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Review

A review on the immobilization of pepsin: A Lys-poor enzyme that is unstable at alkaline pH values

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ABSTRACT

Pepsin is a protease used in many different applications, and in many instances, it is utilized in an immobilized form to prevent contamination of the reaction product. This enzyme has two peculiarities that make its immobilization complex. The first one is related to the poor presence of primary amino groups on its surface (just one Lys and the terminal amino group). The second one is its poor stability at alkaline pH values. Both features make the immobilization of this enzyme to be considered a complicated goal, as most of the immobilization protocols utilize primary amino groups for immobilization. This review presents some of the attempts to get immobilized pepsin biocatalyst and their applications. The high density of anionic groups (Asp and Glu) make the anion exchange of the enzyme simpler, but this makes many of the strategies utilized to immobilize the enzyme (e.g., amino-glutaraldehyde supports) more related to a mixed ion exchange/hydrophobic adsorption than to real covalent immobilization. Finally, we propose some possibilities that can permit not only the covalent immobilization of this enzyme, but also their stabilization via multipoint covalent attachment.

1. Biocatalysis in food technology

Enzyme biocatalysis is gaining interest in many different industrial areas [1–10]. Enzyme selectivity and specificity, together with their capacity to perform their function under mild conditions (atmospheric pressure, aqueous medium, room temperature, etc.) has made them ideal catalysts from the point of view of green chemistry [11–14]. However, enzymes have evolved to fulfil their physiological role in vivo, and that way many of their features do not fit those required for industrial catalysts: moderate stability, saturation kinetics, inhibitions, solubility in aqueous media, etc. [15]. These drawbacks are hampering their industrial implementation. Even enzyme specificity may become a problem in some instances [16–19], like in the full modification of multifunctional or heterogeneous substrates (e.g., oils and fats) [20]. This can also make searching for a new enzyme mandatory if the substrate is slightly different to the natural one [21]. In fact, some current research is focused in strategies to enlarge the enzymes specificities, to utilize them in substrates that differ from the physiological ones [22,23].

Nowadays, there are many different tools to improve the enzyme features and make them fit the industrial requirements better. Metagenomics grants access to all present or even extinct biodiversity [24–28]. Moreover, directed evolution permits to mimic natural

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evolution but focused on the property selected by the researcher in an accelerated way [29–33]. Advances in enzyme modelling are permitting a better design of site-directed mutagenesis to get the desired enzyme improvements [34–39]. Chemical modification becomes more controlled and effective each day [40–43], while enzyme immobilization advances permit to further improve enzyme features [44–49], being compatible with all previous strategies [50–54]. Even the design of new reactors compatible with biocatalysts bearing different mechanical resistance facilitates the final implementation of the enzymatic biocatalyst [55]. In this sense, one of the outstanding developments showing the potential of integrating different strategies has been the design of artificial metal-plurizymes. Researchers generated a second active center in an esterase by using modelling and directed mutagenesis [56]. Next, the activity of this second active center was improved by modelling and site-directed mutagenesis [57]. In a further effort, they designed an organometallic complex coupled to an enzyme irreversible inhibitor intended specifically for one of the enzyme active centers. The coupling of this inhibitor to the enzyme generated an enzyme bearing two different catalytic activities, and it was able to catalyze cascade reactions involving the esterase catalytic center and the organometallic catalyst [57].

In food technology, enzyme specificity is an important feature, as in many instances the researcher only desires to modify one of the very similar compounds that are present in the food [58–60]. Furthermore, enzyme selectivity tends to be a key positive feature, as that way, the production of toxic by-products (or just of some products with undesired organoleptic properties) may be prevented. Standard catalysis is not a competitor of biocatalysis if the modification is carried in the whole food, as a standard catalyst will require incompatible conditions with many of the food components. This will produce a massive modification of all similar compounds and will hardly give only the desired product. That way, implementation of enzymes in food technology is a fact since a long time ago [58–60].

2. Proteases in food technology

Studies on proteases remain numerous and relevant, as they find justifications in broad aspects [61–64]. On the one hand, they comprise studies of their characteristics and functions in their place of production and action, which contribute to the elucidation of essential cycles in living beings and forms of metabolic control [65]. But, in addition, these studies also contribute to an important perspective of application completely outside this natural environment, such as in the cleaning (detergent development) [66–68] and pharmaceutical industries, or in food production stages [62,69,70]. One of the most effective applications of proteases as a biocatalyst in food production is in the generation of protein hydrolysates, either for the direct consumption of these hydrolysates or for the release of bioactive peptides, as it can be inferred from the numerous works in the literature [71].

Food can be seen as complex reaction media, where different types of components and matrix structuring complexities lead to different possibilities of alteration of the proteases when used as industrial biocatalysts, with possible inhibitors or protein denaturing agents, or pH values in the medium that affect enzymatic activity (and that cannot be modified by the researcher) [65]. Proteases can be quite versatile, presenting a huge range of types and specificities, which lead to an expansion of the options of choice. Proteases can be classified by different systems, considering their structures, catalytic mechanism, and their inhibitors. In addition to the classic international system developed in the 1950s [72] for the nomenclature and classification of enzymes (EC number), other databases have been expanded, such as the MEROPS peptidase database. A widely adopted way of grouping sets of proteases with common characteristics is through the focus on their iconic amino acid or metal present in their active sites: cysteine peptidases (C), aspartic peptidases (A), serine peptidases (S), metallo peptidases (M), mixed catalytic type (P) and unknown type (U).

Another useful classification identifies proteases against the optimal pH of the medium, acidic (action at pH < 7.0), neutral (better activity around pH 7.0) and alkaline proteases (pH > 7.0), or even high alkaline proteases (pH > 10.0) [73]. Of course, these classifications can be related, since the different characteristics addressed precisely influence other aspects. For example, acidic proteases usually present aspartate in their triad of amino acids as catalytic groups, which would also classify them as aspartyl proteases. All these features and preferences on catalysis conditions make an enzyme more or less suitable for processing a specific food. In addition to the characteristics that make it more or less susceptible to the food environmental conditions, proteases have an enormous variety of specificities, that is, proteases are able to attack peptides and proteins in very different positions (e.g., endo or *exo*-proteases) [65,74]. This greatly expands the range of choices based on the expected result. However, in addition to the natural characteristics of proteases, when seeking to apply these enzymes, it is possible to consider modifying certain aspects of these catalysts, such as increasing their stability or reducing inhibitions. In this way, in addition to the characterization studies and ways of obtaining it, there are studies of enzymatic modifications that include improving proteases performance via chemical modifications of the protease, ultrasound treatment of the food or immobilization technics [65,74,75].

3. Enzyme immobilization

Enzyme immobilization was developed to solve the problem of enzyme recovery and reuse, as initially the high prices of enzymes became a barrier for their implementation [46,76]. However, nowadays, the price of some enzymes has been reduced to a level that they are now "recommended" to be used in a free way for one cycle, even to produce some relatively cheap products, such as biodiesel [77–79]. Nevertheless, enzyme immobilization is far more than a mere system to recover and reuse enzymes. It has been shown that, if properly performed, enzyme immobilization may be a powerful tool to improve many enzyme features [45,48,49]. Thus, although the main objective of an immobilized protocol remains to maintain the enzyme in a heterogeneous form, an immobilization protocol should, nowadays, improve greatly the enzyme features. One of the enzyme features that is expected to be improved using immobilization is enzyme stability [44]. This may be achieved by different reasons as it has been recently reviewed (e.g., rigidification by multipoint covalent attachment, prevention of enzyme subunit dissociation) [44,80]. Moreover, the enzyme range of operation conditions may be greatly expanded, as a result of the mere immobilization that prevents enzyme precipitation and, more interestingly, by enzyme stabilization [44,81,82]. In some instances, enzyme activity may be increased upon immobilization, due to diverse reasons [47,83]. Immobilized enzyme selectivity and specificity may be randomly tuned, converting an unspecific enzyme in a specific one, or vice versa [47].

In other instances, immobilized enzymes become more resistant to the inactivation by chemicals [83,84], or become more insensitive to inhibitions [49,85–88]. Immobilization may be also coupled to enzyme purification, saving costs and time [83,89–94]. However, all these improvements of the enzyme features lay in the understanding and control of enzyme immobilization. Otherwise, enzyme immobilization may even drive to the worsening of the enzyme features [95]. For example, if the support used in the preparation of the biocatalyst presented a physically no inert surface, it may lead to some undesired enzyme conformational changes during operation [96]. The situation is worse if the support maintains some chemical reactivity after the enzyme immobilization. It has been recently shown that the activity, stability and even the path followed by the enzymes during inactivation may be altered by the support-enzyme interactions [97].

4. Immobilization of proteases

The specific suitability and advantages of the practical use of enzyme

immobilization depends on specific enzyme features, reaction characteristics, and substrate and product properties [76,98–101]. Thus, the formulation of immobilized proteases as immobilized enzymes displays some special advantages and specific problems that require careful and detailed consideration. The special characteristics of the proteases are related to their action on macromolecular substrates (one of the target uses of proteases is in the modification of proteins), the broad molecular diversity of products (from peptides of different sizes to amino-acids) and the possibility of autolysis [48,65,75,102,103]. When considering the potential advantages of immobilization, a distinction between technical and functional advantages is of importance. Technical advantages are associated to the facilitation of enzyme separation, possibility of enzyme reuse or continuous use, which might suggest in many cases a mandatory use of enzyme immobilization. However, this is not true in the case of proteases for all instances. The importance of enzyme separation and reuse is highly dependent on the specific application. For example, application in detergents industry does not need enzyme separation or reuse. Technical benefits of immobilization become more important in food processing that implies a protease-free product formulation or in the manufacturing of specialized proteolytic enzyme-immobilized reactors [76,98–101].

