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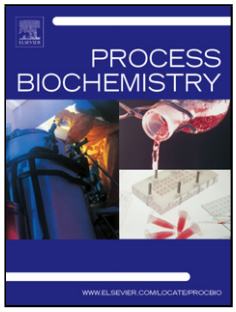

Title: Impact of thermal treatment and hydrolysis by Alcalase and *Cynara cardunculus* enzymes on the functional and nutritional value of Okara

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Impact of thermal treatment and hydrolysis by Alcalase and *Cynara cardunculus* enzymes on the functional and nutritional value of Okara

Running title - Impact of thermal treatment in the enzymatic hydrolysates of Okara

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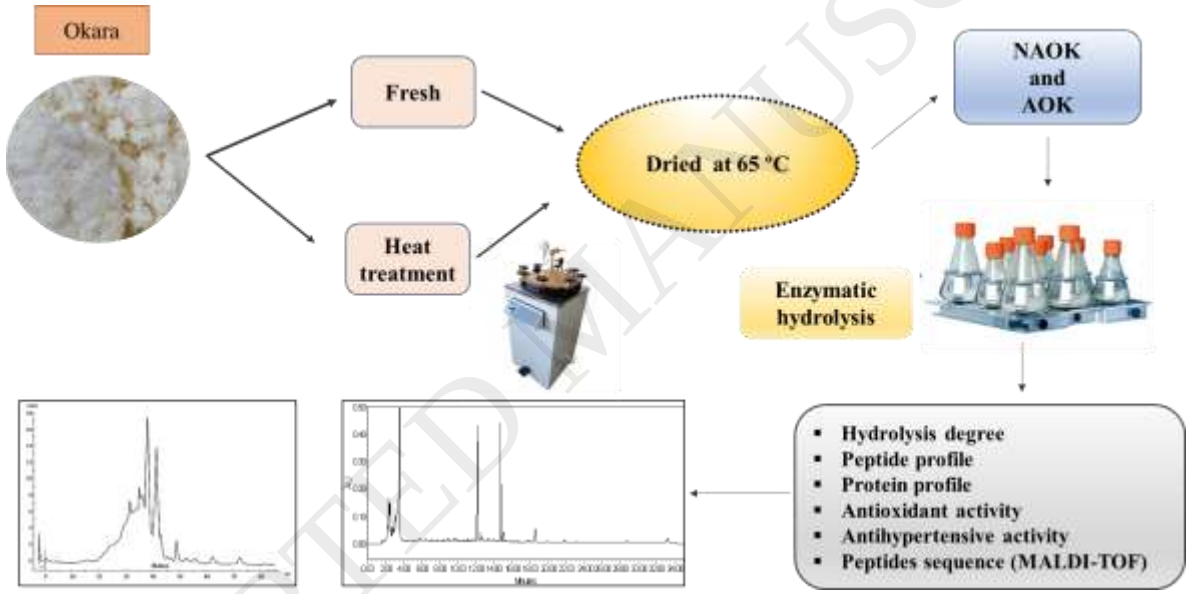
Graphical abstract



Highlights

- Thermal treatment improves the degree of hydrolysis and bioactivities for both enzymes
- Alcalase hydrolysed okara proteins to a greater extent than the *Cynara cardunculus* extract.
- Hydrolysates by Alcalase showed a high correlation between antioxidant activity and degree of hydrolysis.

- Okara hydrolysates with potential anti-hypertensive activity were obtained by *Cynara cardunculus* extract.
- New peptides sequences were identified in okara hydrolysates for both enzymes.

Abstract

Enzymatic hydrolysis of dried okara (autoclaved and non-autoclaved) with Alcalase (AL) and *Cynara cardunculus* extract (CY) was studied, assessing the impact of heat treatment and hydrolysis on potential antioxidant and antihypertensive activities of final hydrolysates. This study showed that the thermal treatment (sterilization at 121 ºC, 1 atm and 15 min) facilitated the enzymatic access to substrate and increased the degree of hydrolysis (DH), especially for AL (37.9%) when compared to CY (3.6%). The antioxidant activity of dried Okara (either autoclaved or not) when hydrolysed with AL was higher (4.2 mg Trolox/mL) than that observed for CY. Additionally, the potential ACE-inhibitory activity was high for samples hydrolysed with both enzymes, however the highest ACE inhibition was also found for AL (IC_{50}= 9.97 µg/mL). This study allowed the identification of new peptide sequences in dried okara hydrolysed with both enzymes, and some sequences that can explain their bioactivities. The results indicate that dried okara hydrolysates can either be used as functional ingredient or as food supplement for blood pressure lowering or antioxidant applications in the future.

Keywords:

Okara, bioactivity, peptide, antioxidant activity, enzymatic hydrolysis, antihypertensive activity.

1. Introduction

The soybean is an important Asia originated grain that has been consumed worldwide [1]. In general, soybean has a great commercial interest and the by-products generated after processing, like okara, are promising sources due to its composition and bioactive compounds. Okara is a soybean by-product that remains after filtering the water soluble fraction during the production of soya beverage or tofu [2].

Considering that about 250 kg of okara can result from the production of 1000 L of soy beverage, and based on soy beverage consumption, approximately 14 million tons of okara are estimated to be produced worldwide annually, which can be associated with serious environmental problems [3] and massive loss of valuable nutrients. Environmental problems caused by okara are mainly related to its highly susceptible to putrefaction. Okara decomposes naturally when not refrigerated due to its high levels of water (~80%), protein, dietary fiber and lipids (MUFA and PUFA) [2], [4]. Thus, okara can be considered a valuable ingredient for animal and human nutrition [1], [5]. Many studies based on okara have also reported its fermentation applications [1] and protein [6], polysaccharides [7], and isoflavones [8] extraction.

Studies performed with okara have shown some bioactivities, especially in protein hydrolysates [9] and fermented products [1]. Other relevant compounds present in okara are isoflavones. Although most soybean fractions are extracted during soymilk production, ca. 12-30% of the isoflavones can be retained in okara [8]. Okara also possesses ca. 30% (dry basis) of two main proteins - β-conglycinin (7S) and glycinin (11S) [2], [9]. These proteins resist to complete digestion by gastrointestinal enzymes and some essential amino acids low bioavailability is due to their poor solubility [11].

Through soy proteins enzymatic hydrolysis, we can obtain bioactive peptides and free amino acids. These peptides are specific protein fragments that possess antioxidant activity[12], [13], i.e. can control oxidative processes in food and in human, and also may have antihypertensive [13], [14] and antidiabetic activities [10] among other potential physiological activities.

In general, the antioxidant activity of proteins has been related to their amino acid composition. Therefore, through the enzymatic hydrolysis it is possible to increase this capacity since the protein cleavage favors the exposure of antioxidant amino acids in proteins [15]. Alcalase (AL) is an enzyme extract from *Bacillus licheniformis*, which presents several proteolytic activities with different specificities [16]. AL has been used extensively to prepare hydrolysates of soy [17], whey [18], sweet potato [19] and fish protein [20] with bioactive peptides. Aqueous extracts from cardoon (*Cynara cardunculus*) flowers can be considered the best-known vegetable coagulant used for production of some traditional ewe´s milk cheeses in Portugal and Spain [21]. The enzymes Cardosin A and Cardosin B, present in the thistle, shown different amino acid sequences but both have proteolytic activity and promote milk clotting [21], [22]. Regarding their activity, Cardosin B has broader specificity than Cardosin A. Cardosin A is comparable with chymosin, while Cardosin B (~25%) is comparable with pepsin. However, Cardosin B is less specific than pepsin and hydrolyses phenylalanine, tyrosine, leucine or valine bonds [22].

