptides and zein peptides, J. Food Biochem. 34 (2010) 44-60, https://doi.org/10.1111/j.1745-4514.2009.00292.x.

[189] X. Tang, Z. He, Y. Dai, Y.L. Xiong, M. Xie, J. Chen, Peptide fractionation and free radical scavenging activity of zein hydrolysate, J. Agric. Food Chem. 58 (2010) 587-593, https://doi.org/10.1021/jf9028656.

[190] X. Ren, Q. Liang, X. Zhang, T. Hou, S. Li, H. Ma, Stability and antioxidant activities of corn protein hydrolysates under simulated gastrointestinal digestion, Cereal Chem. 95 (2018) 760-769, https://doi.org/10.1002/cche.10092.

[191] V.A. Tironi, M.C. Añón, Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis, Food Res. Int. 43 (2010) 315-322, https://doi.org/10.1016/j.foodres.2009.10.001.

[192] C. Pazinatto, L.G. Malta, G.M. Pastore, F. Maria Netto, Antioxidant capacity of amaranth products: effects of thermal and enzymatic treatments, Food Sci. Technol. 33 (2013) 485-493, https://doi.org/10.1590/S0101-20612013005000076.

[193] S.F. Garcia Filleria, V.A. Tironi, Application of amaranth protein isolate and hydrolysate on a reduced salt fish restructured product: antioxidant properties, textural and microbiological effects, Int. J. Food Sci. Technol. 50 (2015) 1452-1460, https://doi.org/10.1111/ijfs.12777.

[194] S. Du, X. Yu, Z. Li, Ultrasonic pretreatment for preparation of antioxidant peptides from chickpea protein, Journal of Chinese Institute of Food Science and Technology 13 (2013) 80-88.

[195] H. Wang, S. Du, Y. Zhao, L. Qiao, Modification of chickpea protein hydrolysates by Alcalase-catalyzed plastein reaction and improvement in antioxidant activity, Journal of Chinese Institute of Food Science and Technology. 15 (2015) 34-40, https://doi.org/10.16429/j.1009-7848.2015.01.006.

[196] A.M. Ghribi, A. Sila, R. Przybylski, N. Nedjar-Arroume, I. Makhlouf, C. Blecker, H. Attia, P. Dhulster, A. Bougatef, S. Besbes, Purification and identification of novel antioxidant peptides from enzymatic hydrolysate of chickpea (*Cicer arietinum* L.) protein concentrate, J. Func. Foods. 12 (2015) 516-525, https://doi.org/10.1016/j.jff.2014.12.011.

[197] L. Wattanasiritham, S. Kubglomsong, C. Theerakulkait, Antioxidant activity of rice bran protein extract, its enzymatic hydrolysates and its combination with commercial antioxidants, Pak. J. Nutr. 14 (2015) 647, https://doi.org/10.3923/pjn.2015.647.652.

[198] W. Sarringkarin, T. Laokuldilok, Optimization of the production conditions of glutinous rice bran protein hydrolysate with antioxidative properties, Chiang Mai University Journal of Natural Sciences. 16 (2017) 1-18, https://doi.org/10.12982/cmujns.2017.0001.

[199] Z. Wang, S. Cui, D. Yuan, X. Zhao, G. An, L. Li, Antioxidant activity and molecular weight distribution of enzymatic hydrolysates of rice protein pretreated with high pressures, J. Chinese Cereals Oils Assoc. 28 (2013) 1-4.

[200] J. Ding, R. Liang, Y. Yang, N. Sun, S. Lin, Optimization of pea protein hydrolysate preparation and purification of antioxidant peptides based on an in silico analytical approach, LWT. 123 (2020) 109126 https://doi.org/10.1016/j.lwt.2020.109126.

[201] A.T. Girgih, D. Chao, L. Lin, R. He, S. Jung, R.E. Aluko, Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment, Food Chem. 188 (2015) 510-516, https://doi.org/10.1016/j.foodchem.2015.05.024.

[202] M. Pan, T.S. Jiang, J.L. Pan, Antioxidant activities of rapeseed protein hydrolysates, Food Bioprocess Technol. 4 (2011) 1144-1152, https://doi.org/10.1007/s11947-009-0206-y.

[203] L. Wang, J. Yang, Y. Wang, J. Zhang, Y. Gao, J. Yuan, A. Su, X. Ju, Study on antioxidant activity and amino acid analysis of rapeseed protein hydrolysates, Int. J. Food Prop. 19 (2016) 1899-1911, https://doi.org/10.1080/10942912.2015.1085397.

[204] F.G.D. e Silva, B. Hernández-Ledesma, L. Amigo, F.M. Netto, B. Miralles, Identification of peptides released from flaxseed (*Linum usitatissimum*) protein by Alcalase® hydrolysis: Antioxidant activity, LWT-Food Sci. Technol. 76 (2017) 140-146, https://doi.org/10.1016/j.lwt.2016.10.049.

[205] F.G.D. Silva, Y. O'callagahan, N.M. O'brien, F.M. Netto, Antioxidant capacity of flaxseed products: the effect of *in vitro* digestion, Plant Foods Hum. Nutr. 68 (2013) 24-30, https://doi.org/10.1007/s11130-012-0329-6.

[206] C.F. Ajibola, J.B. Fashakin, T.N. Fagbemi, R.E. Aluko, Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions, Int. J. Mol. Sci. 12 (2011) 6685-6702, https://doi.org/10.3390/ijms12106685.

[207] X. Li, S. Shen, J. Deng, T. Li, C. Ding, Antioxidant activities and functional properties of tea seed protein hydrolysates (*Camellia oleifera Abel.*) influenced by the degree of enzymatic hydrolysis, Food Sci. Biotechnol. 23 (2014) 2075-2082, https://doi.org/10.1007/s10068-014-0282-2.

[208] N.M. Rafi, N.R.A. Halim, A.M. Amin, N.M. Sarbon, Response surface optimization of enzymatic hydrolysis conditions of lead tree (*Leucaena leucocephala*) seed hydrolysate, Int. Food Res. J. 22 (2015) 1015-1023.

[209] S. Kaveh, A.R. Sadeghimahoonak, M. Ghorbani, M. Jafari, Optimization of factors affecting the antioxidant activity of fenugreek seed's protein hydrolysate by Response Surface Methodology, Iranian J. Nutr. Sci. Food Technol. 14 (2019) 77-88.

[210] S. Xu, Y. Shen, Y. Li, Antioxidant activities of sorghum kafirin Alcalase hydrolysates and membrane/gel filtrated fractions, Antioxidants 8 (2019) 131, https://doi.org/10.3390/antiox8050131.

[211] C. Ning, S. Yi, L. Shu-Ying, Preparation and antioxidant activities of walnut protein hydrolysates, Chem. J. Chinese Univ. 34 (2013) 72-76, https://doi.org/10.7503/cjcu20120277.

[212] F. Zhang, J. Qu, K. Thakur, J.-G. Zhang, A. Mocan, Z.-J. Wei, Purification and identification of an antioxidative peptide from peony (*Paeonia suffruticosa* Andr.) seed dreg, Food Chem. 285 (2019) 266-274, https://doi.org/10.1016/j.foodchem.2019.01.168.

[213] T.Chai, Z.Soo, K. Hsu, J. Li, F. Abd Manan, F. Wong, Antioxidant activity of Semen Cassiae protein hydrolysate: Thermal and gastrointestinal stability, peptide identification, and in silico analysis, Modern Food Sci. Tech. 35 (2019) 38-48, https://doi.org/10.13982/j.mfst.1673-9078.2019.9.004.

[214] S. Ma, M. Zhang, X. Bao, Y. Fu, Preparation of antioxidant peptides from oat globulin, CyTA- J. Food. 18 (2020) 108-115, https://doi.org/10.1080/19476337.2020.1716076.

[215] A. Supriyadi, L.S. Arum, A.S. Nugraha, A.A.I. Ratnadewi, T.A. Siswoyo, Revealing antioxidant and antidiabetic potency of Melinjo (*Gnetum Gnemon*) seed protein hydrolysate

at different stages of seed maturation, Curr. Res. Nutr. Food Sci. 7 (2019) 479-487, http://dx.doi.org/10.12944/CRNFSJ.7.2.17.

[216] M. Mulla, J. Ahmed, Modulating functional and antioxidant properties of proteins from defatted garden cress (*Lepidium sativum*) seed meal by Alcalase hydrolysis, Food Measure. 13 (2019) 3257-3266, https://doi.org/10.1007/s11694-019-00248-8.

[217] R. R. Lu, P. Qian, Z. Sun, X. H. Zhou, T. P. Chen, J. F. He, H. Zhang, J. Wu, Hempseed protein derived antioxidative peptides: purification, identification and protection from hydrogen peroxide-induced apoptosis in PC12 cells, Food Chem. 123 (2010) 1210-1218, https://doi.org/10.1016/j.foodchem.2010.05.089.

[218] E.G. Tovar-Pérez, L. Guerrero-Becerra, F. Lugo-Cervantes, Antioxidant activity of hydrolysates and peptide fractions of glutelin from cocoa (*Theobroma cacao* L.) seed, CyTA-J. Food. 15 (2017) 489-496, https://doi.org/10.1080/19476337.2017.1297963.

[219] K. Thammarat, N. Leena, S. Punnanee, B. Soottawat, Functional and antioxidative properties of bambara groundnut (*Voandzeia subterranea*) protein hydrolysates, Int. Food Res. J. 22 (2015) 1584-1595.

[220] L. Wu, A. Jiang, Y. Jing, Y. Zheng, Y. Yan, Antioxidant properties of protein hydrolysate from Douchi by membrane ultrafiltration, Int. J. Food Prop. 20 (2017) 997-1006, https://doi.org/10.1080/10942912.2016.1192644.

[221] M. Zhang, T. H. Mu, M. J. Sun, Purification and identification of antioxidant peptides from sweet potato protein hydrolysates by Alcalase, J. Func. Foods. 7 (2014) 191-200, https://doi.org/10.1016/j.jff.2014.02.012.

[222] M. Zhang, T. H. Mu, Identification and characterization of antioxidant peptides from sweet potato protein hydrolysates by Alcalase under high hydrostatic pressure, Innov. Food Sci. Emerg. Technol. 43 (2017) 92-101, https://doi.org/10.1016/j.ifset.2017.08.001.

[223] M. Zhang, T.S. Huang, T.H. Mu, Production and characterisation of antioxidant peptides from sweet potato protein by enzymatic hydrolysis with radio frequency pretreatment, Int. J. Food Sci. Technol. 55 (2020) 2352-2358, https://doi.org/10.1111/ijfs.14441.

[224] A. Intiquilla, K. Jiménez-Aliaga, A.I. Zavaleta, B. Hernández-Ledesma, Production of antioxidant hydrolyzates from a *Lupinus mutabilis* (Tarwi) protein concentrate with Alcalase: optimization by Response Surface Methodology, Nat. Prod. Commun. 13 (2018) 751-756, https://doi.org/10.1177/1934578X1801300626.

[225] Y. X. Feng, G. R. Ruan, F. Jin, J. Xu, F. J. Wang, Purification, identification, and synthesis of five novel antioxidant peptides from Chinese chestnut (*Castanea mollissima* Blume) protein hydrolysates, LWT. 92 (2018) 40-46, https://doi.org/10.1016/j.lwt.2018.01.006.

[226] X.h. Dong, J. Li, G. Jiang, H. Li, M. Zhao, Y. Jiang, Effects of combined high pressure and enzymatic treatments on physicochemical and antioxidant properties of peanut proteins, Food Sci. Nutr. 7 (2019) 1417-1425, https://doi.org/10.1002/fsn3.976.

[227] A. Intiquilla, K. Jiménez- Aliaga, F. Guzmán, C.A. Alvarez, A.I. Zavaleta, V. Izaguirre, B. Hernández- Ledesma, Novel antioxidant peptides obtained by Alcalase hydrolysis of *Erythrina edulis* (pajuro) protein, J. Sci. Food Agric. 99 (2019) 2420-2427, https://doi.org/10.1002/jsfa.9449.

[228] Z. Zou, M. Wang, Z. Wang, R.E. Aluko, R. He, Antihypertensive and antioxidant activities of enzymatic wheat bran protein hydrolysates, J. Food Biochem. 44 (2020) e13090, https://doi.org/10.1111/jfbc.13090.

[229] C.M. Montone, R. Zenezini Chiozzi, N. Marchetti, A. Cerrato, M. Antonelli, A.L. Capriotti, C. Cavaliere, S. Piovesana, A. Laganà, Peptidomic approach for the identification of peptides with potential antioxidant and anti-hyperthensive effects derived from Asparagus by-products, Molecules. 24 (2019) 3627, https://doi.org/10.3390/molecules24193627.

[230] S. Ikram, H. Zhang, M.S. Ahmed, J. Wang, Ultrasonic pretreatment improved the antioxidant potential of enzymatic protein hydrolysates from highland barley brewer's spent grain (BSG), J. Food Sci. 85 (2020) 1045-1053, https://doi.org/10.1111/1750-3841.15063.

[231] N. Meshginfar, A.S. Mahoonak, F. Hosseinian, M. Ghorbani, A. Tsopmo, Production of antioxidant peptide fractions from a by-product of tomato processing: mass spectrometry identification of peptides and stability to gastrointestinal digestion, J. Food Sci. Technol. 55 (2018) 3498-3507, https://doi.org/10.1007/s13197-018-3274-z.

[232] E. González-García, P. Puchalska, M.L. Marina, M.C. García, Fractionation and identification of antioxidant and angiotensin-converting enzyme-inhibitory peptides obtained from plum (*Prunus domestica* L.) stones, J. Func. Foods 19 (2015) 376-384, https://doi.org/10.1016/j.jff.2015.08.033.

[233] S. Gallegos- Tintoré, C. Torres- Fuentes, A.L. Martínez- Ayala, J. Solorza- Feria, M. Alaiz, J. Girón- Calle, J. Vioque, Antioxidant and chelating activity of *Jatropha curcas*

L. protein hydrolysates, J. Sci. Food Agric. 91 (2011) 1618-1624, https://doi.org/10.1002/jsfa.4357.

[234] A. Tsopmo, A. Cooper, S. Jodayree, Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties, Advance J. Food Sci. Technol. 2 (2010) 206-212.

[235] M. K. Park, Effect of enzymatic hydrolysis by proteases on antioxidant activity of chungkukjang, J. Korean Soc. Food Sci. Nutr. 40 (2011) 327-333, https://doi.org/10.3746/jkfn.2011.40.2.327.

[236] M.F. Sbroggio, M.S. Montilha, V.R.G.d. Figueiredo, S.R. Georgetti, L.E. Kurozawa, Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates, Food Sci. Technol. Int. 36 (2016) 375-381, https://doi.org/10.1590/1678-457X.000216.

[237] A. Justus, D.G. Pereira, E.L. Ida, L.E. Kurozawa, Combined uses of an endo-and exopeptidase in okara improve the hydrolysates via formation of aglycone isoflavones and antioxidant capacity, LWT. 115 (2019) 108467, https://doi.org/10.1016/j.lwt.2019.108467.

[238] M. Zhang, T.H. Mu, Y.B. Wang, M.J. Sun, Evaluation of free radical- scavenging activities of sweet potato protein and its hydrolysates as affected by single and combination of enzyme systems, Int. J. Food Sci. Technol. 47 (2012) 696-702, https://doi.org/10.1111/j.1365-2621.2011.02895.x.

[239] M. Zhang, T.H. Mu, M.J. Sun, Sweet potato protein hydrolysates: antioxidant activity and protective effects on oxidative DNA damage, Int. J. Food Sci. Technol. 47 (2012) 2304-2310, https://doi.org/10.1111/j.1365-2621.2012.03103.x.

[240] M. Zhang, T. S. Huang, T. H. Mu, Production and *in vitro* gastrointestinal digestion of antioxidant peptides from enzymatic hydrolysates of sweet potato protein affected by pretreatment, Plant Foods Hum. Nutr. 74 (2019) 225-231, https://doi.org/10.1007/s11130-019-00724-y.

[241] L. Popović, D. Peričin, Ž. Vaštag, S. Popović, V. Krimer, A. Torbica, Antioxidative and functional properties of pumpkin oil cake globulin hydrolysates, J. Am. Oil Chem. Soc. 90 (2013) 1157-1165, https://doi.org/10.1007/s11746-013-2257-5.

[242] E. Nourmohammadi, A. SadeghiMahoonak, M. Alami, M. Ghorbani, Amino acid composition and antioxidative properties of hydrolysed pumpkin (*Cucurbita pepo* L.) oil cake protein, Int. J. Food Prop. 20 (2017) 3244-3255, https://doi.org/10.1080/10942912.2017.1283516.

[243] A. Valdez-Ortiz, C.I. Fuentes-Gutiérrez, L.J. Germán-Báez, R. Gutiérrez-Dorado, S. Medina-Godoy, Protein hydrolysates obtained from Azufrado (*sulphur yellow*) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization, LWT-Food Sci. Technol. 46 (2012) 91-96, https://doi.org/10.1016/j.lwt.2011.10.021.

[244] J.A.d. Evangelho, J.d.J. Berrios, V.Z. Pinto, M.D. Antunes, N.L. Vanier, E.d.R. Zavareze, Antioxidant activity of black bean (*Phaseolus vulgaris* L.) protein hydrolysates, Food Sci. Technol. 36 (2016) 23-27, https://doi.org/10.1590/1678-457x.0047.

[245] X. Wang, X. Zheng, N. Kopparapu, W. Cong, Y. Deng, X. Sun, X. Liu, Purification and evaluation of a novel antioxidant peptide from corn protein hydrolysate, Process Biochem. 49 (2014) 1562-1569, https://doi.org/10.1016/j.procbio.2014.05.014.

[246] X. Liu, X. Zheng, Z. Song, X. Liu, N. kumar Kopparapu, X. Wang, Y. Zheng, Preparation of enzymatic pretreated corn gluten meal hydrolysate and *in vivo* evaluation of its antioxidant activity, J. Func. Foods. 18 (2015) 1147-1157, https://doi.org/10.1016/j.jff.2014.10.013.

[247] Y. Liu, H. Zhang, J. Wang, C. Yang, Studies on peanut meal hydrolysis by protease to prepare peanut antioxidative peptides, Journal of Chinese Institute of Food Science and Technology 14 (2014) 62-68.

[248] W.Y. Chen, Y.Y. Chen, L.Y. Liu, Studies on antioxidant activity of defatted peanut meal hydrolysate, Taiwanese Journal of Agricultural Chemistry and Food Science, 53 (2015) 107-116.

[249] P. Thamnarathip, K. Jangchud, S. Nitisinprasert, B. Vardhanabhuti, Identification of peptide molecular weight from rice bran protein hydrolysate with high antioxidant activity, J. Cereal Sci. 69 (2016) 329-335, https://doi.org/10.1016/j.jcs.2016.04.011.

[250] Y. Xia, F. Bamdad, M. Gänzle, L. Chen, Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis, Food Chem. 134 (2012) 1509-1518, https://doi.org/10.1016/j.foodchem.2012.03.063.

[251] R. He, A.T. Girgih, S.A. Malomo, X. Ju, R.E. Aluko, Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions, J. Func. Foods. 5 (2013) 219-227, https://doi.org/10.1016/j.jff.2012.10.008.

[252] P. Guo, Y. Qi, C. Zhu, Q. Wang, Purification and identification of antioxidant peptides from Chinese cherry (*Prunus pseudocerasus* Lindl.) seeds, J. Func. Foods, 19 (2015) 394-403, https://doi.org/10.1016/j.jff.2015.09.003.

[253] B. Jin, M.F. Huang, Z.P. He, S.Q. Shen, Enzymatic hydrolysis of coconut protein and antioxidant activity of the hydrolysates, Modern Food Sci. Tech. 29 (2013) 1826-1831.

[254] E. González-García, M.L. Marina, M.C. García, Plum (*Prunus Domestica* L.) by-product as a new and cheap source of bioactive peptides: Extraction method and peptides characterization, J. Func. Foods. 11 (2014) 428-437, https://doi.org/10.1016/j.jff.2014.10.020.

[255] M.C. García, J. Endermann, E. Gonzalez-Garcia, M.L. Marina, HPLC-Q-TOF-MS identification of antioxidant and antihypertensive peptides recovered from cherry (*Prunus cerasus* L.) subproducts, J. Agric. Food Chem. 63 (2015) 1514-1520, https://doi.org/10.1021/jf505037p.

[256] X. Li, J. Deng, S. Shen, T. Li, M. Yuan, K. Yang, C. Ding, Antioxidant activities and functional properties of enzymatic protein hydrolysates from defatted *Camellia oleifera* seed cake, J. Food Sci. Technol. 52 (2015) 5681-5690, https://doi.org/10.1007/s13197-014-1693-z.

[257] Z. Shahi, S.Z. Sayyed-Alangi, L. Najafian, Effects of enzyme type and process time on hydrolysis degree, electrophoresis bands and antioxidant properties of hydrolyzed proteins derived from defatted *Bunium persicum Bioss.* press cake, Heliyon. 6 (2020) e03365, https://doi.org/10.1016/j.heliyon.2020.e03365.

[258] M. Karamać, A. Kosińska-Cagnazzo, A. Kulczyk, Use of different proteases to obtain flaxseed protein hydrolysates with antioxidant activity, Int. J. Mol. Sci. 17 (2016) 1027, https://doi.org/10.3390/ijms17071027.

[259] M. Mirzapour, K. Rezaei, M.A. Sentandreu, A.A. Moosavi- Movahedi, *In vitro* antioxidant activities of hydrolysates obtained from Iranian wild almond (*Amygdalus scoparia*) protein by several enzymes, Int. J. Food Sci. Technol. 51 (2016) 609-616. https://doi.org/10.1111/ijfs.12996.

[260] A. Intiquilla, K. Jiménez-Aliaga, A.I. Zavaleta, I. Arnao, C. Peña, E.L. Chavez-Hidalgo, B. Hernández-Ledesma, *Erythrina edulis* (Pajuro) seed protein: A new source of antioxidant peptides, Nat. Prod. Commun. 11 (2016) 781-786.

[261] Y. R. Yun, S. H. Park, Antioxidant activities of brown teff hydrolysates produced by protease treatment, J. Nutr. Health. 51 (2018) 599-606, https://doi.org/10.4163/jnh.2018.51.6.599.

[262] D.S. Mohana, T.T. Chai, F. C. Wong, Antioxidant and protein protection potentials of Fennel seed-derived protein hydrolysates and peptides, Modern Food Sci. Tech. 35 (2019) 22-29, https://doi.org/10.13982/j.mfst.1673-9078.2019.9.002.

[263] E.F. Vieira, D.D. da Silva, H. Carmo, I.M. Ferreira, Protective ability against oxidative stress of brewer's spent grain protein hydrolysates, Food Chem. 228 (2017) 602-609, https://doi.org/10.1016/j.foodchem.2017.02.050.

[264] Y. Sadeghian Amin, A.R. Sadeghi Mahoonak, M. Ghorbani, M. Alami, H.R. Joshaghani, Processing time effects on functional and antioxidant properties of the Quinoa proteins hydrolyzed with Alcalase and pancreatin, Iranian J. Nutr. Sci. Food Technol. 14 (2020) 89-102.

[265] N. Ye, P. Hu, S. Xu, M. Chen, S. Wang, J. Hong, T. Chen, T. Cai, Preparation and characterization of antioxidant peptides from carrot seed protein, J. Food Qual. (2018) 8579094, https://doi.org/10.1155/2018/8579094.

[266] J. Zamora-Sillero, P. Ramos, J.M. Monserrat, C. Prentice, Evaluation of the antioxidant activity *in vitro* and in hippocampal HT-22 cells system of protein hydrolysates of common carp (*Cyprinus carpio*) by-product, J. Aquat. Food Prod. Technol. 27 (2018) 21-34, https://doi.org/10.1080/10498850.2017.1390027.

[267] D. Oliveira, D. Bernardi, F. Drummond, F. Dieterich, W. Boscolo, C. Leivas, E. Kiatkoski, N. Waszczynskyj, Potential use of tuna (*Thunnus albacares*) by-product: production of antioxidant peptides and recovery of unsaturated fatty acids from tuna head, Int. J. Food Eng. 13 (2017) 20150365, https://doi.org/10.1515/ijfe-2015-0365.

[268] P. Yang, H. Ke, P. Hong, S. Zeng, W. Cao, Antioxidant activity of bigeye tuna (*Thunnus obesus*) head protein hydrolysate prepared with Alcalase, Int. J. Food Sci. Technol. 46 (2011) 2460-2466, https://doi.org/10.1111/j.1365-2621.2011.02768.x.

[269] S. Saidi, A. Deratani, M.-P. Belleville, R.B. Amar, Antioxidant properties of peptide fractions from tuna dark muscle protein by-product hydrolysate produced by membrane fractionation process, Food Res. Int. 65 (2014) 329-336, https://doi.org/10.1016/j.foodres.2014.09.023.

[270] M. Nurilmala, H.H. Hizbullah, E. Karnia, E. Kusumaningtyas, Y. Ochiai, Characterization and antioxidant activity of collagen, gelatin, and the derived peptides from Yellowfin tuna (*Thunnus albacares*) skin, Mar Drugs. 18 (2020) 98, https://doi.org/10.3390/md18020098.

[271] L. Cai, X. Wu, Y. Zhang, X. Li, S. Ma, J. Li, Purification and characterization of three antioxidant peptides from protein hydrolysate of grass carp (*Ctenopharyngodon idella*) skin, J. Func. Foods. 16 (2015) 234-242, https://doi.org/10.1016/j.jff.2015.04.042.

[272] P. Viji, T.S. Phannendra, D. Jesmi, B. Madhusudana Rao, P.H. Dhiju Das, N. George, Functional and antioxidant properties of gelatin hydrolysates prepared from skin and scale of Sole fish, J. Aquat. Food Prod. Technol. 28 (2019) 976-986, https://doi.org/10.1080/10498850.2019.1672845.

[273] T. Sae- Leaw, S. Karnjanapratum, Y.C. O'Callaghan, M.B. O'Keeffe, R.J. FitzGerald, N.M. O'Brien, S. Benjakul, Purification and identification of antioxidant peptides from gelatin hydrolysate of seabass skin, J. Food Biochem. 41 (2017) e12350, https://doi.org/10.1111/jfbc.12350.

[274] L. Sun, W. Chang, Q. Ma, Y. Zhuang, Purification of antioxidant peptides by high resolution mass spectrometry from simulated gastrointestinal digestion hydrolysates of Alaska Pollock (*Theragra chalcogramma*) skin collagen, Mar. Drugs. 14 (2016) 186, https://doi.org/10.3390/md14100186.

[275] R. Zhang, J. Chen, X. Jiang, L. Yin, X. Zhang, Antioxidant and hypoglycaemic effects of tilapia skin collagen peptide in mice, Int. J. Food Sci. Technol. 51 (2016) 2157-2163, https://doi.org/10.1111/ijfs.13193.

[276] T. Jin, Y. Wu, Effects of production factors on the antioxidant activity of protein hydrolysates from little hairtail (*Trichiurus haumela*) of East China Sea, J. Food Agric. Environ. 11 (2013) 95-98.

[277] T. Jin, Y.-X. Wu, Antioxidant activities of protein hydrolysates from Little Hairtail (*Trichiurus haumela*) of East China Sea, J. Food Agric. Environ. 7 (2015) 354-360, https://doi.org/10.19026/ajfst.7.1324.

[278] L. Zhiyu, Z. Weijing, Z. Ningning, Z. Baodong, Preparation of antioxidative peptide from Pseudosciaena crocea's viscera hydrolysised by Alcalase, Journal of Chinese Institute of Food Science and Technology 16 (8) (2016) 109-117, https://doi.org/10.16429/j.1009-7848.2016.08.016.

