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Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates

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Abstract

The objective of this work was to evaluate the antioxidant activity of protein hydrolysates obtained by the enzymatic hydrolysis of okara using an endopeptidase (Alcalase) and exopeptidase (Flavourzyme). The reaction was monitored by the pH-stat procedure in which five aliquots were collected during the hydrolysis by each enzyme, corresponding to different degrees of hydrolysis (DH). The antioxidant activities of the aliquots were evaluated by the ABTS, DPPH and FRAP methods. For the hydrolysates obtained using Alcalase, the antioxidant activities increased from: 68.6 to 99.5% (ABTS), 14.5 to 17.7% (DPPH) and 222.6 to 684.9 μM Trolox (FRAP), when the DH varied from 0 to 33.6%. With respect to Flavourzyme, the results were: 67.2 to 88.2% (ABTS), 9.5 to 18.5% (DPPH) and 168.0 to 360.3 μM Trolox (FRAP), when the DH increased up to 5.8%. The results showed that the protein hydrolysates had antioxidant capacities, which were influenced by the degree of hydrolysis and the type of enzyme.

Keywords: soy pulp; endopeptidase; exopeptidase; antioxidant capacity; bioactive peptides.

Practical Applications: Okara is a low-value byproduct of soybean obtained from soymilk processing. However, despite having a high protein content, it is usually used as animal feed or discarded, being an industrial problem due to the expensive treatment and final destination. The enzymatic hydrolysis of okara protein is a promising method to obtain value-added peptides and okara protein hydrolysates can be applied as an antioxidant ingredient in processed food products. This work intends to contribute by providing useful information for obtaining okara peptides a with antioxidant activity.

1 Introduction

Approximately 10% of the world soybean production is used directly for human food. Soybean has been commercialized *in natura* and as soy-derived products, such as textured soy, soymilk, *tofu*, fermented products (*miso*, *shoyo*, *tempeh*) and others (Riaz, 2006). The production of soymilk and *tofu* results in an insoluble byproduct called okara with little market value and usually used as animal feed. In this process, about one kilogram of wet okara is generated from every kilogram of soybeans (Wang & Murphy, 1996). Okara has a high nutritive value due to its high quality protein, fat, carbohydrates and fiber. With regard to the proteins initially present in soybeans, about 23% are retained in the okara (Liu, 2008). These proteins have a high nutritive value and superior protein efficiency ratio, indicating that okara is a potential source of low cost vegetable protein for human consumption (O'Toole, 1999). Currently considerable quantities of this material are underutilized as animal feed or discarded, constituting a waste of proteins which could be used in human food.

In order to recover the nutrients normally discarded in okara and decrease its treatment as waste to meet market demands and opportunities, the soymilk and *tofu* industries must develop new products and/or ingredients based on this by-product. The enzymatic hydrolysis of okara proteins could be a potential method to reuse okara and an alternative way of recovering and obtaining value-added bioactive peptides.

Enzymatic protein hydrolysis consists of the cleavage of protein molecules into small peptides of various sizes, and eventually amino acids. Peptides are specific protein fragments that can present biological activity, such as antioxidant, antimicrobial and antihypertensive properties, positively influencing human health (Cumby et al., 2008; Pazinatto et al., 2013; Ranamukhaarachchi et al., 2013; Magaña et al., 2015). According to Elias et al. (2008), antioxidant activity of proteins has been related to their amino acid composition. However, such property is limited by the tertiary structure, because many amino acids with antioxidant potential can be buried within the protein core where they are inaccessible to prooxidants. Therefore, enzymatic hydrolysis favors the exposure of antioxidant amino acids in proteins, increasing antioxidant activity of peptides. Several factors affect the antioxidant properties of protein hydrolysates, and depend on: type of protein and enzyme, degree of hydrolysis and pretreatment of the substrate (Elias et al., 2008; Pazinatto et al., 2013; Polanco-Lugo et al., 2014). This activity occurs by specific mechanisms including the capacity of amino acid residues to donate electrons to free radicals. Bioactive peptides have a great ability to reduce the reactivity of free radicals due to the exposure

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of amino acids that react more effectively with these radicals (Elias et al., 2008). Some authors have observed antioxidant activity in hydrolyzed soybean proteins due to the exposure of amino acids, resulting in high free radical scavenging activity, reducing power, inhibition of lipid oxidation and metal chelation capacity (Sanjukta et al., 2015; Zhao et al., 2013; Zhu et al., 2008b).