Although some studies have been performed with okara, most of them explored its nutritional composition, and only few have tried to valorise the complete byproduct but with scarce applications implemented in the market. A previous study reported that the subcritical water hydrolysis processing of okara protein leaded to the generation of angiotensin-converting enzyme (ACE) inhibitory peptides, which converts angiotensin I to angiotensin II, a potent

vasoconstrictor [23]. Therefore, okara needs to be further valorised in order to transform this byproduct into a food product with added value.

Previous studies have shown that the pre-treatment influences the protein molecular weight. In addition, the heat treatment provides protein denaturation and facilitates the enzymatic attack because the denatured proteins are easier to hydrolyse than when in their native form and the peptide bonds are more accessible in unfolded protein molecules [24]. Moreover, the heat treatment influences the nutritional quality of food protein. Several studies have shown that the wet heat treatment improves the quality compared with dry heat treatments [25]. So, in this study the enzymatic hydrolysis of dried okara (previously autoclaved and not autoclaved) with Alcalase and *C. cardunculus* extract was investigated and the antioxidant capacity and antihypertensive activity of the final hydrolysates were characterised. Furthermore, the peptides profile and respective sequence, as well as free amino acids composition of each hydrolysate were also evaluated.

2. Material and methods

2.1. Materials and chemicals

Okara by-product was provided by Nutre (Aveiro, Portugal), Alcalase 2.4 L FG (a density of 1.17 g/mL) was ordered from Novozymes (Bagsvaerd, Denmark), *Cynara cardunculus* extract (a density of 1.14 g/mL) was obtained from Formulab (Maia, Portugal). Trolox, 2,2′-amino-di (2-ethyl-benzothiazoline sulfonic acid-6) ammonium salt (ABTS), o-Phthalaldehyde, o-Phthalic dicarboxaldehyde, Benzene-1,2-dicarboxaldehyde (OPA), and amino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Proteins standards (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) were purchase from GE Healthcare (USA) and an antihypertensive peptide with the sequence KGYGGVSLPEW (99.7%) (1.2 kDa) was ordered from GenScript (China). All other reagents and chemicals used were of analytical grade.

2.2. Okara samples and drying process

The okara used in this study was kindly provided by a soy beverage producer (NUTRE Industry, Aveiro, Portugal). The collection of okara samples occurred immediately after the beverage production. Samples were readily transported under refrigerated conditions (4 ºC and not more than 2 h) and divided into 2 lots: one was immediately dried at 65 ºC for 68 h, to constant weight, whereas the other was firstly autoclaved (121 ºC, 15 min and 1 atm) and then dried under the same conditions used for the non-autoclaved lot. Both samples were then grounded in a mill (Retsch, Haan, Germany) with 1 mm sieve.

2.3. Proximate composition analysis of okara

Okara samples moisture was determined at 105 ºC for 24 h, according to the Association of Official Analytical Chemists (1995). The crude protein was determined using a nitrogen analyser (N × 6.25, Leco N analyzer, Model FP-528, Leco Corporation, St. Joseph, USA).

2.4. Enzymatic hydrolysis of okara

The hydrolysis of dried okara (autoclaved and not autoclaved) was performed in substrate solutions containing 27 mg protein/mL and using two enzymes - Alcalase 2.4 L FG (a density of 1.17 g/mL) and *Cynara cardunculus* extract (a density of 1.14 g/mL). For both enzymes four different enzyme: substrate (E/S) ratios (1.3; 1.7; 2 and 3.3 % v/v (enzyme volume/ substrate volume (27 mg protein/ mL)) and two reaction times (2.5 and 5 hours) were tested. The enzyme was inactivated by heat treatment at 85 °C 10 min, and the supernatant was separated from precipitate by centrifugation at 1800g for 20 min. The summary of experiment conditions is shown in Table 1. All trials were conducted in duplicate.

The enzymatic hydrolysis with Alcalase (AL) and *Cynara cardunculus* extract (CY) was performed in an orbital shaker (Wiggenhauser, Germany), according to the optimal conditions: AL (phosphate buffer (0.025 M), pH 8.3 at 50 ºC and 120 rpm) CY (citric acid – sodium citrate buffer (0.1 M), pH 5.2 at 55 ºC and 120 rpm).

2.5. Degree of hydrolysis (DH)

The degree of the different okara hydrolysis with CY and AL was determined using the modified OPA method, described by Nielsen et al. [26]. The method was modified concerning the volumes used, so the volume of 200 µl standard, blank (distilled water) or sample were added to each respective tube and 1.5 ml OPA reagents were added to all test tubes and allowed to react for 2 min. The absorbance was read at 340 nm. The % DH was calculated following the equations reported by Adler-Nissen [27], using the control without enzyme as reference.

$$\text{Serine } NH_2 = \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} * 0.9516 * 0.03 * \frac{100}{X + P} \quad (1)$$

$$H = \frac{\text{Serine } NH_2 - \beta}{\alpha} \quad (2)$$

$$DH = \frac{h}{h_{tot}} * 100 \quad (3)$$

Where:

SerineNH_2 = mEq serine NH_2/ g protein; X = sample (g); P = protein (%); 0.9516= mEq serine/ L water; 0.03 is the volume in liter (L).

h = number of hydrolysed bonds (mEq/g protein); β = 0.342 (specific for soy); α = 0.970 (specific for soy).

h_{tot} = total number of peptide bonds per protein equivalent (7.8 specific to soy).

2.6. Molecular weight pattern of okara hydrolysates

The molecular weight of okara hydrolysates was determined by gel filtration chromatography using the FPLC (fast protein liquid chromatography-gel filtration) AKTA Pure 25 system coupled with two gel filtration columns: Superdex 200 increase10/300 GL and Superdex peptide, 10/300 GL (GE Healthcare Life Sciences, Freiburg, Germany). The mobile phase was 25 mM phosphate buffer (pH 7.0), 150 mM sodium chloride and 0.2 g/ L sodium azide. The flow of eluent was 0.5 mL/ min and was monitored by absorbance at 280 nm. Standard proteins with known molecular weights (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotinin, 6.5 kDa) and an antihypertensive peptide with the sequence KGYGGVSLPEW (1.2 kDa) were used to establish the MW standard curve. The software used to evaluate samples was UNICORN 7.0.

2.7. Okara hydrolysates peptides profile

Peptide hydrolysates profile was determined through a modified method described by Garcia et al. [28]. Fifty µL of hydrolysate extract (obtained as explained in 2.3) was analysed by HPLC (Waters Series 2695. Mildford, MA. USA) coupled to a diode array detector, using a Kromasil C-18 column (250 x 4.6 mm) to separate the peptides at 50 ºC. The mobile phase consisted of 0.1% trifluoracetic acid in water (v/v) (solvent A) and 0.1% trifluoracetic acid in acetonitrile (v/v) (solvent B). A linear gradient of 5 steps was used: 5-20% B; 20-25% B and 25-25% B for 10 minutes each, followed by a rapid increase of 35-46% B in 30 seconds and a linear reversed gradient 46%-5% B in 30 seconds to re-equilibrate the column to starting condition. The flow rate was 1 mL/min. The proteins were detected by UV absorption at 254 nm. The data were processed on Empower 3 software. The peptides profile was compared for the different hydrolysates.

2.8. Profile of free amino acids in hydrolysates

Free amino acids content was determined by derivatization using orthophthalaldehyde (OPA) methodology. The amino acids were separated by HPLC (Beckman coulter. California USA) coupled to a fluorescence detector (Waters, Milford, MA, USA), according to the procedure of Proestos et al. [29]. Briefly, 100 µL of each sample, at 10 mg/mL were derivatised according to the described method and with a derivatives injection volume of 20 µL. Free amino acids content was quantified using a calibration curve built with amino acids standards and expressed as mg/g of protein content. Two independent analysis were performed for each condition.