[279] H.L. Jang, S.R. Shin, K.Y. Yoon, Hydrolysis conditions for antioxidant peptides derived from enzymatic hydrolysates of sandfish (*Arctoscopus japonicus*), Food Sci. Biotechnol. 26 (2017) 1191-1197, https://doi.org/10.1007/s10068-017-0178-z.

[280] F. Parthiban, R.J. Shakila, G. Jeyasekaran, R. Shalini, Relationship between antioxidative potential and amino acids composition of the bioactive peptides prepared from Indian squid Uroteuthis (Photololigo) duvaucelii (d'Orbigny, 1835) using Alcalase, Indian Agricultural Research Journals. 64 (2017) 130-138, https://doi.org/10.21077/ijf.2017.64.

[281] S. Bordbar, A. Ebrahimpour, M. Zarei, A. Abdul Hamid, N. Saari, Alcalase-generated proteolysates of stone fish (*Actinopyga lecanora*) flesh as a new source of antioxidant peptides, Int. J. Food Prop. 21 (2018) 1541-1559, https://doi.org/10.1080/10942912.2018.1497060.

[282] P.Y. Kang, N.H. Ishak, N.M. Sarbon, Optimization of enzymatic hydrolysis of shortfin scad (*Decapterus macrosoma*) myofibrillar protein with antioxidant effect using Alcalase, Int. Food Res. J. 25 (2018) 1808-1817.

[283] M.A. Khesal, A. Sharifan, E. Hoseini, A. Ghavami, Optimization of enzymatic hydrolysis conditions of Caspian kutum (*Rutilus frisii kutum*) " by-product for production of bioactive peptides with antioxidative properties, Int. J. Pept. Res. Ther. (2019) 1-10, https://doi.org/10.1007/s10989-019-09981-6.

[284] M.M. Lima, N.L. Vanier, A.R.G. Dias, E. Zavareze, C. Prentice, A.d.S. Moreira, Whitemouth croaker (*Micropogonias furnieri*) protein hydrolysates: chemical composition, molecular mass distribution, antioxidant activity and amino acid profile, Int. Food Res. J. 26 (2019) 247-254.

[285] F. C. Wong, J. Xiao, G. Michelle, L. Ong, M. J. Pang, S. J. Wong, L. K. Teh, T. T. Chai, Identification and characterization of antioxidant peptides from hydrolysate of blue-spotted stingray and their stability against thermal, pH and simulated gastrointestinal digestion treatments, Food Chem. 271 (2019) 614-622, https://doi.org/10.1016/j.foodchem.2018.07.206.

[286] J. Zamora- Sillero, M. Tavares Kütter, M. Borges Tesser, J.M. Monserrat, C. Prentice, Effect of dietary common carp by- product protein hydrolysates on antioxidant status in different organs of zebrafish (*Danio rerio*), Aquacult. Nutr. 25 (2019) 110-118, https://doi.org/10.1111/anu.12835.

[287] H.S. Kim, S.Y. Kim, I.P.S. Fernando, K.K.A. Sanjeewa, L. Wang, S.H. Lee, S.C. Ko, M.C. Kang, T.U. Jayawardena, Y.J. Jeon, Free radical scavenging activity of the peptide from the Alcalase hydrolysate of the edible aquacultural seahorse (*Hippocampus abdominalis*), J. Food Biochem. 43 (2019) e12833, https://doi.org/10.1111/jfbc.12833.

[288] H. Chen, S. Wang, A. Zhou, J. Miao, J. Liu, S. Benjakul, A novel antioxidant peptide purified from defatted round scad (*Decapterus maruadsi*) protein hydrolysate extends lifespan in *Caenorhabditis elegans*, J. Func. Foods. 68 (2020) 103907, https://doi.org/10.1016/j.jff.2020.103907.

[289] D. H. Ngo, Z.J. Qian, B. Ryu, J.W. Park, S.K. Kim, *In vitro* antioxidant activity of a peptide isolated from Nile tilapia (*Oreochromis niloticus*) scale gelatin in free radical-mediated oxidative systems, J. Func. Foods. 2 (2010) 107-117, https://doi.org/10.1016/j.jff.2010.02.001.

[290] S. Choonpicharn, S. Jaturasitha, N. Rakariyatham, N. Suree, H. Niamsup, Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin, J. Food Sci. Technol. 52 (2015) 3134-3139, https://doi.org/10.1007/s13197-014-1581-6.

[291] M. Shamloo, J. Bakar, D. Mat Hashim, A. Khatib, Biochemical properties of red tilapia (*Oreochromis niloticus*) protein hydrolysates, Int. Food Res. J. 19 (2012) 183-188.

[292] X. Li, Y. Luo, H. Shen, J. You, Antioxidant activities and functional properties of grass carp (*Ctenopharyngodon idellus*) protein hydrolysates, J. Sci. Food Agric. 92 (2012) 292-298, https://doi.org/10.1002/jsfa.4574.

[293] S.P. Malaypally, A.M. Liceaga, K.-H. Kim, M. Ferruzzi, F. San Martin, R.R. Goforth, Influence of molecular weight on intracellular antioxidant activity of invasive silver carp (*Hypophthalmichthys molitrix*) protein hydrolysates, J. Func. Foods. 18 (2015) 1158-1166, https://doi.org/10.1016/j.jff.2014.06.011.

[294] M. Chalamaiah, T. Jyothirmayi, P.V. Diwan, B.D. Kumar, Antioxidant activity and functional properties of enzymatic protein hydrolysates from common carp (*Cyprinus*

carpio) roe (egg), J. Food Sci. Technol. 52 (2015) 5817-5825, https://doi.org/10.1007/s13197-015-1714-6.

[295] L. Zhang, Y. Shan, H. Hong, Y. Luo, X. Hong, W. Ye, Prevention of protein and lipid oxidation in freeze-thawed bighead carp (*Hypophthalmichthys nobilis*) fillets using silver carp (*Hypophthalmichthys molitrix*) fin hydrolysates, LWT. 123 (2020) 109050, https://doi.org/10.1016/j.lwt.2020.109050.

[296] M. Ovissipour, B. Rasco, S.G. Shiroodi, M. Modanlow, S. Gholami, M. Nemati, Antioxidant activity of protein hydrolysates from whole anchovy sprat (*Clupeonella engrauliformis*) prepared using endogenous enzymes and commercial proteases, J. Sci. Food Agric. 93 (2013) 1718-1726, https://doi.org/10.1002/jsfa.5957.

[297] I.B.B. Piotrowicz, M.M. Mellado Antioxidant hydrolysates production from Argentine anchovy (*Engraulis anchoita*) with different enzymes, Int. Food Res. J. 22 (2015) 1203-1211.

[298] C. B. Ahn, J. G. Kim, J. Y. Je, Purification and antioxidant properties of octapeptide from salmon byproduct protein hydrolysate by gastrointestinal digestion, Food Chem. 147 (2014) 78-83, https://doi.org/10.1016/j.foodchem.2013.09.136.

[299] A.T. Idowu, S. Benjakul, S. Sinthusamran, P. Sookchoo, H. Kishimura, Protein hydrolysate from salmon frames: Production, characteristics and antioxidative activity, J. Food Biochem. 43 (2019) e12734, https://doi.org/10.1111/jfbc.12734.

[300] Y. Fu, X.-H. Zhao, Utilization of chum salmon (*Oncorhynchus keta*) skin gelatin hydrolysates to attenuate hydrogen peroxide-induced oxidative injury in rat hepatocyte

BRL cell model, J. Aquat. Food Prod. Technol. 24 (2015) 648-660, https://doi.org/10.1080/10498850.2013.804141.

[301] C. Chansuwan, P. Chinachoti, Antioxidative properties and hydrolysis profile of Skipjack tuna dark muscle and skin, Int. Food Res. J. 22 (2015) 1968-1976.

[302] C. F. Chi, F.Y. Hu, B. Wang, Z. R. Li, H. Y. Luo, Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from skipjack tuna (*Katsuwonus pelamis*) dark muscle, Mar. Drugs. 13 (2015) 2580-2601, https://doi.org/10.3390/md13052580.

[303] Y. T. Qiu, Y.M. Wang, X. R. Yang, Y. Q. Zhao, C.-F. Chi, B. Wang, Gelatin and antioxidant peptides from gelatin hydrolysate of skipjack tuna (*Katsuwonus pelamis*) scales: Preparation, identification and activity evaluation, Mar. Drugs. 17 (2019) 565, https://doi.org/10.3390/md17100565.

[304] S. Bordbar, A. Ebrahimpour, A. Abdul Hamid, A. Manap, M. Yazid, F. Anwar, N. Saari, The improvement of the endogenous antioxidant property of stone fish (*Actinopyga lecanora*) tissue using enzymatic proteolysis, Biomed. Res. Int. (2013) 849529, https://doi.org/10.1155/2013/849529.

[305] A. Barkia, A.L.I. Bougatef, H.B. Khaled, M. Nasri, Antioxidant activities of sardinelle heads and/or viscera protein hydrolysates prepared by enzymatic treatment, J. Food Biochem. 34 (2010) 303-320, https://doi.org/10.1111/j.1745-4514.2009.00331.x.

[306] L. Najafian, A.S. Babji, Production of bioactive peptides using enzymatic hydrolysis and identification antioxidative peptides from patin (*Pangasius sutchi*) sarcoplasmic protein hydolysate, J. Func. Foods. 9 (2014) 280-289, https://doi.org/10.1016/j.jff.2014.05.003.

[307] H. Jiang, T. Tong, J. Sun, Y. Xu, Z. Zhao, D. Liao, Purification and characterization of antioxidative peptides from round scad (*Decapterus maruadsi*) muscle protein hydrolysate, Food Chem. 154 (2014) 158-163, https://doi.org/10.1016/j.foodchem.2013.12.074.

[308] T. T. Chai, S. R. Tong, Y. C. Law, N.I.N. Ismail, F.A. Manan, F. C. Wong, Anti-oxidative, metal chelating and radical scavenging effects of protein hydrolysates from blue-spotted stingray, Trop. J. Pharm. Res. 14 (2015) 1349-1355, https://doi.org/10.4314/tjpr.v14i8.5.

[309] Y. Y. Kong, S. S. Chen, J. Q. Wei, Y. P. Chen, W. T. Lan, Q. W. Yang, G. R. Huang, Preparation of antioxidative peptides from spanish mackerel (*Scomberomorus niphonius*) processing byproducts by enzymatic hydrolysis, Biotechnology, 14 (2015) 188-193, https://doi.org/10.3923/biotech.2015.188.193.

[310] M. Nikoo, S. Benjakul, X. Xu, Antioxidant and cryoprotective effects of Amur sturgeon skin gelatin hydrolysate in unwashed fish mince, Food Chem. 181 (2015) 295-303, https://doi.org/10.1016/j.foodchem.2015.02.095.

[311] W. H. Zhao, Q. B. Luo, X. Pan, C. F. Chi, K. L. Sun, B. Wang, Preparation, identification, and activity evaluation of ten antioxidant peptides from protein hydrolysate of swim bladders of miiuy croaker (*Miichthys miiuy*), J. Func. Foods. 47 (2018) 503-511, https://doi.org/10.1016/j.jff.2018.06.014.

[312] I.C. Cheng, J. X. Liao, J. Y. Ciou, L. T. Huang, Y. W. Chen, C. Y. Hou, Characterization of protein hydrolysates from Eel (*Anguilla marmorata*) and their

application in herbal Eel extracts, Catalysts. 10 (2020) 205, https://doi.org/10.3390/catal10020205.

[313] C. Altınelataman, O. Koroleva, T. Fedorova, A. Torkova, K. Lisitskaya, M. Tsentalovich, A. Kononikhin, I. Popov, D. Vasina, L. Kovalyov, An *in vitro* and *in silico* study on the antioxidant and cell culture-based study on the chemoprotective activities of fish muscle protein hydrolysates obtained from European seabass and gilthead seabream, Food Chem. 271 (2019) 724-732, https://doi.org/10.1016/j.foodchem.2018.08.004.

[314] K. M. Kim, D. S. Lee, M. H. Nam, H. S. Yoo, S. B. Kim, B. S. Chun, Y. B. Lee, Optimization of Alcalase for krill byproduct hydrolysis and antioxidative activities by Response surface Methodology, Prev. Nutr. Food Sci. 15 (2010) 316-321, https://doi.org/10.3746/jfn.2010.15.4.316

[315] J. Mamelona, R. Saint- Louis, É. Pelletier, Nutritional composition and antioxidant properties of protein hydrolysates prepared from echinoderm byproducts, Int. J. Food Sci. Technol. 45 (2010) 147-154, https://doi.org/10.1111/j.1365-2621.2009.02114.x.

[316] M.A. Vieira, D.D. Oliveira, L.E. Kurozawa, Production of peptides with radical scavenging activity and recovery of total carotenoids using enzymatic protein hydrolysis of shrimp waste, J. Food Biochem. 40 (2016) 517-525, https://doi.org/10.1111/jfbc.12246.

[317] J. Zhao, G.R. Huang, M.N. Zhang, W.W. Chen, J.X. Jiang, Amino acid composition, molecular weight distribution and antioxidant stability of shrimp processing byproduct hydrolysate, Am. J. Food Technol. 6 (2011) 904-913, https://doi.org/10.3923/ajft.2011.904.913.

[318] A. Sila, N. Sayari, R. Balti, O. Martinez-Alvarez, N. Nedjar-Arroume, N. Moncef, A. Bougatef, Biochemical and antioxidant properties of peptidic fraction of carotenoproteins generated from shrimp by-products by enzymatic hydrolysis, Food Chem. 148 (2014) 445-452, https://doi.org/10.1016/j.foodchem.2013.05.146.

[319] S.S. Dey, K.C. Dora, Antioxidative activity of protein hydrolysate produced by Alcalase hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*), J. Food Sci. Technol. 51 (2014) 449-457, https://doi.org/10.1007/s13197-011-0512-z.

[320] J. Gunasekaran, N. Kannuchamy, S. Kannaiyan, R. Chakraborti, V. Gudipati, Protein hydrolysates from shrimp (*Metapenaeus dobsoni*) head waste: optimization of extraction conditions by Response Surface Methodology J. Aquat. Food Prod. Technol. 24 (2015) 429-442, https://doi.org/10.1080/10498850.2013.787134.

[321] A. Hamzeh, M. Rezaei, S. Khodabandeh, A. Motamedzadegan, M. Noruzinia, Antiproliferative and antioxidative activities of cuttlefish (*Sepia pharaonis*) protein hydrolysates as affected by degree of hydrolysis, Food Measure. 12 (2018) 721-727, https://doi.org/10.1007/s11694-017-9685-0.

[322] A. Hamzeh, M. Rezaei, S. Khodabandeh, A. Motamedzadegan, M. Noruzinia, J.M. Regenstein, Optimization of antioxidant peptides production from the mantle of Cuttlefish (*Sepia pharaonis*) using RSM and fractionation, J. Aquat. Food Prod. Technol. 28 (2019) 392-401, https://doi.org/10.1080/10498850.2019.1594480.

[323] Z. Zhang, G. Su, F. Zhou, L. Lin, X. Liu, M. Zhao, Alcalase-hydrolyzed oyster (*Crassostrea rivularis*) meat enhances antioxidant and aphrodisiac activities in normal male mice, Food Res. Int. 120 (2019) 178-187, https://doi.org/10.1016/j.foodres.2019.02.033.

[324] X.P. Dong, B.W. Zhu, H.X. Zhao, D.Y. Zhou, H.T. Wu, J.F. Yang, D.M. Li, Y. Murata, Preparation and *in vitro* antioxidant activity of enzymatic hydrolysates from oyster (*Crassostrea talienwhannensis*) meat, Int. J. Food Sci. Technol. 45 (2010) 978-984, https://doi.org/10.1111/j.1365-2621.2010.02223.x.

[325] L. Zhang, Y. Liu, X. Tian, Z. Tian, Antimicrobial capacity and antioxidantactivity of enzymatic hydrolysates of protein from Rushan bay oyster (*Crassostrea gigas*), J. Food Process. Pres. 39 (2015) 404-412, https://doi.org/10.1111/jfpp.12245.

[326] G. R. Huang, J. Zhao, J. X. Jiang, Effect of defatting and enzyme type on antioxidative activity of shrimp processing byproducts hydrolysate, Food Sci. Biotechnol. 20 (2011) 651-657, https://doi.org/10.1007/s10068-011-0092-8.

[327] J.M. Latorres, D.G. Rios, G. Saggiomo, W. Wasielesky, C. Prentice-Hernandez, Functional and antioxidant properties of protein hydrolysates obtained from white shrimp (*Litopenaeus vannamei*), J. Food Sci. Technol. 55 (2018) 721-729, https://doi.org/10.1007/s13197-017-2983-z.

[328] M. Yan, H. Tao, S. Qin, Effect of enzyme type on the antioxidant activities and functional properties of enzymatic hydrolysates from sea cucumber (*Cucumaria frondosa*) viscera, J. Aquat. Food Prod. Technol. 25 (2016) 940-952, https://doi.org/10.1080/10498850.2014.994083.

[329] Y. Zhang, S. He, E. Bonneil, B.K. Simpson, Generation of antioxidative peptides from Atlantic sea cucumber using Alcalase versus trypsin: *In vitro* activity, de novo sequencing, and in silico docking for in vivo function prediction, Food Chem. 306 (2020) 125581, https://doi.org/10.1016/j.foodchem.2019.125581.

[330] Z. Zhu, J. Han, Y. Tang, Q. Wang, H. Wu, Studies on radical scavenging activities of hydrolasates from Sea Cucumber gut based on ESR system, Journal of Chinese Institute of Food Science and Technology. 17 (2017) 227-233, https://doi.org/10.16429/j.1009-7848.2017.10.030.

[331] R. Safari, Z. Yaghoubzadeh, Antioxidant Activity of bioactive peptides extracted from sea cucumber (*Holothuria leucospilata*), Int. J. Pept. Res. Ther. In press. https://doi.org/10.1007/s10989-020-10031-9.

[332] M. Jridi, I. Lassoued, R. Nasri, M.A. Ayadi, M. Nasri, N. Souissi, Characterization and potential use of cuttlefish skin gelatin hydrolysates prepared by different microbial proteases, Biomed. Res. Int. (2014) 461728, https://doi.org/10.1155/2014/461728.

[333] J. Y. Je, S.Y. Park, J. Y. Hwang, C. B. Ahn, Amino acid composition and *in vitro* antioxidant and cytoprotective activity of abalone viscera hydrolysate, J. Func. Foods. 16 (2015) 94-103, https://doi.org/10.1016/j.jff.2015.04.023.

[334] J.H. Um, E. A. Kim, W. Lee, N. Kang, E.J. Han, J.Y. Oh, S.Y. Park, Y. J. Jeon, S. H. Lee, G. Ahn, Protective effects of an enzymatic hydrolysate from *Octopus ocellatus* meat against hydrogen peroxide-induced oxidative stress in Chang liver cells and zebrafish embryo, Adv. Exp. Med. Biol. 975 (2017) 603-620, https://doi.org/10.1007/978-94-024-1079-2_47.

[335] Z. Tang, L. Yu, S. Wen, K. Zhang, Y. Sun, J. Lv, B. Ju, Z. Hu, Preparation and antioxidant properties of crayfish (*Procambarus Clarkii*) by-products protein hydrolysates and ultra filtration fractions, Pak. J. Pharm. Sci. 32 (2019) 2391-2397.

[336] X. Peng, B. Kong, X. Xia, Q. Liu, Reducing and radical-scavenging activities of whey protein hydrolysates prepared with Alcalase, Int. Dairy J. 20 (2010) 360-365, https://doi.org/10.1016/j.idairyj.2009.11.019.

[337] B. Kong, X. Peng, Y.L. Xiong, X. Zhao, Protection of lung fibroblast MRC-5 cells against hydrogen peroxide-induced oxidative damage by 0.1–2.8 kDa antioxidative peptides isolated from whey protein hydrolysate, Food Chem. 135 (2012) 540-54, https://doi.org/10.1016/j.foodchem.2012.04.122.

[338] S. Athira, B. Mann, P. Saini, R. Sharma, R. Kumar, A.K. Singh, Production and characterisation of whey protein hydrolysate having antioxidant activity from cheese whey, J. Sci. Food Agric. 95 (2015) 2908-2915, https://doi.org/10.1002/jsfa.7032.

[339] N. Xie, C. Wang, J. Ao, B. Li, Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its *in vitro* gastrointestinal digest, Food Res. Int. 51 (2013) 114-122, https://doi.org/10.1016/j.foodres.2012.12.001.

[340] N. Xie, S. Liu, C. Wang, B. Li, Stability of casein antioxidant peptide fractions during *in vitro* digestion/Caco-2 cell model: characteristics of the resistant peptides, Eur. Food Res. Technol. 239 (2014) 577-586, https://doi.org/10.1007/s00217-014-2253-5.

[341] N. Xie, B. Wang, L. Jiang, C. Liu, B. Li, Hydrophobicity exerts different effects on bioavailability and stability of antioxidant peptide fractions from casein during simulated gastrointestinal digestion and Caco-2 cell absorption, Food Res. Int. 76 (2015) 518-52, https://doi.org/10.1016/j.foodres.2015.06.025.

[342] S. Lin, W. Tian, H. Li, J. Cao, W. Jiang, Improving antioxidant activities of whey protein hydrolysates obtained by thermal preheat treatment of pepsin, trypsin, Alcalase and Flavourzyme, Int. J. Food Sci. Technol. 47 (2012) 2045-2051, https://doi.org/10.1111/j.1365-2621.2012.03068.x.

[343] Q. X. Zhang, H. Wu, Y. F. Ling, R. R. Lu, Isolation and identification of antioxidant peptides derived from whey protein enzymatic hydrolysate by consecutive chromatography and Q-TOF MS, J. Dairy Res. 80 (2013) 367-373, https://doi.org/10.1017/S0022029913000320.

[344] M.B. O'Keeffe, R.J. FitzGerald, Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells, Int. Dairy J. 36 (2014) 128-135, https://doi.org/10.1016/j.idairyj.2014.01.013.

[345] R.S.C.d. Souza, R.V. Tonon, M.P. Stephan, C.M. Silva, A.L. Penteado, L.M.C. Cabral, L.E. Kurozawa, Evaluation of the antioxidant potential of whey protein concentrated by ultrafiltration and hydrolyzed by different commercial proteases, Braz. J. Food Technol. 22 (2019) e2018021, https://doi.org/10.1590/1981-6723.02118.

[346] N.S. Oh, H.A. Lee, J.Y. Lee, J.Y. Joung, K.B. Lee, Y. Kim, K.W. Lee, S.H. Kim, The dual effects of Maillard reaction and enzymatic hydrolysis on the antioxidant activity of milk proteins, J. Dairy Sci. 96 (2013) 4899-491, https://doi.org/10.3168/jds.2013-6613.

[347] X. Y. Mao, X. Cheng, X. Wang, S. J. Wu, Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis, Food Chem. 126 (2011) 484-490, https://doi.org/10.1016/j.foodchem.2010.11.025.

[348] F. Bamdad, S. Bark, C.H. Kwon, J.-W. Suh, H. Sunwoo, Anti-inflammatory and antioxidant properties of peptides released from β-lactoglobulin by high hydrostatic pressure-assisted enzymatic hydrolysis, Molecules. 22 (2017) 949, https://doi.org/10.3390/molecules22060949.

[349] A.B. Shazly, Z. He, M.A. El-Aziz, M. Zeng, S. Zhang, F. Qin, J. Chen, Fractionation and identification of novel antioxidant peptides from buffalo and bovine casein hydrolysates, Food Chem. 232 (2017) 753-762, https://doi.org/10.1016/j.foodchem.2017.04.071.

[350] A.B. Shazly, H. Mu, Z. Liu, M.A. El-Aziz, M. Zeng, F. Qin, S. Zhang, Z. He, J. Chen, Release of antioxidant peptides from buffalo and bovine caseins: Influence of proteases on antioxidant capacities, Food Chem. 274 (2019) 261-267, https://doi.org/10.1016/j.foodchem.2018.08.137.

[351] Q. Liu, B. Kong, Y.L. Xiong, X. Xia, Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis, Food Chem. 118 (2010) 403-410, https://doi.org/10.1016/j.foodchem.2009.05.013.

[352] Q. Liu, B. Kong, G. Li, N. Liu, X. Xia, Hepatoprotective and antioxidant effects of porcine plasma protein hydrolysates on carbon tetrachloride-induced liver damage in rats, Food Chem. Toxicol. 49 (2011) 1316-1321, https://doi.org/10.1016/j.fct.2011.03.013.

[353] H. Yang, Y. Li, P. Li, Q. Liu, B. Kong, X. Huang, Z. Wu, Physicochemical changes of antioxidant peptides hydrolyzed from porcine plasma protein subject to free hydroxyl radical system, Advance J. Food Sci. Technol. 5 (2013) 14-18, http://dx.doi.org/10.19026/ajfst.5.3218.

[354] L.J. Gómez, O.A. Figueroa, J.E. Zapata, Antioxidant activity of bovine plasma enzymatic hydrolysates obtained by effect of Alcalase® 2.4 L, Inf. Tecnol. 24 (2013) 33-42, http://dx.doi.org/10.4067/S0718-07642013000100005.

[355] F.Y. Cheng, I.C. Lai, L.C. Lin, R. Sakata, The *in vitro* antioxidant properties of Alcalase hydrolysate prepared from silkie fowl (*Gallus gallus*) blood protein, Anim. Sci. J. 87 (2016) 921-928, https://doi.org/10.1111/asj.12509.

[356] C. Hou, L. Wu, Z. Wang, E. Saguer, D. Zhang, Purification and identification of antioxidant Alcalase-derived peptides from sheep plasma proteins, Antioxidants, 8 (2019) 592, https://doi.org/10.3390/antiox8120592.

[357] S. Lin, Y. Guo, J. Liu, Q. You, Y. Yin, S. Cheng, Optimized enzymatic hydrolysis and pulsed electric field treatment for production of antioxidant peptides from egg white protein, Afr. J. Biotechnol. 10 (2011) 11648-11657, https://doi.org/10.5897/AJB11.1008.

[358] J. Wang, K. Wang, S. Lin, P. Zhao, G. Jones, H. Trang, J. Liu, H. Ye, Improvement of antioxidant activity of peptides with molecular weights ranging from 1 to 10 kDa by PEF technology, Int. J. Biol. Macromol. 51 (2012) 244-249, https://doi.org/10.1016/j.ijbiomac.2012.05.017.

[359] Y. Ren, H. Wu, X. Li, F. Lai, X. Xiao, Purification and characterization of high antioxidant peptides from duck egg white protein hydrolysates, Biochem. Biophys. Res. Commun. 452 (2014) 888-894, https://doi.org/10.1016/j.bbrc.2014.08.116.

[360] J.R. Jovanović, A.B. Stefanović, M.G. Žuža, S.M. Jakovetić, N.Ž. Šekuljica, B.M. Bugarski, Z.D. Knežević-Jugović, Improvement of antioxidant properties of egg white

protein enzymatic hydrolysates by membrane ultrafiltration, Hem. Ind. 70 (2016) 419-428, https://doi.org/10.2298/HEMIND150506047J.

[361] S.J. Tanasković, N. Luković, S. Grbavčić, A. Stefanović, J. Jovanović, B. Bugarski, Z. Knežević-Jugović, Production of egg white protein hydrolysates with improved antioxidant capacity in a continuous enzymatic membrane reactor: optimization of operating parameters by statistical design, J. Food Sci. Technol. 55 (2018) 128-137, https://doi.org/10.1007/s13197-017-2848-5.