Nevertheless, the total antioxidant activities of vegetables cannot be evaluated by any single method, due to the complex nature of samples. Two or more methods should always be employed in order to evaluate the total antioxidant effects of vegetables (Nuutila et al., 2003, Georgetti et al., 2006). The capacity of antioxidant compound for scavenging free radicals should be assessed by two factors, that is, rate of scavenging radicals and number of radicals each antioxidant molecule can scavenge, which are determined inherently by the chemical structure of the antioxidant compound and also the free radicals. These two parameters can be measured by following the reaction with stable reference free radical such DPPH and ABTS, negatively and positively charged free radicals, respectively. In addition, a potent radical scavenging antioxidant often acts as a potent reductant (Re et al., 1999). The FRAP method measure the ability of antioxidant to reduce ferric Fe(III) ion to their respective lower valency state (Benzie & Strain, 1996).

Thus the objective of this work was to obtain protein hydrolysates from okara proteins by the action of two commercial proteases with distinct catalytic actions (endopeptidase Alcalase and exopeptidase Flavourzyme), with several degrees of hydrolysis, and to evaluate the antioxidant activity by *in vitro* methods.

2 Materials and methods

2.1 Material

The okara was donated by Cocamar Cooperativa Agroindustrial (Maringá, PR, Brazil) and stored in a freezing chamber at -18 °C. The main characteristics of the okara, obtained according to Association of Official Analytical Chemists (2012) procedures, were, on a wet basis: moisture content of 76.7 ± 0.7%, protein content of 6.1 ± 0.4%, fat content of 1.5 ± 0.1%, ash content of 1.2 ± 0.2%, total fiber content of 14.0 ± 0.4%, of which the insoluble and soluble fiber contents were 13.7 ± 0.4% and 0.3 ± 0.1%, respectively. The endopeptidase 2.4L Alcalase® (declared activity of 2.4 AU/g) and the exopeptidase 1000L Flavourzyme® (declared activity of 1000 LAPU/g) (Novozymes, Bagsvaerd, Denmark), obtained from *Bacillus licheniformis* and *Aspergillus oryzae*, respectively, were used for enzymatic hydrolysis. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 6-hydroxy-2,5,7,8- tetramethychroman-2-carboxylic acid (Trolox), were purchased from Sigma-Aldrich (St. Louis, USA). The other reagents used were of analytical grade.

2.2 Enzymatic hydrolysis of okara protein

The hydrolysis experiments were carried out in duplicate according to the pH-stat procedure as described by Adler-Nissen (1986), with modifications. For each experiment, 75 g of okara were thawed overnight and homogenized with 150 g of distilled water (okara:water ratio of 1:2, w/w) in a jacketed 250 mL beaker. The mixture was stirred constantly using a magnetic stirrer and heated using a thermostatically controlled water bath with external circulation (TE-2005 model, Tecnal, Piracicaba, Brazil). After reaching the desired temperature, the pH was adjusted with 1N NaOH. The tests were carried out at 55 °C and pH 9.0 for Alcalase, and 50 °C and pH 7.0 for Flavourzyme. The enzyme (Alcalase or Flavourzyme) was added to the mixture in an enzyme:substrate ratio of 10g/100g protein, corresponding to 0.24 AU/g protein for Alcalase and 100 LAPU/g protein for Flavourzyme, and the hydrolysis was monitored by continuous titration with 1N NaOH in order to maintain the pH constant. The hydrolysis conditions for both enzymes were established according to previous results. The volume of NaOH consumed was recorded at regular time intervals until the end of protein hydrolysis (no change in pH) and used to calculate the degree of hydrolysis (DH, as described below) as a function of reaction time. These data were used to obtain the kinetics of the enzymatic hydrolysis (DH *versus* time). Based on these results, five hydrolysis experiments were carried out with different reaction times in order to obtain protein hydrolysates with different degrees of hydrolysis (Table 1).