2.9. Determination of antioxidant activity

Antioxidant activity of okara hydrolysates obtained was determined using ABTS radical according to the method described by Gião et al. [24]. The ABTS radical cation (7 mmol/L) was mixed with potassium persulfate (2.45 mmol/ L) and kept for 12–16 h at room temperature in the dark. The quantification was performed at 734 nm (mini UV 1240, Shimadzu, Tokyo, Japan) with 10 µl of samples (supernatant from hydrolysed okara) in 1 ml ABTS solution with absorbance limit of 0.7 (± 0.02) at room temperature. The % inhibition of the sample was then compared with a standard curve made from the corresponding readings of Trolox (0.025-0.50 mg/mL) and results expressed as mg Trolox/mL hydrolysate okara.

2.10. Determination of ACE-inhibitory activity

The ACE inhibitory activity was performed based on the method modified by Quirós et al. [31], using the fluorimetric assay. ACE working solution (42 mU/mL) was diluted with 150 mM Tris-HCl (pH 8.3) containing 0.1 µM $ZnCl_2$ and the substrate used was o-Abz-Gly-p-Phe (NO2)-Pro-OH (0.45 mM) dissolved in 150 mM Tris-HCl (pH 8.3) and 1.125 M NaCl. For reaction development, a microplate with ninety-six-well (Porvair, Leatherhead, UK) was used. In each test sample, the assay mixture was composed by the following components: 40 µL of ACE (42 mU/mL), 40 µL of hydrolysate and 160 µL of substrate (o-Abz-Gly-p-Phe(NO2)-Pro-OH, 0.45 mM). Control contained 40 µL of ACE (42 mU/mL), 40 µL of water distilled and 160 µL of substrate. Blank contained 80 µL of distilled water and 160 µL of substrate. The mixture was incubated at 37 °C and the fluorescence generated was measured at 30 min using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) and the wavelengths used were 350 and 420 nm for excitation and emission, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG

Labtech). The activity of each hydrolysate sample was tested in duplicate, where two microplates were analysed for each sample and the inhibitory activity was expressed as the protein concentration required to inhibit the original ACE activity by 50% (IC_{50}). The percentage of ACE-inhibitory activity was calculated using the formula 100 x (*C* - *S*)/(*C* - *B*) and IC_{50} values was calculated using the nonlinear fitting to the data. The protein content in hydrolysate extracts was analysed by bicinchoninic acid, using albumin as standard.

2.11. MALDI-TOF/TOF

For mass spectrometry analysis, the okara hydrolysates were detected and acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF Analyzer, SCIEX, Framingham, MA), equipped with a 200-Hz frequency Nd:YAG laser, operating at a wavelength of 355 nm. Peptides were purified by reversed-phase C18 chromatography (ZipTips, Millipore) following the manufacturer's instructions and eluted in the MALDI sample plate with the MALDI matrix alpha-Cyano-4-hydroxycinnamic acid (CHCA) elution solution at 8 mg/mL in 50% ACN, 0.1% TFA, 6 mM ammonium phosphate. Peptide mass spectra were acquired in reflector positive mode in the mass range of *m/z* 700–5000. Some peptide ions were selected for MS/MS peptide sequencing. Proteins were identified by the combined Peptide Mass Fingerprint (PMF) + MS/MS approach with the Mascot software (Matrix Science) using the UniProt protein sequence database for the taxonomic selection *Glycine max* (2015_12 release). The protein search settings were: methionine oxidation (variable modification), no enzyme specificity, peptide mass tolerance of 20 ppm and fragment mass tolerance of 0.5 Da. Protein scores greater than 61 were considered significant ($P < 0.05$).

2.12. Statistical analysis

The results are expressed as the mean values ± standard deviations. Statistical analysis was performed using SPSS version 23.0 for Windows. Statistical significance of differences among means and group means was analysed by one-way analysis of variance (ANOVA) and student's t-tests. Interaction between factors (reaction time, thermal treatment and E/S ratio) for hydrolysis degree and antioxidant activity were analysed by two-way analysis of variance (ANOVA). Correlations among hydrolysis degree and antioxidant activity were calculated using Pearson's correlation coefficient (r). In all cases a significance level of $P < 0.05$ was considered.

3. Results and discussion

The total protein contents of dried autoclaved okara (AOK) and dried not autoclaved okara (NAOK) were similar, with values of 27.03% ± 0.24 and 26.84% ± 0.05, respectively. This protein content is similar to values reported in previous studies with okara protein showing high nutritive quality [2], [4]. The dried okara products had a moisture content of 3.39% ± 0.14 and 3.80% ± 0.11, for NAOK and AOK, respectively. The drying treatment led to stable powders that maintained stability under controlled storage conditions, overcoming the high moisture content related okara instability [2].

3.1. Enzymatic Hydrolysis

As shown in Table 2, for okara hydrolysates obtained from NAOK and AOK, produced under different enzymes and conditions, it was observed that the DH increased from 12.4% ± 3.1 to 33.8% ± 0.2 and from 21.7% ± 0.8 to 37.9% ± 1.4, for NAOK and AOK, respectively, in 5 hours reaction. DH showed significant differences ($P < 0.05$) with increased E/S ratio for both reaction times evaluated. Hydrolysis of NAOK and AOK for 2.5 hours also showed significant differences ($P < 0.05$) for the different levels of enzyme. The values obtained for NAOK were similar to those observed by Sbroggio et al. [9]. for AL, however, AOK hydrolysate presented higher DH values. Thus, this suggests that okara sterilization heat treatment improves the DH, for both enzymes. Previous studies [24], [25] have also showed that the heat treatment can increase the DH. According to Achouri et al. [24], the thermal denaturation facilitates the enzymatic attack turning the unfolded protein molecules peptide bonds more accessible. Nevertheless, according to previous studies in soy flour and meal, the nutritional value can be increased with the heat treatment, but may also reduce the functionality and bioavailability of amino acids [32].

In relation to hydrolysis performed with CY, for 5 hours of reaction, a lower DH was observed comparing to AL, presenting values of 2.26% ± 0.01 and 3.57% ± 0.18 for NAOK and AOK, respectively. The CY showed significant differences ($P < 0.05$) for DH of individual factors evaluated (E/S ratio, reaction time and thermal treatment of okara) and for the interactions between these factors. Moreover, for NAOK or AOK hydrolysed for 2.5 hours of reaction, no significant differences ($P > 0.05$) were found, whereas for 5 hours, the DH showed significant differences ($P < 0.05$). AL seemed to hydrolyse okara proteins to a greater extent than the CY. AL is an endopeptidase and has a range of specificity of peptide bonds for hydrolysis, especially for aliphatic (Leu and Ala), aromatic (Phe, Trp and Tyr),

sulphur-containing (Met), acidic (Glu), hydroxyl (Ser) and basic (Lys) residues [18]. The CY is constituted by two types of Cardosins, A and B, with similar specificity to chymosin and pepsin, respectively [22]. Both Cardosins have a preference for cleaving peptide bonds just past hydrophobic side chains [22]. In conclusion, the broader specificity of AL may explain a higher DH obtained by AL. Furthermore, in Table 5, it is possible to confirm the specificity of the cleaving though the terminal chain of the peptides obtained in both enzymatic hydrolysis: the CY mainly showed hydrophobic amino acid while AL showed many peptides with glutamic acid in terminal chain.