As explained above, the functional advantages of the immobilization are related to the possibility of modulating/enhancing catalytic functional properties of the enzyme to design the final enzyme-immobilized catalyst: activity and stability [44,47–49]. For example, in some cases the application of the reaction might require some conditions (organic solvents, acidic/basic conditions) that might be incompatible with the use of the free enzyme [76,98–101]. In other cases, it is convenient to use high temperatures or chaotropic agents to re-dissolve protein aggregates, conditions where a non-stabilized enzyme may be unable to perform their function [104,105]. Including the use of proteases to catalyze small peptides synthesis (via thermodynamically controlled synthesis or kinetically controlled synthesis) [106–111], resolution of racemic mixtures [112–116], etc., the range of conditions where proteases can be utilized is quite diverse [65]. In many of these uses, the immobilization of the proteases into a solid matrix is an enabling technology for active product formulation. The activity/stability profile of immobilized proteases is not a trivial feature given the characteristics of the reactions where the enzymes may be used. The immobilized protease must be designed with careful consideration of the different design variables involved: the carrier material properties, the immobilization chemistry and the protease loading [65]. Some basic aspects with the corresponding advantages/disadvantages are commented as follows.

The choice of the material support where the enzyme is immobilized represents a key decision (Fig. 1) [48,96,117]. For example, the immobilization of proteases into the internal surfaces of porous material disables the undesired possibility of autolysis since the immobilized protease molecules cannot interact with each other (Figs. 1A, 2A). This advantage may be extended to avoid any undesired effect due to protein-protein interaction (e.g. aggregation in the presence of organic solvents) or interaction with external interfaces (solvent drops, has bubbles, etc.) [48,118,119]. However, when proteases are immobilized onto non-porous supports, protein-protein interactions are still possible between molecules located in different particles and autolysis might still occur (Figs. 1B, 2B). In those cases, post-immobilization treatments to coat the protease surface with polymers might be a solution, but this could also avoid the hydrolysis of the proteins if it is not carefully designed. The choice between non-porous and porous particles is therefore critical. The use of porous supports displays the additional advantage of the high internal surface area for achieving high loading of catalyst immobilization while a manageable size of particle size (50 micron-500 μm) is used (Fig. 2A). In non-porous particles, the loading capacity decreases reciprocally with particle diameter, so the increase in loading might require too small particle size leading to the colloidal suspensions becoming difficult to separate from the liquid solution unless magnetic particles are used (Fig. 2B) [120]. The prevention of protease autolysis when immobilized on porous supports can give a significant enzyme stabilization, which will not be related to a real enzyme rigidification, but inherent to any immobilization. To really check if the enzyme is stabilized by enzyme rigidification, it is recommended to compare the stability of the new immobilized enzyme with those of an enzyme immobilized via a single point.

The use of porous particles implies an important disadvantage, however. For the catalytic action, the substrate has to diffuse into the porous network where the protease is immobilized (Fig. 2A). Given the macromolecular character of the substrate, the diffusive transport of the substrate might limit or fully disable the reaction. It should be considered that the nature of the substrate where proteases are used will be a collection of different proteins, with different sizes, and that the source of the proteins that we desired to hydrolyze may be diverse. As each protein source may have their own composition, each with different maximum sizes in their components, this can raise some additional problems. Hence, the porous structure of the biocatalyst must be considered to facilitate transport. Once the protein starts to decrease in size by partial hydrolysis, the fragments may diffuse with higher rate and the problems may decrease along time. One support/reactor architecture broadly used in proteolytic reactors is the wall-coated microfluidic protease-immobilized continuous reactors that combined both the positives features of non-porous a porous material supports. In these systems, the protease is immobilized in the inner surface of capillary or monolithic channels where the substrate mixture flows through [55,121–125] (Fig. 1C). The high surface-to-volume ratio of the channels enables a high enzyme loading and thus, high reaction rates, while the channel diameter is in the micrometer scale, and therefore not

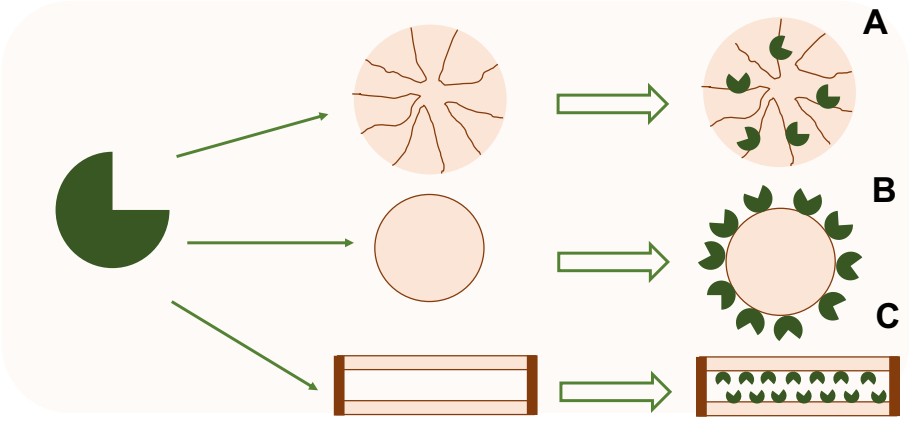

Fig. 1. Different formats of material support used for proteases immobilization. A) Porous particles; B) non-porous particles; C) Microfluidic channels.

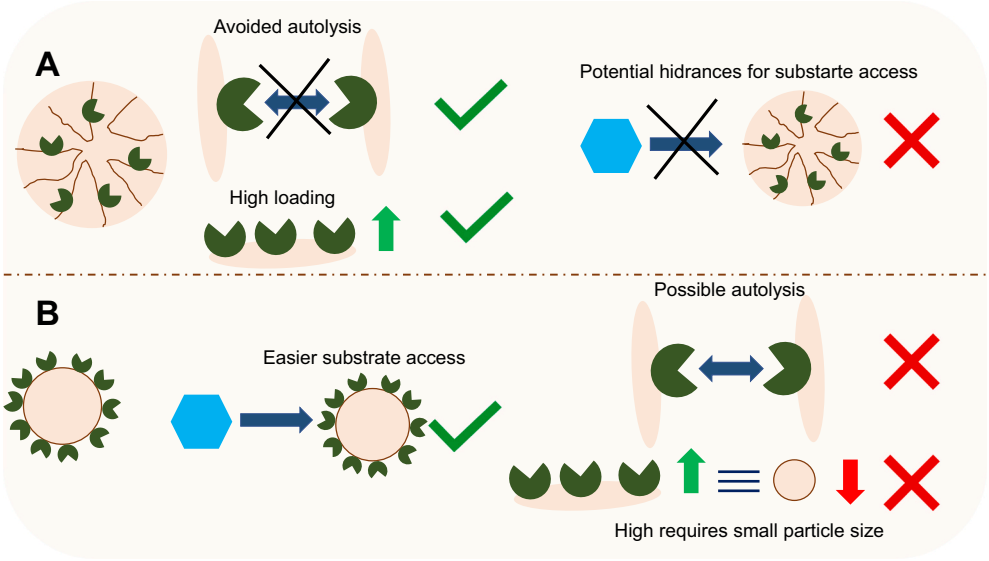

Fig. 2. Disadvantages of using porous (A) or nonporous particles (B) for proteases immobilization.

liming substrate transport.

The choice of the support surface-activation and immobilization protocol has a critical influence on activity/stability since it determines the structural and molecular features of the catalyst: enzyme orientation, enzyme localization, enzyme density, structure distortion, structure rigidification, etc. [65] (Fig. 3).

Enzyme orientation is generally an important factor of the catalytic activity of any immobilized enzyme [97,126]. However, in the case of enzymes acting on macromolecular substrates, the enzyme orientation is critical since it determines the substrate accessibility to the active center of the protease, even if it is immobilized on a non-porous support (Fig. 3A) [48,127]. The importance of this effect will depend on the support surficial geometry. A support formed by fibers with a size similar to that of the protease, will reduce the steric hindrances, but also reduce the possibilities of really improving enzyme stability via multipoint covalent immobilization [44,96]. That way, at first glance, supports forming flat surfaces should be preferred, but these supports can generate some steric undesired problems. Enzyme orientation must facilitate full access of the enzyme to the substrate, Fig. 3. The control of the orientation by immobilization can be based on the control of the immobilization. This may be achieved if the first event of the immobilization aiming to involve different regions of the enzyme to direct the immobilization by different regions (e.g., using heterofunctional supports) [90] or by the use of directed mutagenesis to introduce specific groups that permit the full control of the first groups of the enzyme that will react with the support (e.g., a Cys residue) or by the fusion of the target protease with specific binding modules [127]. Again, the problem is higher using the intact proteins as substrate and will decrease when smaller protein fragments are generated.

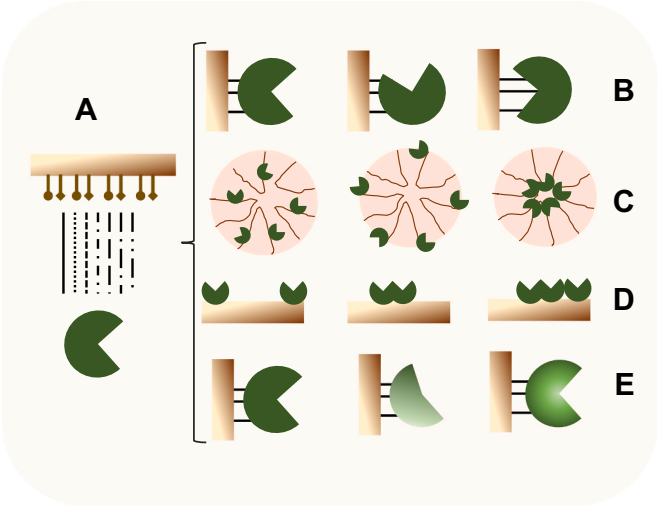

Fig. 3. Importance of the enzyme immobilization design on the final features of immobilized proteases. A) Surface activation degree, immobilization chemistry and protein surface chemistry are the key factors influencing the achievement of different protease orientation (B), protease distribution (C), immobilized enzyme density (D), enzyme structural distortion (E).

In the case of proteases, as other enzymes acting on macromolecular substrate, a correct enzyme orientation is a needed condition but not sufficient to have suitable substrate access. Beyond the commented possible influence of the diffusive hindrances originated in porous supports, the catalyst loading in the surface is another critical factor [128–134].

At low protease surface density, substrate transport to the surface and access to the active site of the protease are enabled (Fig. 4B). On the contrary under conditions of high surface protease density, additional steric hindrances might be created disabling the access to the active site (Fig. 4B) [104]. The undesirable condition of a too high surface density of protease molecules can occur depending on the interplay of some variables: the enzyme loading, the immobilization rate, the enzyme orientation and the size of the substrate.