[362] X. Huang, Y. Zhou, M. Ma, Z. Cai, T. Li, Chemiluminescence evaluation of antioxidant activity and prevention of DNA damage effect of peptides isolated from soluble eggshell membrane protein hydrolysate, J. Agric. Food Chem. 58 (23) (2010) 12137-12142, https://doi.org/10.1021/jf101728d

[363] S. Lin, Y. Guo, Q. You, Y. Yin, J. Liu, Preparation of antioxidant peptide from egg white protein and improvement on its activities assisted by high- intensity pulsed electric field, J. Sci. Food Agric. 92 (2012) 1554-1561, https://doi.org/10.1002/jsfa.4742.

[364] S. Lin, Y. Jin, M. Liu, Y. Yang, M. Zhang, Y. Guo, G. Jones, J. Liu, Y. Yin, Research on the preparation of antioxidant peptides derived from egg white with assisting of high-intensity pulsed electric field, Food Chem. 139 (2013) 300-306, https://doi.org/10.1016/j.foodchem.2013.01.048.

[365] S. Jakovetić, N. Luković, B. Jugović, M. Gvozdenović, S. Grbavčić, J. Jovanović, Z. Knežević-Jugović, Production of antioxidant egg white hydrolysates in a continuous stirred tank enzyme reactor coupled with membrane separation unit, Food Bioprocess Technol. 8 (2015) 287-300, https://doi.org/10.1007/s11947-014-1402-y.

[366] D.O. Noh, H.J. Suh, Preparation of egg white liquid hydrolsate (ELH) and its radical-scavenging activity, Prev. Nutr. Food Sci. 20 (2015) 183-189, https://doi.org/10.3746/pnf.2015.20.3.183.

[367] H. Wang, Y. B. Huang, K.X. Gao, H. Sun, Z. L. Gao, Preparation and purification of velvet antlers peptides and its antioxidant activities, Chem. J. Chinese Univ. 31 (2010) 2390-2395.

[368] S.A. Hamid, N.R.A. Halim, N.M. Sarbon, Optimization of enzymatic hydrolysis conditions of Golden Apple snail (*Pomacea canaliculata*) protein by Alcalase, Int. Food Res. J. 22 (2015) 1615.

[369] M.L. Ling, T.X. Liu, Optimization of enzymatic hydrolysis of polyrhachis vicina roger protein and graded membrane separation of the antioxidant peptides, Modern Food Sci. Tech. 29 (2013) 1089-1092.

[370] A. Alemán, N. Blanco-Pascual, M.P. Montero, M.C. Gómez-Guillén, Simple and efficient hydrolysis procedure for full utilization of the seaweed *Mastocarpus stellatus* to produce antioxidant films, Food Hydrocoll. 56 (2016) 277-284, https://doi.org/10.1016/j.foodhyd.2015.12.024.

[371] Y. Wu, J. Wang, L. Li, X. Yang, J. Wang, X. Hu, Purification and identification of an antioxidant peptide from *Pinctada fucata* muscle, CyTA-J. Food. 16 (2018) 11-19, https://doi.org/10.1080/19476337.2017.1332099.

[372] N.I.M. Saleh, W.A.W.A.K. Ghani, M.R. Harun, S.M.M. Kamal, Optimization of enzymatic hydrolysis for the production of antioxidative peptide from *Nannochloropsis gaditana* using Response Surface Methodology, Pertanika J. Sci. Technol. 27 (2019) 41-55.

[373] T.F. Li, B. Ye, L.Y. Song, R.M. Yu, Isolation and purification of two antioxidant peptides from Alcalase hydrolysate of *Arca subcrenata*, Journal of Chinese medicinal materials, 37 (2014) 1140-1144.

[374] T. Sun, S. Zhang, W. Yang, Z. Zhao, D. Yang, Housefly pupae-derived antioxidant peptides exerting neuroprotective effects on hydrogen peroxide-induced oxidative damage in PC12 Cells, Molecules. 24 (2019) 4486, https://doi.org/10.3390/molecules24244486.

[375] N. Meshginfar, A. Sadeghi-Mahoonak, A.M. Kianfar, M. Ghorbani, M. Kashaninejad, Study of antioxidant activity of sheep visceral protein hydrolysate: Optimization using Response Surface Methodology, ARYA Atheroscler. 10 (2014) 179-184.

[376] K. H. Han, K. Shimada, T. Hayakawa, T.J. Yoon, M. Fukushima, Porcine splenic hydrolysate has antioxidant activity *in vivo* and *in vitro*, Korean J. Food Sci. Anim. Resour. 34 (2014) 325-332, https://doi.org/10.5851/kosfa.2014.34.3.325.

[377] V.G. da Silva, R.J.S. de Castro, Enzymatic hydrolysis of proteins from chicken viscera in the presence of an ionic liquid enhanced their antioxidant properties, Waste Biomass Valor. 11 (2019) 3183–3193, https://doi.org/10.1007/s12649-019-00693-y.

[378] J.B. Fan, L.H. Zheng, F. Wang, H.Y. Guo, L.U. Jiang, F.Z. Ren, Enzymatic hydrolysis of silk sericin by proteases and antioxidant activities of the hydrolysates, J. Food Biochem. 34 (2010) 382-398, https://doi.org/10.1111/j.1745-4514.2009.00286.x.

[379] R. Yang, X. Zhao, Z. Kuang, M. Ye, G. Luo, G. Xiao, S. Liao, L. Li, Z. Xiong, Optimization of antioxidant peptide production in the hydrolysis of silkworm (*Bombyx*

mori L.) pupa protein using Response Surface Methodology, J. Food Agric. Environ. 11 (2013) 952-956.

[380] H. Zhang, P. Wang, A. J. Zhang, X. Li, J. H. Zhang, Q. L. Qin, Y. J. Wu, Antioxidant activities of protein hydrolysates obtained from the housefly larvae, Acta Biol. Hung. 67 (2016) 236-246, https://doi.org/10.1556/018.67.2016.3.2.

[381] M. H. Yu, H. S. Lee, H. R. Cho, S. O. Lee, Enzymatic preparation and antioxidant activities of protein hydrolysates from *Tenebrio molitor* larvae (mealworm), J. Korean Soc. Food Sci. Nutr. 46 (2017) 435-441, https://doi.org/10.3746/jkfn.2017.46.4.435.

[382] H. S. Lee, H. J. Ryu, H. J. Song, S.-O. Lee, Enzymatic preparation and antioxidant activities of protein hydrolysates from *Protaetia brevitarsis* larvae, J. Korean Soc. Food Sci. Nutr. 46 (2017) 1164-1170, https://doi.org/10.3746/jkfn.2017.46.10.1164.

[383] H.J. Ryu, H.J. Song, S.O. Lee, Enzymatic preparation and antioxidant activities of protein hydrolysates from *Allomyrina dichotoma* larvae, J. Korean Soc. Food Sci. Nutr. 48 (2019) 410-417, https://doi.org/10.3746/jkfn.2019.48.4.410.

[384] D. Zhu, X. Huang, F. Tu, C. Wang, F. Yang, Preparation, antioxidant activity evaluation, and identification of antioxidant peptide from black soldier fly (*Hermetia illucens* L.) larvae, J. Food Biochem. 44 (2020) e13186, https://doi.org/10.1111/jfbc.13186.

[385] M.A.J. Alzahrani, C.O. Perera, Y. Hemar, Production of bioactive proteins and peptides from the diatom *Nitzschia laevis* and comparison of their *in vitro* antioxidant activities with those from *Spirulina platensis* and *Chlorella vulgaris*, Int. J. Food Sci. Technol. 53 (2018) 676-682, https://doi.org/10.1111/ijfs.13642.

[386] X. Hu, X. Yang, Q. Wu, L. Li, Y. Wu, S. Chen, R. Li, J. Ren, Purification and identification of antioxidant peptides from *Schizochytrium limacinum* hydrolysates by consecutive chromatography and Electrospray Ionization-Mass Spectrometry, Molecules. 24 (2019) 3004, https://doi.org/10.3390/molecules24163004.

[387] Y. Ding, S. C. Ko, S.-H. Moon, S. H. Lee, Protective effects of novel antioxidant peptide purified from Alcalase hydrolysate of Velvet Antler against oxidative stress in Chang Liver Cells *in vitro* and in a Zebrafish Model *in vivo*, Int. J. Mol. Sci. 20 (2019) 5187, https://doi.org/10.3390/ijms20205187.

[388] S.M. Kim, Antioxidant and anticancer activities of enzymatic hydrolysates of solitary tunicate (*Styela clava*), Food Sci Biotechnol. 20 (2011) 1075, https://doi.org/10.1007/s10068-011-0146-y.

[389] S.Q. Huang, H. Ao, F.K. Zeng, B. Yang, Analysis of enzymatic hydrolysis of *Ganoderma lucidum* protein and antioxidation of its hydrolysates, Modern Food Sci. Tech. 29 (2013) 24-28.

[390] C. F. Chi, B. Wang, F. Y. Hu, Y.M. Wang, B. Zhang, S. G. Deng, C. W. Wu, Purification and identification of three novel antioxidant peptides from protein hydrolysate of bluefin leatherjacket (*Navodon septentrionalis*) skin, Food Res. Int. 73 (2015) 124-129, https://doi.org/10.1016/j.foodres.2014.08.038.

[391] J.O. Onuh, A.T. Girgih, R.E. Aluko, M. Aliani, *In vitro* antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions, Food Chem. 150 (2014) 366-373, https://doi.org/10.1016/j.foodchem.2013.10.107.

[392] N.M. Sarbon, F. Badii, N.K. Howell, Purification and characterization of antioxidative peptides derived from chicken skin gelatin hydrolysate, Food Hydrocoll. 85 (2018) 311-320, https://doi.org/10.1016/j.foodhyd.2018.06.048.

[393] G. P. Hong, S. G. Min, Y. J. Jo, Anti-oxidative and anti-aging activities of porcine by-product collagen hydrolysates produced by commercial proteases: Effect of hydrolysis and ultrafiltration, Molecules. 24 (2019) 1104, https://doi.org/10.3390/molecules24061104.

[394] A.K. Verma, M.K. Chatli, P. Kumar, N. Mehta, Antioxidant and antimicrobial efficacy of peptidic hydrolysate obtained from porcine blood, Agric. Res. 8 (2019) 116-124, https://doi.org/10.1007/s40003-018-0350-6.

[395] S. Arana-Peña, D. Carballares, Á. Berenguer-Murcia, A.R. Alcántara, R.C. Rodrigues, R. Fernandez-Lafuente, One pot use of combilipases for full modification of oils and fats: Multifunctional and heterogeneous substrates, Catalysts. 10 (2020) 605, https://doi.org/10.3390/catal10060605.

[396] G. Chen, L. Cheng, H. Yu, H. Song, Y. Lv, C. Yang, T. Zhu, N. Sun, Functions of different yak bone peptides, Int. J. Food Prop. 14 (2011) 1136-1141, https://doi.org/10.1080/10942911003592753.

[397] Q. Ren, X. Zhang, Studies on enzymatic process of antioxidant peptides from oat protein, Journal of Chinese Institute of Food Science and Technology, 14 (2014) 65-72.

[398] R.J.S. de Castro, H.H. Sato, Synergistic actions of proteolytic enzymes for production of soy protein hydrolysates with antioxidant activities: an approach based on enzymes specificities, Biocatal. Agric. Biotechnol. 4 (2015) 694-702, https://doi.org/10.1016/j.bcab.2015.08.012.

[399] R.J.S. de Castro, V.G. Cason, H.H. Sato, Binary mixture of proteases increases the antioxidant properties of white bean (*Phaseolus vulgaris* L.) protein-derived peptides obtained by enzymatic hydrolysis, Biocatal. Agric. Biotechnol. 10 (2017) 291-297, https://doi.org/10.1016/j.bcab.2017.04.003.

[400] J.G.d.S. Aguilar, V. Granato Cason, R.J.S. de Castro, Improving antioxidant activity of black bean protein by hydrolysis with protease combinations, Int. J. Food Sci. Technol. 54 (2019) 34-41, https://doi.org/10.1111/ijfs.13898.

[401] D. Jin, X. Liu, X. Zheng, X. Wang, J. He, Preparation of antioxidative corn protein hydrolysates, purification and evaluation of three novel corn antioxidant peptides, Food Chem. 204 (2016) 427-436, https://doi.org/10.1016/j.foodchem.2016.02.119.

[402] Z. Wang, Y. Yuan, X. Wang, X. Zhao, G. An, Y. Wang, Optimization of enzymatic hydrolysis of wheat gluten and antioxidant activities of its hydrolysates, J. Chinese Cereals Oils Assoc. 29 (2014) 7-13.

[403] Y. Chim-Chi, S. Gallegos-Tintoré, C. Jiménez-Martínez, G. Dávila-Ortiz, L. Chel-Guerrero, Antioxidant capacity of Mexican chia (*Salvia hispanica* L.) protein hydrolyzates, Food Measure. 12 (2018) 323-331, https://doi.org/10.1007/s11694-017-9644-9.

[404] X. Lu, L. Zhang, Q. Sun, G. Song, J. Huang, Extraction, identification and structure-activity relationship of antioxidant peptides from sesame (*Sesamum indicum* L.) protein hydrolysate, Food Res. Int. 116 (2019) 707-716, https://doi.org/10.1016/j.foodres.2018.09.001.

[405] S. He, F. Wang, Z. Ning, B. Yang, Y. Wang, Preparation of anchovy (*Engraulis japonicus*) protein hydrolysates with high free radical-scavenging activity using

endogenous and commercial enzymes, Food Sci. Technol. Int. 20 (2014) 567-578, https://doi.org/10.1177/1082013213496418.

[406] X. R. Yang, L. Zhang, D. G. Ding, C. F. Chi, B. Wang, J. C. Huo, Preparation, identification, and activity evaluation of eight antioxidant peptides from protein hydrolysate of hairtail (*Trichiurus japonicas*) muscle, Mar. Drugs. 17 (2019) 23, https://doi.org/10.3390/md17010023.

[407] G.V. Marson, R.J.S. de Castro, M.T.d.C. Machado, F. da Silva Zandonadi, H.D.d.F.Q. Barros, M.R. Maróstica Júnior, A. Sussulini, M.D. Hubinger, Proteolytic enzymes positively modulated the physicochemical and antioxidant properties of spent yeast protein hydrolysates, Process Biochem. 91 (2020) 34-45, https://doi.org/10.1016/j.procbio.2019.11.020.

[408] G.V. Marson, M.T. da Costa Machado, R.J.S. de Castro, M.D. Hubinger, Sequential hydrolysis of spent brewer's yeast improved its physico-chemical characteristics and antioxidant properties: A strategy to transform waste into added-value biomolecules, Process Biochem. 84 (2019) 91-102, https://doi.org/10.1016/j.procbio.2019.06.018.

[409] G. Shu, S. Mei, Q. Zhang, N. Xin, H. Chen, Application of the Plackett-Burman design to determine the main factors affecting the anti-oxidative activity of goat's milk casein hydrolyzed by Alcalase and papain, ACTA Sci. Pol. Technol. Aliment. 17 (2018) 257-266, https://doi.org/10.17306/J.AFS.0580.

[410] G. Shu, S. Mei, L. Chen, B. Cao, Q. Zhang, H. Chen, X. Cui, Optimization of the antioxiant peptides production from goat milk casein hydrolyzed by Alcalase and papain using Response Surface Methodology, The Annals of the University Dunarea de Jos of

Galati. Fascicle VI-Food Technology 43 (2019) 24-39, https://doi.org/10.35219/foodtechnology.2019.1.02.

[411] Y. Shi, J. Kovacs-Nolan, B. Jiang, R. Tsao, Y. Mine, Antioxidant activity of enzymatic hydrolysates from eggshell membrane proteins and its protective capacity in human intestinal epithelial Caco-2 cells, J. Func. Foods. 10 (2014) 35-45, https://doi.org/10.1016/j.jff.2014.05.004.

[412] M.d.M. Yust, M.d.C. Millán- Linares, J.M. Alcaide- Hidalgo, F. Millán, J. Pedroche, Hypocholesterolaemic and antioxidant activities of chickpea (*Cicer arietinum* L.) protein hydrolysates, J. Sci. Food Agric. 92 (2012) 1994-2001, https://doi.org/10.1002/jsfa.5573.

[413] X. Kou, J. Gao, Z. Xue, Z. Zhang, H. Wang, X. Wang, Purification and identification of antioxidant peptides from chickpea (*Cicer arietinum* L.) albumin hydrolysates, LWT-Food Sci. Technol. 50 (2013) 591-598, https://doi.org/10.1016/j.lwt.2012.08.002.

[414] H.J. Zhang, J. Wang, B.H. Zhang, H. Zhang, Antioxidant activities of the fractionated protein hydrolysates from heat stable defatted rice bran, Int. J. Food Sci. Technol. 49 (2014) 1330-1336, https://doi.org/10.1111/ijfs.12433.

[415] S.C. Lopez-Garcia, G. Arambula-Villa, J.G. Torruco-Uco, A. Contreras-Oliva, F. Hernandez-Rosas, M. Lopez-Espindola, J.A. Herrera-Corredor, Antioxidant activity of chia (*Salvia hispanica* L.) protein fraction hydrolyzed with Alcalase and Flavourzyme, Agrociencia. 53 (2019) 505-520.

[416] S. Yarnpakdee, S. Benjakul, H.G. Kristinsson, H. Kishimura, Antioxidant and sensory properties of protein hydrolysate derived from Nile tilapia (*Oreochromis niloticus*)

by one-and two-step hydrolysis, J. Food Sci. Technol. 52 (2015) 3336-3349, https://doi.org/10.1007/s13197-014-1394-7.

[417] M. Nurilmala, R.M. Pertiwi, T. Nurhayati, S. Fauzi, I. Batubara, Y. Ochiai, Characterization of collagen and its hydrolysate from yellowfin tuna *Thunnus albacares* skin and their potencies as antioxidant and antiglycation agents, Fish. Sci. 85 (2019) 591-599, https://doi.org/10.1007/s12562-019-01303-5.

[418] X. R. Yang, Y. T. Qiu, Y. Q. Zhao, C. F. Chi, B. Wang, Purification and characterization of antioxidant peptides derived from protein hydrolysate of the marine bivalve mollusk *Tergillarca granosa*, Mar. Drugs. 17 (2019) 251, https://doi.org/10.3390/md17050251.

[419] C. Lan, Y.Q. Zhao, X.R. Li, B. Wang, High Fischer ratio oligopeptides determination from Antartic krill: Preparation, peptides profiles, and *in vitro* antioxidant activity, J. Food Biochem. 43 (2019) e12827, https://doi.org/10.1111/jfbc.12827.

[420] C. Dimou, C. Antza, E. Akrivos, I. Doundoulakis, S. Stabouli, A.B. Haidich, V. Kotsis, A systematic review and network meta-analysis of the comparative efficacy of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in hypertension, J. Hum. Hypertens. 33 (2019) 188-201, https://doi.org/10.1038/s41371-018-0138-y.

[421] H. Meisel, D.J. Walsh, B. Murray, R.J. Fitzgerald, ACE inhibitory peptides, nutraceutical proteins and peptides in health and disease, in: Y. Mine, F. Shahidi (Eds.), Nutraceutical Science and Technology, CRC Press, USA, 2006, pp. 688.

[422] S. H. Lee, Z. J. Qian, S. K. Kim, A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Food Chem. 118 (2010) 96-102, https://doi.org/10.1016/j.foodchem.2009.04.086.

[423] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169, https://doi.org/10.1016/j.copbio.2007.01.013.

[424] B.S. Heran, M.M.Y. Wong, I.K. Heran, J.M. Wright, Blood pressure lowering efficacy of angiotensin converting enzyme (ACE) inhibitors for primary hypertension, Cochrane Database of Syst. Rev. 2008 (2008) CD003823, https://doi.org/10.1002/14651858.CD003823.pub2.

[425] B.M. Hicks, K.B. Filion, H. Yin, L. Sakr, J.A. Udell, L. Azoulay, Angiotensin converting enzyme inhibitors and risk of lung cancer: population based cohort study, BJM. 363 (2018) k4209, https://doi.org/10.1136/bmj.k4209.

[426] M. Ghassem, A.S. Babji, M. Said, F. Mahmoodani, K. Arihara, Angiotensin I–converting enzyme inhibitory peptides from snakehead fish sarcoplasmic protein hydrolysate, J. Food Biochem. 38 (2014) 140-149, https://doi.org/10.1111/jfbc.12031.

[427] Y. Li, Y. Zheng, Y. Zhang, L. Liu, S. Zhao, Purification, characterization, synthesis, *in vivo* and *in vitro* antihypertensive activity of bioactive peptides derived from coconut (*Cocos nucifera* L.) cake globulin hydrolysates, RSC Adv. 6 (2016) 92688-92698, https://doi.org/10.1039/C6RA19971B.

[428] W. Liu, G. Cheng, H. Liu, Y. Kong, Purification and identification of a novel angiotensin I-converting enzyme inhibitory peptide from sesame meal, Int. J. Pept. Res. Ther. 21 (2015) 433-442, https://doi.org/10.1007/s10989-015-9471-y.

[429] M.D. Magaña, M. Segura-Campos, G. Dávila-Ortiz, D. Betancur-Ancona, L. Chel-Guerrero, ACE-I inhibitory properties of hydrolysates from germinated and ungerminated *Phaseolus lunatus* proteins, Food Sci. Technol. 35 (2015) 167-174, https://doi.org/10.1590/1678-457X.6551.

[430] X. Gu, Y. K. Hou, D. Li, J. Z. Wang, F. J. Wang, Separation, purification, and identification of angiotensin I–converting enzyme inhibitory peptides from walnut (*Juglans regia* L.) hydrolyzate, Int. J. Food Prop. 18 (2015) 266-276, https://doi.org/10.1080/10942912.2012.713476.

[431] B. Gao, X. H. Zhao, Modification of soybean protein hydrolysates by Alcalase-catalyzed plastein reaction and the ACE-inhibitory activity of the modified product *in vitro*, Int. J. Food Prop. 15 (2012) 982-996, https://doi.org/10.1080/10942912.2010.511755.

[432] L. Li, X.H. Luo, J. Zhang, Effects of *in vitro* digestion on properties and ACE inhibitory activities of soybean peptides, Journal of South China University of Technology (Natural Science) 42 (3) (2014) 125-130, https://doi.org/10.3969/j.issn.1000-565X.2014.03.020.

[433] Y. Zhang, Y. Zhang, P. Chen, F. Shu, K. Li, L. Qiao, Z. Chen, L. Wang, A novel angiotensin-I converting enzyme inhibitory peptide derived from the glutelin of vinegar soaked black soybean and its antihypertensive effect in spontaneously hypertensive rats, J. Biochem.166 (2019) 223-230, https://doi.org/10.1093/jb/mvz029.

[434] R. He, S.A. Malomo, A. Alashi, A.T. Girgih, X. Ju, R.E. Aluko, Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides, J. Func. Foods. 5 (2013) 781-789, https://doi.org/10.1016/j.jff.2013.01.024.

[435] S. Mäkinen, T. Streng, L.B. Larsen, A. Laine, A. Pihlanto, Angiotensin I-converting enzyme inhibitory and antihypertensive properties of potato and rapeseed protein-derived peptides, J. Func. Foods. 25 (2016) 160-173, https://doi.org/10.1016/j.jff.2016.05.016.

[436] R. He, A.T. Girgih, E. Rozoy, L. Bazinet, X.-R. Ju, R.E. Aluko, Selective separation and concentration of antihypertensive peptides from rapeseed protein hydrolysate by electrodialysis with ultrafiltration membranes, Food Chem. 197 (2016) 1008-1014, https://doi.org/10.1016/j.foodchem.2015.11.081.

[437] R.G. Dadzie, H. Ma, E.E. Abano, W. Qu, S. Mao, Optimization of process conditions for production of angiotensin I-converting enzyme (ACE) inhibitory peptides from vital wheat gluten using Response Surface Methodology, Food Sci. Biotechnol. 22 (2013) 1531-1537, https://doi.org/10.1007/s10068-013-0248-9.

[438] R. He, H. Xing, Z. Wang, W. Ding, P. Zhu, B. Liu, H. Ma, Establishment of an enzymatic membrane reactor for angiotensin- converting enzyme inhibitory peptides preparation from wheat germ protein isolates, J. Food Process Eng. 39 (2016) 296-305, https://doi.org/10.1111/jfpe.12224.

[439] G. Ramírez-Torres, N. Ontiveros, V. Lopez-Teros, J.A. Ibarra-Diarte, C. Reyes-Moreno, E.O. Cuevas-Rodríguez, F. Cabrera-Chávez, Amaranth protein hydrolysates

efficiently reduce systolic blood pressure in spontaneously hypertensive rats, Molecules. 22 (2017) 1905, https://doi.org/10.3390/molecules22111905.

[440] E.E. Valdez-Meza, A. Raymundo, O.G. Figueroa-Salcido, G.I. Ramírez-Torres, P. Fradinho, S. Oliveira, I. de Sousa, M. Suárez-Jiménez, F.I. Cárdenas-Torres, A.R. Islas-Rubio, Pasta enrichment with an Amaranth hydrolysate affects the overall acceptability while maintaining antihypertensive properties, Foods. 8 (2019) 282, https://doi.org/10.3390/foods8080282.

[441] N. Ontiveros, V. López-Teros, M. de Jesús Vergara-Jiménez, A.R. Islas-Rubio, F.I. Cárdenas-Torres, E.-O. Cuevas-Rodríguez, C. Reyes-Moreno, D.M. Granda-Restrepo, S. Lopera-Cardona, G.I. Ramírez-Torres, Amaranth hydrolyzate enriched cookies reduce the systolic blood pressure in spontaneously hypertensive rats, J. Func. Foods. 64 (2020) 103613, https://doi.org/10.1016/j.jff.2019.103613.

[442] Q. Wu, J. Du, J. Jia, C. Kuang, Production of ACE inhibitory peptides from sweet sorghum grain protein using Alcalase: Hydrolysis kinetic, purification and molecular docking study, Food Chem. 199 (2016) 140-149, https://doi.org/10.1016/j.foodchem.2015.12.012.

[443] J. Q. Jia, N. Miao, J. J. Du, Q. Y. Wu, Angiotensin-I converting enzyme inhibitory peptides from Sweet Sorghum grain protein: Optimisation of hydrolysis Conditions and hydrolysate characterization, J. Chem. Soc. Pakistan. 41 (2019) 175-175.

[444] C.F. Ajibola, J.B. Fashakin, T.N. Fagbemi, R.E. Aluko, Renin and angiotensin converting enzyme inhibition with antioxidant properties of African yam bean protein

hydrolysate and reverse-phase HPLC-separated peptide fractions, Food Res. Int. 52 (2013) 437-444, https://doi.org/10.1016/j.foodres.2012.12.003.

[445] P. Valenzuela- García, N.A. Bobadilla, V. Ramírez- González, A. León-Villanueva, I.A. Lares- Asseff, A. Valdez- Ortiz, S. Medina- Godoy, Antihypertensive effect of protein hydrolysate from azufrado beans in spontaneously hypertensive rats, Cereal Chem. 94 (2017) 117-123, https://doi.org/10.1094/CCHEM-04-16-0105-FI.

[446] A.J. Hernández- Álvarez, J. Carrasco- Castilla, G. Dávila- Ortiz, M. Alaiz, J. Girón- Calle, J. Vioque- Peña, C. Jacinto- Hernández, C. Jiménez- Martínez, Angiotensin- converting enzyme- inhibitory activity in protein hydrolysates from normal and anthracnose disease- damaged *Phaseolus vulgaris* seeds, J. Sci. Food Agric. 93 (2013) 961-966, https://doi.org/10.1002/jsfa.5841.

[447] M.R. Segura-Campos, F. Peralta-González, A. Castellanos-Ruelas, L.A. Chel-Guerrero, D.A. Betancur-Ancona, Effect of *Jatropha curcas* peptide fractions on the angiotensin I-converting enzyme inhibitory activity, Biomed. Res. Int. (2013) 541947, https://doi.org/10.1155/2013/541947.