3.2. Molecular weight profiles of okara hydrolysates

The molecular weight distribution of the hydrolysates AOK and NAOK, obtained by AL and CY, and the respective non-hydrolysed samples, were monitored by FPLC, as shown in Fig. 1. Unhydrolysed NAOK and AOK profile, under similar conditions used for hydrolysis by AL (Fig. 1 A and C), exhibited both high and low molecular weight fractions. Unhydrolysed AOK (Fig. 1 A) showed a relevant peak with molecular weight (MW) ranging between 160-22 kDa, (peak 1), a peak 2 with MW lower than 8.5 kDa (average MW of 1.3 kDa) and from the peak 3 representing peptides of low MW (< 1 kDa). The unhydrolysed NAOK showed differences compared to AOK, especially for peak 1 in Fig. 1 (A and C), for which is possible to observe a decrease of 1.5 times after thermal treatment. Thus, it was demonstrated that the applied thermal treatment (121 ºC, 1 atm and 15 minutes) broke part of the high weight proteins present in the okara. When AOK and NAOK were subjected to AL hydrolysis, at high DH, the high molecular weight polypeptides were broken into smaller fragments, releasing smaller peptides (< 2 kDa) as shown in the Fig. 1 (B and D). Both samples (AOK and NAOK) showed a similar profile after hydrolysis. Likewise, in a previous study, Chiang

et al. [33] obtained small peptides with a molecular weight < 4 kDa from a soy protein isolate (SPI), hydrolysed with a mixture of Flavourzyme: Alcalase enzyme. Furthermore, the concentration of peptides after hydrolysis increased as shown in Fig. 1 (B and D) since the peak area increased by 78% and 144%, for AOK (37.9 % DH) and NAOK (33.8%), respectively, compared with unhydrolysed samples.

The enzymatic hydrolysis by CY induced a lower DH. However, based on the observed MW profile, it is evident that the proteins with high MW were broken, generating fragments with lower MW. AOK hydrolyses by this enzyme generated two main peaks, as shown in Fig. 1 (F), peak 3 and 4 (< 1 kDa). In addition, the signal of both peaks increased ca. 1.5 times when compared to unhydrolysed samples. The same was observed for NAOK hydrolysed with CY. The peak 1 (30 KDa) and peak 8 (< 1 KDa) of unhydrolysed samples shown in Fig 1(G) disappeared and the intensity of peak 3 (<1 kDa) in Fig. 1 (H) increased 2.2 times. Studies evaluating okara hydrolysis are scarce, so these results showed for the first time that okara hydrolysed by CY and AL generate smaller fragments, mainly < 1 kDa, confirming previous studies with SPI obtained from soy meal hydrolysed by Corolase PP [13] and with okara protein hydrolysed by Trypsin [11] . Additionally, results with CY are presented for the first time for vegetable protein, considering the fact that CY is an enzyme associated with milk coagulation [21], and applications were only tested on whey protein [34] and yeast hydrolysis [35].

3.3. Free amino acids of okara hydrolysates

Previous studies showed that the ratio of essential amino acids in okara was similar to tofu and soy milk, and this was explained by the fact that some soluble protein may remain in the interstices of the particles [2]. Wang and Cavins [36] analysed the profile of total amino acids

in soybeans, soy beverage and okara, wherein the byproduct presented a similar content for total amino acid analysed.

The free amino acids (FAA) profile analysed in NAOK and AOK, unhydrolysed and hydrolysed with AL and CY (E/S ratio of 3.3 and reaction time 2.5 h and 5 h) are shown in Table 3. The thermal treatment of okara affected the composition of FAA. Unhydrolysed NAOK, for both pH conditions used in enzymatic hydrolysis, presented three essential amino acids (valine, phenylalanine and leucine). While AOK showed only two essential amino acids for each hydrolysis condition: AL (phenylalanine and leucine) and CY (phenylalanine and isoleucine). Besides, the unhydrolysed NAOK showed about two times more FAA than AOK. In previous studies, Fontaine et al. [37] observed that autoclave treatment in soy meal may reduce the total lysine and reactive lysine content. On the other hand, Dajanta et al. [38] compared the FAA in autoclaved and boiled soybeans, and observed that autoclaved soy presented-about two times more FAA than boiled soybeans.

The main FAA identified in both samples of okara were aspartic acid, glutamic acid, arginine and phenylalanine (Table 3). Furthermore, analysis of amino acid profiles presented in this study showed that FAA changed with the thermal treatment applied, with enzyme and hydrolysis time. For example, hydrolysis with CY for 2.5 h presented the higher FAA content for both samples, and the glutamic acid increased with enzymatic hydrolysis for AOK, while the hydrolysis using AL showed decrease in glutamic acid. This may be related to the fact that asparagine and glutamine can be converted to aspartic acid and glutamic acid respectively, while other amino acids are preserved, but this is more evident in strong acidic conditions [39].

The different composition for FAA can be related with the specificity of the enzymes. AL showed a large specificity, for example, due to the presence of a hydrophobic binding area

on the enzyme molecule, it has preference for aromatic and hydrophobic amino acids. But AL also has specificity for glutamic acid [18]. On the other hand, the CY extract has aspartic proteases and cleaves bonds flanked by hydrophobic amino acid residues with large side chains, such phenylalanine and leucine [22].

3.4. RP-HPLC peptide profile

Several studies have focused on bioactive peptides derived from food proteins and physiological effects are related to peptide with low MW [40], [10]. Thus, the hydrolysates obtained in this work were studied by RP- HPLC that allows us a preliminary study of the okara peptide composition [28]. The chromatograms showed differences between the profiles of hydrolysed and unhydrolysed samples, for peak 1, before 5 minutes, and it is more evidenced in hydrolysates of AOK by CY (Fig 2 D). Besides, a major elution of molecules between 0 and 16 minutes (Fig. 2) stage can be observed, where the mobile phase is 75% water, i.e. more hydrophilic. Thus we can predict that the okara hydrolysates present peptides that have in its constitution hydrophilic amino acids such as aspartic acid, glutamic acid, threonine, histidine, serine, lysine, arginine and glutamine. However, AOK hydrolysed by AL (Fig. 2 H) showed peptides with more hydrophobicity (peak 5 and 6). According to previous studies, these peptides are generally associated with biological activities such as antioxidant [40] and antihypertensive [41] activities. This can also be observed in this study, where AOK hydrolysed by AL showed higher antioxidant and antihypertensive activities (Fig. 3 and Table 4) than CY extracts.

3.5. Antioxidant activity (ABTS radical)

Some reactive oxygen species (ROS) are responsible for oxidative reactions in the human body, which are generally converted into harmless species by living cells enzymatic antioxidants. In addition, the intake of antioxidants through food can also help eliminating ROS, producing beneficial health effects [40]. The antioxidant activity of the hydrolysates NAOK and AOK was measured *in vitro* using the ABTS scavenging assay. Fig. 3 shows the antioxidant activity obtained for hydrolysates where is evident the higher antioxidant activity generated by AL compared to CY (3.02-4.27 vs 0.03-0.20 mg Trolox/mL, respectively). Statistical analysis indicated significant differences between the factors analysed (ratio of E/S, thermal treatment, reaction time) and comparing E/S ratio in the same group (reaction time and thermal treatment). CY extracts showed lower antioxidant activity (Fig. 3 B) than AL extracts (ca. 20 times less in AOK and ca. 50 times less in NAOK). In addition, the interaction between reaction time and the E/S ratio showed significant differences ($P < 0.05$) for hydrolysis performed under the same thermal treatment applied in okara (AOK or NAOK). Furthermore, comparing the thermal treatment in okara (NAOK and AOK) for different reaction time and E/S ratio, the antioxidant activity in AOK showed an increase ($P > 0.05$) compared with NAOK. Additionally, the control of AL extracts (without enzyme added) also showed higher antioxidant activity than control of CY extracts, which can be associated with a greater solubilization of the proteins in AL extracts than CY extracts. The high antioxidant activity observed for okara hydrolysates was attributed to peptides generated during the hydrolysis reaction and are related with its structure, composition and hydrophobicity. Peptides could react with free radicals stopping their chain reaction and converting them into more stable products. Furthermore, in AL hydrolysates, the resulting antioxidant activity is highly correlated with DH in both treatments, AOK ($r = 0.91$) and NAOK ($r = 0.88$), corroborating Sbroggio [9], Corrêa et al. [42] and De Oliveira et al.[43],

who showed that the degree of hydrolysis affects the ABTS quenching ability. The extensive hydrolysis induced formation of shorter peptides and free amino acids, that generated smaller and more hydrophilic groups thus being more accessible to water-soluble radical-ABTS, as previously suggested by Sbroggio et al. [9]. On the other hand, as expected, the hydrolysates of CY showed a low correlation between antioxidant activity and DH, AOK ($r = 0.64$) and NAOK ($r = 0.04$).