It is obvious that using a high surface density of the immobilized enzyme, if the enzyme active center is not fully oriented toward the medium, and it is in some way near the support surface, using a very low enzyme surface density, the substrate may access the active center of the enzyme. However, there will be a critical loading beyond which steric hindrances are created by proximity of each immobilized protease molecule with other immobilized enzyme molecules, promoting a drop in the observed enzyme activity, Fig. 4B [104].

If the immobilization rate is low and the protease is permitted to diffuse into the pore of the biocatalysts before immobilization, the average distance between immobilized may be next to the size of the protease. That way, the problem will be more important using substrate proteins larger than the protease, and lower using protein substrate smaller than the protease. If the immobilization rate is very high, the immobilized proteases packing will be more significant, and the steric hindrances will be more significant with all moderately large substrates.

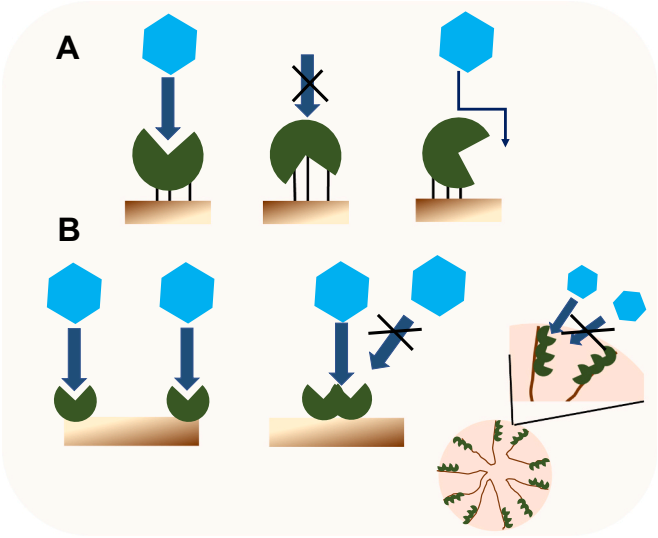

Fig. 4. Importance of protease immobilization features on the performance of the immobilized proteases. A) Different enzyme orientation enables or full disables substrate access. B) Different enzyme density enables unrestricted access of substrate or creates steric hindrances.

In fact, a high surface density of the protease will be also possible even at low enzyme loadings if the enzyme immobilization rate is much quicker that the enzyme diffusion [135]. During the enzyme immobilization process, the protein has to diffuse through the pores to access the support active surface to be immobilized. When the diffusive transport of the protein is significantly slower than the immobilization reaction, the protein will be preferentially located on the more external areas of the material support leading to a high local density of protein and, therefore, steric hindrances may appear far before the support is fully loaded with the enzyme. Therefore, in the case of protease, special care must be maintained for the immobilization rate, that should be sufficiently slow to avoid crowding. Enzyme crowding may also alter enzyme stability [136–138], but in the case of protease use to hydrolyze proteins, the problems may be higher than the benefits. The immobilization rate can be modulated by controlling the surface density of reactive groups on the surface [44], using conditions where the immobilization rate may be reduced (e.g., adding a solvent in hydrophobic immobilization) [135], increasing the ionic strength in ion exchange [139], decreasing the reactivity of the groups in the support (e.g., using borate [140] or aminated compounds [141,142] using glyoxyl supports), using pH values where the reactivity of the support may be reduced [140], etc. These problems can generate interesting reaction profiles. For example, when these problems exist, the protein molecules that go into the support may be fully fragmented (by the action of all enzyme molecules because even if they have not activity versus the large initial protein, they can attack the protein fragments) while a large percentage of initial proteins remain still intact. This will never occur using free proteases.

Using proteases that are intended for protein hydrolysis, it will always be recommended to follow the activity using a small substrate to check that the active center maintains the functionality and a protein to check that the active center remains accessible. That way, if the activity decreases it becomes possible to discriminate if the enzyme is really inactive or just its active center is blocked.

5. Pepsin features

The first studies on pepsin identification, concentrated specifically in the beginning of the 19th century [143–146], evolved around the identification of the component able to covert nitrogenous foodstuffs observed in gastric fluids. Some important moments in the advances of these studies can be highlighted, such as the effective identification of pepsin as a protein with activity among other components of gastric fluids, and its isolation/crystallization after 1930 [147]. With its crystallized form available, in addition to the possibilities of its application, primarily related to medical proposals, there have been advances in the elaboration of specific substrates and thus in deeper understanding of its actions and in kinetic studies [148–152]. Understandably, a huge range of studies with pepsins focused, and still focuses, on their participation in digestive systems, and other relationships with the metabolic situations of living organisms, being one of the most prominent representatives among the aspartyl proteases [147,153]. Aspartyl proteases are of great interest because, in addition to their key gastric digestive action, they exhibit a relationship with virus activities that affect humans, such as HIV [154–157], and mechanisms involved in the generation of Alzheimer's disease [158].

However, its isolation and production, already well mastered, allow its application in different processes such as medical applications (e.g., the treatment of dyspepsia) [159,160], in food quality tests, (e.g., in digestion simulations) [161–164], and more directly in the production of modified foods and nutraceuticals. Many studies point to the promising application of pepsin in the production of protein hydrolysates, and release of peptides [165–167], with differentiated bio and techno functionalities, and, in particular, low allergenicity foods [168–172]. The often observed reduction in the allergenicity of certain proteins after treatment with pepsin has been closely related to one of the functions indicated for this enzyme in the human digestive system [173–175]. Some studies highlight that, despite its low effectiveness in releasing amino acids for absorption in the intestine, pepsin has an important role in reducing the risk of allergenic sequences of proteins reaching the intestinal lumen [173–175]. Although the correlation between resistance to proteolysis and allergenic activity is not absolute, many studies suggest the importance of understanding the role of pepsin in human digestion as a controller of the passage of allergens into the intestinal lumen [176] and it has been proposed that there is a relationship between this escape from hydrolysis by pepsin, that is, a resistance to proteolysis by this enzyme and therefore the absence of amino acid sequences susceptible to catalysis, and the allergenicity of certain proteins [177]. Taking into account and due to these characteristics, studies find the application of these proteases, among others, in the production of hydrolysates with reduced allergenicity. He et al. (2021) demonstrated that pepsin reduced the allergenicity of silkworm pupa protein more strongly than trypsin [168].

Both human and porcine pepsin have a well-demonstrated mechanism of activation by just a change in pH from pepsinogen, which is a molecule originally with two lobes, N and C terminal, which remains stable at alkaline pH value. The auto-catalytic activation occurs when this molecule is brought to a pH below 5, and the removal of the N-terminal section occurs, releasing the pepsins, with molecular weight about 34 and 37 KD. Human and porcine pepsins are very similar, and studies show that human pepsins have a unique sequence of 373 amino acids [178] and porcine has 326 amino acids residues [179] in porcine pepsin, the Asp32 and Asp215 residues belong to the catalytic triad and present dispositions very similar to the equivalent residues in the other aspartic proteinases. Another interesting feature is that there is a phosphoryl group covalently attached to Ser68 and which ensures a net negative charge on porcine pepsin in a strongly acid medium, contributing to the enzyme's behavior in low pH media [179].

Some other residues must contribute to this difference in behavior regarding the pH of the medium between pepsinogen and pepsin, since the first contains 13 cationic residues and pepsin only 4, two Arg, only one Lys and only one His [180].

Although pepsin is, like other proteases, a single-chain enzyme, it is constituted by two homologous lobes, and the denaturation phenomena occurring in the N-terminal lobe are more associated with enzyme instabilities in the face of pH elevations [153].

Although pepsin has a certain range of action between pH 1.0 and

4.0, studies indicate that the best pH of action is around 2.0. As for its specificity, pepsin is mainly influenced by the amino acid residues at position P1 and P1′. However, it is possible to verify that the presence of certain residues in positions such as P2, P2′, and P3, P3′, may or may not be favorable to their actions: phenylalanine, leucine, and methionine are preferred in P1, while histidine, lysine, proline and arginine do not work as P1; pepsin activity is also favored with tyrosine, tryptophan, and phenylalanine at position P1′. Amino acids in P3 also influence the action of the enzyme, with the presence of histidine, lysine, and arginine being unfavorable [181].

6. Immobilization of pepsin

Pepsin surface is quite rich in Asp and Glu groups, (Fig. 5), but it has only this enzyme only possesses one lysine residue [179,182] (Fig. 6). The enzyme has an isoelectric point around 3, while other proteases such as ficin, trypsin or chymotrypsin have an isoelectric point over 9, making that their capabilities to interact with ion exchangers may be fully different. This is very important as most of the immobilization protocols that permit the improvement of the enzyme stability via multipoint covalent immobilization are based on the reaction of the primary amino groups of the enzyme and the support (such as glyoxyl, glutaraldehyde) [44,140,183]. That way, at first glance, other supports able to react with other groups may be more adequate for this enzyme. Pepsin possesses several Tyr residues in its surface, but few Cys or His (Fig. 7). Epoxide activated supports can react with a diversity of groups contained in proteins, such as phenol, thiol, imidazole, or carboxyl group [184]. These supports require the previous fixation of the enzyme on the support due to other reason, because of the poor reactivity of epoxide groups [185]. Vinyl sulfone activated supports can react with a wide variety of groups (similar to epoxy but discarding carboxylic groups), and it is much more reactive [186–191]. Both support require a blocking step, that can become complicated if the immobilzied enzyme is not stable at pHs where the blocking agents and the support are reactive enough.

Another important feature of pepsin when its immobilization is pursued is the low stability of the enzyme at neutral or alkaline pH values [192–195]. This makes it necessary to employ acid pH values during its handling, which are not recommended to get an intense multipoint covalent immobilization [44]. If the enzyme is not going to be highly stabilized by immobilization, the physical and reversible enzyme immobilization may be a better solution, as long as the enzyme is not released to the medium during operation [48]. That way, at least the support may be recovered and reused after enzyme inactivation, after releasing the inactivated enzyme. The low isoelectric point of pepsin (around pH 3) suggests that immobilization via anion exchange may be a good option [196]. Ion change involves the formation of several ion bridges between enzyme and support [197], it may be produced even on supports with mixed anion and cation groups [198], or in conditions where the global ionic nature of the enzyme and support are the same [199,200], requiring just the possibility to establish this multipoint immobilization.