After collection, the hydrolytic process was terminated by heating the aliquots to 85 °C for 20 minutes in a controlled temperature water bath, ensuring inactivation of the enzyme, followed by cooling in an ice bath. The mixture was centrifuged at 5,228 × g (5804R model, Eppendorf, Hamburg, Germany) at 4 °C for 20 minutes to separate the precipitate phase with lipid and fibers from the proteic phase.

The degree of hydrolysis (DH) is the percent ratio between the number of peptide bonds cleaved (h) and the total number of bonds available for proteolytic hydrolysis (h_{total}). In this work, the DH was obtained by the pH-stat method according to Adler-Nissen (1986).

2.3 Hydrogen-donating ability as determined by the DPPH Assay

The ability of protein hydrolysates with different DH values to scavenge DPPH radicals was measured according to Brand-Williams et al. (1995): 50 µL of each sample were added to the reaction mixture containing 1 mL of 0.1 M acetate buffer (pH 5.5), 1 mL of ethanol and 0.5 mL of 250 µM DPPH in an

Table 1. Aliquots with different degrees of hydrolysis (DH) obtained during protein hydrolysis by the enzymes Alcalase and Flavourzyme.

Enzyme	Aliquots	DH (%)	Time (min)
Alcalase	A1	0.0	0
	A2	8.4	5
	A3	16.8	25
	A4	25.2	75
	A5	33.6	180
Flavourzyme	F1	0.0	0
	F2	1.45	5
	F3	2.9	15
	F4	4.35	40
	F5	5.8	110

ethanolic solution. The change in absorbance was measured after 15 min at room temperature. The positive control was prepared in the absence of sample, and it indicated the maximum odd DPPH electrons, which was considered as 100% of free radicals in the solution to calculate the hydrogen-donating ability (%) of protein hydrolysates. The blank was prepared from the reaction mixture without DPPH solution. The DPPH scavenging ability was calculated by Equation 1. All measurements were made in triplicate.

$$\%\ of\ activity = \left(1 - \frac{A_S}{A_C}\right) \times 100 \quad (1)$$

where A_S is the sample absorbance and A_C is the control absorbance.

2.4 Scavenging ability of the protein hydrolysate using the ABTS method

The ability to scavenge the ABTS free radical was measured from the decrease in absorbance due to suppression of the colored radical. The determination was carried out according to Sánchez-Gonzalez et al. (2005) with some modifications. The ABTS radical cation was obtained after the reaction of 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The solution was diluted in phosphate buffer (pH 7.4, 0.1 M) until it reached an absorbance of 0.7 at 730 nm. 20 µL or 10 µL of the samples obtained after the action of the Flavourzyme and Alcalase enzymes, respectively, were added to 4 mL of the diluted ABTS solution. The absorbance was measured in a spectrophotometer at 730 nm after 6 minutes of incubation at RT. The positive control was prepared in the absence of sample and was considered to be 100% of free radicals in the solution in order to calculate the scavenging ability of the protein hydrolysates using Equation 1. All measurements were made in triplicate.

2.5 Evaluation of the ferric reducing antioxidant power (FRAP)

The ferric reduction antioxidant power of the protein hydrolysates was evaluated according to Benzie & Strain (1996), with modifications. The FRAP reagent was prepared using a 2,4,6 tripiridil-S-triazine (TPTZ) solution (10 mM) in HCl (40 mM), $FeCl_3$ ($6H_2O$) solution and acetate buffer (pH 3.6, 0.3 mM). For the assay, 900 µL of FRAP reagent were added to 90 µL of water and 10 µL of Trolox standard or 10 µL of sample. After incubation at 37 °C for 30 minutes, the absorbance was measured in a spectrophotometer at 595 nm. An analytical curve with different concentrations of Trolox (50 to 600 µM) was used for the subsequent calculation of the results in µmol/L of Trolox. All measurements were made in triplicate.