All 20 amino acids found in proteins have the potential to interact with free radicals, but some amino acids have been described as more prone to react, namely aromatic amino acids (tyrosine, phenylalanine and tryptophan) and amino acids with nucleophilic sulphur in side chains (cysteine and methionine). However, the antioxidant activity of the amino acid residues is limited by their tertiary structure, but through the enzymatic hydrolysis it is possible to increase this capacity since the protein cleavage leads to the exposure of antioxidant amino acids [15]. Thus, considering the profile of FAA, AOK hydrolysates produced by AL showed high concentration of tyrosine and phenylalanine. Besides, the main protein in okara is glycinin (11S) that contains cysteine and methionine residues in their structure, and cysteine is considered one of the most active amino acids contributing towards the antioxidant activity assessed by the ABTS [44].

3.6. ACE inhibitory activity

Several studies have evidenced the potential of antihypertensive peptides from foods or byproducts, relating their activity with inhibition of ACE. This is crucial for blood pressure regulation by modulating the renin-angiotensin system. The ACE converts the decapeptide angiotensin I into octapeptide angiotensin II, a potent vasoconstrictor, which leads to an

increase in blood pressure; thus the ACE inhibition has an antihypertensive effect [45]. Previous studies associated the positive health effects of soy with its antihypertensive activity, either fermented and hydrolysed soy protein [13], [41]. For characterization of ACE-inhibition activity of NAOK and AOK hydrolysed (with AL and CY) and non-hydrolysed, IC_{50} was determined and results are presented in Table 4. The unhydrolysed samples showed low or no significant inhibition ($IC_{50} \geq 530$ μg protein/mL), but a significant increase in ACE-inhibition (lowering IC_{50} values) was observed for all samples hydrolysed with AL and CY, indicating a potential for ACE-inhibition. Considering that low values of IC_{50} represent high ACE-inhibitory activity, a small amount of protein was required to produce 50% ACE inhibition. Although all hydrolysates showed very good ACE inhibitory activity (IC_{50} <100 μg protein/mL), those from AL showed higher ACE inhibitory activity (22.11 to 9.97 μg protein/mL) than CY (48.59-62.55 μg protein/mL) (see Table 4). In addition, in a previous study, [46] related antihypertensive activity using an aqueous extract from okara sterilized and their oligopeptides fractions.

Potential antihypertensive activity has been reported in previous studies for soy protein hydrolysates, with values for ACE inhibitory activity between 78 to 177 μg protein/mL [13,47]. These values are lower than those observed in the present study, which showed higher potential of ACE inhibition especially when AL enzyme was used. But Chiang et al. [47] reported that SPI hydrolysates obtained with AL showed an increase of ACE inhibitory activity from 668 to78 μg protein/mL in 6 h of hydrolysis. Additionally, the okara hydrolysates, obtained for the first time with CY have shown higher inhibition activity compared with hydrolysates obtained from whey protein concentrate using the same enzyme (48.6 vs 72 μg protein/mL, respectively) [34]. However, it is important to highlight that the antihypertensive activity reported in most previous studies using AL correspond to protein

concentrates or isolates, while the present activity is associated with total okara. In fact, this is a relevant result for a complete food ingredient that combines other health benefits such us functional lipids and fiber. It can then be concluded that okara hydrolysates produced with AL or CY have a potential antihypertensive activity that has never been reported before.

3.7. Identification of peptide in hydrolysed from okara by mass spectrometry

Samples with the highest DH and bioactivity values, from each analysed condition, were selected for peptide sequencing by MALDI stabilities via resonance structures. This property improves the radical scavenging capacity of the amino acid residues. Thus, as depicted in Table 5, aromatic amino acids-TOF/TOF: NAOK and AOK either hydrolysed by AL and CY (5 h of reaction time and an E/S ratio of 3.3). The main peptides were identified in each sample and the majority has never been previously reported in the literature (Table 5). In general, peptides from AL and CY hydrolysates were rich in hydrophobic amino acids, such as proline (P), leucine (L) and isoleucine (I). According to Sarmadi and Ismail [48] the presence of certain amino acids, their hydrophobicity and positioning in the peptide sequence determine the antioxidant activity. Some examples of amino acids that can have antioxidant activity are tyrosine (Y), tryptophan (W), methionine (M), lysine (K) and cysteine (C). The aromatic residues in amino acids can donate protons to electron deficient radicals maintaining their stabilities via resonance structures. This property improves the radical scavenging capacity of the amino acid residues. Thus, as depicted in Table 5, aromatic amino acids were present in both enzymatic hydrolysates and some peptides presented the aromatic residues located in the carboxyl terminus. Furthermore, the higher antioxidant activity found in hydrolysates obtained of AL compared with CY may be related with proline, which is present

in greater quantity in the peptides generated by AL, as shown in a previous study by Kitts and Weiler [49] for antioxidant peptides isolated from soybean.

On other hand the antihypertensive activity is the most studied bioactivity in peptides generated from food. The ACE-inhibitory peptides are usually rich in hydrophobic amino acid residues and have proline (P), lysine (L), arginine (R) or aromatic amino acids (F, Y, W) as a carboxyl terminal. In addition amino acids as R, H, W, Y and F, have important characteristics for the ACE inhibitory properties, especially for peptides with more than four amino acids residues [49]. Thus, most of the peptides identified (Table 5) shared part of specific structural characteristics described above and their composition was rich in proline, arginine, leucine or phenylalanine. Furthermore, the positive charge on the C-terminal, contribute substantially to inhibitory potency, and according to Quirós et al. [31], the substitution of proline for arginine in a model peptide increased twice the activity. Thus, this can explain the ACE inhibitory found for hydrolysates obtained by AL and CY.

4. Conclusions

The selected thermal treatment (sterilization at 121 ºC, 1 atm and 15 min) facilitated the enzymatic attack and the DH (degree of hydrolysis), particularly when AL was used: a 1.7 time increase in DH was observed in autoclaved okara. The antioxidant activity of dried Okara (either autoclaved or not) hydrolysed with AL was higher (4.2 mg Trolox/ mL) than that observed when CY was used (0.12 mg Trolox/ mL). On the other hand, the potential antihypertensive activity was high for samples hydrolysed with both enzymes, AL and CY, however higher ACE inhibitory activity was also found for AL (22.11-9.97 µg /mL) than for CY (62.55-48.59 µg/mL).

The current study also enabled the identification of several new peptide sequences in dried okara (autoclaved and not autoclaved) hydrolysed with AL and CY, and some sequences could explain the bioactivities found for these hydrolysates.

These results indicate that is possible to obtain valuable extracts from okara with different biological properties depending on the enzyme used to promote the hydrolysis. This suggests okara hydrolysates can either be used as functional ingredient or as possible food supplement for blood pressure lowering or antioxidant applications in future.