Next, we will present a summary of the main efforts in the immobilization of this complex protease.

7. Covalent immobilization of pepsin

7.1. Use of glutaraldehyde activated supports for pepsin immobilization

One of the most employed tools to achieve an enzyme covalent immobilization has typically been the use of glutaraldehyde chemistry, usually employing aminated supports activated with glutaraldehyde [183]. These supports have an intrinsic heterofunctionality, which can permit the first protein immobilization via different events (ion exchange, hydrophobic adsorption or covalent reaction), with quite different results depending on the first cause of immobilization [81,201–205]. However, this means that enzyme immobilization is not compulsory meaning that the enzyme has been covalently immobilized; it may be only physically adsorbed.

Another possibility of using glutaraldehyde is the treatment of previously adsorbed enzymes, that can permit the covalent immobilization or the promotion of large intermolecular protein aggregates that make enzyme release almost impossible [206]. This method is regarded as a very effective one to get enzyme stabilization via multipoint covalent attachment [44].

It is possible to find many examples in the literature where glutaraldehyde has been used for the covalent immobilization of pepsin, as presented below. Unfortunately, the heterofunctionality of the support is usually ignored, and the fact that the enzyme has only two available residues to covalently react with the support is hardly considered.

One of the most typical aminated supports used to immobilize enzyme is chitosan. It is an accessible support, cheap and easy to use [207,208]. There are several studies where it has been used together with glutaraldehyde in order to immobilize pepsin. In one work, for instance, pepsin was immobilized on chitosan and then modified with glutaraldehyde [209]. The method was optimized and the properties of the immobilized enzyme were studied. The recovery of the immobilized pepsin reached 74%, the optimum reactive temperature and pH were 55 °C and 3.0 respectively. The enzyme remained active after five weeks stored at 4 °C [209]. In another study, pepsin immobilization on glutaraldehyde crosslinked chitosan - silica nanobeads was characterized and the effect of pH, pepsin concentration, and temperature were evaluated [210]. The results showed that pH range, performance and stability to high temperatures were improved on the immobilized pepsin with respect to the free enzyme. The biocatalyst can be reused up to 10 times, maintaining 20% of the initial activity [210]. Another interesting research describes how pepsin was immobilized on chitosan beads using glyoxal hydrate and glutaraldehyde as crosslinkers [211]. The

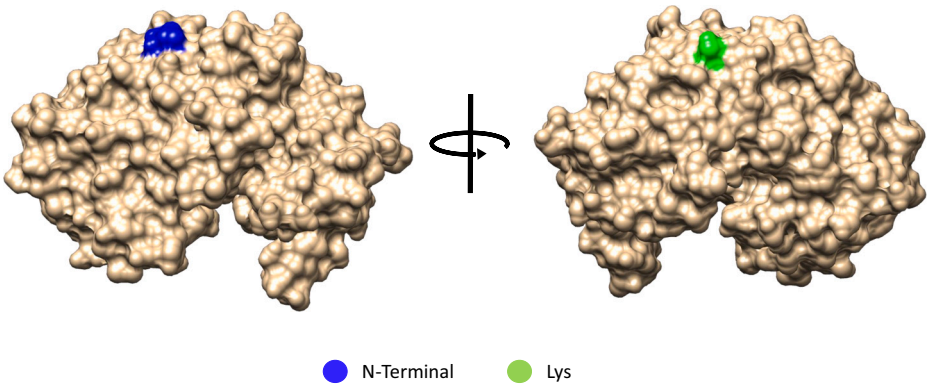

Fig. 5. Tridimensional structure (based on pdb code "4PEP") of porcine pepsin (EC: 3.4.23.1) showing the presence of amine groups on the surface of the enzyme.

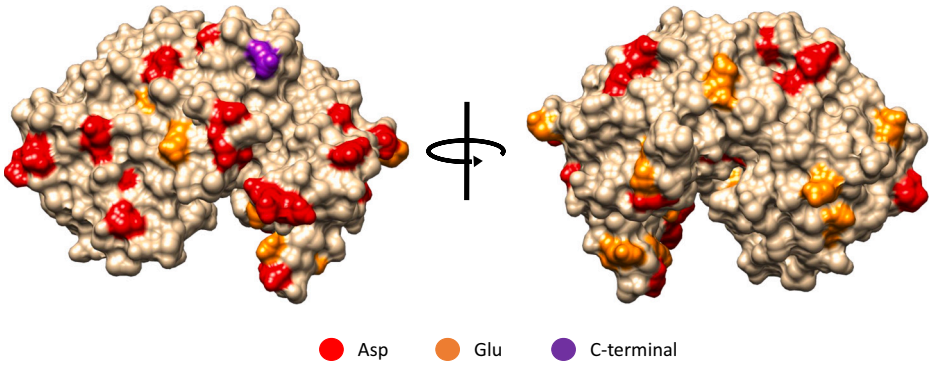

Fig. 6. Tridimensional structure (based on pdb code "4PEP") of porcine pepsin (EC: 3.4.23.1) showing the presence of carboxylic groups on the surface of the enzyme.

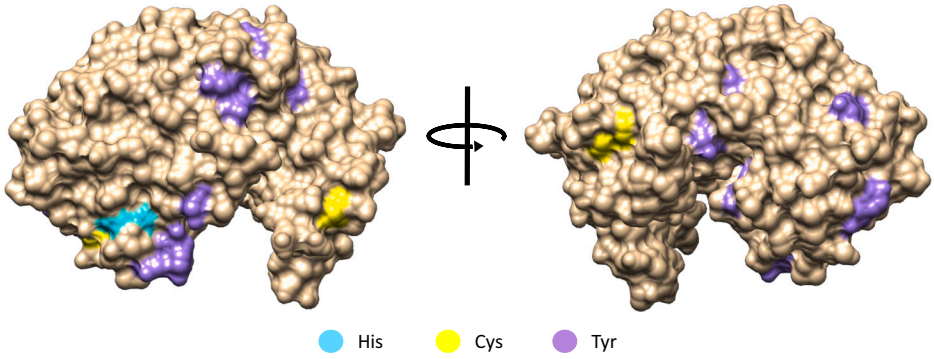

Fig. 7. Tridimensional structure (based on pdb code "4PEP") of porcine pepsin (EC: 3.4.23.1) showing some important residues on the surface of the enzyme interesting for enzyme immobilization.

immobilized pepsin showed some improved properties with respect to the free enzyme, such an increased thermal stability and storage stability. The biocatalyst was then applied to the milk clotting process. It was found that the optimum temperature for the enzyme increased from 40 to 50 °C after pepsin immobilization [211]. Pepsin was also successfully immobilized on chitosan-acrolein and chitosan-glutaraldehyde gels together with many other enzymes [212].

Glutaraldehyde and chitosan have also typically been used together to silica supports. For instance, Daisogel silica microparticles covered by chitosan were employed to immobilize pepsin and lysozyme, using glutaraldehyde or 3,3′,4,4'benzophenonetetracarboxylic-dianhydride (BTCDA) as crosslinkers. It was found that pepsin was immobilized onto Daisogel//(chitosan -BTCDA)- glutaraldehyde in a lower amount than lysozyme, and that the amount of immobilized enzymes depended significantly on the isoelectric point of the enzyme. [213]. The same group also studied how pepsin an lysozyme were immobilized, using the glutaraldehyde chemistry as well, on silica microparticles of different sizes initially treated with poly(ethylenimine) and then modified with BTCDA to generate carboxylic groups on the surface [214]. The amount of the attached enzyme significantly depended on the isoelectric point of the enzyme [214]. This suggests that the enzyme ionic exchange may take a more relevant role than the direct covalent immobilization.

In other instances, glutaraldehyde is used just as a crosslinker. For example, pepsin was immobilized together with chicken ovomucoid on porous aminopropyl-silica activated with N,N′-disuccinimidyl carbonate and later on, the bioconjugate was cross-linked with glutaraldehyde for a further stabilization of the immobilized enzyme [215].

In a different study, silica gel carrier, acrylic beads, and a cellulose-based carrier – Granocel, were employed to immobilize pepsin via covalent attachment. The different supports were previously functionalized to present -OH, -COOH, -NH_2, or glycidyl groups on their surfaces [216]. Three different crosslinkers were used. As a result, pepsin was successfully immobilized on Granocel activated by glutaraldehyde or carbodiimide and on silica gel activated by glutaraldehyde [216].

Pepsin was immobilized via absorption and covalent attachment, using the glutaraldehyde chemistry, on functionalized biochar support [217]. The objective was to employ this biocatalyst in the hydrolysis of casein. The covalently immobilized pepsin showed a higher casein hydrolysis rate when compared to the enzyme immobilized only by adsorption, maintaining 95% of the initial activity after 60 min [217]. In another paper, Jasim et al. (1987) immobilized five proteases (bromelain, ficin, papain, pepsin and trypsin) on cellulose supports using two different protocols: cyanogen bromide or glutaraldehyde. However in this instance cyanogen bromide proved to be the most suitable technique in terms of immobilization yield, activity and stability of the enzymes [218]. Pepsin was also immobilized onto aminohexyl-Sepharose activated with glutaraldehyde under acidic conditions [219]. The immobilized enzyme maintained >40% of its initial activity against a synthetic substrate, and 30% against IgG. The stability of the biocatalyst also was increased with respect to the free enzyme. The preparation was employed to obtain an efficacious and safe immunoglobulin G preparation for intravenous use [219]. In other instance, synthetic zeolites were used to immobilize pepsin using glutaraldehyde as crosslinking agent [220]. This biocatalyst presented higher retention of activity and higher thermal stability in comparison to pepsin immobilized in absence of glutaraldehyde [220].