[448] Y. Xu, Y. Li, T. Bao, X. Zheng, W. Chen, J. Wang, A recyclable protein resource derived from cauliflower by-products: Potential biological activities of protein hydrolysates, Food Chem. 221 (2017) 114-122, https://doi.org/10.1016/j.foodchem.2016.10.053.

[449] H. J. Lim, M. S. Kim, D. S. Kim, Y.J. Kim, J.H. Lee, J.H. Pan, E. C. Shin, J.K. Kim, Blood pressure-lowering effects of Alcalase-hydrolyzed Camellia seed hull *in vitro* and in

spontaneous hypertensive rats, J. Med. Food. 20 (2017) 720-723, https://doi.org/10.1089/jmf.2016.0175.

[450] B. Bhaskar, L. Ananthanarayan, S. Jamdar, Purification, identification, and characterization of novel angiotensin I-converting enzyme (ACE) inhibitory peptides from Alcalase digested horse gram flour, LWT. 103 (2019) 155-161, https://doi.org/10.1016/j.lwt.2018.12.059.

[451] H. Li, R.E. Aluko, Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate, J. Agric. Food Chem. 58 (2010) 11471-11476, https://doi.org/10.1021/jf102538g.

[452] N. Gupta, S.S. Bhagyawant, Enzymatic treatment improves ACE-I inhibiton and antiproliferative potential of chickpea, Vegetos. 32 (2019) 363-369, https://doi.org/10.1007/s42535-019-00031-6.

[453] C. Guang, R.D. Phillips, Angiotensin I-converting enzyme inhibitory peptides from hydrolyzed cowpea flour, J. Food Agric. Environ. 10 (2012) 55-59.

[454] Z. Zhu, N. Qiu, J. Yi, Production and characterization of angiotensin converting enzyme (ACE) inhibitory peptides from apricot (*Prunus armeniaca* L.) kernel protein hydrolysate, Eur. Food Res. Technol. 231 (2010) 13-19, https://doi.org/10.1007/s00217-010-1235-5.

[455] S. Y. Back, J. R. Do, G. P. Do, H. K. Kim, Effect of angiotensin-I converting enzyme inhibitory from hydrolysate of soybean protein isolate, J. Korean Soc. Food Sci. Nutr. 39 (2010) 8-13, https://doi.org/10.3746/jkfn.2010.39.1.008.

[456] M.A. Hanafi, S.N. Hashim, S.Y. Chay, A. Ebrahimpour, M. Zarei, K. Muhammad, A. Abdul-Hamid, N. Saari, High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (*Glycine max*) hydrolysates, Food Res. Int. 106 (2018) 589-597, https://doi.org/10.1016/j.foodres.2018.01.030.

[457] M.R. Segura Campos, L.A. Chel Guerrero, D.A. Betancur Ancona, Angiotensin- I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea Vigna unguiculata hydrolysates, J. Sci. Food Agric. 90 (2010) 2512-2518, https://doi.org/10.1002/jsfa.4114.

[458] Y.Y. Chin, L.Y. Chew, G.T. Toh, J. Salampessy, A. Azlan, A. Ismail, Nutritional composition and angiotensin converting enzyme inhibitory activity of blue lupin (*Lupinus angustifolius*), Food Biosci. 31 (2019) 100401, https://doi.org/10.1016/j.fbio.2019.04.002.

[459] Ž. Vaštag, L. Popović, S. Popović, V. Krimer, D. Peričin, Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate, Food Chem. 124 (2011) 1316-1321, https://doi.org/10.1016/j.foodchem.2010.07.062.

[460] S. Medina- Godoy, D.L. Ambriz- Pérez, C.I. Fuentes- Gutiérrez, L.J. Germán-Báez, R. Gutiérrez- Dorado, C. Reyes- Moreno, A. Valdez- Ortiz, Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard- to- cook chickpeas, J. Sci. Food Agric. 92 (2012) 1974-1981, https://doi.org/10.1002/jsfa.5570.

[461] R. He, A. Alashi, S.A. Malomo, A.T. Girgih, D. Chao, X. Ju, R.E. Aluko, Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein

hydrolysates, Food Chem. 141 (2013) 153-159, https://doi.org/10.1016/j.foodchem.2013.02.087.

[462] S. Mäkinen, T. Johannson, E.V. Gerd, J.M. Pihlava, A. Pihlanto, Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates, J. Func. Foods 4 (2012) 575-583, https://doi.org/10.1016/j.jff.2012.03.003.

[463] A.M. Alashi, C.L. Blanchard, R.J. Mailer, S.O. Agboola, A.J. Mawson, R. He, S.A. Malomo, A.T. Girgih, R.E. Aluko, Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats, Food Res. Int. 55 (2014) 281-287, https://doi.org/10.1016/j.foodres.2013.11.015.

[464] D.M. Marrufo Estrada, L. Chel Guerrero, D. Betancur Ancona, V. Hernandez Escalante, Angiotensin I-Converting Enzyme inhibition with protein hydrolysates from *Jatropha curcas*, Acta bioquím. clín. latinoam. 46 (2012) 385-392.

[465] D.M. Marrufo-Estrada, M.P. Segura-Campos, L.A. Chel-Guerrero, D.A. Betancur-Ancona, Defatted *Jatropha curcas* flour and protein isolate as materials for protein hydrolysates with biological activity, Food Chem. 138 (2013) 77-83, https://doi.org/10.1016/j.foodchem.2012.09.033.

[466] M. Mirzapour, K. Rezaei, M.A. Sentandreu, Identification of potent ACE inhibitory peptides from wild almond proteins, J. Food Sci. 82 (2017) 2421-2431, https://doi.org/10.1111/1750-3841.13840.

[467] S.A. Malomo, J.O. Onuh, A.T. Girgih, R.E. Aluko, Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates, Nutrients. 7 (2015) 7616-7632, https://doi.org/10.3390/nu7095358.

[468] N. Gupta, N. Srivastava, S.S. Bhagyawant, Vicilin—A major storage protein of mungbean exhibits antioxidative potential, antiproliferative effects and ace inhibitory activity, PloS One. 13 (2018) e0191265, https://doi.org/10.1371/journal.pone.0191265.

[469] M.A. Nazir, T.H. Mu, M. Zhang, Preparation and identification of angiotensin I-converting enzyme inhibitory peptides from sweet potato protein by enzymatic hydrolysis under high hydrostatic pressure, Int. J. Food Sci. Technol. 55 (2020) 482-489, https://doi.org/10.1111/ijfs.14291.

[470] Y. Xu, T. Bao, W. Han, W. Chen, X. Zheng, J. Wang, Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from cauliflower by-products protein hydrolysate, Process Biochem. 51 (2016) 1299-1305, https://doi.org/10.1016/j.procbio.2016.05.023.

[471] A.K. Arise, A.M. Alashi, I.D. Nwachukwu, S.A. Malomo, R.E. Aluko, E.O. Amonsou, Inhibitory properties of bambara groundnut protein hydrolysate and peptide fractions against angiotensin converting enzymes, renin and free radicals, J. Sci. Food Agric. 97 (2017) 2834-2841, https://doi.org/10.1002/jsfa.8112.

[472] F. F. Ma, H. Wang, C. K. Wei, K. Thakur, Z. J. Wei, L. Jiang, Three novel ACE inhibitory peptides isolated from *Ginkgo biloba* seeds: Purification, inhibitory kinetic and mechanism, Front. Pharmacol. 9 (2019) 1579, https://doi.org/10.3389/fphar.2018.01579.

[473] C. B. Ahn, Y. J. Jeon, Y. T. Kim, J. Y. Je, Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis, Process Biochem. 47 (2012) 2240-2245, https://doi.org/10.1016/j.procbio.2012.08.019.

[474] R. Intarasirisawat, S. Benjakul, J. Wu, W. Visessanguan, Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe, J. Func. Foods. 5 (2013) 1854-1862, https://doi.org/10.1016/j.jff.2013.09.006.

[475] Y. Zhuang, L. Sun, B. Li, Production of the angiotensin-I-converting enzyme (ACE)-inhibitory peptide from hydrolysates of jellyfish (*Rhopilema esculentum*) collagen, Food Bioprocess Technol. 5 (2012) 1622-1629, https://doi.org/10.1007/s11947-010-0439-9.

[476] I.R. Amado, J.A. Vázquez, P. González, D. Esteban-Fernández, M. Carrera, C. Piñeiro, Identification of the major ACE-inhibitory peptides produced by enzymatic hydrolysis of a protein concentrate from cuttlefish wastewater, Mar. Drugs. 12 (2014) 1390-1405, https://doi.org/10.3390/md12031390.

[477] J. Kasiwut, W. Youravong, N. Srinupong, Angiotensin I- converting enzyme inhibitory peptides produced from tuna cooking juice hydrolysate by continuous enzymatic membrane reactor, J. Food Biochem. 43 (2019) e13058, https://doi.org/10.1111/jfbc.13058.

[478] A. Sila, A. Haddar, C. Martinez-Alvarez, A. Bougatef, Angiotensin-I-converting enzyme inhibitory and antioxidant activities of protein hydrolysate from muscle of barbel (*Barbus callensis*), J. Chem (2013) 1- 6, https://doi.org/10.1155/2013/545303.

[479] F. Mahmoodani, M. Ghassem, A.S. Babji, S.M. Yusop, R. Khosrokhavar, ACE inhibitory activity of pangasius catfish (*Pangasius sutchi*) skin and bone gelatin hydrolysate, J. Food Sci. Technol. 51 (2014) 1847-1856, https://doi.org/10.1007/s13197-012-0742-8 .

[480] J. Li, Q. Li, J. Li, B. Zhou, Peptides derived from *Rhopilema esculentum* hydrolysate exhibit angiotensin converting enzyme (ACE) inhibitory and antioxidant abilities, Molecules. 19 (2014) 13587-13602, https://doi.org/10.3390/molecules190913587.

[481] B. Forghani, M. Zarei, A. Ebrahimpour, R. Philip, J. Bakar, A.A. Hamid, N. Saari, Purification and characterization of angiotensin converting enzyme-inhibitory peptides derived from *Stichopus horrens*: Stability study against the ACE and inhibition kinetics, J. Func. Foods. 20 (2016) 276-290, https://doi.org/10.1016/j.jff.2015.10.025.

[482] H. Rasli, N.M. Sarbon, Optimization of enzymatic hydrolysis conditions and characterization of Shortfin scad (*Decapterus macrosoma*) skin gelatin hydrolysate using Response Surface Methodology, Int. Food Res. J. 25 (2018) 1541-1549.

[483] J. Roslan, S.M.M. Kamal, K.F.M. Yunos, N. Abdullah, A comparative study between Tilapia (*Oreochromis niloticus*) by-product and Tilapia protein hydrolysate on angiotensin I-converting enzyme (ACE) inhibition activities and functional properties, Sains Malaysiana. 47 (2018) 309-318, https://doi.org/10.17576/jsm-2018-4702-13.

[484] I. Wijesekara, Z. J. Qian, B. Ryu, D. H. Ngo, S. K. Kim, Purification and identification of antihypertensive peptides from seaweed pipefish (*Syngnathus schlegeli*) muscle protein hydrolysate, Food Res. Int. 44 (2011) 703-707, https://doi.org/10.1016/j.foodres.2010.12.022.

[485] A. Alemán, E. Pérez-Santín, S. Bordenave-Juchereau, I. Arnaudin, M.C. Gómez-Guillén, P. Montero, Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity, Food Res. Int. 44 (2011) 1044-1051, https://doi.org/10.1016/j.foodres.2011.03.010.

[486] Z.Y. Dai, Y.P. Zhang, H. Zhang, Y.B. Lu, Preparation and characterization of mussel (*mytilus edulis*) protein hydrolysates with angiotensin- i- converting enzyme (ace) inhibitory activity by enzymatic hydrolysis, J. Food Biochem. 36 (2012) 66-74, https://doi.org/10.1111/j.1745-4514.2010.00505.x.

[487] B. Forghani, A. Ebrahimpour, J. Bakar, A. Abdul Hamid, Z. Hassan, N. Saari, Enzyme hydrolysates from *Stichopus horrens* as a new source for angiotensin-converting enzyme inhibitory peptides, Evid. Based Complement. Alternat. Med. (2012), https://doi.org/10.1155/2012/236384.

[488] T. S. Vo, D. H. Ngo, J. A. Kim, B. Ryu, S. K. Kim, An antihypertensive peptide from tilapia gelatin diminishes free radical formation in murine microglial cells, J. Agric. Food Chem. 59 (2011) 12193-12197, https://doi.org/10.1021/jf202837g.

[489] D. H. Ngo, B. Ryu, S. K. Kim, Active peptides from skate (*Okamejei kenojei*) skin gelatin diminish angiotensin-I converting enzyme activity and intracellular free radical-mediated oxidation, Food Chem. 143 (2014) 246-255, https://doi.org/10.1016/j.foodchem.2013.07.067.

[490] S. Feng, J. Limwachiranon, Z. Luo, X. Shi, Q. Ru, Preparation and purification of angiotensin- converting enzyme inhibitory peptides from hydrolysate of shrimp (*Litopenaeus vannamei*) shell waste, Int. J. Food Sci. Technol. 51 (2016) 1610-1617, https://doi.org/10.1111/ijfs.13131.

[491] I. Lassoued, L. Mora, R. Nasri, M. Jridi, F. Toldrá, M. C. Aristoy, A. Barkia, M. Nasri, Characterization and comparative assessment of antioxidant and ACE inhibitory

activities of thornback ray gelatin hydrolysates, J. Func. Foods. 13 (2015) 225-238, https://doi.org/10.1016/j.jff.2014.12.042.

[492] R. Ghanbari, M. Zarei, A. Ebrahimpour, A. Abdul-Hamid, A. Ismail, N. Saari, Angiotensin-I converting enzyme (ACE) inhibitory and anti-oxidant activities of sea cucumber (*Actinopyga lecanora*) hydrolysates, Int. J. Mol. Sci. 16 (2015) 28870-28885, https://doi.org/10.3390/ijms161226140.

[493] J. Yi, C. De Gobba, L.H. Skibsted, J. Otte, Angiotensin-I converting enzyme inhibitory and antioxidant activity of bioactive peptides produced by enzymatic hydrolysis of skin from grass carp (*Ctenopharyngodon idella*), Int. J. Food Prop. 20 (2017) 1129-1144, https://doi.org/10.1080/10942912.2016.1202932.

[494] J. Kasiwut, N. Sirinupong, W. Youravong, The anticoagulant and angiotensin I-Converting Enzyme (ACE) Inhibitory peptides from tuna cooking juice produced by Alcalase, Curr. Nutr. Food Sci. 14 (2018) 225-234, https://doi.org/10.2174/1573401313666170427122708.

[495] B. Borges-Contreras, C.E. Martínez-Sánchez, E. Herman-Lara, J. Rodríguez-Miranda, B. Hernández-Santos, J.M. Juárez-Barrientos, C.M. Guerra-Almonacid, D.A. Betancur-Ancona, J.G. Torruco-Uco, Angiotensin-converting enzyme inhibition in vitro by protein hydrolysates and peptide fractions from Mojarra of Nile Tilapia (*Oreochromis niloticus*) Skeleton, J. Med. Food. 22 (2019) 286-293, https://doi.org/10.1089/jmf.2018.0163.

[496] A.S. Dewi, G. Patantis, Y.N. Fawzya, H.E. Irianto, S. Sa'diah, Angiotensin-converting enzyme (ACE) inhibitory activities of protein hydrolysates from Indonesian sea cucumbers, Int. J. Pept. Res. Ther. In press, https://doi.org/10.1007/s10989-020-10035-5.

[497] K.P.M. Noorani, R.A. Nazeer, Enzymatic production of two tri-peptides on ACE-I inhibition and antioxidant activities, Int. J. Pept. Res. Ther. In press, https://doi.org/10.1007/s10989-020-10037-3.

[498] Y. Y. Li, T. J. Li, X. H. Zhao, Preparation of Alcalase-catalyzed casein plasteins in the presence of proline addition and the ACE-inhibitory activity of the plasteins *in vitro*, Eur. Food Res. Technol. 231 (2010) 197-207, https://doi.org/10.1007/s00217-010-1270-2.

[499] S.J. Jiang, F. Qian, X. Shen, G. Mu, Separation and purification of angiotensin converting enzyme inhibitory peptides derived from bovine casein, J. Pure Appl. Microbiol. 7 (2013) 789-793.

[500] X. Wei, T. J. Li, X. H. Zhao, Coupled Neutrase–catalyzed plastein reaction mediated the ACE-inhibitory activity *in vitro* of casein hydrolysates prepared by Alcalase, Int. J. Food Prop. 16 (2013) 433-443, https://doi.org/10.1080/10942912.2011.553759.

[501] H. Sun, T. J. Li, X. H. Zhao, ACE inhibition and enzymatic resistance *in vitro* of a casein hydrolysate subjected to plastein reaction in the presence of extrinsic proline and ethanol-or methanol-water fractionation, Int. J. Food Prop. 17 (2014) 386-398, https://doi.org/10.1080/10942912.2011.642048.

[502] Y. Zhang, X. H. Zhao, Properties of casein hydrolysate as affected by plastein reaction in ethanol-water medium, Czech J. Food Sci. 31 (2013) 559-567, https://doi.org/10.17221/480/2012-CJFS.

[503] Y. Zhang, X. H. Zhao, *In vitro* Angiotensin I-converting enzyme inhibition of casein hydrolysate responsible for plastein reaction in ethanol-water medium, solvent fractionation, and protease digestion, Int. J. Food Prop. 17 (2014) 1577-1590, https://doi.org/10.1080/10942912.2013.768269.

[504] B. Zhao, X. H. Zhao, *In vitro* angiotensin-converting enzyme inhibition or digestive stability of casein hydrolysates treated by plastein reaction in propanol–water medium, CyTA-J. Food. 11 (2013) 293-299, https://doi.org/10.1080/19476337.2012.748693.

[505] M.B. O'Keeffe, R.J. FitzGerald, Whey protein hydrolysate induced modulation of endothelial cell gene expression, J. Funct. Foods. 40 (2018) 102-109, https://doi.org/10.1016/j.jff.2017.11.001.

[506] S. M. Lim, N. K. Lee, K. K. Park, Y. C. Yoon, H. D. Paik, ACE-inhibitory effect and physicochemical characteristics of yogurt beverage fortified with whey protein hydrolysates, Korean J. Food Sci. Ani. Resour. 31 (2011) 886-892, http://dx.do.org/10.5851/kosfa.2011.31.6.886.

[507] P. Mudgil, B. Baby, T. Y. Ngoh, H. Kamal, R. Vijayan, C. Y. Gan, S. Maqsood, Molecular binding mechanism and identification of novel anti-hypertensive and anti-inflammatory bioactive peptides from camel milk protein hydrolysates, LWT. 112 (2019) 108193, https://doi.org/10.1016/j.lwt.2019.05.091.

[508] F. Zhou, Z. Xue, J. Wang, Antihypertensive effects of silk fibroin hydrolysate by alcalase and purification of an ACE inhibitory dipeptide, J. Agric. Food Chem. 58 (2010) 6735-6740, https://doi.org/10.1021/jf101101r.

[509] J. Lu, D. F. Ren, Y. L. Xue, Y. Sawano, T. Miyakawa, M. Tanokura, Isolation of an antihypertensive peptide from Alcalase digest of *Spirulina platensis*, J. Agric. Food Chem. 58 (2010) 7166-7171, https://doi.org/10.1021/jf100193f.

[510] S. C. Huang, P. J. Liu, Inhibition of angiotensin I-converting enzymes by enzymatic hydrolysates from chicken blood, J. Food Drug Anal. 18 (2010) 458-463.

[511] L.J. Gómez Sampedro, J.E. Zapata Montoya, Effects of hydrolysis and digestion *in vitro* on the activity of bovine plasma hydrolysates as inhibitors of the angiotensin I converting enzyme, Braz. Arch. Biol. Technol. 57 (2014) 386-393, https://doi.org/10.1590/S1516-89132014005000004

[512] S. Y. Back, H. K. Kim, S. D. Lim, G. P. Do, J. R. Do, Development of antihypertensive natural seasoning using beef hydrolyzate, J. Korean Soc. Appl. Biol. Chem. 56 (2013) 201-206, https://doi.org/10.1007/s13765-011-3023-8.

[513] C. Dai, H. Ma, L. Luo, X. Yin, Angiotensin I-converting enzyme (ACE) inhibitory peptide derived from *Tenebrio molitor* (L.) larva protein hydrolysate, Eur. Food Res. Technol. 236 (2013) 691-698, https://doi.org/10.1007/s00217-013-1923-z.

[514] J. Jia, Q. Wu, H. Yan, Z. Gui, Purification and molecular docking study of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide from Alcalase hydrolysate of ultrasonic-pretreated silkworm pupa (*Bombyx mori*) protein, Process Biochem. 50 (2015) 876-883, https://doi.org/10.1016/j.procbio.2014.12.030.

[515] S. Pan, S. Wang, L. Jing, D. Yao, Purification and characterisation of a novel angiotensin-I converting enzyme (ACE)-inhibitory peptide derived from the enzymatic

hydrolysate of *Enteromorpha clathrata* protein, Food Chem. 211 (2016) 423-430, https://doi.org/10.1016/j.foodchem.2016.05.087.

[516] J. Liu, Z. Yu, W. Zhao, S. Lin, E. Wang, Y. Zhang, H. Hao, Z. Wang, F. Chen, Isolation and identification of angiotensin-converting enzyme inhibitory peptides from egg white protein hydrolysates, Food Chem. 122 (2010) 1159-1163, https://doi.org/10.1016/j.foodchem.2010.03.108.

[517] Z. Yu, W. Zhao, J. Liu, J. Lu, F. Chen, QIGLF, a novel angiotensin I- converting enzyme- inhibitory peptide from egg white protein, J. Sci. Food Agric. 91 (2011) 921-926, https://doi.org/10.1002/jsfa.4266.

[518] W. Qu, H. Ma, Z. Pan, L. Luo, Z. Wang, R. He, Preparation and antihypertensive activity of peptides from *Porphyra yezoensis*, Food Chem. 123 (2010) 14-20, https://doi.org/10.1016/j.foodchem.2010.03.091.

[519] Z. J. Qian, S. J. Heo, C.H. Oh, D. H. Kang, S.H. Jeong, W.S. Park, I. W. Choi, Y.J. Jeon, W. K. Jung, Angiotensin I-converting enzyme (ACE) inhibitory peptide isolated from biodiesel byproducts of marine microalgae, *Nannochloropsis oculata*, J. Biobased Mater. Bioenergy. 7 (2013) 135-142, https://doi.org/10.1166/jbmb.2013.1264.

[520] S. C. Ko, N. Kang, E. A. Kim, M.C. Kang, S. H. Lee, S. M. Kang, J. B. Lee, B. T. Jeon, S. K. Kim, S. J. Park, A novel angiotensin I-converting enzyme (ACE) inhibitory peptide from a marine *Chlorella ellipsoidea* and its antihypertensive effect in spontaneously hypertensive rats, Process Biochem. 47 (2012) 2005-2011, https://doi.org/10.1016/j.procbio.2012.07.015.

[521] J.O. Onuh, A.T. Girgih, R.E. Aluko, M. Aliani, Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates, Food Res. Int. 53 (2013) 260-267, https://doi.org/10.1016/j.foodres.2013.05.010.

[522] J.O. Onuh, A.T. Girgih, S.A. Malomo, R.E. Aluko, M. Aliani, Kinetics of *in vitro* renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats, J. Func. Foods. 14 (2015) 133-143, https://doi.org/10.1016/j.jff.2015.01.031.

[523] S. Nm, W.A. Wan, Angiotensin-I converting enzyme (ACE) inhibitory peptides from chicken skin gelatin hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Int. Food Res. J. 26 (2019) 903-911.

[524] P. Mudgil, B. Jobe, H. Kamal, M. Alameri, N. Al Ahbabi, S. Maqsood, Dipeptidyl peptidase-IV, α-amylase, and angiotensin I converting enzyme inhibitory properties of novel camel skin gelatin hydrolysates, LWT. 101 (2019) 251-258, https://doi.org/10.1016/j.lwt.2018.11.014.

[525] Y. Fu, J.F. Young, T.K. Dalsgaard, M. Therkildsen, Separation of angiotensin I-converting enzyme inhibitory peptides from bovine connective tissue and their stability towards temperature, pH and digestive enzymes, Int. J. Food Sci. Technol. 50 (2015) 1234-1243, https://doi.org/10.1111/ijfs.12771.

[526] M.H. Nurfatin, I.K.E. Syarmila, D.N. Aliah, M.K. Zalifah, A.S. Babji, M.K. Ayob, Effect of enzymatic hydrolysis on Angiotensin converting enzyme (ACE) inhibitory activity in swiftlet saliva, Int. Food Res. J. 23 (2016) 141-146.

[527] Y. L. Huang, M. F. Ma, C. J. Chow, Y. H. Tsai, Angiotensin I-converting enzyme inhibitory and hypocholesterolemic activities: Effects of protein hydrolysates prepared from *Achatina fulica* snail foot muscle, Int. J. Food Prop. 20 (2017) 3102-3111, https://doi.org/10.1080/10942912.2016.1274904.

[528] S.J. You, J. Wu, Angiotensin- I converting enzyme inhibitory and antioxidant activities of egg protein hydrolysates produced with gastrointestinal and nongastrointestinal enzymes, J. Food Sci. 76 (2011) C801-C807, https://doi.org/10.1111/j.1750-3841.2011.02228.x.

[529] L. Chen, J. Wang, G. Shu, H. Chen, Production of Angiotensin-I-Converting Enzyme inhibitory peptide from goat milk casein: optimization conditions of complex protease hydrolysate by Response Surface Methodology and purification, Emir. J. Food Agric. 30 (2018) 742-749, https://doi.org/10.9755/ejfa.2018.v30.i9.1795.

[530] L. Chen, W. Shangguan, C. Bao, G. Shu, H. Chen, Collaborative optimization and molecular docking exploration of novel ACE-inhibitory peptides from bovine milk by complex proteases hydrolysis, Artif. Cells Nanomed. Biotechnol. 48 (2020) 180-187, https://doi.org/10.1080/21691401.2019.1699824.

[531] P. Ambigaipalan, A.S. Al-Khalifa, F. Shahidi, Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin, J. Func. Foods. 18 (2015) 1125-1137, https://doi.org/10.1016/j.jff.2015.01.021.

[532] L. Wang, X. Mao, X. Cheng, X. Xiong, F. Ren, Effect of enzyme type and hydrolysis conditions on the *in vitro* angiotensin I- converting enzyme inhibitory activity and ash

content of hydrolysed whey protein isolate, Int. J. Food Sci. Technol. 45 (2010) 807-812, https://doi.org/10.1111/j.1365-2621.2010.02210.x.

[533] X. Rui, J.I. Boye, B.K. Simpson, S.O. Prasher, Angiotensin I-converting enzyme inhibitory properties of *Phaseolus vulgaris* bean hydrolysates: Effects of different thermal and enzymatic digestion treatments, Food Res. Int. 49 (2012) 739-746, https://doi.org/10.1016/j.foodres.2012.09.025.

[534] X. Rui, J.I. Boye, B.K. Simpson, S.O. Prasher, Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phaseolus vulgaris*) hydrolysates, J. Func. Foods. 5 (2013) 1116-1124, https://doi.org/10.1016/j.jff.2013.03.008.

[535] Y. Zheng, Y. Li, Y. Zhang, X. Ruan, R. Zhang, Purification, characterization, synthesis, *in vitro* ACE inhibition and *in vivo* antihypertensive activity of bioactive peptides derived from oil palm kernel glutelin-2 hydrolysates, J. Func. Foods. 28 (2017) 48-58, https://doi.org/10.1016/j.jff.2016.11.021.