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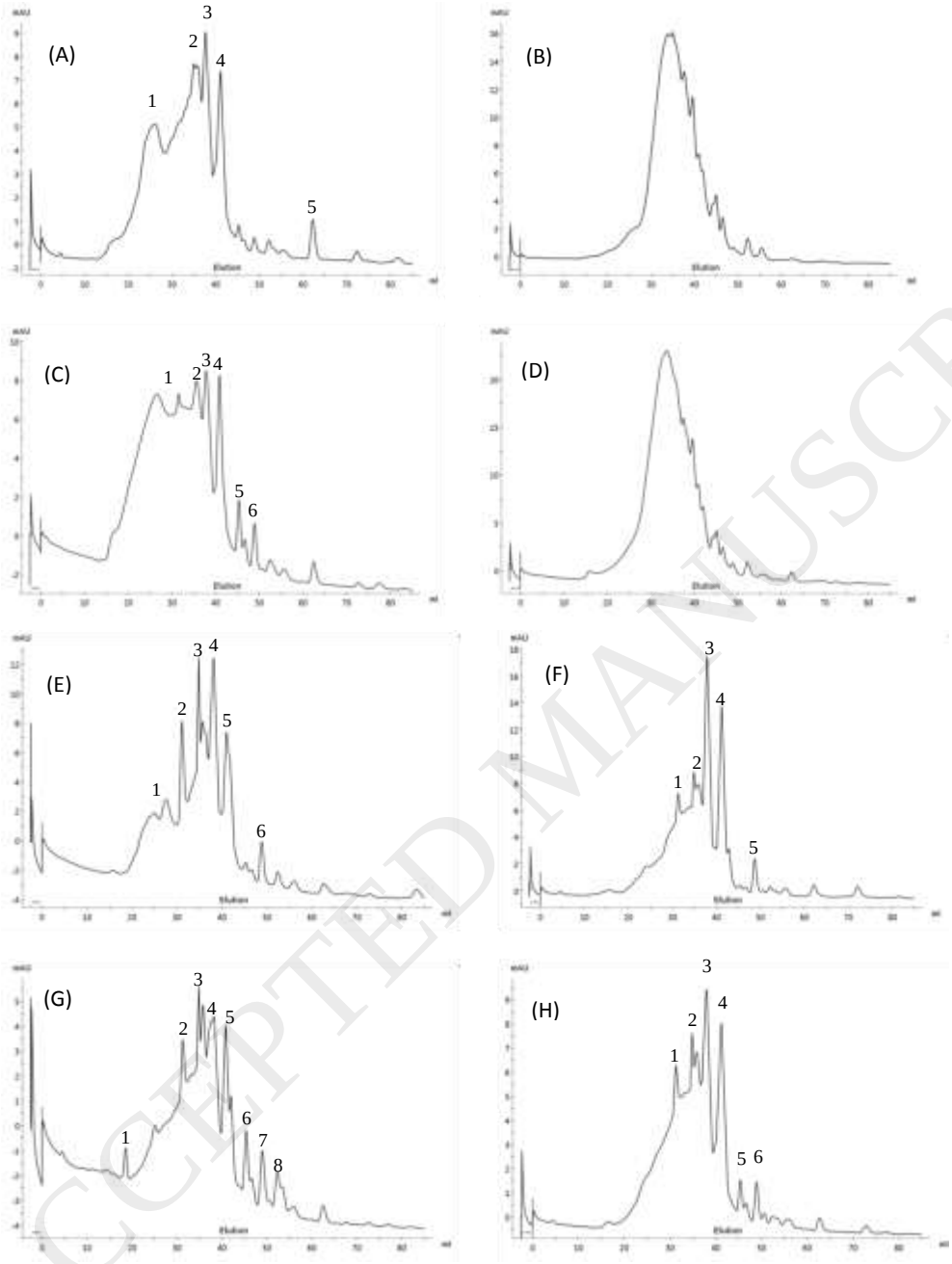
Figure 1. Typical size exclusion chromatograms of hydrolysate produced by Alcalase (AL) (A) control AOK, (B) AOK (37.9 % DH), (C) control NAOK and (D) NAOK (33.8 % DH) and by *Cynara cardunculus* extract (CY) (E) control AOK, (F) AOK (3.6 % DH), (G) control NAOK and (H) NAOK (2.3 % DH).

Figure 2. Peptide profile of hydrolysate produced by *Cynara cardunculus* extract (CY) (A) control NAOK, (B) NAOK (5 h and 3.3 E/S ratio), (C) control AOK and (D) AOK (5 h and 3.3 E/S ratio) and Alcalase (AL) (E) control NAOK, (F) NAOK (5 h and 3.3 E/S ratio), (G) control AOK and (H) AOK (5 h and 3.3 E/S ratio).

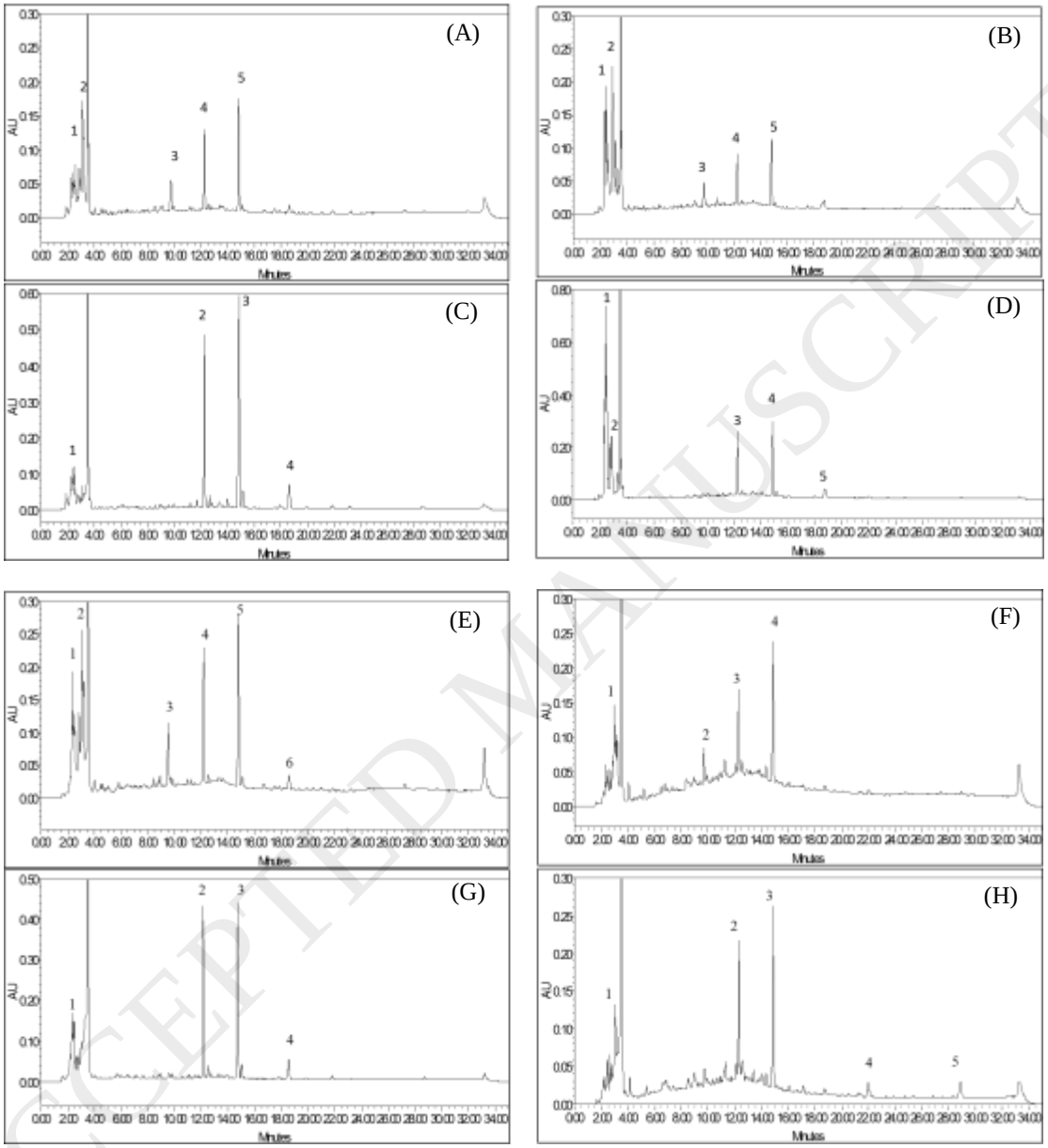
Figure 3. Antioxidant activity of hydrolysates from okara using (A) Alcalase (AL) and (B) *Cynara cardunculus* extract (CY).

