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Comparing the hydrolysis degree of industrialization byproducts of Withemouth croaker (*Micropogonias furnieri*) using microbial enzymes

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Abstract

Enzymatic modification of proteins is being increasingly studied, with the aim of adding commercial and nutritional values to byproducts. Thus this work aims to study the hydrolysis degree obtained by different enzymes and compare among different Withemouth croaker (*Micropogonias furnieri*) byproducts: mechanically separated meat, protein isolate and bones. To obtain the peptides, an enzymatic hydrolysis was performed, the reaction occurred in a thermostated bath using three separate enzymes under optimal conditions of each: Alcalase (pH 8.0, 50°C), Flavourzyme (pH 7.0, 50°C) and Protamex (pH 7.0, 40°C), the concentration of enzyme and protein substrate was 1:10 (U/g) according to the specific activity of each enzyme and when the hydrolysis process became constant the reaction was stopped by inactivating the enzyme at 85°C/10 min. The different enzymes tested showed the ability to hydrolyze proteins from both protein isolate and the demineralized croaker bones (DCB), with the DCB showing a higher hydrolysis degree, using the enzyme Protamex, and showing higher affinity for this enzyme by the substrate. It was also noted that the degree of hydrolysis is not correlated with the percentage of free tyrosine.

Introduction

Protein hydrolysis can occur by different sources of protein, in this work croaker (*Micropogonias furnieri*) which is the main species processed by the Rio Grande industries was chosen due to this species being captured in all four seasons, but despite of its wide availability, it reaches the lower market prices compared to other regional species, especially those of smaller size (Centenaro *et al*., 2009) and also because of the large amount of byproduct generated by fish processing industries, thus this fish being destined especially for the manufacture of flour or else discarded into the environment.

Hydrolyzed protein is defined as proteins that are cleaved chemically or enzymatically into peptides of various sizes to be produced for use in a wide variety of food products. However, if you want products with high functionality and nutritional value, the biological processes that utilize enzymes are considered the most promising (Martins *et al*., 2009). There are proteolytic enzymes classified as endo and exopeptidases. Endopeptidases hydrolyze the peptide bonds, usually at specific residues, producing large peptides. On the other hand, exopeptidases act on the terminal nitrogen of the amino acids forming the aminopeptidases or terminal carbon, producing carboxypeptidases (Torruco-Uco *et al.*, 2008).

Alcalase (endopeptidase) is an enzyme produced by submerged fermentation of the microorganism *Bacillus licheniformis*. Many researchers have proved that it is the best enzyme used in the preparation of various protein hydrolysates (Kristinsson and Rasco, 2000). Flavourzyme (a mixture of endoprotease and exopeptidase) is a complex fungal protease produced by submerged fermentation of a selected strain of *Aspergillus oryzae* that has not been genetically modified, acting on the hydrolysis under neutral or slightly acidic conditions (Slyzyte *et al*., 2005). Protamex is a bacterial protease from *Bacillus* sp. and is a mixture of endo and exopeptidases. It is used in the food industry to improve the functional, nutritional and flavoring properties of the protein (Novozymes, 2012).

Thus, the study of the hydrolysis conditions leads to obtaining a specific hydrolyzate, important for the preparation of products with unique functional properties and health-related beneficial characteristics being an indispensable tool to identify different bioactive compounds (Pacheco *et al.*, 2002). From that, this work aims to study the hydrolysis degree obtained by different enzymes and compare among different byproducts: mechanically recovered meat, protein isolate and bones of Whitemouth croaker.

Material and Methods

Enzymes and reagents

The enzymes used were Alcalase (endopeptidase of *Bacillus licheniformis*), and Flavourzyme (mixture

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of exopeptidase and endoprotease from *Aspergillus oryzae*), provided by Novozymes Latin America Brazil; and Protamex enzyme (a mixture of endo and exopeptidase from *Bacillus* sp.), obtained from bovine pancreas supplied by Sigma - Aldrich Co (St. Louis, MO, USA). The other reagents were of analytical grade (A.R.).

Obtaining the protein isolate from mechanically separated meat of Withemouth croaker

To obtain the mechanically separated meat, a byproduct of Withemouth croaker industrialization was used. It was transported to the FURG Laboratory of Food Technology, where it was washed with chlorinated water (2 g/L), gutted and separated in a mechanical meat separator, to remove the skin and spines, thus obtaining the MSM.