[536] M.R. Segura Campos, F. Peralta González, L. Chel Guerrero, D. Betancur Ancona, Angiotensin I-converting enzyme inhibitory peptides of chia (*Salvia hispanica*) produced by enzymatic hydrolysis, Int. J. Food Sci. (2013) 1-8, https://doi.org/10.1155/2013/158482.

[537] R. L. Liu, X. L. Ge, X. Y. Gao, H. Y. Zhan, T. Shi, N. Su, Z.-Q. Zhang, Two angiotensin-converting enzyme-inhibitory peptides from almond protein and the protective action on vascular endothelial function, Food Funct. 7 (2016) 3733-3739, https://doi.org/10.1039/C6FO00654J.

[538] Y. Zheng, X. Wang, Y. Zhuang, Y. Li, H. Tian, P. Shi, G. Li, Isolation of novel ACE-inhibitory and antioxidant peptides from quinoa bran albumin assisted with an in

silico approach: Characterization, *in vivo* antihypertension, and molecular docking, Molecules. 24 (24) (2019) 4562, https://doi.org/10.3390/molecules24244562.

[539] R. Z. Gu, C. Y. Li, W. Y. Liu, W. X. Yi, M. Y. Cai, Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (*Salmo salar* L.) skin, Food Res. Int. 44 (2011) 1536-1540, https://doi.org/10.1016/j.foodres.2011.04.006.

[540] Q. Wu, Q. Cai, L. Weng, J. Shen, Q. Zhang, M. Cao, Study on preparation of Angiotensin-I converting enzyme (ACE) inhibitory peptides derived from abalone (*Haliotis discus hannai*) gonads, Journal of Chinese Institute of Food Science and Technology 15 (2015) 91-99, https://doi.org/10.16429/j.1009-7848.2015.10.013.

[541] Q. Wu, Q. F. Cai, Z. P. Tao, L. C. Sun, J. D. Shen, L. J. Zhang, G. M. Liu, M. J. Cao, Purification and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from abalone (*Haliotis discus hannai Ino*) gonads, Eur. Food Res. Technol. 240 (2015) 137-145, https://doi.org/10.1007/s00217-014-2315-8.

[542] D. H. Ngo, K. H. Kang, B. Ryu, T. S. Vo, W. K. Jung, H. G. Byun, S. K. Kim, Angiotensin-I converting enzyme inhibitory peptides from antihypertensive skate (*Okamejei kenojei*) skin gelatin hydrolysate in spontaneously hypertensive rats, Food Chem. 174 (2015) 37-43, https://doi.org/10.1016/j.foodchem.2014.11.013.

[543] Y. Chim-Chi, L. Olivera-Castillo, D. Betancur-Ancona, L. Chel-Guerrero, Protein hydrolysate fractions from sea cucumber (*Isostichopus badionotus*) inhibit angiotensin-converting enzyme, J. Aquat. Food Prod. Technol. 26 (2017) 1199-1209, https://doi.org/10.1080/10498850.2015.1080775.

[544] F. Ghibaudo, E. Gerbino, A.A. Hugo, M.G. Simões, P. Alves, B.F.O. Costa, V.C.D. Orto, A. Gómez-Zavaglia, P.N. Simoes, Development and characterization of iron-pectin beads as a novel system for iron delivery to intestinal cells, Colloids Surf. B Biointerfaces. 170 (2018) 538-543, https://doi.org/10.1016/j.colsurfb.2018.06.052.

[545] J.-A. Fernández-López, M. Esteve, I. Rafecas, X. Remesar, M. Alemany, Management of dietary essential metals (iron, copper, zinc, chromium and manganese) by Wistar and Zucker obese rats fed a self-selected high-energy diet, Biometals. 7 (1994) 117-129, https://doi.org/10.1007/BF00140481.

[546] L. Guo, H. Hou, B. Li, Z. Zhang, S. Wang, X. Zhao, Preparation, isolation and identification of iron-chelating peptides derived from Alaska pollock skin, Process Biochem. 48 (2013) 988-993, https://doi.org/10.1016/j.procbio.2013.04.013.

[547] T.A. Rouault, The role of iron regulatory proteins in mammalian iron homeostasis and disease, Nat Chem Biol. 2 (2006) 406-414, https://doi.org/10.1038/nchembio807.

[548] A.Z. Cdc, Recommendations to prevent and control iron deficiency in the United States, MMWR Recomm Rep. 47 (1998) 1-29.

[549] W. Zhang, Y. Li, J. Zhang, G. Huang, Optimization of hydrolysis conditions for the production of iron-binding peptides from Scad (*Decapterus maruadsi*) processing byproducts, Am. J. Biochem. Biotechnol. 12 (2016) 220-229, https://doi.org/10.3844/ajbbsp.2016.220.229.

[550] J.H. Swain, L.B. Tabatabai, M.B. Reddy, Histidine content of low-molecular-weight beef proteins influences nonheme iron bioavailability in Caco-2 cells, J. Nutr. 132 (2002) 245-251, https://doi.org/10.1093/jn/132.2.245.

[551] R.W. Hardy, C.V. Sullivan, A.M. Koziol, Absorption, body distribution, and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*), Fish Physiol. Biochem. 3 (1987) 133-143, https://doi.org/10.1007/BF02180415.

[552] W.J. Miller, Absorption, tissue distribution, endogenous excretion, and homeostatic control of zinc in ruminants, Am. J. Clin. Nutr. 22 (1969) 1323-1331, https://doi.org/10.1093/ajcn/22.10.1323.

[553] G.C. Sturniolo, M.M. Molokhia, R. Shields, L.A. Turnberg, Zinc absorption in Crohn's disease, Gut. 21 (1980) 387-391, https://doi.org/10.1136/gut.21.5.387.

[554] E. John, T.C. Laskow, W.J. Buchser, B.R. Pitt, P.H. Basse, L.H. Butterfield, P. Kalinski, M.T. Lotze, Zinc in innate and adaptive tumor immunity, J. Transl. Med. 8 (2010) 118, https://doi.org/10.1186/1479-5876-8-118.

[555] A. Brocard, B. Dreno, Innate immunity: a crucial target for zinc in the treatment of inflammatory dermatosis, J. Eur. Acad. Dermatol. Venereol. 25 (2011) 1146-1152, https://doi.org/10.1111/j.1468-3083.2010.03934.x.

[556] N. Xie, J. Huang, B. Li, J. Cheng, Z. Wang, J. Yin, X. Yan, Affinity purification and characterisation of zinc chelating peptides from rapeseed protein hydrolysates: Possible contribution of characteristic amino acid residues, Food Chem. 173 (2015) 210-217, https://doi.org/10.1016/j.foodchem.2014.10.030.

[557] F.J.M. Maathuis, Physiological functions of mineral macronutrients, Curr. Opin. Plant Biol. 12 (2009) 250-258, https://doi.org/10.1016/j.pbi.2009.04.003.

[558] F. R. Liu, L. Wang, R. Wang, Z. X. Chen, Calcium-binding capacity of wheat germ protein hydrolysate and characterization of peptide–calcium complex, J. Agric. Food Chem. 61 (2013) 7537-7544, https://doi.org/10.1021/jf401868z.

[559] D.W. Choi, N.H. Kim, K.B. Son, Isolation of iron-binding peptides from sunflower (*Helianthus annuus* L.) seed protein hydrolysates, J. Korean Soc. Food Sci. Nutr. 42 (2013) 1162-1166, https://doi.org/10.3746/jkfn.2013.42.7.1162.

[560] T. Fu, S. Zhang, Y. Sheng, Y. Feng, Y. Jiang, Y. Zhang, M. Yu, C. Wang, Isolation and characterization of zinc-binding peptides from mung bean protein hydrolysates, Eur. Food Res. Technol. 246 (2020) 113-124, https://doi.org/10.1007/s00217-019-03397-8.

[561] J. Huang, N. Xie, B. Li, J. Cheng, Z. Wang, J. Yin, X. Yan, Preparation, purification and structure analysis of rapeseed source zinc chelating peptide, J. Chinese Cereals Oils Assoc. 31 (2016) 68-73.

[562] K. X. Zhu, X. P. Wang, X. N. Guo, Isolation and characterization of zinc-chelating peptides from wheat germ protein hydrolysates, J. Func. Foods. 12 (2015) 23-32, https://doi.org/10.1016/j.jff.2014.10.030.

[563] W. Xiaoping, C. Xiaona, Z. Kexue, P. Wei, Z. Huiming, Enrichment of metal chelating peptides from wheat germ protein by immobilized-metal Ion Affinity Chromatography, J. Chinese Cereals Oils Assoc. 30 (2015) 101-105.

[564] M.L. Zhang, X.H. Zhao, *In vitro* calcium- chelating and platelet anti- aggregation activities of soy protein hydrolysate modified by the Alcalase- catalyzed plastein reaction, J. Food Biochem. 38 (2014) 374-380, https://doi.org/10.1111/jfbc.12063.

[565] S.K. Devaraju, P. Thatte, J. Prakash, J.A. Lakshmi, Bioaccessible iron and zinc in native and fortified enzyme hydrolyzed casein and soya protein matrices, Food Biotechnol. 30 (2016) 233-248, https://doi.org/10.1080/08905436.2016.1233887.

[566] N. Sun, P. Cui, Z. Jin, H. Wu, Y. Wang, S. Lin, Contributions of molecular size, charge distribution, and specific amino acids to the iron-binding capacity of sea cucumber (*Stichopus japonicus*) ovum hydrolysates, Food Chem. 230 (2017) 627-636, https://doi.org/10.1016/j.foodchem.2017.03.077.

[567] X. Liu, Z. Wang, J. Zhang, L. Song, D. Li, Z. Wu, P. Zhu, Y. Nakamura, F. Shahidi, C. Yu, Isolation and identification of zinc- chelating peptides from sea cucumber (*Stichopus japonicus*) protein hydrolysate, J. Sci. Food Agric. 99 (2019) 6400-6407, https://doi.org/10.1002/jsfa.9919.

[568] S. B. Kim, M. J. Ku, W. M. Cho, K. S. Ki, H. S. Kim, M. S. Nam, Production of iron-binding peptides from colostral whey by enzymatic hydrolysis, Korean J. Food Sci. Ani. Resour. 30 (2010) 923-929.

[569] L. Zhao, S. Wang, L. Guo, S. Huang, Y. Huang, Study on the preparation of whey protein chelating calcium and characterization of the complexes, Journal of Chinese Institute of Food Science and Technology, 15 (2015) 160-167, https://doi.org/10.16429/j.1009-7848.2015.07.023.

[570] J. Zhou, X. Wang, T. Ai, X. Cheng, H.Y. Guo, G.X. Teng, X.Y. Mao, Preparation and characterization of β-lactoglobulin hydrolysate-iron complexes, J. Dairy Sci. 95 (2012) 4230-4236, https://doi.org/10.3168/jds.2011-5282.

[571] H. Jiang, W. Zhang, F. Chen, J. Zou, W. Chen, G. Huang, Purification of an iron-binding peptide from scad (*Decapterus maruadsi*) processing by- products and its effects on iron absorption by Caco- 2 cells, J. Food Biochem. 43 (2019) e12876, https://doi.org/10.1111/jfbc.12876.

[572] A. Jaiswal, R. Bajaj, B. Mann, K. Lata, Iron (II)-chelating activity of buffalo α S-casein hydrolysed by corolase PP, Alcalase and Flavourzyme, J. Food Sci. Technol. 52 (2015) 3911-3918, https://doi.org/10.1007/s13197-014-1626-x.

[573] P. F. Wang, G. R. Huang, J. X. Jiang, Optimization of hydrolysis conditions for the production of iron-binding peptides from Mackerel processing byproducts, Adv. J. Food Sci. Technol. 5 (2013) 921-925, https://doi.org/10.19026/ajfst.5.3183.

[574] X. Wang, J. Zhou, P.S. Tong, X.Y. Mao, Zinc-binding capacity of yak casein hydrolysate and the zinc-releasing characteristics of casein hydrolysate-zinc complexes, J. Dairy Sci. 94 (2011) 2731-2740, https://doi.org/10.3168/jds.2010-3900.

[575] N. Charoenphun, B. Cheirsilp, N. Sirinupong, W. Youravong, Calcium-binding peptides derived from tilapia (*Oreochromis niloticus*) protein hydrolysate, Eur. Food Res. Technol. 236 (2013) 57-63, https://doi.org/10.1007/s00217-012-1860-2.

[576] D. W. Choi, J. H. Lee, H. H. Chun, K.B. Song, Isolation of a calcium-binding peptide from bovine serum protein hydrolysates, Food Sci. Biotechnol. 21 (2012) 1663-1667, https://doi.org/10.1007/s10068-012-0221-z.

[577] X. Lin, L. Yang, M. Wang, T. Zhang, M. Liang, E. Yuan, J. Ren, Preparation, purification and identification of cadmium-induced osteoporosis-protective peptides from

chicken sternal cartilage, J. Func. Foods. 51 (2018) 130-141, https://doi.org/10.1016/j.jff.2018.09.036.

[578] H.J. Hong, E.j. Kim, I.S. Park, J.H. Ryu, T. Ryu, B.G. Kim, K.S. Chung, Preparation and characterisation of an easily absorbabl Mg- casein hydrolysate complex produced through enzymatic hydrolysis and ultrafiltration, Int. J. Food Sci. Technol. 50 (2015) 365-371, https://doi.org/10.1111/ijfs.12650.

[579] D. Prakash, A.J. Lakshmi, Preparation of caseinophosphopeptides and assessing their efficacy in enhancing the bioaccessibility of iron and zinc, J. Food Sci. Technol. 52 (2015) 7493-7499, https://doi.org/10.1007/s13197-015-1864-6.

[580] N. H. Kim, S. H. Jung, J. Kim, S. H. Kim, H. J. Ahn, K.B. Song, Purification of an iron-chelating peptide from spirulina protein hydrolysates, J. Korean Soc. Appl. Biol. Chem. 57 ((2014) 91-95, https://doi.org/10.1007/s13765-013-4211-5.

[581] B. Yuan, C. Zhao, C. Cheng, D.-c. Huang, S.-j. Cheng, C. Cao, G. Chen, A peptide-Fe (II) complex from *Grifola frondosa* protein hydrolysates and its immunomodulatory activity, Food Biosci. 32 (2019) 100459, https://doi.org/10.1016/j.fbio.2019.100459.

[582] E. Abeyrathne, H.Y. Lee, C. Jo, J.W. Suh, D.U. Ahn, Enzymatic hydrolysis of ovomucoid and the functional properties of its hydrolysates, Poultry Sci. 94 (2015) 2280-2287, https://doi.org/10.3382/ps/pev196.

[583] W. Wu, L. He, Y. Liang, L. Yue, W. Peng, G. Jin, M. Ma, Preparation process optimization of pig bone collagen peptide-calcium chelate using Response Surface Methodology and its structural characterization and stability analysis, Food Chem. 284 (2019) 80-89, https://doi.org/10.1016/j.foodchem.2019.01.103.

[584] L. C. Foong, M.U. Imam, M. Ismail, Iron-binding capacity of defatted rice bran hydrolysate and bioavailability of iron in Caco-2 cells, J. Agric. Food Chem. 63 (2015) 9029-9036, https://doi.org/10.1021/acs.jafc.5b03420.

[585] A.D. Shah, C. Langenberg, E. Rapsomaniki, S. Denaxas, M. Pujades-Rodriguez, C.P. Gale, J. Deanfield, L. Smeeth, A. Timmis, H. Hemingway, Type 2 diabetes and incidence of cardiovascular diseases: a cohort study in 1· 9 million people, Lancet Diabetes Endocrinol. 3 (2015) 105-113, https://doi.org/10.1016/S2213-8587(14)70219-0.

[586] S. Chatterjee, K. Khunti, M.J. Davies, Type 2 diabetes, Lancet. 389 (2017) 2239-2251, https://doi.org/10.1016/S0140-6736(17)30058-2.

[587] J.E. Shaw, R.A. Sicree, P.Z. Zimmet, Global estimates of the prevalence of diabetes for 2010 and 2030, Diabetes Res. Clin. Pract. 87 (2010) 4-14, https://doi.org/10.1016/j.diabres.2009.10.007.

[588] E. Sebokova, A.D. Christ, M. Boehringer, J. Mizrahi, Dipeptidyl peptidase IV inhibitors: the next generation of new promising therapies for the management of type 2 diabetes, Curr. Top. Med. Chem. 7 (2007) 547-555, https://doi.org/10.2174/156802607780091019.

[589] A. Ceriello, Postprandial hyperglycemia and diabetes complications: is it time to treat?, Diabetes. 54 (2005) 1-7, https://doi.org/10.2337/diabetes.54.1.1.

[590] M.R. Bhandari, N. Jong-Anurakkun, G. Hong, J. Kawabata, α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata*, Haw.), Food Chem. 106 (2008) 247-252, https://doi.org/10.1016/j.foodchem.2007.05.077.

[591] M.D.P.T. Gunawan-Puteri, J. Kawabata, Novel α-glucosidase inhibitors from Macaranga tanarius leaves, Food Chem. 123 (2010) 384-389, https://doi.org/10.1016/j.foodchem.2010.04.050.

[592] H.E. Lebovitz, Alpha-glucosidase inhibitors, Endocrinol. Metab. Clin. North Am. 26 (1997) 539-551, https://doi.org/10.1016/S0889-8529(05)70266-8.

[593] M.H. Johnson, A. Lucius, T. Meyer, E. Gonzalez de Mejia, Cultivar evaluation and effect of fermentation on antioxidant capacity and in vitro inhibition of α-amylase and α-glucosidase by highbush blueberry (*Vaccinium corombosum*), J. Agric. Food Chem. 59 (2011) 8923-8930, https://doi.org/10.1021/jf201720z.

[594] Z. Yu, Y. Yin, W. Zhao, Y. Yu, B. Liu, J. Liu, F. Chen, Novel peptides derived from egg white protein inhibiting alpha-glucosidase, Food Chem. 129 (2011) 1376-1382, https://doi.org/10.1016/j.foodchem.2011.05.067.

[595] C.F. Deacon, Dipeptidyl peptidase- 4 inhibitors in the treatment of type 2 diabetes: a comparative review, Diabetes Obes. Metab. 13 (2011) 7-18, https://doi.org/10.1111/j.1463-1326.2010.01306.x.

[596] L. Juillerat-Jeanneret, Dipeptidyl peptidase IV and its inhibitors: therapeutics for type 2 diabetes and what else?, J. Med. Chem. 57 (2014) 2197-2212, https://doi.org/10.1021/jm400658e.

[597] B.D. Green, V.A. Gault, F.P.M. O'Harte, P.R. Flatt, Structurally modified analogues of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) as future antidiabetic agents, Curr. Pharm. Des. 10 (2004) 3651-3662, https://doi.org/10.2174/1381612043382774.

[598] A. Connolly, M.B. O'Keeffe, A.B. Nongonierma, C.O. Piggott, R.J. FitzGerald, Isolation of peptides from a novel brewers spent grain protein isolate with potential to modulate glycaemic response, Int. J. Food Sci. Technol. 52 (2017) 146-153, https://doi.org/10.1111/ijfs.13260.

[599] G. Tulipano, V. Sibilia, A.M. Caroli, D. Cocchi, Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors, Peptides 32 (2011) 835-838, https://doi.org/10.1016/j.peptides.2011.01.002.

[600] A.R. Aroor, C. Manrique-Acevedo, V.G. DeMarco, The role of dipeptidylpeptidase-4 inhibitors in management of cardiovascular disease in diabetes; focus on linagliptin, Cardiovasc. Diabetol. 17 (2018) 59, https://doi.org/10.1186/s12933-018-0704-1 .

[601] P.A. Harnedy-Rothwell, C.M. McLaughlin, M.B. O'Keeffe, A.V. Le Gouic, P.J. Allsopp, E.M. McSorley, S. Sharkey, J. Whooley, B. McGovern, F.P.M. O'Harte, Identification and characterisation of peptides from a boarfish (*Capros aper*) protein hydrolysate displaying in vitro dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulinotropic activity, Food Res. Int. 131 (2020) 108989, https://doi.org/10.1016/j.foodres.2020.108989.

[602] S. El- Guendouz, S. Aazza, B. Lyoussi, M.D. Antunes, M.L. Faleiro, M.G. Miguel, Anti- acetylcholinesterase, antidiabetic, anti- inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis, Int. J. Food Sci. Technol. 51 (2016) 1762-1773, https://doi.org/10.1111/ijfs.13133.

[603] T. de Souza Rocha, L.M.R. Hernandez, Y.K. Chang, E.G. de Mejía, Impact of germination and enzymatic hydrolysis of cowpea bean (*Vigna unguiculata*) on the

generation of peptides capable of inhibiting dipeptidyl peptidase IV, Food Res. Int. 64 (2014) 799-809, https://doi.org/10.1016/j.foodres.2014.08.016.

[604] T. de Souza Rocha, L.M.R. Hernandez, L. Mojica, M.H. Johnson, Y.K. Chang, E.G. de Mejia, Germination of *Phaseolus vulgaris* and Alcalase hydrolysis of its proteins produced bioactive peptides capable of improving markers related to type-2 diabetes *in vitro*, Food Res. Int. 76 (2015) 150-159, https://doi.org/10.1016/j.foodres.2015.04.041.

[605] C.Y. Huang, W. D. Chiang, P. Pai, W. T. Lin, Potato protein hydrolysate attenuates high fat diet-induced cardiac apoptosis through SIRT1/PGC-1a/Akt signalling, J. Func. Foods. 12 (2015) 389-398, https://doi.org/10.1016/j.jff.2014.11.027.

[606] S. Marthandam Asokan, T. Wang, W. T. Su, W. T. Lin, Antidiabetic effects of a short peptide of potato protein hydrolysate in STZ-induced diabetic mice, Nutrients. 11 (2019) 779, https://doi.org/10.3390/nu11040779.

[607] M.E. Oseguera-Toledo, E.G. de Mejia, S.L. Amaya-Llano, Hard-to-cook bean (*Phaseolus vulgaris* L.) proteins hydrolyzed by alcalase and bromelain produced bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative stress, Food Res. Int. 76 (2015) 839-851, https://doi.org/10.1016/j.foodres.2015.07.046.

[608] F. Wang, G. Yu, Y. Zhang, B. Zhang, J. Fan, Dipeptidyl peptidase IV inhibitory peptides derived from oat (avena sativa L.), buckwheat (*fagopyrum esculentum*), and highland barley (*hordeum vulgare trifurcatum* (L.) trofim) proteins, J. Agric. Food Chem. 63 (2015) 9543-9549, https://doi.org/10.1021/acs.jafc.5b04016.

[609] L. Mojica, E.G. de Mejía, Optimization of enzymatic production of anti-diabetic peptides from black bean (*Phaseolus vulgaris* L.) proteins, their characterization and biological potential, Food Funct. 7 (2016) 713-727, https://doi.org/10.1039/c5fo01204j.

[610] C. Uraipong, J. Zhao, Rice bran protein hydrolysates exhibit strong *in vitro* α-amylase, β- glucosidase and ACE- inhibition activities, J. Sci. Food Agric. 96 (2016) https://doi.org/10.1002/jsfa.7182.

[611] T.O. Awosika, R.E. Aluko, Inhibition of the *in vitro* activities of α- amylase, α-glucosidase and pancreatic lipase by yellow field pea (*Pisum sativum* L.) protein hydrolysates, Int. J. Food Sci. Technol. 54 (2019) 2021-2034, https://doi.org/10.1111/ijfs.14087.

[612] M.E.O. Toledo, E.G. de Mejia, M. Sivaguru, S.L. Amaya-Llano, Common bean (*Phaseolus vulgaris* L.) protein-derived peptides increased insulin secretion, inhibited lipid accumulation, increased glucose uptake and reduced the phosphatase and tensin homologue activation *in vitro*, J. Func. Foods. 27 (2016) 160-177, https://doi.org/10.1016/j.jff.2016.09.001.

[613] K. Su, X. Mao, L. Ai, X. Zhang, *In vitro* assessment of anti-diabetic potential of four kinds of dark tea (*Camellia sinensis* L.) protein hydrolysates, J. Appl. Bot. Food Qual. 92 (2019) 57-63, https://doi.org/10.5073/JABFQ.2019.092.008.

[614] P.N. Nuñez- Aragón, M. Segura- Campos, E. Negrete- León, J.J. Acevedo-Fernández, D. Betancur- Ancona, L. Chel- Guerrero, G. Castañeda- Corral, Protein hydrolysates and ultrafiltered <1 KDa fractions from *Phaseolus lunatus*, *Phaseolus vulgaris* and *Mucuna pruriens* exhibit antihyperglycemic activity, intestinal glucose absorption and

α- glucosidase inhibition with no acute toxicity in rodents, J. Sci. Food Agric. 99 (2019) 587-595, https://doi.org/10.1002/jsfa.9219.

[615] F. Xu, Y. Yao, X. Xu, M. Wang, M. Pan, S. Ji, J. Wu, D. Jiang, X. Ju, L. Wang, Identification and quantification of DPP-IV-inhibitory peptides from hydrolyzed-rapeseed-protein-derived Napin with analysis of the interactions between key residues and protein domains, J. Agric. Food Chem. 67 (2019) 3679-3690, https://doi.org/10.1021/acs.jafc.9b01069.

[616] E. Castañeda-Pérez, K. Jiménez-Morales, C. Quintal-Novelo, R. Moo-Puc, L. Chel-Guerrero, D. Betancur-Ancona, Enzymatic protein hydrolysates and ultrafiltered peptide fractions from Cowpea *Vigna unguiculata* L bean with *in vitro* antidiabetic potential, J. Iran. Chem. Soc. 16 (2019) 1773-1781, https://doi.org/10.1007/s13738-019-01651-0.

[617] P.A. Harnedy, V. Parthsarathy, C.M. McLaughlin, M.B. O'Keeffe, P.J. Allsopp, E.M. McSorley, F.P.M. O'Harte, R.J. FitzGerald, Blue whiting (*Micromesistius poutassou*) muscle protein hydrolysate with *in vitro* and *in vivo* antidiabetic properties, J. Func. Foods. 40 (2018) 137-145, https://doi.org/10.1016/j.jff.2017.10.045.

[618] P.A. Harnedy, V. Parthsarathy, C.M. McLaughlin, M.B. O'Keeffe, P.J. Allsopp, E.M. McSorley, F.P.M. O'Harte, R.J. FitzGerald, Atlantic salmon (*Salmo salar*) co-product-derived protein hydrolysates: A source of antidiabetic peptides, Food Res. Int. 106 (2018) 598-606, https://doi.org/10.1016/j.foodres.2018.01.025.

[619] V. Parthsarathy, C.M. McLaughlin, P.A. Harnedy, P.J. Allsopp, W. Crowe, E.M. McSorley, R.J. FitzGerald, F.P.M. O'Harte, Boarfish (*Capros aper*) protein hydrolysate has potent insulinotropic and GLP- 1 secretory activity *in vitro* and acute glucose lowering

effects in mice, Int. J. Food Sci. Technol. 54 (2019) 271-281, https://doi.org/10.1111/ijfs.13975.

[620] R. Medzhitov, Inflammation 2010: New Adventures of an Old Flame, Cell. 140 (2010) 771-776, https://doi.org/10.1016/j.cell.2010.03.006.

[621] R. Medzhitov, Origin and physiological roles of inflammation, Nature. 454 (2008) 428-435, https://doi.org/10.1038/nature07201.

[622] M.A. Sugimoto, J.P. Vago, M. Perretti, M.M. Teixeira, Mediators of the resolution of the inflammatory response, Trends Immunol. 40 (2019) 212-227, https://doi.org/10.1016/j.it.2019.01.007.

