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Antioxidant hydrolysates production from Argentine anchovy *(Engraulis anchoita)* with different enzymes

Piotrowicz, I. B. B. and *Mellado, M. M. S.

Federal University of Rio Grande, Food and Chemistry School, Rio Grande, Brazil

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Abstract

Hydrolysates of Argentine anchovy were produced with Alcalase, Flavourzyme and Protamex, checking the degree of hydrolysis and antioxidant activity through inhibition of lipid peroxidation, scavenger effect of free radical DPPH and reducing power. The maximum value of the degree of hydrolysis obtained was 78.26 ± 1.66%, with the hydrolysate obtained in 5 hours of reaction with Alcalase, that also showed the greatest inhibition of lipid peroxidation (23.38%). The hydrolysate obtained with Protamex in 3 hours of reaction was more effective for scavenger free radical DPPH (53.32 ± 0.44%), whereas in the reducing power, the enzyme Alcalase with 3 and 5 hours of reaction produced the most active hydrolysates. The Protamex hydrolysate had a high percentage of essential amino acids compared with the Alcalase hydrolysate, and the presence of hydrophobic amino acids like histidine, proline, methionine, tyrosine and phenylalanine could be the reason for the bioactivity of hydrolysates increase.

Introduction

Argentine anchovy *(Engraulis anchoita)* has coastal habits and is distributed mainly from the San Jorge Gulf (Argentina) to Rio de Janeiro (Brazil), where it has been little explored. One of the reasons why some species of fish are discarded or considered low commercial value products, is specially due to the dark meat they present, susceptible to oxidation and residual taste (Thiansilakul *et al*., 2007). Fish protein hydrolysed is obtained by a process in which the plant and / or microbial enzymes act as biological catalysts that accelerate the hydrolysis of proteins by promoting its isolation from different species. Proteins as a result of cleavage of their peptide bonds are processed into peptides of different sizes and free amino acids (Zavareze *et al*., 2009). Different proteases have been investigated for the production of protein hydrolysates such as Alcalase, Flavourzyme and Protamex, utilized by Cheung and Li-Chan (2010) in hydrolysates of shrimp *(Pandalopsis dispar)*, verifying that the type of enzyme affects the bioactivity of the peptides.

Many proteins have been shown to present strong antioxidant activity against lipid peroxidation in fatty acids system. Thus, hydrolysates of different protein sources have received increased attention because they are sources of bioactive peptides (Je *et al*., 2005). Different raw materials like egg-yolk (Sakanaka *et al*., 2003), egg white (Chen *et al*., 2012), the pine nut *(Pinus gerardiana)* (Cai *et al*., 2013), blue mussel *(Mytilus edulis)* (Wang *et al*., 2013), the skipjack roe *(Katsuwonous pelamis)* (Intarasirisawat *et al*., 2012), Alaska Pollack (Je *et al*., 2005), herring *(Clupea harengus)* (Sathivel *et al*., 2003), horse mackerel *(Magalaspis cordyla)* and croaker *(Otolithes ruber)* (Kumar *et al*., 2012), mackerel *(Scomber austriasicus)* (Wu *et al*., 2003), silver carp *(Hypophthalmichthys molitrix)* (Zhong *et al*., 2011), skipjack tuna *(Nemipterus hexodon)* (Nalinanon *et al*., 2011), sardinelle *(Sardinella aurita)* (Khaled *et al*., 2011), pacific hake *(Merluccius productus)* (Cheung *et al*., 2012), among others, have shown antioxidant activity.

There are several methods to evaluate the in vitro antioxidant activity of biologically active substances, ranging from chemical assays with lipid substrates to more complex assays (Picot *et al*., 2010; Zhong *et al*., 2011). The scavenger effect of free radicals is determined not only by the reactivity of the radical antioxidant but also by its concentration in the medium (Alves *et al*., 2010).