Figure 1



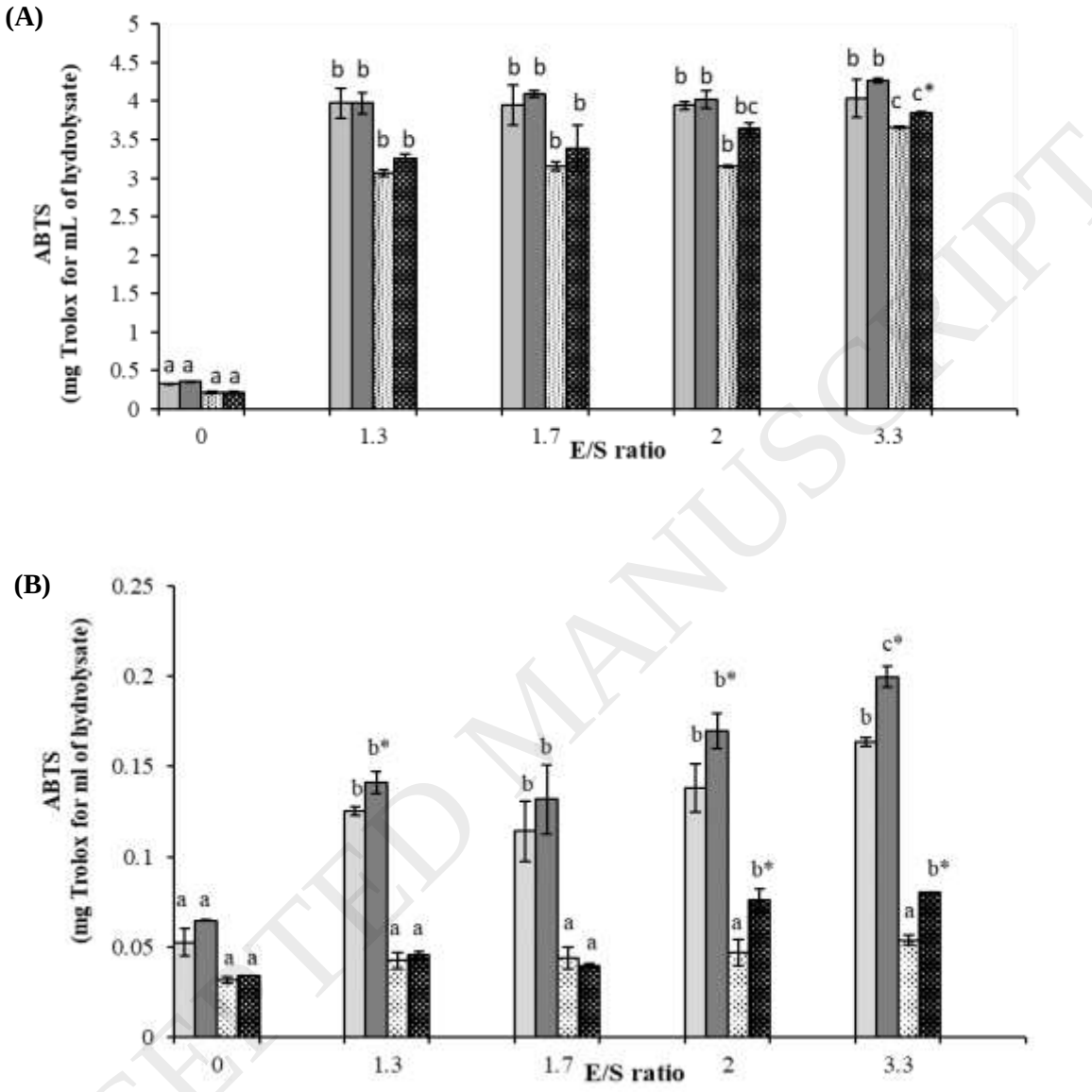
* Different numbers identify the peaks with different molecular weights for each sample.

Figure 2



* Different numbers identify the different peaks of peptide profile for each sample.

Figure 3



[a] Different lower letter between different E/S ratio in the same group (reaction time and thermal treatment) have significative difference. *Difference between reaction time (2.5 and 5 h) analyzing same group (thermal treatment and E/S ratio).

Table 1. Summary of experiment conditions tested: okara sample, type and concentration of enzyme and time of hydrolysis.

Table 2. Degree of hydrolysis (%) of hydrolysates obtained from dry okara autoclaved (AOK) and dry okara not autoclaved (NAOK) using Alcalase (AL) and *Cynara Cardunculus* extract (CY).

Table 3. Free amino acids in unhydrolysed and hydrolysed NAOK and AOK with Alcalase and *Cynara Cardunculus* extract for 2.5 and 5 hours.

Table 4. ACE-inhibitory activity IC_{50} (µg protein/mL) for NAOK and AOK hydrolysed with Alcalase and *Cynara Cardunculus* extract and unhydrolysed (control).

Table 5. Proteins and major peptide sequences identified by MALDI-TOF/TOF using PMF or PMF+MS/MS approaches in AOK and NAOK hydrolysed with Alcalase and *Cynara cardunculus* extract.

Table 1

Sample	Enzyme	E/S	Time (h)	Sample	Enzyme	E/S	Time (h)
NAOK	Control	0	0	AOK	Control	0	0
			2.5				2.5
			5.0				5.0
	AL	1.3	2.5		AL	1.3	2.5
			5.0				5.0
		1.7	2.5			1.7	2.5
			5.0				5.0
		2.0	2.5			2.0	2.5
			5.0				5.0
		3.3	2.5			3.3	2.5
			5.0				5.0
	CY	1.3	2.5		CY	1.3	2.5
			5.0				5.0
		1.7	2.5			1.7	2.5
			5.0				5.0
		2.0	2.5			2.0	2.5
			5.0				5.0
		3.3	2.5			3.3	2.5
			5.0				5.0

E/S – ratio of enzyme: substrate (% v/v); NAOK- dry okara not autoclaved; AOK- dry okara previously autoclaved; AL – Alcalase; CY - *Cynara cardunculus* extract.

Table 2

Enzyme	Time (h)	Sample	E/S			
			1.3	1.7	2.0	3.3
AL	2.5	NAOK	13.14 ± 1.05^{a}	15.38 ± 0.19^{a}	15.65 ± 0.26^{a}	28.56 ± 1.28^{b}
		AOK	22.03 ± 0.27^{a}	21.08 ± 0.12^{a}	21.54 ± 0.54^{a}	33.69 ± 2.33^{b}
	5.0	NAOK	12.42 ± 3.11^{a}	24.98 ± 2.23^{b}	28.11± 0.10^{bc}	33.80 ± 0.18^{c}
		AOK	21.72 ± 0.81^{a}	31.25 ± 1.64^{b}	31.99 ± 0.49 ^{bc}	37.87 ± 1.43^{c}
CY	2.5	NAOK	1.87 ± 0.05^{a}	2.02 ± 0.09^{a}	1.80 ± 0.11^{a}	2.00 ± 0.10^{a}
		AOK	1.93 ± 0.47^{a}	1.52 ± 0.05^{a}	1.94 ± 0.11^{a}	2.49 ± 0.11^{a}
	5.0	NAOK	2.16 ± 0.02^{a}	1.42 ± 0.06^{b}	1.85 ± 0.24^{b}	2.26 ± 0.01^{a}
		AOK	3.36 ± 0.04^{a}	1.72 ± 0.04^{b}	2.28 ± 0.23^{c}	3.57 ± 0.18^{a}

Means in the same line with common lower letters are not significantly different ($P < 0.05$).