[623] T.J. Koh, L.A. DiPietro, Inflammation and wound healing: the role of the macrophage, Expert Rev. Mol. Med. 13 (2011) e23, https://doi.org/10.1017/S1462399411001943.

[624] C.N. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, Nat. Immunol. 6 (2005) 1191-1197, https://doi.org/10.1038/ni1276.

[625] A. Murakami, H. Ohigashi, Targeting NOX, INOS and COX-2 in inflammatory cells: Chemoprevention using food phytochemicals, Int. J. Cancer. 121 (2007) 2357-2363, https://doi.org/10.1002/ijc.23161.

[626] C. Meram, J. Wu, Anti-inflammatory effects of egg yolk livetins (α, β, and γ-livetin) fraction and its enzymatic hydrolysates in lipopolysaccharide-induced RAW 264.7 macrophages, Food Res. Int. 100 (2017) 449-459, https://doi.org/10.1016/j.foodres.2017.07.032.

[627] J.L. Wallace, Pathogenesis of NSAID-induced gastroduodenal mucosal injury, Best Pract. Res. Clin. Gastroenterol. 15 (2001) 691-703, https://doi.org/10.1053/bega.2001.0229.

[628] I. Joshi, S. Sudhakar, R.A. Nazeer, Anti-inflammatory properties of bioactive peptide derived from gastropod influenced by enzymatic hydrolysis, Appl. Biochem. Biotechnol. 180 (2016) 1128-1140, https://doi.org/10.1007/s12010-016-2156-y.

[629] M. L. Cheng, H. C. Wang, K. C. Hsu, J. S. Hwang, Anti-inflammatory peptides from enzymatic hydrolysates of tuna cooking juice, Food Agric. Immunol. 26 (2015) 770-781, https://doi.org/10.1080/09540105.2015.1036352.

[630] K. Majumder, Y. Mine, J. Wu, The potential of food protein- derived anti-inflammatory peptides against various chronic inflammatory diseases, J. Sci. Food Agric. 96 (2016) 2303-2311, https://doi.org/10.1002/jsfa.7600.

[631] M.E. Oseguera-Toledo, E.G. de Mejia, V.P. Dia, S.L. Amaya-Llano, Common bean (*Phaseolus vulgaris* L.) hydrolysates inhibit inflammation in LPS-induced macrophages through suppression of NF-κB pathways, Food Chem. 127 (2011) 1175-1185, https://doi.org/10.1016/j.foodchem.2011.01.121.

[632] M. Oseguera-Toledo, V.P. Dia, E.G. de Mejia, S.L. Amaya Llano, Bean concentrates and inflammation reduction, Hispanic Foods: Chemistry and Bioactive Compounds, ACS Publications 2012, 217-231, https://doi.org/10.1021/bk-2012-1109.ch014.

[633] M.d.C. Millán-Linares, F. Millán, J. Pedroche, M.d.M. Yust, GPETAFLR: A new anti-inflammatory peptide from *Lupinus angustifolius* L. protein hydrolysate, J. Func. Foods. 18 (2015) 358-367, https://doi.org/10.1016/j.jff.2015.07.016.

[634] S.H. Lee, H.W. Yang, Y. Ding, Y. Wang, Y.J. Jeon, S.H. Moon, B.T. Jeon, S.H. Sung, Anti-inflammatory effects of enzymatic hydrolysates of velvet antler in RAW 264.7 cells *in vitro* and zebrafish model, Excli J. 14 (2015) 1122-32, https://doi.org/10.17179/excli2015-481.

[635] X. Sun, S. Chakrabarti, J. Fang, Y. Yin, J. Wu, Low-molecular-weight fractions of Alcalase hydrolyzed egg ovomucin extract exert anti-inflammatory activity in human dermal fibroblasts through the inhibition of tumor necrosis factor-mediated nuclear factor κB pathway, Nutr. Res. 36 (2016) 648-657, https://doi.org 10.1016/j.nutres.2016.03.006.

[636] Y. Ma, J. Liu, H. Shi, L. Yu, Isolation and characterization of anti-inflammatory peptides derived from whey protein, J. Dairy Sci. 99 (2016) 6902-6912, https://doi.org/10.3168/jds.2016-11186.

[637] S. J. Lee, E. K. Kim, Y. S. Kim, J. W. Hwang, K.H. Lee, D. K. Choi, H. Kang, S. H. Moon, B. T. Jeon, P. J. Park, Purification and characterization of a nitric oxide inhibitory peptide from *Ruditapes philippinarum*, Food Chem. Toxicol. 50 (2012) 1660-1666, https://doi.org/10.1016/j.fct.2012.02.021.

[638] S.M. O'Sullivan, T. Lafarga, M. Hayes, N.M. O'Brien, Bioactivity of bovine lung hydrolysates prepared using papain, pepsin, and Alcalase, J. Food Biochem. 41 (2017) e12406, https://doi.org/10.1111/jfbc.12406.

[639] M.d.C. Millán-Linares, M.d.M. Yust, J.M. Alcaide-Hidalgo, F. Millán, J. Pedroche, Lupine protein hydrolysates inhibit enzymes involved in the inflammatory pathway, Food Chem. 151 (2014) 141-147, https://doi.org/10.1016/j.foodchem.2013.11.053.

[640] A. Yépez, C. Luz, G. Meca, G. Vignolo, J. Mañes, R. Aznar, Biopreservation potential of lactic acid bacteria from Andean fermented food of vegetal origin, Food Control. 78 (2017) 393-400, https://doi.org/10.1016/j.foodcont.2017.03.009.

[641] L. Tamkutė, B.M. Gil, J.R. Carballido, M. Pukalskienė, P.R. Venskutonis, Effect of cranberry pomace extracts isolated by pressurized ethanol and water on the inhibition of food pathogenic/spoilage bacteria and the quality of pork products, Food Res. Int. 120 (2019) 38-51, https://doi.org/10.1016/j.foodres.2019.02.025.

[642] C.M.A.P. Franz, H.M.W. den Besten, C. Böhnlein, M. Gareis, M.H. Zwietering, V. Fusco, Microbial food safety in the 21st century: Emerging challenges and foodborne pathogenic bacteria, Trends Food Sci. Technol. 81 (2018) 155-158, https://doi.org/10.1016/j.tifs.2018.09.019

[643] A. Osman, H.A. Goda, M. Abdel-Hamid, S.M. Badran, J. Otte, Antibacterial peptides generated by Alcalase hydrolysis of goat whey, LWT-Food Sci. Technol. 65 (2016) 480-486, https://doi.org/10.1016/j.lwt.2015.08.043.

[644] A. Sila, A. Bougatef, Antioxidant peptides from marine by-products: Isolation, identification and application in food systems. A review, J. Func. Foods. 21 (2016) 10-26, https://doi.org/10.1016/j.jff.2015.11.007.

[645] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238-250, https://doi.org/110.1038/nrmicro1098.

[646] L. Najafian, A.S. Babji, A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications, Peptides. 33 (2012) 178-185, https://doi.org/10.1016/j.peptides.2011.11.013.

[647] D. Kumar, M.K. Chatli, R. Singh, N. Mehta, P. Kumar, Antioxidant and antimicrobial activity of camel milk casein hydrolysates and its fractions, Small Rumin. Res. 139 (2016) 20-25, https://doi.org/10.1016/j.smallrumres.2016.05.002.

[648] A.K. Verma, M.K. Chatli, N. Mehta, P. Kumar, Efficacy of antioxidant and antimicrobial activity of whole porcine blood hydrolysates and its fractions under *in vitro* conditions, Anim. Prod. Sci. 58 (2018) 2084-2090, https://doi.org/10.1071/AN16804.

[649] W. Song, X. Kong, Y. Hua, Y. Chen, C. Zhang Y. Chen, Identification of antibacterial peptides generated from enzymatic hydrolysis of cottonseed proteins, LWT. (2020) 109199, https://doi.org/10.1016/j.lwt.2020.109199.

[650] Y.N. Tan, M.K. Ayob, M.A. Osman, K.F. Matthews, Antibacterial activity of different degree of hydrolysis of palm kernel expeller peptides against spore- forming and non- spore- forming bacteria, Lett. Appl. Microbiol. 53 (2011) 509-517, https://doi.org/10.1111/j.1472-765X.2011.03137.x.

[651] Y.N. Tan, M.K. Ayob, W.A.W. Yaacob, Purification and characterisation of antibacterial peptide-containing compound derived from palm kernel cake, Food Chem. 136 (2013) 279-284, https://doi.org/10.1016/j.foodchem.2012.08.012.

[652] A. Sila, N. Nedjar-Arroume, K. Hedhili, G. Chataigné, R. Balti, M. Nasri, P. Dhulster, A. Bougatef, Antibacterial peptides from barbel muscle protein hydrolysates: Activity against some pathogenic bacteria, LWT-Food Sci. Technol. 55 (2014) 183-188, https://doi.org/10.1016/j.lwt.2013.07.021.

[653] K.O. Lima, C.d.C. de Quadros, M. da Rocha, J.T.J.G. de Lacerda, M.A. Juliano, M. Dias, M.A. Mendes, C. Prentice, Bioactivity and bioaccessibility of protein hydrolyzates

from industrial byproducts of Stripped weakfish (*Cynoscion guatucupa*), LWT. 111 (2019) 408-413, https://doi.org/10.1016/j.lwt.2019.05.043.

[654] F.S. Taha, S.S. Mohamed, S.M. Wagdy, G.F. Mohamed, Antioxidant and antimicrobial activities of enzymatic hydrolysis products from sunflower protein isolate, World Appl. Sci. J. 21 (2013) 651-658, https://doi.org/10.5829/idosi.wasj.2013.21.5.2879.

[655] M.S. Coelho, R.A.M. Soares-Freitas, J.A.G. Arêas, E.A. Gandra, M. de las Mercedes Salas-Mellado, Peptides from chia present antibacterial activity and inhibit cholesterol synthesis, Plant Foods Hum. Nutr. 73 (2018) 101-107, https://doi.org/10.1007/s11130-018-0668-z.

[656] Z.M.L. Dong-Hui, Z.Q.L. Xiao-Tao, 7S-rich fractions hydrolyzed by Alcalase and functional properties of the hydrolysates, Journal of South China University of Technology (Natural Science). 38 (2010) 45, https://doi.org/10.3969/j.issn.1000-565X.2010.04.009.

[657] R. Horax, M.S. Vallecios, N. Hettiarachchy, L.F. Osorio, P. Chen, Solubility, functional properties, ACE-I inhibitory and DPPH scavenging activities of Alcalase hydrolysed soy protein hydrolysates, Int. J. Food Sci. Technol. 52 (2017) 196-204, https://doi.org/10.1111/ijfs.13267.

[658] H.J. Zhang, H. Zhang, L. Wang, X.N. Guo, Preparation and functional properties of rice bran proteins from heat-stabilized defatted rice bran, Food Res. Int. 47 (2012) 359-363, https://doi.org/10.1016/j.foodres.2011.08.014.

[659] S. Arsa, C. Theerakulkait, Sensory aroma characteristics of Alcalase hydrolyzed rice bran protein concentrate as affected by spray drying and sugar addition, J. Food Sci. Technol. 52 (2015) 5285-5291, https://doi.org/10.1007/s13197-014-1610-5.

[660] H. Zhang, X. Xia, J. Wang, Y. Liu, Y. Li, Functional properties of rice protein and its enzymatic hydrolysates, Journal of Chinese Institute of Food Science and Technology, 15 (2015) 63-70, https://doi.org/10.16429/j.1009-7848.2015.08.010.

[661] J. Miedzianka, A. Pęksa, M. Pokora, E. Rytel, A. Tajner-Czopek, A. Kita, Improving the properties of fodder potato protein concentrate by enzymatic hydrolysis, Food Chem. 159 (2014) 512-518, https://doi.org/10.1016/j.foodchem.2014.03.054.

[662] N. Akbari, J. Mohammadzadeh Milani, P. Biparva, Functional and conformational properties of proteolytic enzyme- modified potato protein isolate, J. Sci. Food Agric. 100 (2020) 1320-1327, https://doi.org/10.1002/jsfa.10148.

[663] J. Ren, C. Song, P. Wang, S. Li, N. Kopparapu, X. Zheng, Modification of structural and functional properties of sunflower 11S globulin hydrolysates, Czech J. Food Sci. 33 (2015) 474-479, https://doi.org/10.17221/154/2015-CJFS.

[664] J. Ren, C.L. Song, H.Y. Zhang, N.K. Kopparapu, X.Q. Zheng, Effect of hydrolysis degree on structural and interfacial properties of sunflower protein isolates, J. Food Process. Pres. 41 (2017) e13092, https://doi.org/10.1111/jfpp.13092.

[665] W. He, R. Yang, W. Zhao, Effect of acid deamidation-Alcalase hydrolysis induced modification on functional and bitter-masking properties of wheat gluten hydrolysates, Food Chem. 277 (2019) 655-663, https://doi.org/10.1016/j.foodchem.2018.11.004.

[666] M.B. Elmalimadi, A.B. Stefanović, N.Ž. Šekuljica, M.G. Žuža, N.D. Luković, J.R. Jovanović, Z.D. Knežević- Jugović, The synergistic effect of heat treatment on Alcalase-assisted hydrolysis of wheat gluten proteins: Functional and antioxidant properties, J. Food Process. Pres. 41 (2017) e13207, https://doi.org/10.1111/jfpp.13207.

[667] I. Normah, M.P. Nashrah, Evaluation on the properties of Mentarang (*Pholas orientalis*) protein hydrolysate, Pertanika Journal of Tropical Agricultural Science 36 (2013), 199-210.

[668] L. Cai, L. Xiao, C. Liu, T. Ying, Functional properties and bioactivities of pine nut (*Pinus gerardiana*) protein isolates and its enzymatic hydrolysates, Food Bioprocess Technol. 6 (2013) 2109-2117, https://doi.org/10.1007/s11947-012-0885-7.

[669] E. Demirhan, B. Özbek, Influence of enzymatic hydrolysis on the functional properties of sesame cake protein, Chem. Eng. Commun. 200 (2013) 655-666, https://doi.org/10.1080/00986445.2012.717316.

[670] A.M. Ghribi, I.M. Gafsi, A. Sila, C. Blecker, S. Danthine, H. Attia, A. Bougatef, S. Besbes, Effects of enzymatic hydrolysis on conformational and functional properties of chickpea protein isolate, Food chemistry 187 (2015) 322-330, https://doi.org/10.1016/j.foodchem.2015.04.109.

[671] Y. Zheng, Y. Li, Y. Zhang, R. Zhang, Y. Zhang, S. Zhao, Effects of limited enzymatic hydrolysis, pH, ionic strength and temperature on physicochemical and functional properties of palm (*Elaeis guineensis* Jacq.) kernel expeller protein, J. Food Sci. Technol. 52 (2015) 6940-6952, https://doi.org/10.1007/s13197-015-1839-7.

[672] S. Thaiphanit, G. Schleining, P. Anprung, Effects of coconut (*Cocos nucifera* L.) protein hydrolysates obtained from enzymatic hydrolysis on the stability and rheological properties of oil-in-water emulsions, Food Hydrocoll. 60 (2016) 252-264, https://doi.org/10.1016/j.foodhyd.2016.03.035.

[673] Q. Guo, N. Zhang, Q. Fu, C. Zou, Z. Zhang, Evaluation of amino acid nutritional composition and total reducing power of *Corylus Mandshurica* Maxim Hazelnut Peptides, J. Chinese Cereals Oils Assoc. 32 (2017) 64-70.

[674] C. Liu, M. Bhattarai, K.S. Mikkonen, M. Heinonen, Effects of enzymatic hydrolysis of fava bean protein isolate by alcalase on the physical and oxidative stability of oil-in-water emulsions, J. Agric. Food Chem. 67 (2019) 6625-6632, https://doi.org/10.1021/acs.jafc.9b00914.

[675] B. Bhaskar, L. Ananthanarayan, S.N. Jamdar, Effect of enzymatic hydrolysis on the functional, antioxidant, and angiotensin I-converting enzyme (ACE) inhibitory properties of whole horse gram flour, Food Sci. Biotechnol. 28 (2019) 43-52, https://doi.org/10.1007/s10068-018-0440-z.

[676] V. Muhamyankaka, C.F. Shoemaker, M. Nalwoga, X.M. Zhang, Physicochemical properties of hydrolysates from enzymatic hydrolysis of pumpkin (*Cucurbita moschata*) protein meal, Int. Food Res. J. 20 (2013) 2227.

[677] M. Venuste, X. Zhang, C.F. Shoemaker, E. Karangwa, S. Abbas, P.E. Kamdem, Influence of enzymatic hydrolysis and enzyme type on the nutritional and antioxidant properties of pumpkin meal hydrolysates, Food Funct. 4 (2013) 811-820, https://doi.org/10.1039/C3FO30347K.

[678] S. Bučko, J. Katona, L. Popović, L. Petrović, J. Milinković, Influence of enzymatic hydrolysis on solubility, interfacial and emulsifying properties of pumpkin (*Cucurbita pepo*) seed protein isolate, Food Hydrocoll. 60 (2016) 271-278, https://doi.org/10.1016/j.foodhyd.2016.04.005.

[679] S.H. Koo, I.Y. Bae, S. Lee, D.-H. Lee, B.-S. Hur, H.G. Lee, Evaluation of wheat gluten hydrolysates as taste-active compounds with antioxidant activity, J. Food Sci. Technol. 51 (2014) 535-542, https://doi.org/10.1007/s13197-011-0515-9.

[680] L. Liao, Z. Xie, L. Ni, Studies on fermentation characteristic of yogurt using wheat gluten peptides, Journal of Chinese Institute of Food Science and Technology 17 (2017) 126-131, https://doi.org/10.16429/j.1009-7848.2017.08.017.

[681] X. Guo, J. Zhang, Y. Ma, S. Tian, Optimization of limited hydrolysis of proteins in rice residue and characterization of the functional properties of the products, J. Food Process. Pres. 37 (2013) 245-253, https://doi.org/10.1111/j.1745-4549.2011.00641.x.

[682] G. Zhao, Y. Liu, J. Ren, M. Zhao, B. Yang, Effect of protease pretreatment on the functional properties of protein concentrate from defatted peanut flour, J. Food Process Eng. 36 (1) (2013) 9-17, https://doi.org/10.1111/j.1745-4530.2011.00646.x.

[683] J.A. do Evangelho, N.L. Vanier, V.Z. Pinto, J.J. De Berrios, A.R.G. Dias, E. da Rosa Zavareze, Black bean (*Phaseolus vulgaris* L.) protein hydrolysates: Physicochemical and functional properties, Food chemistry 214 (2017) 460-467, https://doi.org/10.1016/j.foodchem.2016.07.046.

[684] M. Geirsdottir, S. Sigurgisladottir, P.Y. Hamaguchi, G. Thorkelsson, R. Johannsson, H.G. Kristinsson, M.M. Kristjansson, Enzymatic hydrolysis of blue whiting (*Micromesistius poutassou*); functional and bioactive properties, J. Food Sci. 76 (2011) C14-C20, https://doi.org/10.1111/j.1750-3841.2010.01877.x.

[685] K. Balaswamy, P.G. Prabhakara Rao, G. Narsing Rao, T. Jyothirmayi, Functional properties of roe protein hydrolysates from Catla catla, Electronic Journal of Environmental Agricultural and Food Chemistry 10 (2011) 2139-2147.

[686] M.A. Amiza, Y.L. Kong, A.L. Faazaz, Effects of degree of hydrolysis on physicochemical properties of Cobia (*Rachycentron canadum*) frame hydrolysate, Int. Food Res. J. 19 (2012) 199-206.

[687] R. Intarasirisawat, S. Benjakul, W. Visessanguan J. Wu, Antioxidative and functional properties of protein hydrolysate from defatted skipjack (*Katsuwonous pelamis*) roe, Food Chem. 135 (2012) 3039-3048, https://doi.org/10.1016/j.foodchem.2012.06.076.

[688] S. Klomklao, S. Benjakul, Utilization of tuna processing byproducts: Protein hydrolysate from skipjack tuna (*Katsuwonus pelamis*) viscera, J. Food Process. Pres. 41 (2017) e12970, https://doi.org/10.1111/jfpp.12970.

[689] A. Taheri, S.A.A. Anvar, R. Ahari, V. Fogliano, Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (*Onchorhynchus mykiss*) viscera, Iran. J. Fish. Sci. 12 (2013) 154-169.

[690] E. Nguyen, O. Jones, Y.H.B. Kim, F. San Martin-Gonzalez, A.M. Liceaga, Impact of microwave-assisted enzymatic hydrolysis on functional and antioxidant properties of rainbow trout *Oncorhynchus mykiss* by-products, Fish. Sci. 83 (2017) 317-331, https://doi.org/10.1007/s12562-017-1067-3.

[691] M. Nikoo, S. Benjakul, H.A. Gavlighi, X. Xu, J.M. Regenstein, Hydrolysates from rainbow trout (*Oncorhynchus mykiss*) processing by-products: Properties when added to

fish mince with different freeze-thaw cycles, Food Biosci. 30 (2019) 100418, https://doi.org/10.1016/j.fbio.2019.100418.

[692] M.A. Amiza, Y.W. Ow, A.L. Faazaz, Physicochemical properties of silver catfish (*Pangasius* sp.) frame hydrolysate, Int. Food Res. J. 20 (2013), 1255-1262.

[693] N.H. Jamil, N.R.A. Halim, N.M. Sarbon, Optimization of enzymatic hydrolysis condition and functional properties of eel (*Monopterus* sp.) protein using Response Surface Methodology (RSM), Int. Food Res. J. 23 (2016) 1-9.

[694] N.R.A. Halim, N.M. Sarbon, Characterization of Asian swamp eel (*Monopterus* sp.) protein hydrolysate functional properties prepared using Alcalase® enzyme, Food Res. 4 (1) (2020) 207-215, https://doi.org/10.26656/fr.2017.4(1).205.

[695] Ş. Yilmaz, Ş. ÇAkli, N. Erol, Ö. Erdem, B. Sen Yilmaz, A. Yavuz, Determination of some functional properties of enzymatically hydrolyzed fish protein from Sea Bass (*Dicentrarchus labrax*) by-products and its effect in Whiting Mince (*Merlangius merlangus*), Deutsche Lebensmittel-Rundschau, 112 (2016) 261-269.

[696] T.H. Cao, T.T.O. Nguyen, T.M.H. Nguyen, N.T. Le, R.G. Razumovskaya, Characteristics and physicochemical properties of gelatin extracted from scales of Seabass (*Lates calcarifer*) and Grey Mullet (*Mugil cephalus*) in Vietnam, J. Aquat. Food Prod. Technol. 26 (2017) 1293-1302, https://doi.org/10.1080/10498850.2017.1390026.

[697] Q. Zhao, Q. Shen, R. Guo, J. Wu, Z.-y. Dai, Characterization of flavor properties from fish (*Collichthys niveatus*) through enzymatic hydrolysis and the maillard reaction, J. Aquat. Food Prod. Technol. 25 (2016) 482-495, https://doi.org/10.1080/10498850.2013.873965.

[698] N.H. Ishak, N.M. Sarbon, Physicochemical characterization of enzymatically prepared fish protein hydrolysate from waste of shortfin scad (*Decapterus macrosoma*), Int. Food Res. J. 25 (2018) 2593-2600.

[699] H.I. Rasli, N.M. Sarbon, Preparation and physicochemical characterization of fish skin gelatine hydrolysate from shortfin scad (*Decapterus macrosoma*), Int. Food Res. J. 26 (2019) 287-294.

[700] S. Ghelichi, A.-D.M. Sørensen, P.J. García-Moreno, M. Hajfathalian, C. Jacobsen, Physical and oxidative stability of fish oil-in-water emulsions fortified with enzymatic hydrolysates from common carp (*Cyprinus carpio*) roe, Food chemistry 237 (2017) 1048-1057, https://doi.org/10.1016/j.foodchem.2017.06.048.

[701] Y. Zhang, P. Dutilleul, V. Orsat, B.K. Simpson, Alcalase assisted production of novel high alpha-chain gelatin and the functional stability of its hydrogel as influenced by thermal treatment, Int. J. Biol. Macromol. 118 (2018) 2278-2286, https://doi.org/10.1016/j.ijbiomac.2018.07.114.

[702] E.H. Hau, M.Z. Zia, N. Zuraidah, N.A. Shaharudin, M.K. Zainol, Physicochemical properties of powdered protein hydrolysate from Yellowstripe scad (*Selaroides leptolepis*) fish, Int. Food Res. J. 25 (2018) 2553-2559.

[703] Y. Zhang, P. Dutilleul, C. Li, B.K. Simpson, Alcalase-assisted production of fish skin gelatin rich in high molecular weight (HMW) polypeptide chains and their characterization for film forming capacity, LWT. 110 (2019) 117-125, https://doi.org/10.1016/j.lwt.2018.12.012.

[704] A. Noman, J. Qixing, Y. Xu, A.H. Ali, W.Q. Al-Bukhaiti, S.M. Abed, W. Xia, Influence of degree of hydrolysis on chemical composition, functional properties, and antioxidant activities of chinese sturgeon (*Acipenser sinensis*) hydrolysates obtained by using Alcalase 2.4 L, J. Aquat. Food Prod. Technol. 28 (2019) 583-597, https://doi.org/10.1080/10498850.2019.1626523.

[705] S.D.A. dos Santos, V.G. Martins, M. Salas-Mellado, C. Prentice, Evaluation of functional properties in protein hydrolysates from bluewing searobin (*Prionotus punctatus*) obtained with different microbial enzymes, Food Bioprocess Technol. 4 (2011) 1399-1406, https://doi.org/10.1007/s11947-009-0301-0.

[706] M.B.K. Foh, I. Amadou, M.T. Kamara, B.M. Foh, W. Xia, Effect of enzymatic hydrolysis on the nutritional and functional properties of nile tilapia (*Oreochromis niloticus*) proteins, Am. J. Biochem. Mol. Biol. 1 (2011) 54-67, https://doi.org/10.3923/ajbmb.2011.54.67.

[707] M. Chalamaiah, G.N. Rao, D.G. Rao, T. Jyothirmayi, Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties, Food Chem. 120 (2010) 652-657, https://doi.org/10.1016/j.foodchem.2009.10.057.

[708] M. Muzaifa, N. Safriani, F. Zakaria, Production of protein hydrolysates from fish by-product prepared by enzymatic hydrolysis, Aquaculture, Aquarium, Conservation & Legislation 5 (2012) 36-39.

[709] R. Balti, A. Bougatef, P. Dhulster, M. Nasri, N. Nedjar-Arroume, Effect of enzymatic hydrolysis on the interfacial and surface properties of Cuttlefish (*Sepia officinalis*) muscle proteins, Nat. Prod. J. 3 (2013) 115-124, https://doi.org/10.2174/2210315511303020006.

[710] M. Rajabzadeh, P. Pourashouri, B. Shabanpour, A. Alishahi, Amino acid composition, antioxidant and functional properties of protein hydrolysates from the roe of rainbow trout (*Oncorhynchus mykiss*), Int. J. Food Sci. Technol. 53 (2018) 313-319, https://doi.org/10.1111/ijfs.13587.

[711] H.D. Yoon, E.P. Karaulova, L.V. Shulgina, E.V. Yakush, J.S. Mok, S.S. Lee, C. Xie, J.G. Kim, Nutritional value and bioactive properties of enzymatic hydrolysates prepared from the livers of *Oncorhynchus keta* and *Oncorhynchus gorbuscha* (Pacific Salmon), Fisheries and aquatic sciences 18 (2015) 13-20, http://dx.doi.org/10.5657/FAS.2015.0013.