The amino acid composition of many protein foods has a significant role in various physiological activities of the human body and affects others directly or indirectly in maintaining health. The amino acids are essential for the synthesis of proteins with a variety of important functions, including transport of oxygen, vitamins, CO_2, enzymes and structural proteins (Chalamaiah *et al*., 2012). The aim of this study was to obtain protein hydrolysates of Argentine anchovy by the action of different

*Corresponding author.
Email: *mysame@yahoo.com*
Tel: 55-53-32338621; Fax: 55-53-32338745

proteases in different hydrolysis times, to check the degree of hydrolysis, antioxidant activity and amino acids composition.

Material and Methods

Material

The raw material used in this work was Argentine anchovy, caught in southern Rio Grande do Sul (Brazil) from where it was taken directly to the industry for washing and removal of head and viscera. The fish was grinded (HI TECH HT/2500-Brasil) thus obtaining the Argentine anchovy minced, and stored at -20°C (Refer CHB/53) until use. The proteolytic enzymes used were Alcalase® 2.4L and Flavourzyme ® 1000L, obtained from Novozymes Latin America Ltda and the enzyme Protamex from *Bacillus* sp. purchased from Sigma-Aldrich. Other reagents, such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid and 2,4,6-Trinitrobenzenesulfonic acid were from Sigma-Aldrich.

Proximal composition

Proximal composition of raw material was carried out according AOAC (2000). The moisture content (method n° 935.29), ashes (method n° 923.03), lipids (method nº 920.85), proteins (micro-Kjeldahl method, n° 920.87).

Protein hydrolysis process

To obtain the Argentine anchovy hydrolysates, a substrate was used at a concentration of 2.0% (wprotein / vbuffer) and enzyme (1.0% wenzyme / wprotein). The enzymes were applied at the respective optimum pH, for Alcalase (pH 8.0), Flavourzyme (pH 7.0) and Protamex (pH 6.5). The reaction was conducted in a double wall glass reactor, connected to a thermostatic bath (BROOKFIELD TC/102 – USA), maintained at temperature of 50°C for all experiments. Three reaction times: 1, 3 and 5 hours were tested. After the indicated period of hydrolysis, the inactivation of the enzyme was done by heating the medium at 90°C for 15 minutes. After cooling to room temperature, the hydrolysate was centrifuged (3220 xg/15 min) (Biosystems MPW-350/350-R - Brazil). The supernatant was subjected to filtration (WHATMAN N°1) and the filtrate was lyophilized (LIOTOP L108, Brazil), obtaining different dried hydrolysates.

Degree of hydrolysis of the minced fish hydrolyzed

The degree of hydrolysis (DH) was determined using the method described by Adler-Nissen (1979), using the 2,4,6-trinitrobenzenesulfonic acid (TNBS). Solutions of 125 mg hydrolysate diluted in 25 ml of 1.0% SDS was used. The solution was heated at 50°C for 15 minutes (QUIMIS Q/215-1/2 - Brazil) and then cooled to room temperature. 250 µl of the sample, added with 2 mL of 0.2 M phosphate buffer pH 8.2 and 2 ml of 0.1% TNBS were placed in amber vials, mixed and heated in a water bath at 50°C for 60 minutes. The reaction was inhibited by the addition of 4 ml of 0.1M hydrochloric acid. The samples were allowed to stand at room temperature for 30 minutes and were analyzed by UV-visible spectrophotometer at 340 nm (Varian UV-VISIBLE CARY/100 - USA). A standard curve of L-leucine was using for quantification, applying the following equation.

$$\%DH = (x_1.14{,}007.V_1.d.100)/(w.V_2.10000)$$

Where the listed ηmol of leucine was read from the standard curve (x_1), the volume (V_1) of the initial sample prepared (25 mL) and the dilution factor (d) with the mass (w) used in the test (125 mg) and the volume (V_2) used for the reaction (250 µl).