2.6 Statistical analysis

The results obtained for the chemical composition and antioxidant activities of the protein hydrolysates with various DH values represent the means of three determinations ± mean standard deviation. The antioxidant activity values were analyzed by the one-way analysis of variance (ANOVA) and Bonferroni multiple comparisons t-tests, using the software GraphPad Prism (version 6.00) at 5% significance.

3 Results and discussion

3.1 Kinetics of the enzymatic hydrolysis of the okara protein

The overall rate of enzymatic hydrolysis of the okara protein was evaluated, since the control of the reaction process is necessary to design and optimize the batch bioreactors and to predict the extension of protein bond cleavage (Kurozawa et al., 2009). Kinetic curves of DH *versus* time were drawn for both enzymes in duplicate (Figure 1) up to the end of protein hydrolysis. The mean deviation values of the experimental data for hydrolysis using Alcalase or Flavourzyme ranged from 0.3 to 0.6% and from 0.2 to 0.5%, respectively. As expected, an analysis of Figure 1 showed that the DH increased with hydrolysis time, showing a gradual release of peptide fragments during protein hydrolysis. The curves had a high initial reaction rate followed by a decrease up to the stationary period, in which the degree of hydrolysis became constant at 33.6% and 5.8% for Alcalase and Flavourzyme, respectively.

Similar hydrolysis curves have been reported for several protein substrates, such as peanut (Jamdar et al., 2010), *Jatropha curcas* seed cake (Selanon et al., 2014), rice protein (Li et al., 2016) and corn protein (Jin et al., 2016). Such behavior can be related to the reduction in peptide bonds capable of being cleaved, competition between the substrate and the hydrolysis products and enzyme denaturation that decreases its activity (Adler-Nissen, 1986).

At the end of the hydrolysis reaction, as characterized by a stationary phase, the protein hydrolysates produced by Alcalase and Flavourzyme presented different DH values of 33.6% and 5.8%, respectively, indicating that Alcalase cleaved more peptide bonds than Flavourzyme. Higher DH values were also obtained with Alcalase in the hydrolysis of corn protein (Jin et al., 2016), pumpkin oil cake protein isolate (Vaštag et al., 2011) and canola proteins (Cumby et al., 2008). However, several studies reported the opposite result: a greater DH being obtained for Flavourzyme. For instance, Bamdad et al. (2011) studied the effect of the type of protease on the hydrolysis process. Using the same enzyme concentrations as used in the present work, the authors verified that the Flavourzyme hydrolysis (100 LAPU/g protein) of hordein was relatively more extensive than the hydrolysis by Alcalase

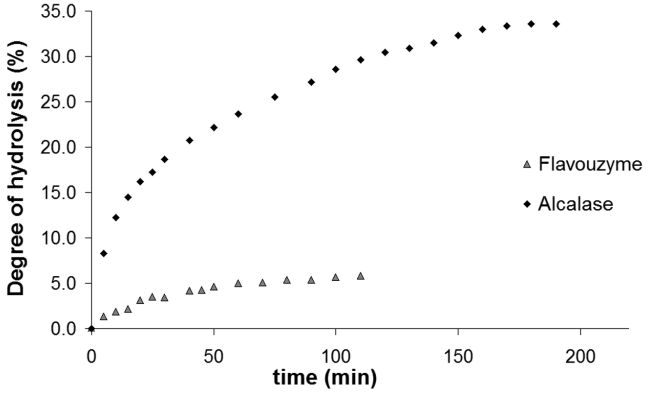

Figure 1. Kinetics of the hydrolysis of okara protein by Alcalase or Flavourzyme.