The protein isolate from the croaker MSM (Figure 1) was obtained by Fontana *et al.* (2009), this was homogenized with distilled water in the ratio 1:9 (w:v) at 2°C temperature controlled through an thermostated bath (QUIMIS model 214 D2, Brazil) for 5 min with stirrer propeller shaft (IKA model RW 20DZM.n, Brazil). After this process the solubilization of proteins was performed at pH 11.2 with addition of 1M NaOH for 20 min, the samples were then centrifuged at 7500 g for 15 min in the centrifuge cup in order to separate the lipid and insoluble proteins (precipitated) from the soluble proteins (supernatant). In the supernatant, isoelectric precipitation of solubilized proteins was carried out at pH 5.2 by adding 1 M HCl for 20 min and centrifugation was then held (7500 g for 15 min) to separate the insoluble fraction from the soluble fraction, facilitating the collection of the precipitated proteins. After these steps, the samples were dried at 35°C for 48 h, ground in a homogenizer (TECNAL, model TE -645, Brazil), sieved through 100 mesh sieve, and then stored at -18 ± 2°C.

Demineralization process of the croaker bones (CB)

Bones were obtained from the croaker, filleted and the meat separated manually from the muscle and left in a solution of HCl 0.1 mol/L for 24 h at 4°C for best removal of the adhered meat and then dried for 48 h in an incubator at 35°C, then this was ground in a Wiley mill (Thomas Scientific, Wiley Mill model, USA).

The process of demineralization of the bones was performed according to Alfaro *et al*. (2010) with modifications. The bones were mixed with 3% HCl solution in 1:5 (Bone:HCl) ratio, homogenized at 600 rpm propeller shaft homogenizer for 5 min and kept at 4°C for 24 h. The samples were washed with distilled H_2O at 35°C, the mixture was centrifuged at 9000 g for 20 min. This process was repeated again. The sedimented material was washed with distilled H_2O at 35°C until pH 6.0.

Obtaining enzymatic hydrolysates

The enzymatic hydrolysis of the different samples was based on the procedure described by Kristinsson and Rasco (2000), using enzyme concentration and protein substrate 1:10, depending on the specific activity of each enzyme.

Prior to the hydrolysis process, the endogenous enzymes contained in the MSM and DCB were inactivated in a water bath at 85°C for 15 min. The hydrolysis reaction was conducted in a glass reactor, double wall, connected to a thermostatic bath (TC/102 BROOKFIELD - USA), using three separate enzymes under optimal conditions of each: Alcalase (pH 8.0, 50°C), Flavourzyme (pH 7.0, 50°C) and Protamex (pH 7.0, 40°C) (Jung *et al*., 2006), and the enzyme concentration of protein substrate was 1:10 (U/g protein) according to the specific activity (Alcalase 99.75 U/g, Flavourzyme 2.07 U/g and Protamex 8.41 U/g). The hydrolysis degree was monitored throughout the process, this was determined according to item 2.7, when it became constant, the reaction was stopped by inactivating the enzyme (90°C/10 min) in a thermostated bath (QUIMIS, model 218.2 - Brazil). After cooling to room temperature, the supernatants from each hydrolysate were centrifuged (3220 g for 20 min) (BIOSYSTEMS MPW-350/350-R - Brazil), lyophilized (Liotop L108, Brazil) and stored at -18 ± 2°C for further analysis.

Degree of hydrolysis (DH)

DH was determined by the pH-stat method, according to Adler-Nissen (1986) apud Geirsdottir (2009) and calculated according to equation 1.

$$GH(\%) = \frac{BN_B}{\alpha h_{tot} \times MP} \times 100 \qquad (1)$$

where: h_{tot} is the number of peptide bonds, with 8.6 mol equiv/kg for fish (Adler - Nissen, 1986) and 8.41 mol equiv/kg for collagen (Zhuang *et al.*, 2009), B (ml) is the volume of base consumed during hydrolysis to maintain constant pH, and N_B the normality of base; MP is the protein mass (g, determined by N X the Kjeldahl factor), and α is the degree of dissociation.