Inhibition of lipid peroxidation

This analysis was performed by the method described by Osawa and Namiki (1985) using a model system containing linoleic acid. 5 mg of the hydrolysate were dissolved in 10 ml of sodium phosphate buffer 50 mM (pH 7.0). An aliquot of 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol was added. The suspension was homogenized under stirring (PHOENIX AP56 - Brazil) and completed to 25 mL. The commercial antioxidants α-tocopherol (diluted in 99.5% ethanol) and ascorbic acid (diluted in buffer) at the same concentrations were used as standards. A control sample was prepared without hydrolysate or commercial antioxidant, containing only the phosphate buffer. The set was incubated in the dark at 40°C and then analyzed for 24-hour period for seven days. The daily analysis consisted of measuring the oxidation of linoleic acid according to Mitsuda *et al*. (1966). Thus, 100µL incubated sample was mixed with 4.7 mL of 75% ethanol, 100µL of 30% ammonium thiocyanate and 100 µL of 0.02 M of ferrous chloride in 3.5% HCl in amber bottles. This mixture was allowed to stand for 3 minutes, and read in a spectrophotometer (BIOSPECTRO SP/22, Brazil) at 500 nm. The percentage of inhibition shown by oxidation hydrolysates was calculated using the following equation.

$$\%Inhibition = [(1-ABS_{sample})/(ABS_{control})]x100$$

The inhibition percentage is connected with the absorbance of the sample (ABS_{sample}) with the control ($ABS_{control}$).

Scavenger effect of free radical DPPH

The measure of antioxidant activity was performed according to Shimada *et al.* (1992) based on the scavenger effect of hydrolysates on free radical 2,2 - diphenyl - 1 - picrilidrazil (DPPH). A volume of 1.5 mL of hydrolysate diluted in water (5.0 mg/mL) was mixed with 1.5 mL of 0.1 mM DPPH solution in 95% ethanol. After 30 minute the samples were analyzed in a spectrophotometer (BIOSPECTRO SP/22, Brazil) at 517 nm. The lower absorbance means the greater scavenger effect of the hydrolysate by DPPH radical. Results were determined using the following equation:

$$\%SE=[1 - (ABS_{sample} / ABS_{blank}k)]x100$$

The scavenger effect (SE) is connected with the absorbance of the blank (ABS_{blank}) and the absorbance of the sample (ABS_{sample}).

Reducing power

The reducing power was measured according to the method performed by Oyazu (1988). A sample of 2 mL of hydrolysate solution (5.0 mg/mL) was mixed with 2 mL of sodium phosphate buffer 0.2M (pH 6.6) and 2 ml potassium ferricyanide 1.0%. This mixture was incubated in a water bath at 50°C (QUIMIS Q-215-1/2 - Brazil) for 20 minutes, 2 mL of solution of TCA 10.0% was then added, 2 mL aliquots of the incubated samples were mixed with 2 mL of distilled water and 0.4 mL of solution 0.1% ferric chloride. After 10 minutes, the absorbance was determined at 700 nm in a spectrophotometer (BIOSPECTRO SP/22, Brazil). The increase in absorbance of the mixture indicates an increase of reducing power.

Amino acid profile

The composition of total amino acids was determined in hydrolyzed samples with 6N HCl at constant boiling point, containing 0.01% phenol, for 22h at 110°C. The amino acids were separated according to the separation protocol developed by Bidlingmeyer *et al.* (1984). A C18 Waters Pico-Tag with dimensions of 3.9 x150 mm was used. The chromatographic step was carried out at a constant temperature of 38.0 ± 0.1°C (CM4000-Brazil).

Statistical analysis

Data on degree of hydrolysis, scavenger effect of free radical DPPH and reducing power were analyzed using ANOVA and Tukey test (α=0,05) using the Statistica 7.0 program.

Results and Discussion

Proximal composition of raw material

The proximate composition of minced fish was 79.7% moisture, 16.5% protein, 2.0% fat and 1.2% ash. Furlan *et al*. (2009) obtained similar values (78,1% moisture, 17,5% protein, 2,4% fat and 2,0% ash) considering that the composition could varied with the sex, age of the fish and seasons (Yannes and Almandos, 2003).