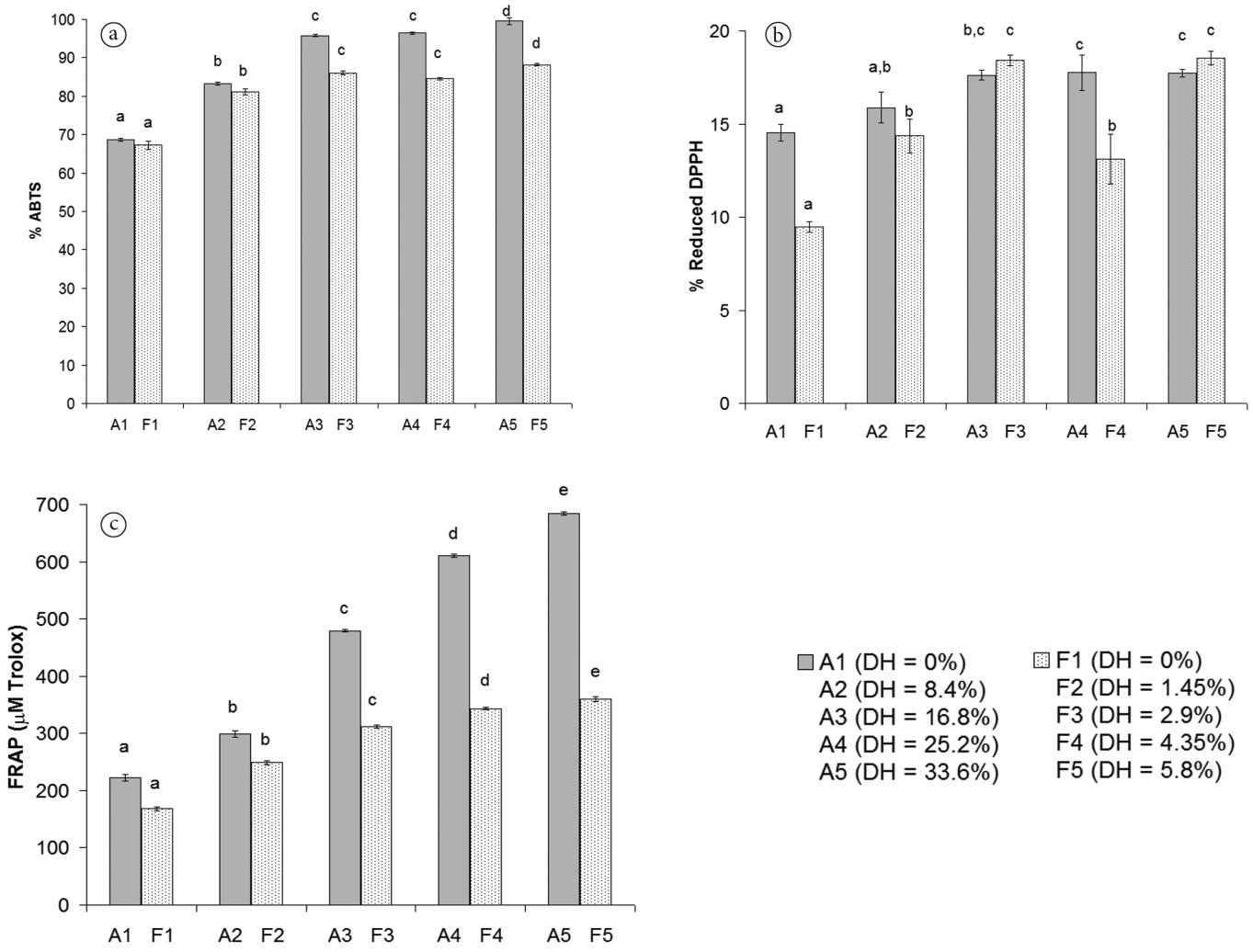

Figure 2. Scavenging abilities for the ABTS radical (a) and DPPH radical (b) and the reducing power activity (c) of the proteins (DH 0%) and protein hydrolysates with several degrees of hydrolysis (DH) obtained by the action of the enzymes Alcalase (A) and Flavourzyme (F). Different letters indicate that the samples were considered significantly different at the 5% level ($p < 0.05$).