Glutaraldehyde has also been used together with diverse inorganic supports. For instance, various different inorganic supports were used in order to immobilize pepsin and later, used on a milk coagulation continuous process [221]. The chosen supports for this study were beads of alumina, titania, glass, stainless steel, iron oxide, and Teflon. Two strategies were employed to activate the supports: glutaraldehyde and

silanized supports. Titania and glass resulted to be the best supports for the immobilization of pepsin [221]. Another research group was able to immobilize pepsin on a porous membrane, coated with polydopamine, via three different mechanisms: hydrophobic adsorption, electrostatic attraction and covalent bonding [222]. This last procedure was achieved by means of the glutaraldehyde chemistry, activating the support and attaching the enzyme to it. Results showed that except for dextranase, enzyme immobilization via electrostatic attraction retained the most activity, whereas covalent bonding and hydrophobic adsorption were detrimental to enzyme conformation [222]. In another example, pepsin was immobilized on magnetic nanoparticles via two different approaches: by covalent immobilization activating the nanoparticles with glutaraldehyde, and by physical adsorption on amino-functionalized nanoparticles. In this instance, the best results were obtained employing the adsorbed pepsin, with a loading of 2 mg/g [223]. Finally, pepsin was immobilized via the glutaraldehyde chemistry on particles of gelatin insolubilized by formaldehyde treatment [224]. The biocatalyst was used to reduce the haze formation during beer production, finding that good protein stability of the beverage results when at least 50 g immobilized enzyme per hl beer per h are applied in a special type of packed bed reactor [224].

As explained above, the fact that the enzyme has only two primary amino residues and that the researchers have not included studies to assess the real covalent immobilization of the enzyme does not clarify if in all the above examples the enzyme has been immobilized even by just one covalent immobilization.

7.2. Use of methacrylate as support for pepsin immobilization

Another kind of support to immobilize enzymes is the methacrylate-derived supports [184,225–228]. Methacrylate can adopt different shapes (monoliths, gels, microspheres…) depending on the desired properties and the planned usage of the biocatalyst, and may be activated using different groups. One characteristic is a moderate hydrophobicity of the matrix, which coupled to some physical characteristics of the active groups, can convert this in heterofunctional supports. The use of an hydrophobic supports may be negative for the enzyme stability, as it the interactions with partially unfolded enzyme structures can drive to their stabilization [96].

For instance, pepsin was immobilized via reductive amination on a poly(glycidyl methacrylate-*co*-methyl methacrylate) monolith modified with aminoacetal to produce aldehyde groups on the surface [229]. Immobilized pepsin showed better pH and thermal stability compared with free pepsin. This biocatalyst was later employed for online protein digestion [229]. As another example of the use of methacrylate monoliths, pepsin was covalently immobilized on a monolith prepared in situ as a copolymerization of 2-vinyl-4,4-dimethylazlactone and ethylene dimethacrylate. The biocatalyst was successfully used in the online digestion of myoglobin, albumin, and haemoglobin [230].,

There are also several examples of the use of methacrylate microspheres. In one study, pepsin was covalently immobilized on poly (methyl methacrylate)/acrylaldehyde microspheres [231]. This support possesses aldehyde groups on the surface, which offer a simple immobilization. With this protocol, it was possible to immobilize up to 82 mg of enzyme per g of support Although the Km increased and the Vmax decreased, the activity decreased only by 50% of the free enzyme, and the optimum temperature increased from 43 to 47 °C [231]. Modified poly(methyl methacrylate) microspheres were employed by the same research group in another study for the immobilization of pepsin. This support also has aldehyde groups on the surface, which react with the amino groups of the enzyme forming covalent bonds. The Km value increased and Vmax decreased respect to the free enzyme. However, the temperature resistance was improved after immobilization, making this protocol useful for pepsin immobilization-stabilization [232,233].

In another study, chitosan was introduced in poly(styrene-*co*-glycidyl methacrylate) (PSt-GMA) microspheres with/without spacer-arms in order to immobilize pepsin [234]. The different microspheres proportioned different mechanisms of interaction with the enzyme, as it was discussed before, chitosan is a complex polymer that can react in many different ways with the enzyme (covalent bonding, hydrogen bonding, electrostatic interaction and hydrophobic interaction, etc.). Thermal and storage stabilities of immobilized pepsin were enhanced respect to the free enzyme [234].

Methacrylate has also been used in the form of gels to immobilize pepsin. In one work, pepsin was immobilized on hydrogel films by radical polymerization with poly-(ethylene glycol) dimethacrylate as crosslinking agent [235]. Hydrogels showed the ability to preserve the catalytic sites at temperatures above the lower critical solution temperature, and the immobilization process allowed pepsin to preserve its activity after several cycles of reuse. [235]. Another example of covalent attachment of pepsin, is its immobilization on hydroxyalkyl methacrylate gels modified with 1,6-diaminohexane or ε-aminocaproic acid. After immobilization, parameters such as proteolytic activity, pH activity curves and stability depending on the pH and time were improved regarding the free enzyme [236].

In another study, pepsin was immobilized on a copolymer of acrylamide and 2-hydroxyethyl methacrylate. Covalent bonds were produced due to the simultaneous activation of the hydroxyl and amino groups of the support, although the order of activation was relevant to permit pepsin immobilization. The study of the kinetic variables showed that there were no conformational changes on the enzyme. This biocatalyst was successfully employed on the hydrolysis of casein [237].

In a different approach, a wide number of enzymes, including pepsin, were covalently immobilized on copolymers of 2-hydroxyethyl methacrylate and ethylene dimethacrylate (Separon HEMA), containing epoxy groups, in order to study the effect of the addition of salts in the immobilization efficiency. It was observed how the immobilization yields depended on the concentration and type of the ions added [238]. In this instance, the blocking steo was not utilized making complex to understand the results.

Finally, a composite of chitosan on Fe_3O_4 nanoparticles was the chosen support to immobilize pepsin via imine linkages. A biocatalyst with a loading of 99 mg/g of pepsin was achieved. With this biocatalyst, the deacetylation of various amides was prosperously accomplished. Hydrolysis yields of 56–98% were obtained using chitosan magnetic nanoparticle-supported pepsin and it could be reused for at least three cycles [239].

7.3. Use of the carbodiimide chemistry in the covalent immobilization of pepsin

Carbodiimide is a reagent widely employed as an carboxylic activator to form covalent bonds between a carboxylic group and a primary amine group [240]. As pepsin has plenty more Asp and Glu groups than Lys (see Fig. 5), it seems a possible good strategy to get an intense multipoint covalent attachment. Unfortunately, the poor stability of these groups makes to reach high stabilization degrees complex, although it may be quite efficient to immobilize an enzyme, that requires long enzyme-support reaction time [44]. This also produces the chemical modification of groups of the enzyme do not involved in the enzyme immobilization.

In this regard, many studies employ this resource to immobilize pepsin via covalent attachment. In one study, pepsin was immobilized on Duolite amine resin and later treated with carbodiimide to get some covalent attachments [241]. The biocatalyst presented a half-life of 15 days and it was used for the hydrolysis of 2.5% haemoglobin solution in a column reactor. The degree of hydrolysis could be modulated by changing the flow rate of the reactor [241].

Another research shows how carbodiimide was employed to obtain a covalent immobilization of pepsin to wide-porous and nonporous ω-aminoalkyl derivatized inorganic supports [242]. The determination of the enzyme loading on the support and proteolytic activity of the

biocatalyst was carried out. All the tested biocatalysts were stable on storage at 8 °C and pH 4.5 for 1 month [242].

In another research, pepsin together with trypsin, α-chymotrypsin, or some other intestinal mucosal peptidases were covalently immobilized on porous succinylated aminopropyl-glass beads, using carbodiimide as activator of the groups in the support [243]. The biocatalysts were employed to determine the digestibility of several animal and plant proteins, finding that the combined proteolysis using various enzymes produced a more rapid digestion [243]. However, for pepsin, we cannot see the advantage of using their two amino groups for the immobilization. At first glance, it had been more productive to adsorb the enzyme in an aminated support. A comparison of both strategies could be interesting.

In another work, pepsin was immobilized via its carboxyl groups on an ethylene maleic anhydride copolymer presenting 1,6-diamino-hexane, in presence of dicyclohexylcarbodiimide [244]. On lyophilisation, the immobilized pepsin preparations retained 70–80% of their activity. The preparations were stored at 4 °C for four months without any remarkable loss in their activity [244]. Alternatively, the immobilization of pepsin on N-(carboxyphenylcarbamoylmethyl) cellulose was studied [245]. In this case, the activating reagent to produce covalent bonds was dicyclohexylcarbodiimide. The researchers analyzed how the enzyme/support and activator/support ratios, pH, and immobilization time influenced the immobilization process. It was also observed that the Km was lower for the immobilized enzyme [245]. Another study employed BIOZAN R to immobilize pepsin, using dicyclohexylcarbodiimide as activator [246]. A pepsin loading of 35–110 mg/g of polymer and a proteolytic activity between 20.85 and 28.75 μmol tyrosine/L·min·g of polymer was obtained [246].

Pepsin was also immobilized on agarose supports. They were obtained through the modification of Sepharose 4B with cyanogen bromide, then treated with 3,3′-diaminodipropylamine to finally produce carboxyl groups with succinic anhydride [247]. The covalent attachment of the enzyme was achieved by the use of carbodiimide as activator. The biocatalyst was successfully employed in the digestion of haemoglobin and rabbit IgG [247]. Carbodiimide has been also used in chitosan supports to immobilize pepsin [248]. To reach this goal, chitosan was previously succinylated, forming amide bonds with the enzyme after condensation with carbodiimide (again, using the amino groups of the enzyme and not the carboxylic groups). At the end of the process, the preparation retained 80% specific activity, presenting a higher storage stability than the free enzyme [248].

Finally, Kurth and Bein (2003) immobilized pepsin on oxidized aluminum substrates functionalized with thin layers of (3-aminopropyl) triethoxysilane, (3-mercaptopropyl)trimethoxysilane, (3-bromopropyl) trimethoxysilane, and (8-bromooctyl)trimethoxysilane by means of the carbodiimide chemistry. The results showed that, even though the packing structures may differ to a certain extent, the attachment of a single pepsin layer with a 100% surface coverage was produced. [249].