Degree of hydrolysis

The extent of protein degradation by proteolytic enzymes was measured by assessing the degree of hydrolysis (DH), which is the most widely used indicator for the comparison of different protein hydrolysates (Bougatef *et al.*, 2010). Figure 1 shows the values of DH in percentage of hydrolyzed Argentine anchovy minced.

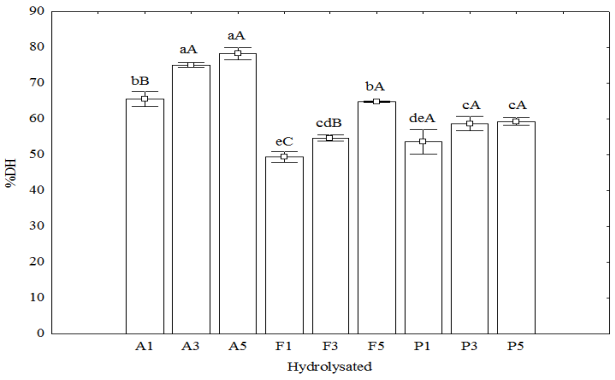

Figure 1. Degree of hydrolysis (mean ± standard deviation) of Argentine anchovy hydrolysates obtained with Alcalase (A), Flavourzyme (F) and Protamex (P) and different reaction times (A1, F1 and P1: 1 hour; A3, F3 and P3: 3 hours; A5, F5 and P5: 5 hours)
(a-e) significant differences in the degree of hydrolysis (% DH) among all the samples; (A-C) significant differences of degree of hydrolysis (% DH) between hydrolysed samples for the same enzyme at different hydrolysis times ($p < 0.05$).

Degree of hydrolysis increased in over reaction time. Alcalase produced one hydrolysate with highest DH (A5 = 78.6 ± 1.7%) compared to the others, with no significant difference with the hydrolysate A3. Hydrolysates with Flavourzyme presented significant difference in DH at different times, where the sample F1 showed the lowest value (49.42 ± 1.46%) among all hydrolysates. The hydrolysates produced with Protamex showed no significant difference with reaction time. Kechaou *et al.* (2009) with guts of cuttlefish and sardines used these same enzymes