(0.24 AU/g protein), which resulted in lower DH values. Alcalase is an endopeptidase and has a range of specificity of peptide bonds for hydrolysis. On the other hand, Flavourzyme is a mixture of an endoprotease and exopeptidase, produced by *A. oryzae*, giving it a broader range of action and thus a higher DH is expected when compared with hydrolysis by Alcalase (Pedroche et al., 2002). However, Vaštag et al., (2011) reported that the limited hydrolysis (lower DH) and the presence of both high and low molecular weight bands in the electrophoretic profile could indicate that the effectiveness of Flavourzyme towards pumpkin oil cake protein was due to exopeptidase activity.

3.2 Antioxidant activity

Two or more methods should always be employed to evaluate the antioxidant potential of any substance, since oxidative stress depends on the type of ROS generated, how and where it is generated, and the oxidative target evaluated (Georgetti et al., 2006; Nuutila et al., 2003). The ABTS and DPPH methods consist of verifying suppression of the colored radical in the medium, decreasing the absorbance. In the FRAP assay, antioxidants in the sample reduce the Fe^{+3}–TPTZ complex to form a blue-colored Fe^{+2}–TPTZ complex, which results in an increase in absorbance (Campanini et al., 2014).

As shown in Figure 2a, the protein hydrolysates showed higher ABTS free radical scavenging activity than the non-hydrolyzed samples A1 and F1. When the DH was increased from 0 (A1) to 33.6% (A5), the antioxidant activity values increased significantly ($p \leq 0.5$) from 68.6 to 99.5% for samples hydrolyzed with Alcalase, representing an increase of 45%. For Flavourzyme, the same behavior was observed, reaching a maximal value of 88.2% at a DH of 5.8% (F5), an increase of 30% when compared with the control sample F1.

The DPPH radical scavenging activities of the protein hydrolysates with different DH values can be seen in Figure 2b. This method is based on the hydrogen atom donation ability of the antioxidant, donating to the stable free radical DPPH• which has one unpaired electron (Brand-Williams et al., 1995). Higher

values were obtained for hydrolysates A3 and F3, with no significant differences between these samples and others with greater DH values (A4, A5, F5). For Alcalase, as the DH rose from 8.4% to 16.8%, the DPPH radical scavenging activity increased from 15.9 to 17.6% ($p < 0.05$). Similarly, the antioxidant activity of samples hydrolyzed with Flavourzyme reached maximum values of 18.4% at a DH of 2.9% (F3). Protein hydrolysis with Alcalase and Flavourzyme contributed to enhancing the DPPH radical scavenging activity, increasing about 20 and 95%, respectively, when compared with the non-hydrolyzed samples. The improvement of the ABTS and DPPH free radical scavenging activity of the protein hydrolysates at higher DH values could be attributed to the increase in the electron and hydrogen donating properties of the active peptides, respectively. These peptides could react with free radicals to convert them to more stable products and stop the radical chain reaction (Pazinatto et al., 2013).

However, the results obtained for the protein hydrolysates of both enzymes exhibited greater free radical scavenging activity with the positively rather than the negatively charged free radicals, ABTS and DPPH, respectively. According to Zhu et al. (2008a), these differences in scavenging patterns for the DPPH and ABTS radicals were possible associated with the structures of the peptides produced. Extensive hydrolysis would lead to the formation of shorter peptides (tri- and dipeptides) and free amino acids. Thus, the peptides became more hydrophilic and more accessible to the ABTS radical, which is water-soluble. Chen et al. (1998) verified that soybean peptides could not interact properly with hydrophobic peroxyl radicals such as DPPH, due to the hydrophilicity of the peptides.

The capacity of the okara protein hydrolysates to act as a reducing component was assessed by the ferric reducing antioxidant power (FRAP) assay (Figure 2c). The protein hydrolyzed by Alcalase presented higher ferric-reducing antioxidant power than that hydrolyzed by Flavourzyme. In addition, the reducing power increased significantly with DH, reaching 684.9 μM Trolox for A5 (DH 33.6%) and 360.3 μM Trolox for F5 (DH 5.8%). In other words, the antioxidant activities of the protein hydrolysates obtained using Alcalase and Flavourzyme (A5 and F5) were 3 and 2-fold higher than those of the non-hydrolyzed proteins (A1 and F1), respectively. According to Wang and Xiong (2005), the strong reducing power of the protein hydrolysates could be a result of the increase in availability of hydrogen ions (protons and electrons) due to cleavage of the peptide bonds. Proton donation could occur at specific side-chain groups or specific peptide structures. Wiriyaphan et al. (2011) reported that the protein hydrolysate of surimi presented an antioxidant activity of only 60 μM Trolox. Thus the results found in the present work showed that the okara peptides had a high iron ion reducing capacity.