Table 3

		NAOK[a]						AOK[a]					
		Unhydrolysed		2.5 h[b]		5.0 h[c]		Unhydrolysed		2.5 h[b]		5.0 h[c]	
Enzyme	**Amino acid**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**	**mg of amino acid g^{-1} protein**	**SD**
Alcalase (AL)	Aspartic acid	1.96	0.05	1.60	0.28	1.51	0.10	1.28	0.00	1.66	0.08	1.49	0.08
	Glutamic acid	3.30	0.67	1.84	0.05	0.68	0.04	2.02	0.02	2.03	0.01	1.25	0.41
	Arginine	1.09	0.03	0.88	0.20	0.71	0.01	0.64	0.04	1.17	0.11	0.75	0.01
	Tyrosine	-	-	0.29	0.09	0.50	0.01	-	-	1.00	0.06	0.54	0.03
	Valine	0.19	0.03	0.25	0.09	1.37	<0.01	-	-	0.80	0.02	0.25	0.05
	Methionine	-	-	-	-	1.01	<0.01	-	-	0.52	0.03	0.09	0.01
	Phenylalanine	1.48	0.08	1.07	0.39	1.83	0.65	0.64	<0.01	6.53	0.14	1.77	<0.01
	Leucine	0.09	0.01	0.09	0.02	0.30	0.01	0.06	<0.01	0.28	<0.01	0.18	0.01
	Total	8.11		6.02		7.90		4.64		13.98		6.32	
***Cynara cardunculus* extracts (CY)**	Aspartic acid	2.10	0.05	2.34	0.07	2.08	0.03	1.40	<0.01	1.55	0.08	1.10	0.03
	Glutamic acid	3.53	0.71	3.25	0.23	3.44	0.04	0.56	<0.01	1.81	0.07	1.42	0.09
	Arginine	1.17	0.03	1.42	<0.01	1.24	0.04	0.78	0.05	0.72	<0.01	0.53	0.01
	Tyrosine	-	-	0.18	0.01	0.17	0.02	-	-	0.08	0.03	0.06	0.02
	Valine	0.21	0.03	-	-	0.17	0.07	-	-	-	-	-	-
	Phenylalanine	1.58	0.08	1.85	0.28	1.25	0.08	0.77	<0.01	1.00	0.02	0.77	0.05
	Isoleucine	-	-	-	-	0.31	0.02	0.20	<0.01	-	-	-	-
	Leucine	0.10	0.01	0.13	<0.01	0.11	0.01	-	-	0.09	<0.01	0.06	<0.01
	Total	8.69		9.24		8.77		3.72		5.18		3.95	

(-) Not detected. [a] NAOK (dry okara not autoclaved) and [a] AOK (dry okara autoclaved). [b] Enzymatic hydrolysis with E/S ratio of 3.3 % v/v and 2.5 hours. [c] Enzymatic hydrolysis with E/S ratio of 3.3 % v/v and 5 hours.

Table 4

ACE-inhibitory activity IC_{50} (µg protein/mL)				
Sample	**AOK**		**NAOK**	
	AL	**CY**	**AL**	**CY**
Control	536.90 ± 0.91		>800	
E/S 3.3 and 2.5 h	21.87 ± 0.51	48.59 ± 7.22	22.11 ± 5.31	59.09 ± 6.70
E/S 3.3 and 5 h	9.97 ± 0.63	54.30 ± 0.70	12.33 ± 3.97	62.55 ± 3.74

Values are expressed as average ± standard error (n= 2). AOK: dry okara previously autoclaved, NAOK: dry okara not autoclaved, AL: Alcalase and CY: *Cynara cardunculus* extract.

Table 5

	AOK				NAOK			
	MW (DA)	**PEPTIDES**	**PROTEIN**	**MS/MS SCORE**	**MW (DA)**	**PEPTIDES**	**PROTEIN**	**MS/MS SCORE**
Cynara cardunculus extract (CY)	862.44	V.SIIDTNSL.E	Glycinin G1-P04776		862.44	V.SIIDTNSL.E	Glycinin G2-P04405 and Proglycinin-Q549Z4	
	949.47	L.DQMPRRF.Y						
	1209.57	F.LVPPQESQRR.A	Glycinin G2-P04405		906.49	H.RVEFEGGL.I and S.GFSKHFLA.Q	Glycinin-Q43452	
	1331.67	Y.NFREGDLIAVPT.G			949,47	L.DQMPRRF.Y		
	1387,63	P.TDEQQQRPQEE.E			1149.52	S.EGGFIETWNP.N		
	1402.73	N.NPFKFLVPPQES.Q			1190,62	N.QLDQMPRRF.Y		
	1433.71	R.GSQSKSRRNGIDE.T			1331.68	F.NNQLDQTPRVF.Y and E.DEQIPSHPPRR.P		55
	1486.71	G.KGIFGMIYPGCPST.F	Glycinin G1-P04776		1515,82	R.SQRPQDRHQKVH.R		
	1532,79	Y.TNGPQEIYIQQGKG.I			1523.76	L.IAVPTGVAWWMYN.N		
	1541,82	Y.ALNGRALIQVVNCNG.E			1607.81	M.PRRFYLAGNQEQE.F	Glycinin G2-P04405 and Proglycinin-Q549Z4	
	1633.82	P.QDRHQKIYNFRE.G			1628,82	L.NGRALVQVVNCNGER.V		
	1699.91	V.FDGELQEGRVLIVPQ.N			1699.92	Q.EGGVLIVPQNFAVAAKS.Q		
	1741.91	L.NGRALIQVVNCNGERV.F			1741.92	A.LNGRALVQVVNCNGER.V		
	1892.97	L.KSQQARQVKNNNPFSF.L	Glycinin G2-P04405	77	1892.99	L.KSQQARQVKNNNPFSF.L		58
	1948.05	L.KSQQARQIKNNNPFKF.L		83	1892.99	S.KRSRNGIDETICTMRL.R		
	2075.08	A.NSIIYALNGRALIQVVNCN.G	Glycinin G1-P04776		1948.96	I.TTATSLDFPALWLLKLSA.Q		

	703,36	P.HFNSKA.I	β-conglycinin:Q948X9, O22120, P13916	63	718.39	V.NKPGRF.E			
	774.386	P.SQVQELA.F	and Q94LX2		750.38	S.RDPIYS.N			
	795.38	P.QHPERE.P			755.44	E.KNPQLR.D			
	718.4	V.NKPGRF.E			985,52	R.QFPFPRPP.H			
	750.38	S.RDPIYS.N			1068.56	E.REPQQPGEK.E			
	879.43	H.ADADYLIV.I			1098.64	L.AIPVNKPGRF.E			
	1048,55	V.LQRFNQRS.P			1181.64	L.FKNQYGRIR.V			20
	1068.57	E.REPQQPGEK.E			1361.71	F.PFPRPPHQKEE.R	β-conglycinin: P13916, Q94LX2. F7J075 and Q4LER5		
Alcalase (AL)	1302.74	E.GEIPRPRPRPQ.H		17	1378,7356	R.QFPFPRPPHQK.E			
	1361.72	S.QVQELAFLGSAQA.V	β-conglycinin: Q948X9		1411.76	N.PDNNENLRLITL.A and S.EDSELRRHKNK.N			36
	1378,74	R.QFPFPRPPHQK.E	β-conglycinin:Q948X9, O22120, P13916	34					
	1665.9	E.GEIPRPRPRPQHPE.R	and Q94LX2	27	1665.9	E.GEIPRPRPRPQHPE.R			
	1829.98	E.DEQPRPIPFPRPRQP.R	β-conglycinin: Q948X9	45	1829.98	E.DEQPRPIPFPRPQPR.Q			
	1829.98	E.DEQPRPIPFPRPQPR.Q	β-conglycinin:Q948X9, O22120, P13916	45	2074.05	E.DEDEQPRPIPFPRPQPR.Q			38
	2074.05	E.DEDEQPRPIPFPRPQPR.Q	and Q94LX2	63	2200.12	F.SREEGQQQGEQRLQESVIV.E			