7.4. Gold nanoparticles as a flexible tool for pepsin immobilization

Gold nanoparticles have become popular enzyme carriers [250–254]. Gold surfaces allows ionic and hydrophobic interactions, but also, covalent attachment can be achieved by different mechanisms, such as the strong chemo-adsorption of thiol groups [255]. This means that the enzyme will be immobilized in an always active surface, that can be negative for the final enzyme stability [96].

Regarding pepsin, there are some studies that utilize this material. In a study to determine if gold nanoparticles, with a carboxy-terminated hydrophilic PEG7 shell, were suitable as a heterogeneous support to be employed in protein sequence determination, pepsin was immobilized via amide coupling [255]. Turnover rates, kcat and enhanced catalytic efficiencies were essentially equivalent compared to the homogeneous catalyst. The catalyst was stable enough for more than a month [255]. In another work, the covalent immobilization of pepsin onto a free-standing gold nanoparticle membrane was successfully achieved [256]. The catalytic activity of the immobilized enzyme was similar to the free enzyme, and the biocatalyst could be reused over ten successive cycles. The stability against pH and temperature was enhanced as well [256]. In another paper, it is described how pepsin was immobilized on gold nanoparticles assembled in a polyurethane surface [253]. This bioconjugate material showed a slightly higher biocatalytic activity and significantly enhanced pH and temperature stability. It also permitted to separate the enzyme form the medium and reuse it over six reaction cycles [253]. The same research group, in a different study, was able to assemble the gold nanoparticles on a different surface, 3-aminopropyltrimethoxysilane (APTS)-functionalized Na - Y zeolites, allowing the recovery of the bioconjugate from the medium [257]. Once again, pepsin was the enzyme employed to test this method, obtaining excellent results, since the catalytic activity of pepsin in the bioconjugate was comparable to that of the free enzyme in solution, and it could be reused several times [257].

7.5. Other methods for the covalent immobilization of pepsin

Apart from all the methodologies explained above, many different strategies exist to immobilize pepsin through covalent attachment. Next, we present all the different approaches we have been able to find in the literature about this topic.

In one work, pepsin was immobilized, via covalent attachment, on agarose beads through the amino groups of the enzyme (and the enzyme has only one Lys and the terminal amino group, Fig. 5). The objective of the immobilization in the study was to eliminate the intermolecular interactions in order to be able to refold the enzyme. Finally, pepsin could be refolded, with a higher activity recovery in presence of salts that did not affect to the refolding rate [258]. In another study, cod pepsin was successfully immobilized via covalent attachment on Sepharose activated with cyanogen bromide [259], again a good method to immobilize the enzyme but that hardly will permit an intense multipoint covalent immobilization, and less considering the limitaitons promoted by the pepsin stability at alkaline pH values. The process increased the Arrhenius activation energy for haemoglobin hydrolysis from 8.5 Kcal/mol to 12.8 Kcal/mol. The biocatalyst was tested in milk clotting [259]. Sepharose activated with cyanogen bromide was employed to immobilize 43 mg porcine pepsinogen per g of support [260]. The specific activity of the immobilized enzyme was similar to that of the soluble one, and the biocatalyst was stable on exposure to protein substrates [260].

Cellulose has also been employed as support to immobilize many proteins [261] pepsin being among them. Pepsin and trypsin were immobilized in two steps on aminoethyl-cellulose [262]. The first step consisted on the chemical modification of the enzymes with bifunctional isothiocyanates. After the modification, the enzymes were covalently attached to the support, which presented amino groups on the surface. The results of the immobilization were satisfactory [262]. In another research, the authors immobilized pepsin together with beta-galactosidase on gel film of bacterial cellulose [263]. In this case the aldehyde groups of the support, obtained by oxidation with sodium periodate, interact with the amino groups of the enzyme, forming azomethine bonds. Although both enzymes were successfully immobilized on the gel film, it was confirmed that the gel film binds more β-galactosidase than pepsin [263]. This could be explained by the poor content of primary amino groups of pepsin.

Pepsin was also immobilized on POROS 20 resin beads through Schiff base bond (again, hardly an intense multipoint covalent immobilization may be expected, considering that the enzyme has only two primary amino groups). The biocatalyst was employed to compare the protease cleavage specificities of pepsin with Nepenthesin II, a brand new protease with the potential to be used as the proteolytic agent in Hydrogen/Deuterium Exchange coupled with Mass Spectrometry studies. The cleavage of pepsin was much influenced by the amino acid

residue at position P1 while NepII offers advantageous cleavage to all basic residues and produces shortened peptides that could improve the spatial resolution in HDX-MS studies. [264].

In another paper, three proteases: chymosin, pepsin or trypsin were covalently immobilized on oxirane-acrylic beads in order to obtain the phosphopeptide-rich fractions from casein [265]. Some diffusional limitations were detected that influenced the degree of hydrolysis. In addition, the adsorption of small peptides to the biocatalyst produced a decrease in the detectable activity values. Up to 50% of the initial enzyme activity was recovered after immobilization [265]. The hydrophobicity of the support may also give some stability problems [96], but this was not studied in this paper.

Pepsin was also immobilized on in situ synthesized urea-formaldehyde microspheres, obtained through a dispersion polycondensation polymerization method [266]. These microspheres presented reactive groups capable to produce a condensation reaction with the amino groups of the enzyme. The immobilized pepsin stability against temperature and pH was enhanced, and the biocatalyst could be reused at least eight times maintaining the 505 of the initial activity [266].

In a different study, pepsin was covalently immobilized on two different supports: acidic alumina and on 2-ethanolamine-O-phosphate (2-EAOP)-modified acidic alumina, obtaining similar results [267]. These biocatalysts were then applied to the hydrolysis of bovine haemoglobin for the production of bioactive peptides. Fixation of 2-EAOP on a pepsin-alumina complex gave hydrolysis kinetics of urea-denatured haemoglobin close to that obtained with the same amount of pepsin in solution, but with comparatively less adsorption of peptides and complete adsorption of haem, while heterogeneous hydrolyses of haemoglobin with pepsin, immobilized on functionalized alumina, resulted in the presence of VV-haemorphin-4, VV-haemorphin-7 and neokyotorphin in the supernatants without haem [267].

A new type of porous zirconia support activated with 3-isothiocyanatopropyltriethoxy silane was proposed as an alternative to the extensively used porous silica supports [268]. To show the usefulness of the new support, four proteases, including pepsin, were immobilized via covalent attachment. The results indicate that immobilization in this new support is suitable, providing a different support with clear advantages in terms of density and chemical robustness [268].

In a different work, a copolymer of acrylonitrile and acrylamide was used to immobilize pepsin to ultrafiltration membrane, via covalent bonds to the hydroxymethyl groups of the support [269]. A high relative activity was observed after immobilization (75%). The conjugate was employed for the hydrolysis of wine proteins in limited proteolysis conditions [269].

In another interesting research, poultry bone residue was employed as support matrix to immobilize different enzymes, including pepsin [270]. Both covalent attachment and adsorption were observed. The study describes the advantages of using bone as supports, since they are inexpensive, abundant, chemically functional, porous, non-toxic and mechanically strong [270].

A novel technology was applied to covalently immobilize a large diversity of enzymes, including pepsin [271]. The technology consists on the light-induced breakage of disulfide bridges in proteins, producing reactive thiols, which can react with thiol reactive surfaces. This protocol allows obtaining a spatially oriented and spatially localized covalent coupling of the enzymes with potential for biomedical, bioelectronic, nanotechnology, and therapeutic applications [271]. In another work, the authors propose a protocol to use photoactivatable polymers, containing diazirine photophore, on a polypropylene surface to immobilize various enzymes, including pepsin [272]. Upon irradiation, the photoactivatable polymer successfully immobilized the enzymes covalently, acting as crosslinkers, in a one-step reaction. These polymers were synthesized from polyamine or polycarboxylic acid and supplied a hydrophilic environment around the enzymes that enhanced their activity [272].

In general, the use of methods involving amino groups should not permit a high rigidification of the enzyme, because of the restriction of the pH values that can be used by the enzyme stability and the poor number of reactive amine groups in pepsin.

8. Immobilization of pepsin via ion exchange

Ion exchange is a simple and utilized immobilization method [196], that even though it cannot promote a high rigidification of the enzyme, permits the reuse of the support after release of the inactivated enzymes [48]. One of the risks of this method is the enzyme release during operation. This can be favored using protease in hydrolysis of proteins, as the final concentration of ionically active species (even free amino acids) will be very high at the end of the process. Considering the low isoelectric point and the great excess of anionic groups of pepsin, immobilization using anion exchangers should be preferred, However, this did not occur in all cases.

Pepsin and trypsin were immobilized onto resinous salicylic acid-resorcinol-formaldehyde and used in a fluidized-bed reactor for the study of milk coagulation [273]. Immobilized proteolytic enzymes showed improved thermal and chemical stabilities. The maximum activity occurred between 30 and 40 °C, and the operational half-life of the systems was from 15 to 22 days [273]. Zhukovskii (1996) proposed a method for giving polypropylene thread ion-exchange properties, in which gentamicin, kanamycin, monomycin, tetracycline, trypsin, pepsin, or novocain were adsorbed separately and together [274]. It was found that when antibiotics and enzymes were combined, the activity of the immobilized enzymes was synergistically potentiated, and the modified polypropylene surgical thread demonstrated high efficacy on antimicrobial, proteolytic, and anaesthetic action [274].

In another work, it was demonstrated that modified proteases such as pepsin adsorbed on celite, a noncovalent complex of subtilisin with sodium dodecylsulfate, and subtilisin or thermolysin covalently on a cryogel of polyvinyl alcohol presented a good activity [275]. These biocatalysts could catalyze the synthesis of a wide variety of peptides of various lengths and structures both in solution and on solid phase in organic solvents [275].

Porcine pepsin was immobilized in SBA-15 mesoporous silica through physical adsorption. In order to reduce the pore openings of the host material for minimization of the enzyme leaching, a grafting step with 3-aminopropryltriethoxysilane was performed [276]. The catalytic activity of the hybrid bioinorganic material, confirmed that pepsin was located inside the pore/channels of the silica material and that the grafting process did not affect the enzyme structure [276]. Porcine pepsin was also immobilized by physical adsorption inside of short, channelled Zr-Ce-SBA-15 mesoporous materials with hexagonal platelet morphologies [277]. A grafting process with [1-(2-amino-ethyl)-3-aminopropryl] trimethoxysilane was performed to minimize the enzyme leaching, without affecting the mesoporous structure of the host material. The results confirmed that pepsin was located inside the channels of the mesoporous materials and maintained the necessary degree of freedom to fulfil its catalytic activity [277].