Generally, the results demonstrated that higher antioxidant activities were obtained for both Alcalase and Flavourzyme protein hydrolysates with greater DH values. Several authors have reported the positive effect of DH on antioxidant activity. For instance, Jamdar et al. (2010) and Karamać et al. (2014) verified that, as the DH increased, so the ferrous ion chelating activity and DPPH radical scavenging activity of peanut and flaxseed cake proteins hydrolysates increased. According to Elias et al. (2008), the antioxidant activity of proteins can be related to their amino acid compositions. However, this property of the amino acid residues is limited by their tertiary structure, because many amino acids with antioxidant potential can be buried within the protein core, where they are inaccessible to pro-oxidants. Therefore enzymatic hydrolysis favors the exposure of the antioxidant amino acids in proteins, increasing the antioxidant activity of the peptides. All 20 amino acids found in proteins have the potential to interact with free radicals; however, the most reactive amino acids tend to be those containing either nucleophilic sulfur-containing side chains (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine and phenylalanine) (Elias et al., 2008). For Polanco-Lugo et al. (2014), the high exposure of hydrophobic amino acids residues in *Phaseolus lunatus* protein hydrolysate may have an impact on antioxidant activity. Stanojevic et al., (2012) reported that the main protein found in okara is glycinin (11S protein), whose peptides contain cysteine and methionine residues (Murphy, 2008). Waliszewski et al. (2002) verified the presence of the following antioxidative amino acids in okara: cysteine (12.5 g/kg protein), methionine (10.6 g/kg protein), tyrosine (34.3 g/kg protein), phenylalanine (48.4 g/kg protein) and tryptophan (11.4 g/kg protein). Thus the presence or absence of such amino acids in the peptides would affect the antioxidant activity of the hydrolysate.

In the present work, higher antioxidant activity values were observed for the okara proteins hydrolyzed by Alcalase. However, comparing hydrolysates with similar DH values for both enzymes, samples A2 (DH 8.4%) and F5 (DH 5.8%), higher antioxidant activity values were verified for sample F5 (ABTS 88.2%, DPPH 18.5% and FRAP 360.3 μM Trolox) than for hydrolysate A2 (ABTS 83.3%, DPPH 15.9% and FRAP 299.5 μM Trolox). The differences in the antioxidant activities of the hydrolysates with similar DH could be due to different catalytic actions and enzyme specificity, which can affect the number and location of the peptide linkages hydrolyzed. Thus, there is a release of different peptides in terms of size, level and amino acid composition (Polanco-Lugo et al., 2014). Alcalase is an endopeptidase that cleaves a protein bond located on the inside of the molecule, releasing two peptides of relatively substantial molecular weight. On the other hand, Flavourzyme is an exopeptidase that releases small molecular weight peptides or free amino acids, because it is capable of removing one, two, or three amino acid residues at a time from the N terminus, or one or two at a time from the C terminus (McDonald, 1985). Cumby et al. (2008) observed the Flavourzyme hydrolysate (DH 6.3%) of canola protein showed greater scavenging activity against the DPPH radical and reducing power than the Alcalase hydrolysate (DH 20.6%).

4 Conclusion

The okara protein hydrolysates presented higher ABTS and DPPH free radical scavenging activities and ferric reducing antioxidant powers than the non-hydrolyzed okara protein. The antioxidant activities were more visible when using the ABTS and FRAP assays than when using the DPPH method and protein hydrolysates with higher DH values had greater antioxidant activity. This study demonstrated that the enzymatic hydrolysis of okara protein was a potential process for obtaining

bioactive peptides with high antioxidant activity from a by-product normally discarded or underutilized as animal feed.

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