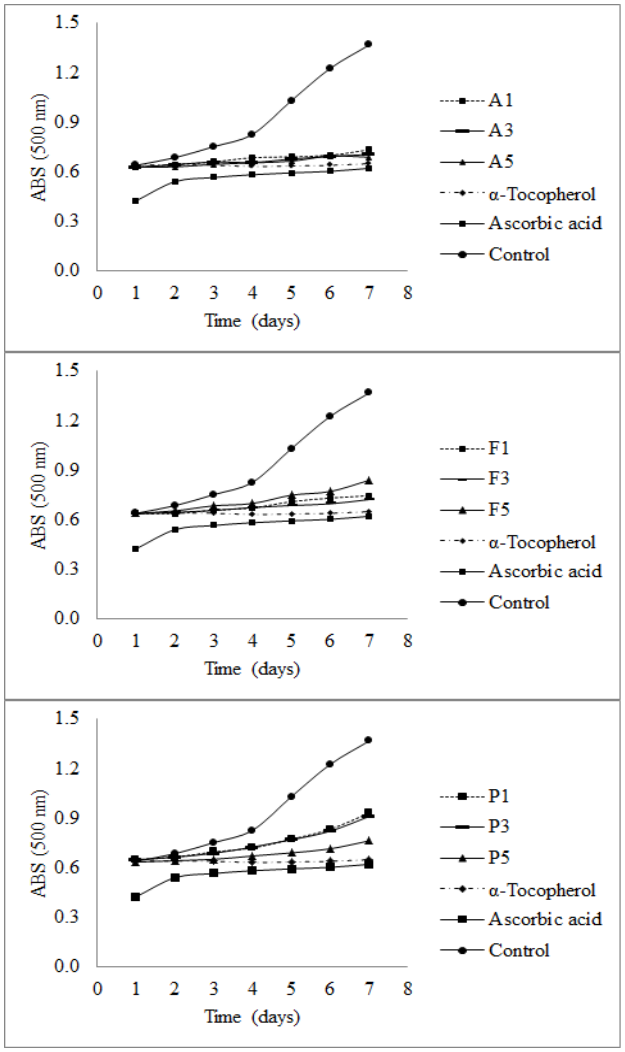

Figure 2. Peroxidation of linoleic acid expressed in ABS presented by hydrolysates obtained with Alcalase (A), Flavourzyme (F) and Protamex (P) and different reaction times (A1, F1 and P1: 1 hour; A3, F3 and P3: 3 hours; A5, F5 and P5: 5 hours), by α-tocopherol, ascorbic acid and control.

and obtained a similar relation, because the Alcalase was more active than Protamex and Flavourzyme enzymes, obtaining hydrolysates with higher DH, as Ovissipour *et al*. (2012) with the guts of persian sturgeon *(Acipenser persicus)*.

Alcalase, an endopeptidase, causes release of peptides of different sizes with amino groups exposed to act with trinitrobenzenesulfonic acid, having in the initial periods (1 to 3 hours), an increase of approximately 10% of DH with a smaller increase between 3 and 5 hours. Both enzymes, Flavourzyme and Protamex, are a mixtures of endopeptidases and exopeptidases, and work differently in peptide chain compared to the Alcalase, which had a slower action (Santos *et al*., 2011), which explains the low range, approximately 5%, in the initial hydrolysis.

The rate of increase of DH is greater at the initial time of reaction, decreasing over time. According to Guerard *et al*. (2002) the reduction of the reaction rate is due to the limitation of the activity of the enzyme, and the decrease in the concentration of available substrate. There was an increase in DH of the hydrolyzates with Flavourzyme in the reaction times, while with Protamex the hydrolysates of 1, 3 and 5 hours showed the same degree of hydrolysis. The specificity of the enzyme to the substrate can explain this fact, requiring more time to reach the same DH of other hydrolysates.

Liceaga-Gesualdo and Li-Chan (1999) obtained hydrolysates of herring *(Cuplea harengus)* using the enzyme Alcalase with DH of 60.0% after 1-hour reaction, and in this work for the same time it was obtained a value of 65.6 ± 2.1%, showing greater susceptibility of Argentine anchovy to the action of the enzyme Alcalase.

Thiansilakul *et al*. (2007) obtained hydrolysates of scad (*Decapetrus maruadsi)* with the enzyme Flavourzyme acting for 1 hour, with DH of 60.0%. The difference in the DH values between different species may be related to the activity of the enzyme on protein chains as well as the substrate upon which the enzyme will act is closely related to the final DH value (Santos *et al*., 2011).

With the enzyme Protamex the DH ranged from 53.6 ± 3.5% to 59.3 ± 1.1% for hydrolysates obtained after 1 and 5 hours of reaction, respectively. Hydrolysates of Protamex enzyme have been studied presenting various degrees of hydrolysis, depending on the type of raw material, concentration of substrate and enzyme, hydrolysis time, and the type of method for its determination. Kechaou *et al.* (2009) found DH values of 3.2% and 3.1% for squid and sardines viscera hydrolysates, produced by the enzyme Protamex in 24 hours of reaction, respectively. The low value related to Argentine anchovy hydrolysates, comes from the lower enzyme concentration (0.1%), which was ten times less than that used in this study.