[712] T. Aspevik, C. Totland, P. Lea, Å. Oterhals, Sensory and surface-active properties of protein hydrolysates based on Atlantic salmon (*Salmo salar*) by-products, Process Biochem. 51 (2016) 1006-1014, https://doi.org/10.1016/j.procbio.2016.04.015.

[713] N. Kim, S.H. Son, J.S. Maeng, Y.J. Cho, C.T. Kim, Enzymatic hydrolysis of anchovy fine powder at high and ambient pressure, and characterization of the hydrolyzates, J. Sci. Food Agric. 96 (2016) 970-978, https://doi.org/10.1002/jsfa.7173.

[714] E.F. Vieira, O. Ferreira, I.M. Ferreira, Bio- functional properties of sardine protein hydrolysates obtained by brewer's spent yeast and commercial proteases, J. Sci. Food Agric. 97 (2017) 5414-5422, https://doi.org/10.1002/jsfa.8432.

[715] I. Normah, M.S.S. Hafsah, A.N. Izzaira, Bitterness of green mussel (*Perna viridis*) hydrolysate as influenced by the degree of hydrolysis, Int. Food Res. J. 20 (2013) 2261-2268.

[716] F.G. Hall, O.G. Jones, M.E. O'Haire, A.M. Liceaga, Functional properties of tropical banded cricket (*Gryllodes sigillatus*) protein hydrolysates, Food chemistry 224 (2017) 414-422, https://doi.org/10.1016/j.foodchem.2016.11.138.

[717] J.D.F. da Silva, A.P.F. Correa, C.P. Kechinski, A. Brandelli, Buffalo cheese whey hydrolyzed with Alcalase as an antibrowning agent in minimally processed apple, J. Food Sci. Technol. 55 (2018) 3731-3738, https://doi.org/10.1007/s13197-018-3303-y.

[718] L. Wu, C. Hou, B. Xi, L.A.I. Boga, D. Zhang, Sheep plasma hydrolysate inhibits lipid and protein oxidation to improve color stability in mutton patties, Food Sci. Technol. Res. 24 (2018) 661-668, https://doi.org/10.3136/fstr24.661.

[719] Y. S. Ma, H. J. Zhao, X. H. Zhao, Comparison of the effects of the Alcalase-hydrolysates of caseinate, and of fish and bovine gelatins on the acidification and textural features of set-style skimmed yogurt-type products, Foods. 8 (2019) 501, https://doi.org/10.3390/foods8100501.

[720] V. Dhanabalan, M. Xavier, L.N. Murthy, K.K. Asha, A.K. Balange, B.B. Nayak, Evaluation of physicochemical and functional properties of spray- dried protein hydrolysate from non- penaeid shrimp (*Acetes indicus*), J. Sci. Food Agric. 100 (2020) 50-58, https://doi.org/10.1002/jsfa.9992.

[721] Y. Schober, S.H. Yoo, H.D. Paik, E.J. Park, B. Spengler, A. Römpp, H.M. Jayaprakasha, Y.C. Yoon, Characterization of bioactive peptides derived by enzymatic hydrolysis of whey protein concentrate, Milchwissenschaft. 67 (2012) 55-57.

[722] R. Kankanamge, C. Jeewanthi, H.-D. Paik, M.-H. Kim, N.-K. Lee, S.-Y. Kim, Y.C. Yoon, Characteristics of whey protein hydrolysates from cheese whey, favors on various

food application, Chem. Ind. Chem. Eng. Q. 20 (2014) 503-509, https://doi.org/10.2298/CICEQ130221032J.

[723] D. Pein, I. Clawin- Rädecker, P.C. Lorenzen, Peptic treatment of beta- lactoglobulin improves foaming properties substantially, J. Food Process. Pres. 42 (2018) e13543, https://doi.org/10.1111/jfpp.13543.

[724] Z. Barzideh, A.A. Latiff, C.-Y. Gan, M.Z. Abedin, A.A. Karim, Functional properties of collagen hydrolysates from the jellyfish, Agro. Food Ind. Hi Tec. 25 (2014) 27-32.

[725] N. Ismail, N. Mustakim, Sensory Characteristics of Mud Clam (*Polymesoda Erosa*) Hydrolysate, Malaysian J. Anal. Sci. 20 (2016) 812-819, http://dx.doi.org/10.17576/mjas-2016-2004-14.

[726] A.L.M. de Queiroz, T.K.A. Bezerra, S. de Freitas Pereira, M.E.C. da Silva, C.A. de Almeida Gadelha, T.S. Gadelha, M.T.B. Pacheco, M.S. Madruga, Functional protein hydrolysate from goat by-product: Optimization and characterization studies, Food Biosci. 20 (2017) 19-27, https://doi.org/10.1016/j.fbio.2017.07.009.

[727] Z. Bao, Y. Zhao, X. Wang, Y. Chi, Effects of degree of hydrolysis (DH) on the functional properties of egg yolk hydrolysate with Alcalase, J. Food Sci. Technol. 54 (2017) 669-678, https://doi.org/10.1007/s13197-017-2504-0.

[728] G. Linkai, L. Binbin, H. Dongyi, C. Shengyang, W. Shaoyun, Optimized preparation of enzymatic flavors for the offal of Octopus and Abalone and its component analysis, Journal of Chinese Institute of Food Science and Technology 18 (2018) 167-174, https://doi.org/10.16429/j.1009-7848.2018.09.021.

[729] M. Fallah- Delavar, J. Farmani, Recovery and characterization of enzymatic protein hydrolyzates and fat from chicken skin, J. Am. Oil Chem. Soc. 95 (2018) 1151-1161, https://doi.org/10.1002/aocs.12131.

[730] S. Putra, N.H. Ishak, N.M. Sarbon, Preparation and characterization of physicochemical properties of golden apple snail (*Pomacea canaliculata*) protein hydrolysate as affected by different proteases, Biocatal. Agric. Biotechnol. 13 (2018) 123-128, https://doi.org/10.1016/j.bcab.2017.12.002.

[731] A. Singh, S. Benjakul, Effect of partial enzymatic hydrolysis on physicochemical and foaming properties of ovary from squid *Loligo formosana*, Waste Biomass Valor. 10 (2019) 3351-3361, https://doi.org/10.1007/s12649-018-0348-0.

[732] Z. Ling, M. Ai, Q. Zhou, S. Guo, L. Zhou, H. Fan, Y. Cao, A. Jiang, Fabrication egg white gel hydrolysates-stabilized oil-in-water emulsion and characterization of its stability and digestibility, Food Hydrocoll. 102 (2020) 105621, https://doi.org/10.1016/j.foodhyd.2019.105621.

[733] T. Jin, W. Li, Y. Wu, Production and characteristics of protein hydrolysates from little hairtail (*Trichiurus haumela*) of East China sea, Journal of Food, Agriculture & Environment 10 (2012) 81-85.

[734] Y. Xu, M. Galanopoulos, E. Sismour, S. Ren, Z. Mersha, P. Lynch, A. Almutaimi, Effect of enzymatic hydrolysis using endo-and exo-proteases on secondary structure, functional, and antioxidant properties of chickpea protein hydrolysates, Food Measure. 14 (2020) 343-352, https://doi.org/10.1007/s11694-019-00296-0.

[735] M.R. Segura-Campos, K. García-Rodríguez, J.C. Ruiz-Ruiz, L. Chel-Guerrero, D. Betancur-Ancona, *In vitro* bioactivity, nutritional and sensory properties of semolina pasta added with hard-to-cook bean (*Phaseolus vulgaris* L.) protein hydrolysate, J. Func. Foods. 8 (2014) 1-8, https://doi.org/10.1016/j.jff.2014.02.016.

[736] M.R. Segura- Campos, K. García- Rodríguez, J.C. Ruiz- Ruiz, L. Chel- Guerrero, D. Betancur- Ancona, Effect of incorporation of hard- to- cook bean (*Phaseolus vulgaris* L.) protein hydrolysate on physical properties and starch and dietary fiber components of semolina pasta, J. Food Process. Pres. 39 (2015) 1159-1165, https://doi.org/10.1111/jfpp.12330.

[737] K. Schlegel, K. Sontheimer, P. Eisner, U. Schweiggert- Weisz, Effect of enzyme-assisted hydrolysis on protein pattern, technofunctional, and sensory properties of lupin protein isolates using enzyme combinations, Food Sci. Nutr. In press, https://doi.org/10.1002/fsn3.1286.

[738] S.B. Zhang, *In vitro* antithrombotic activities of peanut protein hydrolysates, Food Chem. 202 (2016) 1-8, https://doi.org/10.1016/j.foodchem.2016.01.108.

[739] Y. Ren, Y. Yang, W. Wu, M. Zhang, H. Wu, X. Li, Identification and characterization of novel anticoagulant peptide with thrombolytic effect and nutrient oligopeptides with high branched chain amino acid from *Whitmania pigra* protein, Amino Acids 48 (2016) 2657-2670, https://doi.org/10.1007/s00726-016-2299-8.

[740] J. Liu, F. Wang, Y. Zhang, E. Wang, Z. Wang, Y. Jiang, Purification of anticoagulant peptides derived from egg white powder, Journal of Jilin University. 42 (2012) 466-469.

[741] J. B. Liu, F. Wang, C. N. Wang, J. Liu, Z. Z. Wang, E. L. Wang, Y. Zhang, S. Y. Lin, Optimization of preparation for anticoagulant peptide from egg white powder by Alcalase, Journal of Jilin University. 42 (2012) 250-255.

[742] Y. Ren, H. Wu, F. Lai, M. Yang, X. Li, Y. Tang, Isolation and identification of a novel anticoagulant peptide from enzymatic hydrolysates of scorpion (*Buthus martensii* Karsch) protein, Food Res. Int. 64 (2014) 931-938, https://doi.org/10.1016/j.foodres.2014.08.031.

[743] A.C. Sabbione, A. Scilingo, M.C. Añón, Potential antithrombotic activity detected in amaranth proteins and its hydrolysates, LWT-Food Sci. Technol. 60 (2015) 171-177, https://doi.org/10.1016/j.lwt.2014.07.015.

[744] D. Ding, T. Yu, B. Du, Y. Huang, Collagen hydrolysate from *Thunnus orientalis* bone induces osteoblast proliferation and differentiation, Chem. Eng. Sci. 205 (2019) 143-150, https://doi.org/10.1016/j.ces.2019.04.040.

[745] S. Y. Kim, H. S. Kim, M. Cho, Y. J. Jeon, Enzymatic hydrolysates of *Hippocampus abdominalis* regulates the skeletal muscle growth in C2C12 cells and Zebrafish model, J. Aquat. Food Prod. Technol. 28 (2019) 264-274, https://doi.org/10.1080/10498850.2019.1575940.

[746] C.S. Yang, L.G. Zhang, Y.H. Zhao, Preparation collagen peptides from antlers of Red Deer (*Cervus elaphus*) with the activity of promoting proliferation of human skin fibroblasts by bi-enzymatic hydrolysis, Modern Food Sci. Tech. 34 (2018) 119-126, https://doi.org/10.13982/j.mfst.1673-9078.2018.1.019.

[747] Y. Fang, X. Pan, E. Zhao, Y. Shi, X. Shen, J. Wu, F. Pei, Q. Hu, W. Qiu, Isolation and identification of immunomodulatory selenium-containing peptides from selenium-enriched rice protein hydrolysates, Food Chem. 275 (2019) 696-702, https://doi.org/10.1016/j.foodchem.2018.09.115.

[748] D. Crowley, Y. O'Callaghan, A. McCarthy, A. Connolly, C.O. Piggott, R.J. FitzGerald, N.M. O'Brien, Immunomodulatory potential of a brewers' spent grain protein hydrolysate incorporated into low-fat milk following *in vitro* gastrointestinal digestion, Int. J. Food Sci. Nutr. 66 (2015) 672-676, https://doi.org/10.3109/09637486.2015.1077788.

[749] J. Diao, Z. Chi, Z. Guo, L. Zhang, Mung bean protein hydrolysate modulates the immune response through NF- κB pathway in lipopolysaccharide- stimulated RAW 264.7 macrophages, J. Food Sci. 84 (2019) 2652-2657, https://doi.org/10.1111/1750-3841.14691.

[750] W. Wu, M. Zhang, C. Sun, M. Brennan, H. Li, G. Wang, F. Lai, H. Wu, Enzymatic preparation of immunomodulatory hydrolysates from defatted wheat germ (*Triticum Vulgare*) globulin, Int. J. Food Sci. Technol. 51 (2016) 2556-2566, https://doi.org/10.1111/ijfs.13238.

[751] Z. Li, S. Zhao, X. Xin, B. Zhang, A. Thomas, A. Charles, K.S. Lee, B.R. Jin, Z. Gui, Purification and characterization of a novel immunomodulatory hexapeptide from Alcalase hydrolysate of ultramicro-pretreated silkworm (*Bombyx mori*) pupa protein, J. Asia-Pac. Entomol. 22 (2019) 633-637, https://doi.org/10.1016/j.aspen.2019.04.005.

[752] Z. Li, S. Zhao, X. Xin, B. Zhang, A. Thomas, A. Charles, K.S. Lee, B.R. Jin, Z. Gui, Purification, identification and functional analysis of a novel immunomodulatory peptide

from Silkworm pupa protein, Int. J. Pept. Res. Ther. 26 (2020) 243-249, https://doi.org/10.1007/s10989-019-09832-4.

[753] D. Lozano-Ojalvo, E. Molina, R. López-Fandiño, Hydrolysates of egg white proteins modulate T-and B-cell responses in mitogen-stimulated murine cells, Food Funct. 7 (2016) 1048-1056, https://doi.org/10.1039/c5fo00614g.

[754] D. Lozano-Ojalvo, E. Molina, R. Lopez-Fandino, Regulation of exacerbated immune responses in human peripheral blood cells by hydrolysed egg white proteins, PloS One. 11 (2016) e0151813, https://doi.org/10.1371/journal.pone.0151813.

[755] W. He, G. Su, D. Sun-Waterhouse, G.I.N. Waterhouse, M. Zhao, Y. Liu, *In vivo* anti-hyperuricemic and xanthine oxidase inhibitory properties of tuna protein hydrolysates and its isolated fractions, Food Chem. 272 (2019) 453-461, https://doi.org/10.1016/j.foodchem.2018.08.057.

[756] I. Murota, S. Taguchi, N. Sato, E.Y. Park, Y. Nakamura, K. Sato, Identification of antihyperuricemic peptides in the proteolytic digest of shark cartilage water extract using in vivo activity-guided fractionation, J. Agric. Food Chem. 62 (2014) 2392-2397, https://doi.org/10.1021/jf405504u.

[757] C. Xiao, M. Zhao, F. Zhou, M. Gallego, J. Gao, F. Toldrá, L. Mora, Isolation and identification of alcohol dehydrogenase stabilizing peptides from Alcalase digested chicken breast hydrolysates, J. Func. Foods. 64 (2020) 103617, https://doi.org/10.1016/j.jff.2019.103617.

[758] N. Sun, T. Xu, Y. Liu, C. Ye, Z. Jiang, F. Du, Y. Wang, Preparation of two oligopeptides from corn protein and their protective effect on acute alcohol intoxication in mice, Biomed. Res, 29 (2018) 1284-1289.

[759] G.C. Yu, J.T. Li, H.U.I. He, W.H. Huang, W.J. Zhang, Ultrafiltration preparation of potent bioactive corn peptide as alcohol metabolism stimulator *in vivo* and study on its mechanism of action, J. Food Biochem. 37 (2013) 161-167, https://doi.org/10.1111/j.1745-4514.2011.00613.x.

[760] M. Son, J. Moon, S. Park, M. Cho, Hepatoprotective effect of *Hippocampus abdominalis* hydrolysate, J. Appl. Biol. Chem. 59 (2016) 265-271, https://doi.org/10.3839/jabc.2016.046.

[761] H.J. Suh, B. Kang, C.-Y. Kim, H.-S. Choi, Enzyme hydrolysate of silk protein suppresses tert-butyl hydroperoxide-induced hepatotoxicity by enhancing antioxidant activity in rats, Korean J Food Preserv. 24 (2017) 550-558, https://doi.org/10.11002/kjfp.2017.24.4.550.

[762] S. Luo, H. Guo, L. Zhang, F. Wang, X. Tan, L. He, Preparation and *in vitro* sobering activity of wheat germ protein hydrolysates by double enzymes, J. Chinese Cereals Oils Assoc. 33 (2018) 87-93.

[763] X. Cai, A. Yan, N. Fu, S. Wang, *In vitro* antioxidant activities of enzymatic hydrolysate from *Schizochytrium* Sp. and its hepatoprotective effects on acute alcohol-induced liver injury *in vivo*, Mar. Drugs 15 (2017) 115, https://doi.org/10.3390/md15040115.

[764] S. Dumeus, M.A. Shibu, W. T. Lin, M. F. Wang, C. H. Lai, C. Y. Shen, Y. M. Lin, V.P. Viswanadha, W. W. Kuo, C. Y. Huang, Bioactive peptide improves diet-induced hepatic fat deposition and hepatocyte proinflammatory response in SAMP8 ageing mice, Cell Physiol. Biochem. 48 (2018) 1942-1952, https://doi.org/10.1159/000492518.

[765] W. D. Chiang, C.Y. Huang, C.R. Paul, Z. Y. Lee, W. T. Lin, Lipolysis stimulating peptides of potato protein hydrolysate effectively suppresses high-fat-diet-induced hepatocyte apoptosis and fibrosis in aging rats, Food Nutr. Res. 60 (2016) 31417, https://doi.org/10.3402/fnr.v60.31417.

[766] B.K. Han, Y. Park, H.S. Choi, H.J. Suh, Hepatoprotective effects of soluble rice protein in primary hepatocytes and in mice, J. Sci. Food Agric. 96 (2016) 685-694, https://doi.org/10.1002/jsfa.7153.

[767] G.C. Yu, J. Lv, H. He, W. Huang, Y. Han, Hepatoprotective effects of corn peptides against carbon tetrachloride- induce liver injury in mice, J. Food Biochem. 36 (2012) 458-464, https://doi.org/10.1111/j.1745-4514.2011.00551.x.

[768] B.C. K. Tsai, D.J. Y. Hsieh, W. T. Lin, S. Tamilselvi, C.H. Day, T. J. Ho, R. L. Chang, V.P. Viswanadha, C. H. Kuo, C. Y. Huang, Functional potato bioactive peptide intensifies Nrf2-dependent antioxidant defense against renal damage in hypertensive rats, Food Res. Int. 129 (2020) 108862, https://doi.org/10.1016/j.foodres.2019.108862.

[769] Y. Oh, C.-B. Ahn, N.Y. Yoon, K.H. Nam, Y. K. Kim, J. Y. Je, Protective effect of enzymatic hydrolysates from seahorse (*Hippocampus abdominalis*) against H_2O_2-mediated human umbilical vein endothelial cell injury, Biomed Pharmacother. 108 (2018) 103-110, https://doi.org/10.1016/j.biopha.2018.08.143.

[770] M. Ortiz-Martinez, E.G. de Mejia, S. García-Lara, O. Aguilar, L.M. Lopez-Castillo, J.T. Otero-Pappatheodorou, Antiproliferative effect of peptide fractions isolated from a quality protein maize, a white hybrid maize, and their derived peptides on hepatocarcinoma human HepG2 cells, J. Func. Foods. 34 (2017) 36-48, https://doi.org/10.1016/j.jff.2017.04.015.

[771] S.J. Rayaprolu, N.S. Hettiarachchy, P. Chen, A. Kannan, A. Mauromostakos, Peptides derived from high oleic acid soybean meals inhibit colon, liver and lung cancer cell growth, Food Res. Int. 50 (2013) 282-288, https://doi.org/10.1016/j.foodres.2012.10.021.

[772] M. Zhang, T.H. Mu, Contribution of different molecular weight fractions to anticancer effect of sweet potato protein hydrolysates by six proteases on HT- 29 colon cancer cells, Int. J. Food Sci. Technol. 53 (2018) 525-532, https://doi.org/10.1111/ijfs.13625.

[773] Q. Zheng, D. Qiu, X. Liu, L. Zhang, S. Cai, X. Zhang, Antiproliferative effect of *Dendrobium catenatum* Lindley polypeptides against human liver, gastric and breast cancer cell lines, Food Funct. 6 (2015) 1489-1495, https://doi.org/10.1039/c5fo00060b.

[774] X. Li, H. Xie, Y. Chen, M. Lang, Y. Chen, L. Shi, Silkworm pupa protein hydrolysate induces mitochondria-dependent apoptosis and S phase cell cycle arrest in human gastric cancer SGC-7901 cells, Int. J. Mol. Sci. 19 (2018) 1013, https://doi.org/10.3390/ijms19041013.

[775] Z. Wang, X. Zhang, Characterization and antitumor activity of protein hydrolysates from Arthrospira platensis (*Spirulina platensis*) using two-step hydrolysis, J. Appl. Phycol. 28 (2016) 3379-3385, https://doi.org/10.1007/s10811-016-0881-9.

[776] J. Luo, K. Mills, S. le Cessie, R. Noordam, D. van Heemst, Ageing, age-related diseases and oxidative stress: What to do next?, Ageing Res. Rev. 57 (2020) 100982, https://doi.org/10.1016/j.arr.2019.100982.

[777] B. Wang, N. Xie, B. Li, Charge properties of peptides derived from casein affect their bioavailability and cytoprotection against H_2O_2-induced oxidative stress, J. Dairy Sci. 99 (2016) 2468-2479, https://doi.org/10.3168/jds.2015-10029.

[778] Y. Zou, W. Feng, W. Wang, Y. Chen, Z. Zhou, Q. Li, T. Zhao, G. Mao, X. Wu, L. Yang, Protective effect of porcine cerebral hydrolysate peptides on learning and memory deficits and oxidative stress in lead-exposed mice, Biol. Trace Elem. Res. 168 (2015) 429-440, https://doi.org/10.1007/s12011-015-0329-0.

[779] S. H. Chiang, S. Y. Wang, C. Y. Chang, C. W. Chen, Bovine colostrum whey protein hydrolysate inhibits cell DNA damage and LDL oxidation *in vitro*, Molecules. 22 (2017) 456, https://doi.org/10.3390/molecules22030456.

[780] A.M. Alashi, C.L. Blanchard, R.J. Mailer, S.O. Agboola, A.J. Mawson, R.E. Aluko, P. Strappe, Effects of canola proteins and hydrolysates on adipogenic differentiation of C3H10T/2 mesenchymal stem cells, Food Chem. 185 (2015) 226-232, http://doi.org/10.1016/j.foodchem.2015.03.054.

[781] Y. Yao, J. Zhang, H. Hui, X. Hao, H. Tao, W. Chi, Preparation and bioactivity of chickpea hypocholesterolemic peptides, J. Chinese Cereals Oils Assoc. 30 (2015) 33-38.

[782] W. T. Lin, K. I. Lee, M. C. Hong, W.-D. Chiang, Production of potato protein hydrolysates with lipolysis-stimulating activity and study of their possible regulation mechanism, Taiwanese Journal of Agricultural Chemistry & Food Science. 54 (2016) 63-73.

[783] W. S. Hu, W. J. Ting, W. D. Chiang, P. Pai, Y. L. Yeh, C. H. Chang, W. T. Lin, C. Y. Huang, The heart protection effect of Alcalase potato protein hydrolysate is through IGF1R-PI3K-Akt compensatory reactivation in aging rats on high fat diets, Int. J. Mol. Sci. 16 (2015) 10158-10172, https://doi.org/10.3390/ijms160510158.

[784] H. Zhang, W.H. Yokoyama, H. Zhang, Concentration- dependent displacement of cholesterol in micelles by hydrophobic rice bran protein hydrolysates, J. Sci. Food Agric. 92 (2012) 1395-1401, https://doi.org/10.1002/jsfa.4713.

[785] F.S. Taha, G.A. Yamamah, G.F. Mohamed, S.S. Mohamed, S.M. Wagdy, Biological activities of sunflower protein hydrolysate, World Appl. Sci. J. 30 (2014) 1462-1470, https://doi.org/10.5829/idosi.wasj.2014.30.11.14185.

[786] L.J.W.Z.H. Qingbo, Anti-fatigue effect of rice residue peptide and isolation and identification of anti-fatigue peptide, J. Chinese Cereals Oils Assoc. 28 (2013) 1-5.

[787] T. Vijitpunyaruk, C. Theerakulkait, Preparation of Alcalase hydrolysed rice bran protein concentrate and its inhibitory effect on soybean lipoxygenase activity, Int. J. Food Sci. Technol. 49 (2014) 501-507, https://doi.org/10.1111/ijfs.12329.

[788] J. Ren, B. Yang, Y. Lv, S. Guo, Protective and reparative effects of peptides from soybean β-conglycinin on mice intestinal mucosa injury, Int. J. Food Sci. Nutr. 65 (2014) 345-350, https://doi.org/10.3109/09637486.2013.854748.

[789] A. Puangphet, W. Tiyaboonchai, T. Thongsook, Inhibitory effect of sericin hydrolysate on polyphenol oxidase and browning of fresh-cut products, Int. Food Res. J. 22 (2015) 1623-1630.

[790] J.I. Kwon, Y. Park, S.H. Han, H.J. Suh, Hydrolysate preparation with high content of 5-hydroxytryptophan from liquid egg protein and its sleep-potentiating activity, Korean J. Food Sci. Anim. Resour. 37 (2017) 646-653, https://doi.org/10.5851/kosfa.2017.37.5.646.

[791] K. Limpisophon, H. Iguchi, M. Tanaka, T. Suzuki, E. Okazaki, T. Saito, K. Takahashi, K. Osako, Cryoprotective effect of gelatin hydrolysate from shark skin on denaturation of frozen surimi compared with that from bovine skin, Fish. Sci. 81 (2015) 383-392, https://doi.org/10.1007/s12562-014-0844-5.

[792] C. Sutthiwanjampa, S.M. Kim, Production and characterisation of hyaluronidase and elastase inhibitory protein hydrolysates from *Venus clam*, Nat. Prod. Res. 29 (2015) 1614-1623, https://doi.org/10.1080/14786419.2014.990903.

[793] L. Ugolini, S. Cinti, L. Righetti, A. Stefan, R. Matteo, L. D'Avino, L. Lazzeri, Production of an enzymatic protein hydrolyzate from defatted sunflower seed meal for potential application as a plant biostimulant, Ind. Crop. Prod. 75 (2015) 15-23, https://doi.org/10.1016/j.indcrop.2014.11.026.

[794] N.M. Rodriguez-Martin, R. Toscano, A. Villanueva, J. Pedroche, F. Millan, S. Montserrat-de la Paz, M.C. Millan-Linares, Neuroprotective protein hydrolysates from hemp (*Cannabis sativa* L.) seeds, Food Funct. 10 (2019) 6732-6739, https://doi.org/10.1039/c9fo01904a.

Figure legends

Figure 1. Effect of the enzyme rigidification on the activity under drastic conditions of immobilized enzymes. The immobilized/stabilized by multipoint covalent immobilization enzyme structure is not altered by the experimental conditions and retains full activity, while the free enzyme becomes inactive.

Figure 2. Possibility of protease autolysis under different circumstances. A: Free enzyme B: Protease immobilized on non-porous nanoparticle C: Immobilization of proteases inside porous supports.

Figure 3. Effect of the orientation on the activity of the immobilized proteases versus small and large substrates. A. Small substrate, active center oriented towards the support surface. B Large substrate, active center orientated out of the support surface. C. Large substrate, active center oriented towards the support surface.

Figure 4. Effect of protease support loading on the expressed activity versus large substrate when the active center orientation is not perfectly out of the support surface. A: A lowly loaded biocatalyst with the enzyme not fully properly oriented remains active because the substrate may reach the active center. B: A highly loaded biocatalyst, with the enzyme molecules near each other, will be not accessible for large subsrates.