Composition proximal

The proximate composition of the samples was performed as recommended by the AOAC (2000) methodologies. The analyses were conducted for moisture, protein, lipid and ash, with n° 960.39,

992.15, 925.30 and 923.03, respectively.

Statistical analysis

The results were expressed as mean ± standard deviation, submitted to Analysis of Variance (ANOVA) and Tukey's test applied for comparison of means ($p < 0.05$).

Results and Discussion

The proximal composition of mechanically separated croaker meat, the protein isolate of mechanically separated croaker meat (Table 1), bones and demineralized croaker bone (Table 2). Analyzing Table 1 and 2, we can see that for the MSM and IMSM samples, protein content increased from 81.2% to 93.1%, thus obtaining a protein isolate, because according to Uzzan (1998) apud Frota (2007) to be considered as protein isolate the protein content should be greater than 90%. The content of lipids and ash decreased 98.5% and 76.8%, respectively. Fontana *et al*. (2009) found values similar to those obtained in this work, reaching values of 97.7% protein, 1.5 % ash and 1.1 % fat for croaker pulp. With the process of bone demineralization of sea bass, the ash content was reduced by 32.8%, we can also see a decrease in the lipid content 22.7% and an increase in protein content in 27.8 % reaching 44.5 %. Alfaro *et al*. (2010) used bones from Macrodon ancylodon to study the effect of extraction parameters on the properties of gelatin and found lower protein values (28.08%) and higher ash (63%). Jung *et al.* (2006) using Alaska pollock (*Theragra chalcogramma*), found similar amounts of protein (43.5%), higher ash (42.2%) and lower lipid (13.3%).

We can observe in Figure 1, that for the enzymes Alcalase and Flavourzyme the HD obtained was 7.7 and 5.9% of IMSM and 14.7 and 15.5% for DCB. When they hydrolyzed defatted silver carp (*Hypophthalmichthys molitrix*) muscle using Alcalase and Flavourzyme (0.5:100w/w) enzymes, Dong *et al.* (2008) reached HD values of approximately 25 and 10%, respectively, and it was observed that the period of rapid reaction occurred within 60 min of hydrolysis. Zavareze *et al.* (2009) using Bluewing searobin (*Prionotus punctatus*) minced got 25.41% of HD with Alcalase enzyme and 23.68% with Flavourzyme, higher than those found in this study, with the HD measured for soluble protein and enzyme/substrate concentration of 0.5% for Alcalase and 2% for Flavourzyme, Alcalase and Flavourzyme with specific activity of 36.0 and 22.2 U/mg protein (activity unit (U) corresponds to 1 μmol of tyrosine /h).

Table 1. Proximal composition of mechanically separated croaker meat (MSM) the protein isolate of mechanically separated croaker meat (IMSM)

	MSM (%)	IMSM (%)
Moisture	78.5 ± 0.12	7.4 ± 0.11
Protein *	81.2 ± 0.19^a	93.1 ± 1.75^b
Lipids *	13.2 ± 0.11^a	0.2 ± 0.06^b
Ash *	5.6 ± 0.09^a	1.3 ± 0.09^b

All data was expressed as average (mean ± standard deviation, n = 3). values. *results expressed on a dry basis. Equal letters on the same line indicate that there is no significant difference between the samples ($p < 0.05$).

Table 2. Proximal composition of the bones (B) and demineralized croaker bone (DCB)

	B (%)	DCB (%)
Moisture	9.2 ± 0.8	6.4 ± 0.38
Protein *	34.8 ± 2.17^a	44.5 ± 0.26^b
Lipids *	25.5 ± 2.54^a	19.7 ± 0.43^b
Ash *	41.1 ± 0.51^a	27.6 ± 0.66^b

All data was expressed as average (mean ± standard deviation, n = 3) values. *results expressed on a dry basis. Equal letters on the same line indicate that there is no significant difference between the samples ($p < 0.05$).

Protamex enzyme showed higher HD reaching values of 30.5% and 21.5% for IMSM and DCB, respectively. Nguyen *et al.* (2011) hydrolyzed byproducts of yellow fin tuna (*Thunnus thynnus*) using the enzyme Protamex (0.1% w/w), and found similar behavior, where the period of intense hydrolysis reaction was 120 min, after the HD decreased to constant phase, reaching HD values of 32.3, 22.2 and 16.8% for the head, tail and viscera, respectively.