Inhibition of lipid peroxidation

Figure 2 shows the absorbance values expressing the inhibition of lipid peroxidation for each hydrolysate, for α-tocopherol ,ascorbic acid and control, it was observed that the higher the absorbance the higher oxidation of the linoleic acid. It can be seen that with the increase of incubation time the system experienced higher oxidation. It was observed that the hydrolysates of Protamex and Alcalase enzymes showed a direct relationship between inhibition of peroxidation of linoleic acid and degree of hydrolysis, being the hydrolysates A5 and P5 the most active. This results is in accordance to some studies that reported the higher DH and the lower

Table 1. Scavenger effect of DPPH % (mean ± standard deviation) presented by Argentine anchovy minced hydrolysates obtained by Alcalase, Flavourzyme and Protamex enzymes, at different hydrolysis times

	Time		
	1 hour	3 hours	5 hours
Alcalase	20.7 ± 0.1^{aC}	38.9 ± 0.1^{bB}	43.4 ± 0.1^{bA}
Flavourzyme	37.0 ± 0.2^{bB}	20.81 ± 0.4^{cC}	45.4 ± 0.4^{aA}
Protamex	39.7 ± 0.1^{aB}	53.3 ± 0.4^{aA}	34.9 ± 0.4^{cC}

(a-c) in the same column indicate a significant difference between the hydrolysates obtained at different hydrolysis times. (A-C) in the same line indicate significant difference between the hydrolysates obtained with different enzymes ($p < 0.05$).

Table 2. Reduction power (mean ± standard deviation) expressed as absorbance (700 nm) of the hydrolysates from Argentine anchovy obtained with different enzymes and different hydrolysis times.

	Time		
	1 hour	3 hours	5 hours
Alcalase	0.175 ± 0.004^{aB}	0.269 ± 0.008^{aA}	0.262 ± 0.001^{aA}
Flavourzyme	0.088 ± 0.006^{bA}	0.012 ± 0.001^{cC}	0.050 ± 0.010^{bB}
Protamex	0.028 ± 0.005^{cB}	0.110 ± 0.006^{bA}	0.017 ± 0.003^{cB}

(a-c) in the same column indicate a significant difference between the hydrolysates obtained with the different enzymes. (A-C) in the same line indicate significant difference at different times of hydrolysis ($p < 0.05$)

peptide molecular weight, better is its antioxidant action (Je *et al*., 2005). Nevertheless, the amino acid composition often prevails and characterizes the best antioxidant activity, which may be evidenced by the hydrolysates obtained with Flavourzyme enzyme, which presented the hydrolysate with the highest degree of hydrolysis and with the highest antioxidant activity.

Zhong *et al*. (2011) produced hydrolysates of silver carp *(Hypophthalmichthys molitrix)* protein isolate using ultrafiltration for the separation of different fractions of peptides in which the inhibitory activity of lipid peroxidation was tested. The values obtained by these authors ranged from 10.9% to 69.8% of inhibition, while those found in hydrolysates of Argentine anchovy ranged from 5.2% to 23.4% for the hydrolysate obtained with Protamex enzymes for 1 hour and with Alcalase after 5 hours of reaction, respectively.

The ultrafiltration applied by Zhong *et al.* (2011) may have provided a greater purification and antioxidant activity of the peptides obtained, because studies showed that peptides purified from fish of different species, like horse mackerel *(Magalaspis cordyla)*, croaker *(Otolithes ruber)* (Kumar *et al*., 2012) and black scabbard *(Aphanopus carbo)* (Batista *et al*., 2010) showed high antioxidant activities. Moreover, according to Picot *et al*. (2010), several studies refer to the use of ultrafiltration to refine hydrolysates, to increase their activities, and to produce specific bioactive food ingredients.

Effect of the radical scavenger 2,2 - diphenyl - 1 - picrilidrazil (DPPH)

Table 1 shows the values of radical scavenger DPPH of hydrolysates with enzymes Alcalase, Flavourzyme and Protamex, in relation to the different reaction times. Hydrolysates with different DPPH radical scavenging effect were obtained with different enzymes and reaction times, with significant differences between all samples. For Flavourzyme and Alcalase enzymes, the hydrolysates obtained in 5 hours of hydrolysis were more effective. The greatest scavenger effect was found in the hydrolysates produced in 3 hours of reaction with Protamex.