Figure 5. Effect of the support pore diameter in the activity of immobilized proteases versus substrates larger than the immobilized enzymes. **A:** Large pore diameter supports: large substrates may reach the enzyme. **B**: Small pore diameter supports: large substrates cannot reach the enzyme in the core of the support, just in the external support particle surface.

Figure 6. Hydrolysis of solid substrates using enzymes immobilized inside porous supports: only the enzyme molecules immobilized on the support surface (a minimal percentage) will be active.

Figure 7. Hydrolysis of solid substrates by enzymes immobilized on non-porous nanomaterials: the correct enzyme orientation remains critical. A: Fully correctly oriented immobilized enzymes exhibiting activity versus solid substrates. B: Incorrectly oriented immobilized enzymes will not be active versus solid substrates even using a nanoparticle for its immobilization.

Figure 8. Schematic representation of the progressive reduction of the size of the substrate during hydrolysis of large substrates: when the reaction progresses and the substrate size decreases, more immobilized enzyme molecules can exert their catalytic function.

Figure 9. Analyzing the effects of the multipoint covalent attachment on enzyme stability using free or one-point immobilized proteases: artifacts versus a fairer comparison. A direct comparision between free and immobilized enzyme will include effects of autolysis in the free enzyme. Moreover, if the enzyme extract has some stabilizing agents (e.g., protease inhibitors), this can increase the stability of the free enzyme. That way, a fairer comparison will be a comparion between one point covalenatly immobilized enzyme (without the risks described but with identical rigidity to the free enzyme) and the biocatalysts where a multipoint covalent attachment has been intended.

Figure 10. Representation of the use and comparison of Alcalase and other different individual proteases a hydrolysis of a protein (A), sequential hydrolysis of a protein by

Alcalase and other proteases (B) and simultaneous hydrolysis of a protein by Alcalase and other proteases.

author statement

This is review paper, RFL and VTP designed the structure and supervised the writing, editing the final version, ABM edited the final version, VTP, RMS and HES performed the bibliographic search and write the preliminary draft, OLT write the general introduction and help in the final editing of the review.

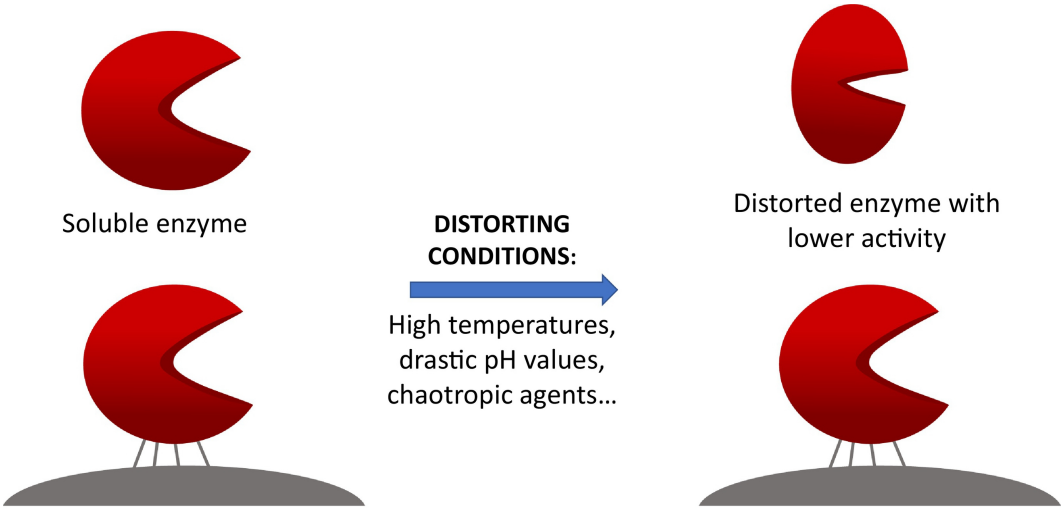
Soluble enzyme
DISTORTING CONDITIONS:
High temperatures, drastic pH values, chaotropic agents…
Distorted enzyme with lower activity
Multi-point covalently attached enzyme
Non-distorted enzyme maintaining the activity

Figure 1

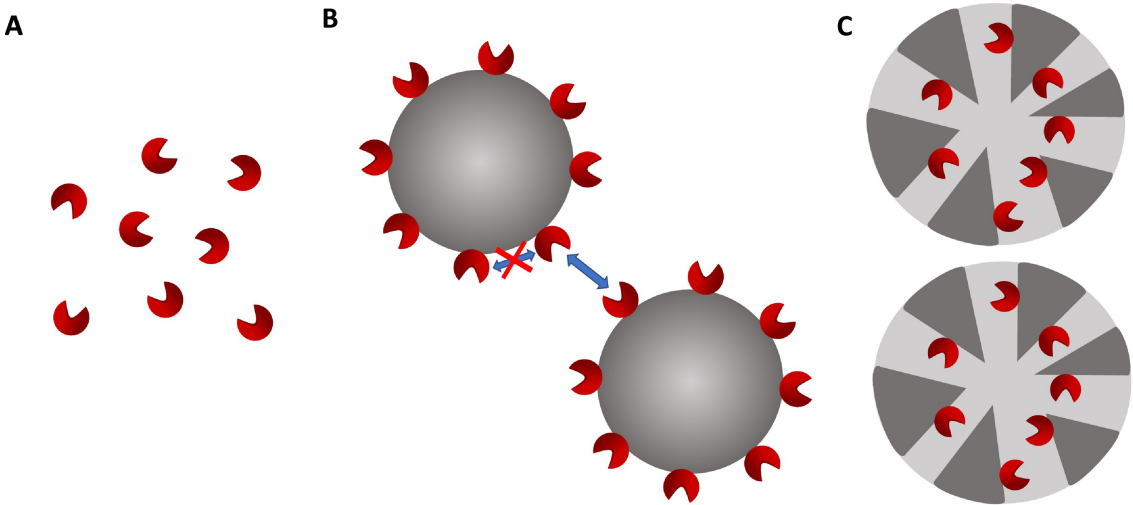
A
B
C
All enzyme molecules can attach to other enzyme molecules
Proteases can only attach to proteases immobilized on another nanoparticle
Enzyme molecules cannot attach to other enzyme molecules

Figure 2

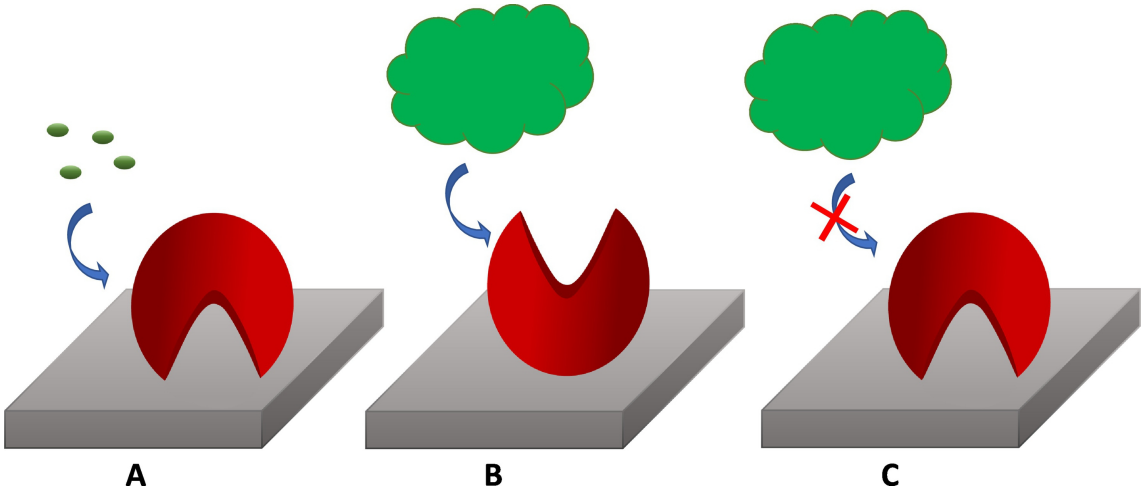

Figure 3

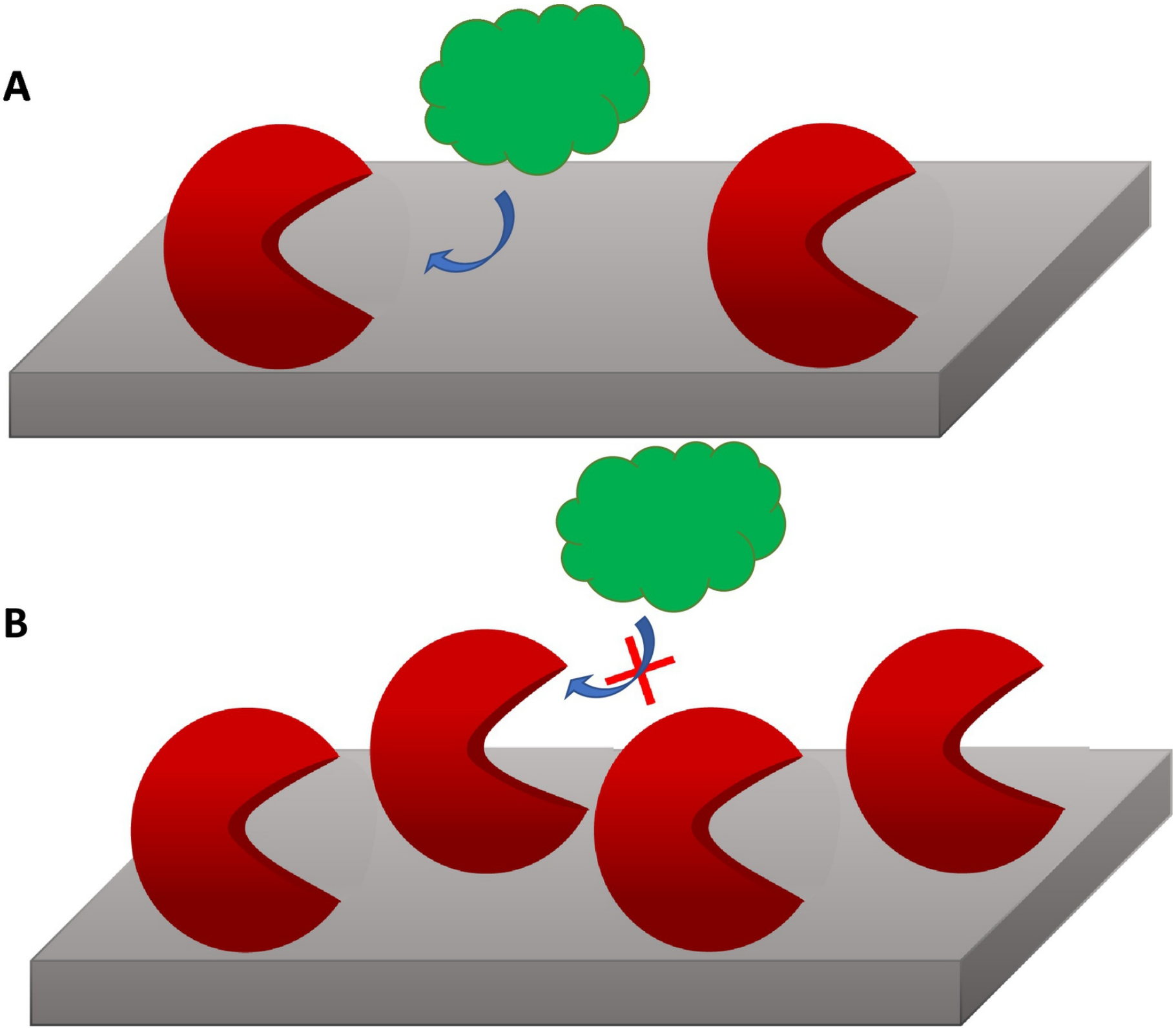

Figure 4

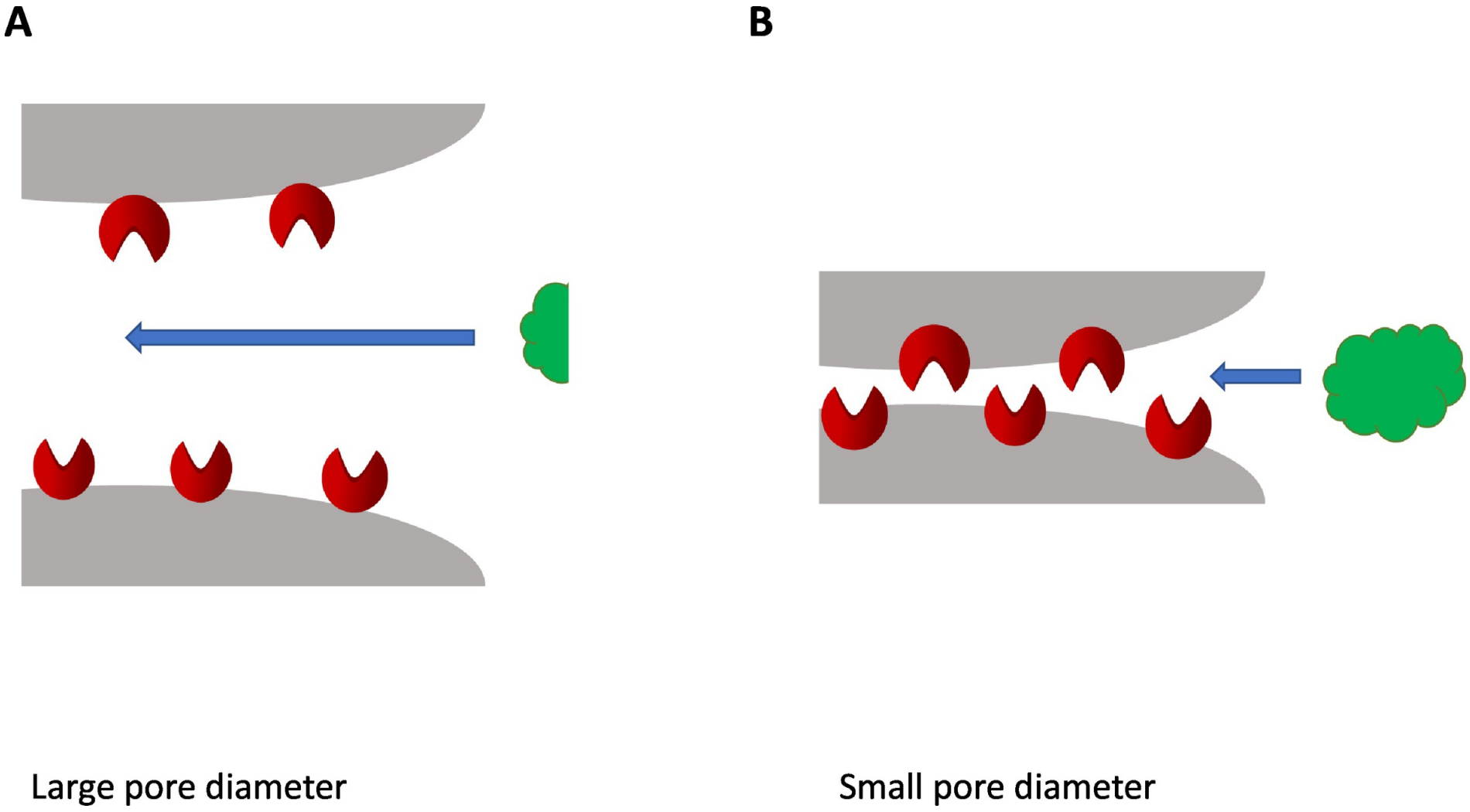

Figure 5

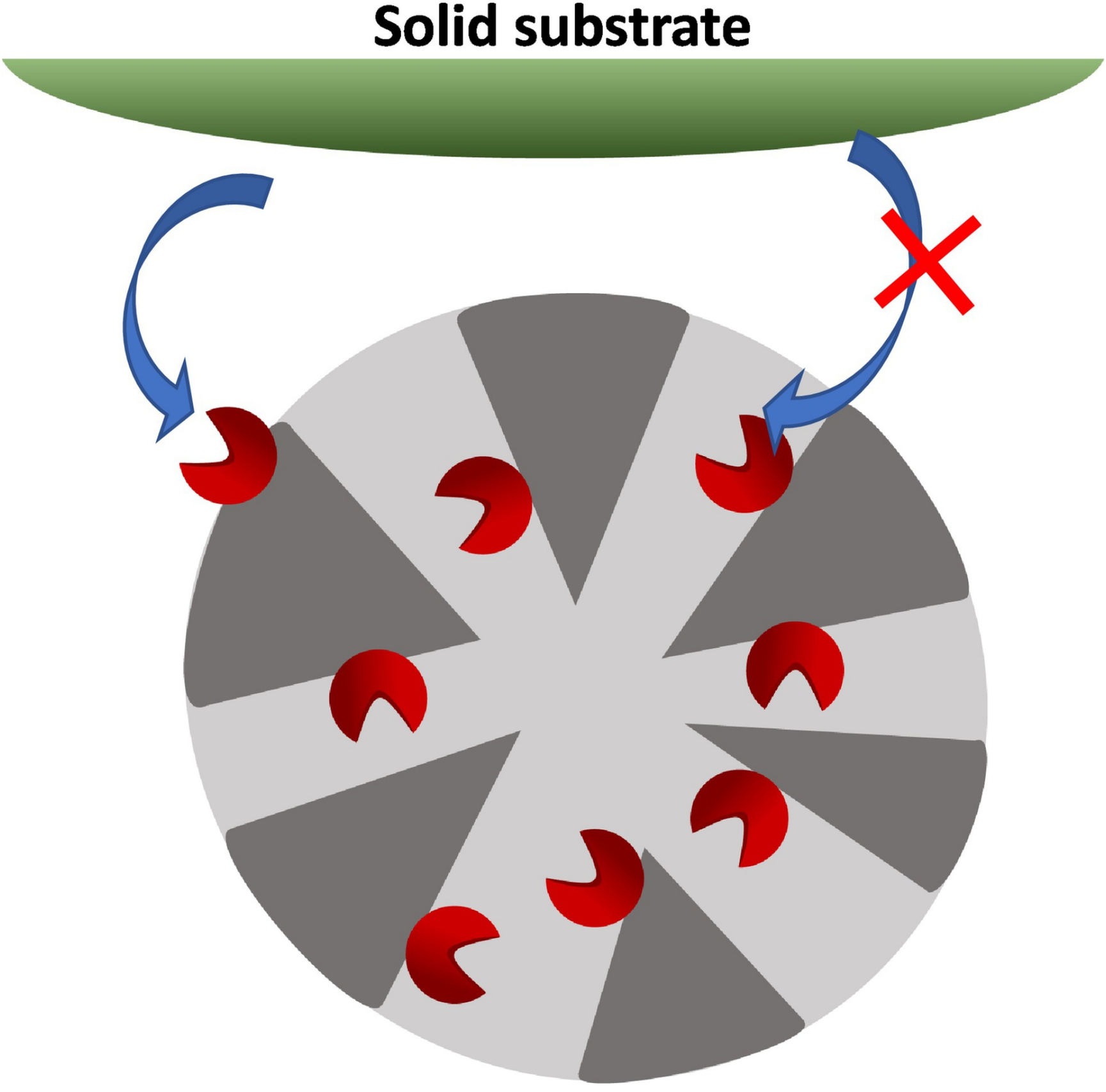

Figure 6

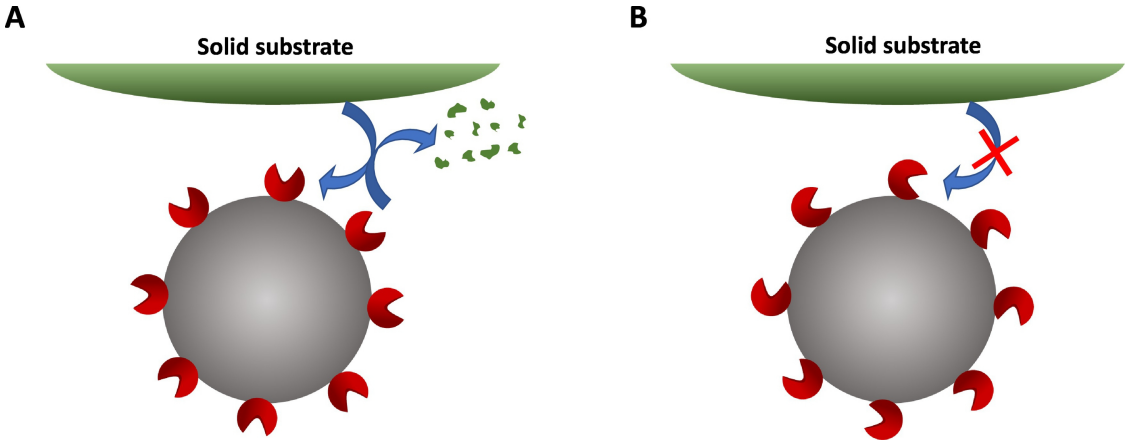

Figure 7

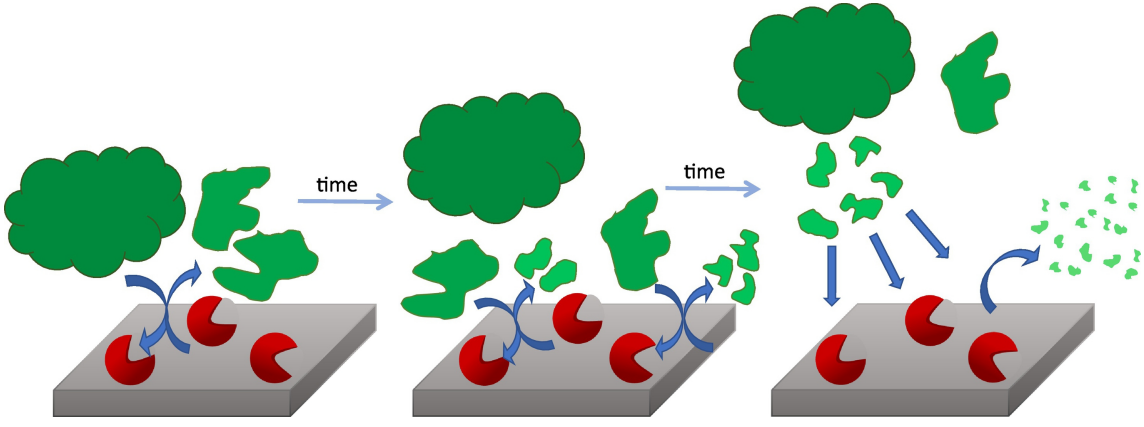

Figure 8

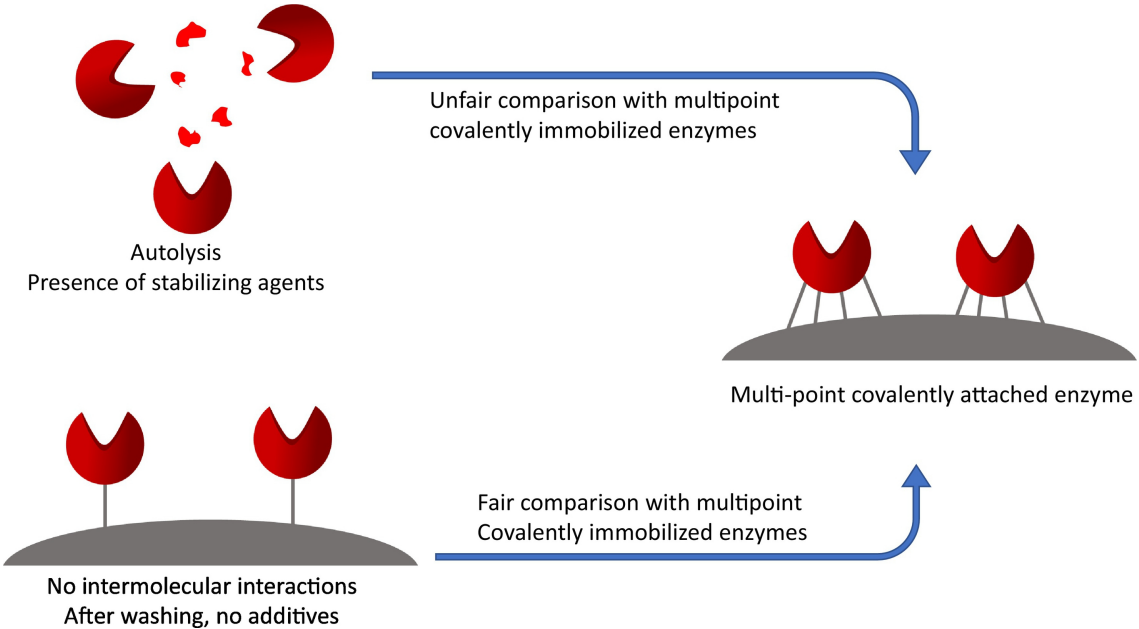
Unfair comparison with multipoint
covalently immobilized enzymes
Autolysis
Presence of stabilizing agents
Multi-point covalently attached enzyme
Fair comparison with multipoint
Covalently immobilized enzymes
No intermolecular interactions
After washing, no additives

Figure 9

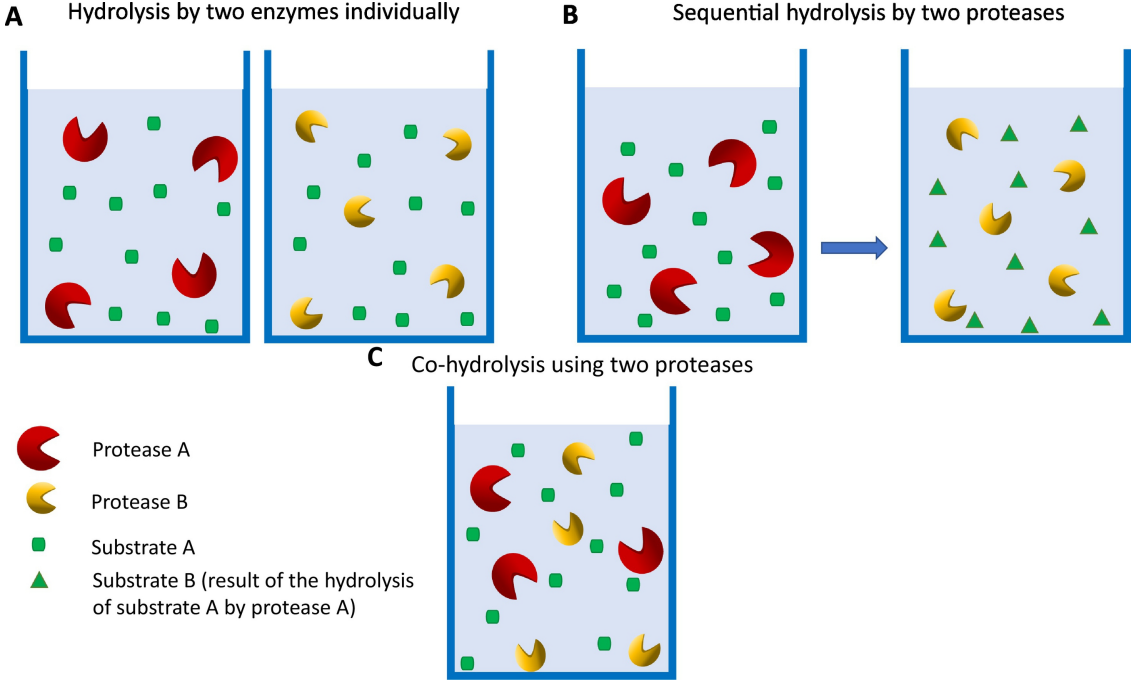
A
Hydrolysis by two enzymes individually
B
Sequential hydrolysis by two proteases
C
Co-hydrolysis using two proteases
Protease A
Protease B
Substrate A
Substrate B (result of the hydrolysis of substrate A by protease A)

Figure 10