Nanoparticles of pepsin generated in an aqueous solution were immobilized on both low-density polyethylene films, on polycarbonate plates or on microscope glass slides [277]. Pepsin coated onto polyethylene showed the best catalytic activity in all the examined parameters, compared to native pepsin. At high temperatures, pepsin immobilized on glass exhibited better activity than the native enzyme. Enzyme activity of pepsin immobilized on polycarbonate was no better than native enzyme activity. A very surprising result was that immobilized pepsin on all the surfaces was still active to some extent even at pH 7, while free pepsin was completely inactive [278].

The surface of SBA-15 mesoporous aminated silica was methylated by treatment with methyltrimethoxysilane, and then pepsin was immobilized by physical adsorption on the obtained materials giving host-guest composite materials (SBA-15)-pepsin and (methylated SBA-

15)-pepsin. Methylated SBA-15 resulted in an improved immobilization efficiency of enzyme compared to initial SBA-15 silica. It was demonstrated that pepsin was immobilized in the host pore channels without affecting the framework structure of the carriers while retaining its structure and conformation [279].

Pepsin was immobilized on Sepabeads functionalized with polyethyleneimine polymers of varying molecular sizes [280]. The results showed that the immobilized pepsin had good storage and operational stability, which were dependent on polyethyleneimine molecular weight and also on the ionic strength of medium, used for immobilization and protein hydrolysis [280]. In another research, pepsin and trypsin were immobilized on amino-functionalized sol-gel-coated magnetic nanoparticles, and then lightly crosslinked fluorescently doped polyacrylamide nanogels were subsequently produced by high-dilution polymerization of monomers in the presence of the amino-functionalized solgel-coated magnetic nanoparticles [281]. Both nanogels exhibited $K_d < 10$ pM for their respective target protein and low cross-reactivity with five reference proteins [281]. Later, pepsin from porcine gastric mucosa was immobilized by physical adsorption on amino-functionalized magnetite nanoparticles with the aim to obtain active and reusable biocatalytical constructs [223]. A total amount of 2 mg pepsin was loaded on each mg of magnetite nanoparticles, and the immobilized pepsin was able to catalyze several cycles of haemoglobin hydrolysis after successive recovery through magnetic separation [223]. In another study, pepsin was encapsulated by electrostatically controlled diffusion into thermally evaporated fatty amine films [282]. The catalytic activity of the pepsin after immobilization was then evaluated using haemoglobin as substrate, showing that the activity of pepsin in the matrix activated with octadecylamine is slightly lower than that of the enzyme in solution [282]. Finally, pepsin was also adsorbed on Celite and Chromosorb [283]. The resulting biocatalyst was employed to study the specificity of the enzyme in organic media, using ethyl acetate and acetonitrile. It was observed that the most hydrophobic solvents produced the best yields, despite the low solubility of substrates in these media [283].

The high density of anionic groups in pepsin (Fig. 6) can give strong enough strength anion exchange if the supports are properly designed. Considering the difficulties to reach an intense multipoint covalent immobilization, this reversible immobilization method may be a suitable one for pepsin [48].

9. Pepsin immobilization by hydrophobic interactions

Immobilization of enzymes in hydrophobic supports tends to be problematic, as in many instances the near presence of a hydrophobic surface may stabilize incorrect enzyme forms [96,284]. Lipases are the exception to this rule [83]. In any case, this strategy is used to immobilize enzyme in different instances [285,286].

This strategy has also been used to immobilize pepsin. Thus, Marakova et al. (1980) studied the effect of the macromolecular structure and the halogen content of the copolymers' brom- and iodoacetyl derivatives which were used as polymeric carriers for the immobilization of glucoamylase and pepsin [287]. They found that the iodoacetyl derivatives of the styrene-divinylbenzene copolymers with a high iodine content and a macro-porous structure are the most suitable for immobilizing these enzymes [287]. In another study, the milk-clotting enzymes chymosin and pepsin were immobilized on various hydrophobic carriers (hexyl-substituted Sepharose 6B, phenoxyacetyl cellulose, activated carbon) and utilized in the continuous coagulation of skim milk in cheese production [288]. All enzyme-carrier preparations exhibited high initial activity on exposure to milk, but also high deactivation rates mainly by the loss of enzyme from the carriers due to physical desorption of enzyme, as well as from the relatively rapid leakage of the ligand from the carrier [288]. Zhang et al. (2018) proposed a platform for biocatalytic membrane preparation and provided a novel methodology to evaluate the effect of immobilization mechanisms on enzyme performance based on polydopamine coating [288]. Nemat-Gorgani and Karimian (1982) proposed a method to immobilize pepsin (among other enzymes) via hydrophobic adsorption on palmityl-substituted Sepharose 4B containing long hydrophobic ligands. Although other enzymes were desorbed at increasing NaCl concentrations, pepsin remained adsorbed even at NaCl 1 M. The catalytic activity of pepsin was slightly increased upon immobilization [289].

10. Other immobilization strategies

Monoliths represent a novel technology on the field of enzyme immobilization, but their utility due to the significant acceleration of the reaction rate probably caused by very fast mass transfer of the substrate to the immobilized enzyme has been proven more than enough [290]. This methodology can also be applied to the separation or concentration of proteins from a mixture, including pepsin. For instance, one research group was able to prepare inorganic monoliths covered with gold nanoparticles to retain standard proteins, like pepsin and haemoglobin, with the objective of getting a high effective isolation of these analytes. Preconcentration from a real sample was achieved [291,292].

Another research group developed a spongy monolith consisting of poly(ethylene-*co*-glycidyl methacrylate) with continuous macropores presenting epoxy groups on the surface [293]. The preparation was used to immobilize protein A with the objective of obtaining a high-yield collection of immunoglobulin G from cell culture supernatant, an also to immobilize pepsin for online digestion at high flow rate. Actually, longer reaction in solution yielded larger peptide fragments, but, in online digestion with the pepsin-immobilized spongy monolith, the peptide fragments were much larger, even though a faster flow rate (100 mL h − 1) was employed. [293].

11. Immobilization of specific ligands for pepsin selective immobilization/purification

A strategy used to separate a certain enzyme from a mixture consists on the covalent immobilization of the inhibitor of that enzyme, and then present the protein containing the target enzyme mixture to the support, so the desired enzyme attaches to the support via interaction with the inhibitor. Using substrate analogs, or other compounds that directly interact with the active center, this is valid for enzyme fixation and purification, but the enzyme activity is blocked, that way, it should be released to the medium to have catalytic activity. This methodology was used in several studies regarding pepsin [294]. A research developed a synthetic protein scavenger material based on imprinted amino polystyrene microbeads and the pepsin inhibitor pepstatin, attached via carbodiimide linker reaction [295,296], to achieve the solid phase extraction of pepsin. Homogeneous pepsin binding sites on the surface of the imprinted microbeads was reported [295,296]. Tichá et al. (2004) used different specific pepsin substrates immobilized on divinyl sulfone activated Sepharose (if the molecule presented a free amino group) or in Sepharose via ethylene diamine spacer using carbodiimide reaction for molecules with blocked amino group but free carboxyl. Affinity chromatography of pepsin was carried out employing the previous supports, with excellent results. Pepsin could be later released just by increasing the ionic strength [297]. Later on, the same group reported the use of a synthetic heptapeptide containing D-amino acid residues coupled to glyoxal-activated magnetic agarose particles via the free peptide amino group to separate pepsins of different species. This technology can be useful in combination with mass spectrometry, for the enzyme detection and determination [298]. That way, this is more related to enzyme purification via strong interaction with the support that a real strategy to develop an industrial pepsin biocatalyst.

12. Some practical applications of immobilized pepsin

12.1. Immobilized enzyme reactors (IMERs)

Apart from the advantages that immobilization may present related to the protease itself (reusability, enhanced stability and avoidance of proteolysis among many others), it can provide other improvements to the reaction, such as an easier purification of the products or a reduced reaction time due to the high enzyme concentration on the support. These advantages take a special relevance in the field of proteomics, where an undefined protein mixture has to be hydrolyzed and the peptides analyzed afterwards [299]. This is where the immobilized enzyme reactors (IMERs) take a major relevance, especially because they can be coupled with other analyzing technologies like mass spectrometry (MS), capillary electrophoresis or high performance liquid chromatography (HPLC) and provide a continuous, online digestion [300]. Pepsin has proven to be a good alternative to trypsin in the protein digestion on IMERs since it is less specific. Thus, a pepsin microreactor was employed to analyze myoglobin sequence coverage, which was accomplished in a thousandth the time of solution digestion [301].

A bioinspired proteolytic membrane was built via layered immobilization of pepsin forming a bilayer structure (polydopamine coating and polyethyleneimine grafting) to produce peptides from food proteins. The proteolytic membrane exhibited great pepsin immobilization amount and substrate accessibility to pepsin, and excellent filtration performance (permeate flux = 6.5 L m^2/h bar and DH = 45.7%) during long-term tests at optimized operation condition (transmembrane pressure = 3 bar and pepsin concentration = 0.5 mg/mL. [302]. A high-surface monolithic support made of thiol-ene was employed to immobilize pepsin via covalent linkage with ascorbic acid, to produce a microfluidic chip combined with an enzyme microreactor capable of separating and collecting the peptidic fragments in a single step. The online coupling of this all- thiol-ene-based system demonstrates its potential for avoiding tedious and time-consuming sample transfer, decreasing incubation time compared with traditional enzymatic approach, minimizing sample volume consumption, and allowing repeated usage. [303].