Li *et al.* (2012) working with carp hydrolysate using the enzyme Alcalase with 2 hours of reaction and a sample of 7 mg/mL presented a value of 49.5% of scavenger effect, which is close to that achieved with 5 mg/mL of Argentine anchovy hydrolysate produced in 5 hours of reaction. Yellow stripe *(Selaroides leptolepis)* hydrolysates with Flavourzyme, obtained scavenger effect values close to 80% (Klompong *et al.,* 2007), almost twice the maximum amount found for Argentine anchovy with this enzyme. This may be due to the proportion of sample and DPPH used in the reaction, which was 4:1 (v: v) as compared with that of this study which was 1:1 (v: v). Thus, with a larger amount of sample present, the greater is the amount of hydrolysate available for DPPH radical scavenging, thus presenting a greater scavenger effect.

You *et al*. (2009) with loach *(Misgurnusanguilli caudatus)* hydrolysates with the enzyme Protamex obtained radical scavenger DPPH values between 80% and 90%, but with a solution concentration of 40 mg/mL, higher than that used in this work (5 mg/mL), which would explain the big difference between the results.

Reduction power

Table 2 presents the values of absorbance (ABS 700 nm) presented by the samples, being considered as reducing power. The higher the absorbance the greater the reduction of ferricyanide, thus correlating with a higher antioxidant capacity. Considering

Table 3. Profile of aminoacids (mean ± standard deviation) of Argentine anchovy hydrolysates produced by Alcalase and Protamex enzymes

Amino acids	mg /g protein					
	A5	P3	Infant mean*	2-5 years*	10-12 years*	Adults*
Asp	124.51±0.65	113.65±2.69	-	-	-	-
Gln	155.07±1.37	172.92±2.57	-	-	-	-
Ser	41.92±1.00	43.49±0.45	-	-	-	-
Gly	42.45±1.89	45.41±1.33	-	-	-	-
His	24.59±4.03	33.08±0.22	26	19	19	16
Arg	72.01±0.84	53.64±2.61	-	-	-	-
Thr**	47.85±0.30	45.88±1.94	43	34	28	9
Ala	66.39±1.48	61.95±0.70	-	-	-	-
Pro	26.54±0.93	39.12±7.69	-	-	-	-
Tyr	35.67±1.10	29.36±0.79	-	-	-	-
Val	47.15±1.52	49.53±0.83	55	35	25	13
Met**	43.34±3.85	40.25±0.36	42[a]	25[a]	22[a]	17[a]
Ile**	36.88±2.75	39.03±1.48	46	28	28	13
Leu**	91.07±0.14	99.08±0.76	93	66	44	19
Phe**	39.78±0.32	41.82±0.19	72[b]	63[b]	22[b]	19[b]
Lys**	103.11±3.61	111.39±2.0	66	58	44	16
Cys	nd	nd	-	-	-	-
Trp	nd	nd	17	11	9	5

hydrolysate with the Protamex enzyme in 3 hours of reaction. *Profile of essential amino acids suggested (FAO/WHO, 1991). **Essential Amino Acids. a: related to methionine + cysteine, b: related to phenylalanine + tyrosine; nd: amino acid not detected.

absorbance values, it was found that the enzymes produced protein hydrolysates with different reducing activities, and the products obtained with Alcalase in 3 and 5 hours were the most efficient in the reduction of ferric ion. It was found that the enzyme type influenced the reducing activity of hydrolysates, because for the same period of reaction it was verified a significant difference between the values.

This difference in the responses of radical scavenger DPPH and reduction power may have been due to different factors. As indicated by Rajapakse *et al. (*2005) and Chen *et al*. (1996) to explain the type of enzyme and substrates, their concentrations and the reaction medium during the process of hydrolysis, as well as composition, structure, hydrophobicity, and the presence of the amino acid in position within the sequence forming the peptide is very important for the presence of antioxidant activity in hydrolysates.

It was also observed that there was no correlation between the degree of hydrolysis and antioxidant power. This also occurred with Klompong *et al.* (2007) with yellow stripe *(Selaroides leptolepis)* hydrolysates where the product with the highest degree of hydrolysis showed a lower reducing power. This difference can be attributed to the composition of peptides and specific amino acids present in the hydrolysates (Wu *et al.*, 2003).

Amino acid profile

The amino acid composition of hydrolysed proteins is important due to nutritional value (amino acids), and can have an effect on its properties (Santos *et al*., 2011). Table 3 shows the amino acid belonging the two hydrolysates of Argentine anchovy minced, in which enzymes Alcalase (A5) and Protamex (P3) were used. These two hydrolysates produced with Argentine anchovy minced, presented the best results in antioxidant activity.

The essential amino acids were present in greater quantities in the hydrolysate with the enzyme Protamex, compared with the hydrolysate of Alcalase enzyme. Both samples have the amino acid profile above the threshold suggested by FAO / WHO (1991) for the consumption by children of 2-5 and 10-12 years and for adults. Hydrolysate with Alcalase showed the amino acids threonine and lysine above the limit for babies requirements, but with the hydrolysate P3, the amino acids histidine, threonine, leucine and lysine were higher than the rates recommended by the FAO.

There are many studies on the production of hydrolysates using the enzyme Alcalase, varying the substrate concentration, enzyme/substrate ratio, pH, temperature and hydrolysis time, and evaluating the amino acid profile of products obtained (Sathivel *et al*., 2005; Wasswa *et al*., 2007; Dong, *et al*., 2008; Ovissipour *et al*., 2009; Cheung *et al.*, 2010;

Ovissipour *et al*., 2012; Bougatef *et al.*, 2012).

Sathivel *et al*. (2005), with hydrolyzed salmon *(Oncorhynchus nerka)*, showed a lower value observed in most amino acids compared with the hydrolysate A5. This may be due to the lower concentration of enzyme, and shorter hydrolysis time (75 minutes). Total of hydrophobic amino acid content of A5 and P3 hydrolysates was 35.17 and 35.53 %, respectively. These values were similar to the study conducted by Bougatef *et al.* (2012) with tuna *(Thunnus thynnus)* heads hydrolysate using Alcalase, which was 34.8 % of the total amino acids.

Cheung and Li-Chan (2010) produced hydrolysates of shrimp by-products using both Alcalase and Protamex enzymes. With respect to Alcalase hydrolysate, only the amino acid proline showed a greater amount than the Argentine anchovy hydrolysate. For the hydrolysate from Protamex enzyme, two amino acids (arginine and tyrosine) were present in higher quantities in the shrimp waste hydrolysate, both being considered conditionally essential. These variations may be due to lower enzyme concentration and reaction time.

Based on total amino acid content, essential amino acids comprised 43.45% and 45.12% of the Alcalase and Protamex protein hydrolysates, respectively. The constituent amino acids and peptides in the sequence are important for their antioxidant activity. This has been demonstrated by the presence of hydrophobic amino acids and one or more residues of histidine, proline, methionine, cysteine, tyrosine, phenylalanine and tryptophan, which may increase the bioactivity of pept*ides (Je et al., 2007; Ren et al., 2008; You et al*., 2010). These amino acids were present in both hydrolysates, except cystein, which was not detected. The existence of hydrophobic sequences could interact with lipid molecules and could sequester, donating protons to the radicals derived from lipids (Je *et al*., 2007).

Conclusion

The antioxidant activity of Argentine anchovy hydrolysates produced with different proteolytic enzymes at different times of hydrolysis was studied, obtaining products with different degrees of hydrolysis and bioactive capability. The hydrolysates obtained with Alcalase in 3 h of hydrolysis and with Protamex in 5 h of hydrolysis, presented better activity and showed satisfactory values in the content of amino acids and may be beneficial for being functional products, due to its amino acid profile.

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