The specific enzyme-inhibitor bonding can be employed to detect potential pepsin inhibitory molecules, as proved by Cancilla et al. (2000). Enzyme immobilization was carried out using Aminolink coupling gel. They used immobilized pepsin and employed electrospray ionization ion cyclotron resonance mass spectrometry to detect what molecules from a library expressed a pepsin inhibitor activity by being attached to the immobilized enzyme upon incubation. The enzyme was recycled for continuous screening of new libraries [304]. This strategy permitted to identify strong binding targets from complex mixtures, being able to differentiate strong and weak binding ligands from a pool of potential target compounds. In another research, an IMER was fabricated by immobilizing pepsin onto an organic polymer monolith using glutaraldehyde as crosslinking agent [305] Immobilized pepsin maintained >95% of the initial activity after 40 cycles. The preparation could be stored at 4 °C and retaining around 85% of the initial activity after 36 days. The system was employed in the screening of pepsin inhibitors [305].

Bonichon et al. (2016) developed a pepsin-based immobilized enzyme microreactor, using CNBr-sepharose gels, coupled to nano-LC–MS/MS for the faster hydrolysis of human butyrylcholinesterase in order to detect exposure to toxic organophosphorus compounds [306]. Later on, the same group, used the same methodology to develop a fast, selective and sensitive on-line set-up for the analysis of human butyrylcholinesterase from plasma based on immunoextraction by anti-HuBuChE antibodies [307]. This idea was improved even further, being able to detect sarin and soman adducts in plasma, by means of three different monoclonal antibodies, covalently grafted on Sepharose to achieve the extraction of the enzyme [308]. In all of the studies, immobilized pepsin was used to digest the samples [308].

Möller et al. (2019) compared the use of pepsin with other aspartic proteases on the hydrolysis of four integral membrane proteins immobilized on-column and coupled to hydrogen/deuterium exchange mass spectrometry [309]. Pepsin provided the highest coverage for two of the proteins with 82.2 and 33.2% of the sequence respectively. However, the other proteases provided a better coverage for assayed other proteins. These results highlight the importance of using different proteases to optimize the digestion for mass spectrometry analysis.

A polymer microfluidic chip-based enzyme reactor, coupled to LC-MS, was developed by immobilization of pepsin via thiol-ene chemistry on a monolithic stationary phase to improve the sample handling, and reduce cost and preparation time of peptide mapping on protein analytical research. Chip-immobilized pepsin show virtually identical apparent activity to the commercially available agarose packing material, but with a 70× higher surface coverage, the result of which is an almost 3× higher activity per volume of solid phase at the tested conditions. [310]. In another study, human serum albumin or pepsin were chosen as chiral selectors, and were immobilized on graphene oxide-modified affinity capillary monoliths. The effect of three spacer arms (ammonium hydroxide, ethanediamine and polyethyleneimine) on the chiral recognition of nine pairs of enantiomers was studied. Unfortunately, pepsin- graphene oxide-polyethyleneimine-based affinity capillary monoliths possessed the highest protein digestion capacity, which was different from the effect of amino donors on enantiorecognition [311].

A pepsin IMER was employed to analyze the proteome of cellular membranes coupled to microflow reversed-phase liquid chromatography with electrospray ionization tandem mass spectrometry, being able to identify 235 unique proteins [312]. In another work a pepsin IMER was coupled on-line to a selective immunoextraction step, using specific antibodies covalently immobilized, to analyze a target protein from a plasma sample. The whole on-line device (immunosorbent-IMER-LC-MS/MS) allowed the quantification of cytochrome *c* from 8.5 pmol to 1.7 nmol in buffer medium [313].

The milk and cheese industry has also taken advantage of the strategy of immobilizing pepsin on a continuous reactor. In this case, the objective is the milk coagulation, that has been accomplished in different ways, like immobilizing pepsin on porous glass beads [314], on porous glass in a fluidized bed reactor [315], pepsin immobilized on glass on a column configuration [316] or on porous, alkylamine glass and incorporated into a fluidized-bed continuous coagulation scheme [317].

Ticu et al. (2005) designed a continuous stirred tank reactor using immobilized pepsin. The immobilization was performed on acidic alumina treated with 2-ethanolamine-O-phosphate. This biocatalyst was employed in the preparation of two pure transient and hydrophobic opioid peptides (LVV-haemorphin-7 and VV-haemorphin-7) together with other peptides of interest obtained from the hydrolysis of bovine haemoglobin [318]. Later on, the same group, prepared an IMER by immobilizing pepsin on A568 Duolite resin to perform the continuous hydrolysis of bovine haemoglobin in order to prepare the same two peptides from the previous work in one step [319]. On a subsequent research, the same group immobilized pepsin also on a Duolite resin column for the continuous hydrolysis of bovine haemoglobin. The resulting peptides were simultaneously extracted by adsorption on an aluminum oxide column and the eluted with a volatile ethanolamine solution [320].

On another work, pepsin was covalently immobilized on dextran-modified fused-silica capillaries to carry out the on-line digestion of proteins under acidic conditions [321]. The digested samples were analyzed by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS). The reactor was stable for at least 40 days of continuous use [321].

A pepsin microreactor, where pepsin was immobilized on poly (methyl methacrylate) activated with water soluble carbodiimide and

NHS (N-hydroxysuccinimide), was prepared. Enzyme immobilized with water soluble carbodiimide decreased over a period of days, whereas the enzyme immobilized with both water soluble carbodiimide and N-hydroxysuccinimide remained active even after a month of use [322]. Ethyl-bridged hybrid particles in columns were used to immobilize pepsin, and then the system was evaluated in terms of its digestion performance in a completely online format, with the specific intent of using the particles for hydrogen-deuterium exchange mass spectrometry (HDX MS) analysis. Prototype columns made with the ethyl-bridged hybrid pepsin particles exhibited robust performance, and deuterium back-exchange was similar to that of other immobilized pepsin particles [323]. The use of open tubular capillary pepsin and trypsin reactors for rapid digestion, coupled on-line to capillary electrophoresis and mass spectrometry for the rapid protein digestion was reported. It was found that complete on-line digestion can be performed in a matter of seconds, basically without adding any extra time to the MS analysis [324]. Other examples where immobilized pepsin is used in a reactor for diverse protein hydrolysis are the extraction of gluten peptides with foaming properties by continuous hydrolysis in a pepsin bioreactor system employing porous chitosan beads as support [325] and pepsin and trypsin immobilized on hydrogels in digital microfluidic systems, on a shape of cylindrical agarose discs bearing the enzymes, coupled to mass spectrometry analysis of the products [326].

12.2. Production of drugs and peptides with industrial biological interest

Pepsin has also been employed on pharmaceutical studies for the discovery and development of new drugs. The interest of the enzyme immobilization in these cases is associated to the simplification of the purification process of the products from the enzyme, which also avoids an excessive proteolysis of the substrate (permitting to optimize the duration of the process) [327]. The fact of having the enzyme immobilized onto a column or on a continuous reactors, permits the coupling with the chosen analysis method (mass spectrometry for instance).

Immobilized pepsin was employed for the digestion of Rituximab to obtain different fragments as radioimmunoscintigraphic agents for the treatment of Non-Hodgkin's Lymphoma [327,328]. IgG1 monoclonal antibody fragments were also obtained using immobilized pepsin and purified by affinity chromatography to use them as targets for phage display [329].

Lysobactin is a cyclic peptide with great antibiotic activity against Gram-positive aerobic and anaerobic bacteria. Some immobilized gastric and intestinal proteases, including pepsin, were used to verify its resistance to degradation after oral administration. In the study it was shown that Lysobactin was not directly hydrolyzed by immobilized pepsin, chymotrypsin, trypsin, nor mucosal peptidases [330]. The same methodology has been used on other fields, as is the cellular biology, represented in this study, where immobilized pepsin was employed to study binding domains of antibodies to annexin-A1, a phospholipid-binding protein involved on process like cell proliferation, apoptosis, metastasis, and the inflammatory response, by means of hydrogen–deuterium exchange mass spectrometry. With this work it was demonstrated that even cryptic and flexible binding regions can be studied by HDX-MS, allowing a fast and efficient determination of the binding sites of antibodies which will help to define a mode of action profile for their use in therapy [331].

Rémigy et al. (1997) characterized, by mass spectrometry, the hydrolysis peptides of ferredoxin from the cyanobacterium *Anabaena* sp., obtained by digestion with immobilized pepsin [332]. Although this study is more focused on the method of analysis than on pepsin digestion, this is one more study in which the versatility and usefulness of this enzyme can be appreciated.

A more industrial application was given to pepsin, employed in a different approach where lucerne leaf protein, a protein-rich concentrate of high nutritive value, was hydrolyzed by pepsin immobilized on parafilm wax to modify its unfavorable organoleptic properties [333].

13. Future trends

The examples given in this review show the great interest in the immobilization of pepsin, even though it is a very difficult task to get the covalent immobilization, it is not complex to get the ion exchange thanks to the great density of anionic groups on its surface (Figs. 5 and 6). The reported stabilizations may be consequence of the prevention of intermolecular phenomena discussed in introduction, more than due to multipoint covalent immobilization.

In this regard, we would like to remark two different possibilities to increase the prospects of getting an intense multipoint immobilization of pepsin, which to our understanding has not been tried with this enzyme.

The first one is to increase the number of primary amino groups, chemically (aminating the carboxylic acids) (Fig. 5) or genetically, in order to increase the possibilities of achieving an intense multipoint covalent [53]. The potential of this strategy has already been showed with some other enzymes, and in this instance it may permit to give a qualitative leap regarding the final rigidification of the enzyme considering the high density of carboxylic groups on the enzyme surface [105,334–337].

The second is the use of supports with active groups able to react with other groups different to the primary amino groups, such as Tyr (Fig. 7). Although epoxy supports have been tested with this enzyme, and they may involve many groups of the enzyme surface [184], the utilized protocols are not adequate to achieve an optimized multipoint covalent immobilization of the enzyme [284,338]. Alternatively, supports activated with vinyl sulfone have been regarded as very suitable ones to give intense multipoint covalent immobilization [44,186].

Considering that the utilized methodologies have already permitted to prepare pepsin biocatalysts with improved performance, it is not difficult to foresee the improvements in the processes development that advances in the pepsin immobilization may produce.

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CRediT authorship contribution statement

Roberto Morellon-Sterling, Olga Tavano and Juan M. Bolivar performed the initial literature search. Veymar G. Tacias-Pascacio and Roberto Fernandez-Lafuente designed the paper and supervised the writing. All authors contributed to the writing and final editing of the paper.

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