The best HD results were obtained when the DCB was used as substrate, compared with the ISMS despite this presenting less medium interfering for the enzymatic reaction, showing that the high lipid content seemed not to influence the enzyme activity, since demineralized bones have higher lipid content compared with the protein isolate. According to Matsushita *et al.* (1970) any enzyme inhibition can occur due to lipid oxidation. What may have happened for the IMSM to present lower values is the process of protein isolation, because it works with pH changes which may damage the protein structure and hinder the action of the enzyme. According to Mariotto (2008), the structure and shape of the active site are a result of the three-dimensional structure of the enzyme and may be affected by any agents capable of causing conformational changes in the structure.

The enzymes show different HD when the same raw material was used, which can be explained by the affinity of the enzyme by the substrate. Enzymes are highly efficient catalysts because they bind to the substrate (and cofactors) in the active site of the peptide bond stereospecifically oriented in the proximity of groups that perform the catalytic reaction, forming the enzyme substrate complex

Table 3. Percentage of free tyrosine protein hydrolysates of protein isolate of mechanically separated croaker meat demineralized croaker bone

	IMSM	DCB
	Tyrosine (%)	
Alcalase	2.8	9.0
Flavourzyme	3.0	11.5
Protamex	6.7	5.7

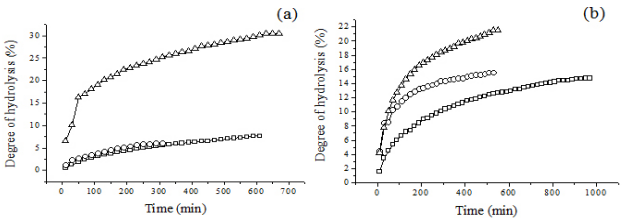

Figure 1. Graph of the hydrolysis degree of (a) the IMSM and (b) of the CB, with: (□)Alcalase, (○)Flavourzyme and (Δ) Protamex enzymes

(Whitaker *et al*., 2003).

The curves of degree of hydrolysis showed intense reaction to a period of 100 min for all the enzymes when DCB was used as substrate (Figure 1b), then a decrease in HD can be seen over time. As for the substrate IMSM, Protamex was the only enzyme to exhibit the same behavior (Figure 1a). The Alcalase and Flavourzyme enzymes exhibited a reaction, but less intense when the IMSM was used (Figure 1a). According to Guérard *et al.* (2002) the reaction kinetics can be divided into two different phases: a first phase in which the reaction rate is fast, which is an easy cleavage of the peptide, and the second stage where the reaction rate decreases due to hydrolysis of more compact proteins, or also due to the increased peptides. The lowest reaction may have occurred by enzymatic inhibition, where substances are capable of interfering, in a specific manner, in the rate of a reaction of enzymatic catalysis, reducing or delaying the process or biological specificity of the reaction (Marques and Hideko Yamanaka, 2008).

The initial heating of raw materials for inactivation of endogenous enzymes may also have affected the enzyme activity when IMSM was used a reason for the lower HD, this may already contain proteins of the sarcoplasmic fraction, unlike the DCB where collagen is the predominant protein. Mohr, apud Martins (2009) noted that, during heating to the temperature of hydrolysis, sarcoplasmic fraction proteins may denature and precipitate. This is most evident when the material in natura is heated before to inactivate the endogenous enzymes of fish. These denatured proteins are apparently highly resistant to enzymatic breakdown, consequently, only a minority of the denatured protein is solubilized during the subsequent enzymatic hydrolysis.

The percentage of free tyrosine (Table 3) was also higher when the substrate CB was used for all enzymes tested. Looking at Figure 1 and Table 3, we note that the HD and the percentage of free tyrosine are not proportional.

Thus the observed difference in cleavage of protein for each enzyme, with endopeptidases Alcalase and Protamex and Flavourzyme, a mixture of endo-and exopeptidase. Endopeptidases hydrolyze peptide bonds in protein molecules randomly to produce relatively large peptides. Exoproteases systematically remove amino acids from the N-terminus or C-terminus or by hydrolysis of peptide bonds in the terminals (Clement, 2000). According to Guérard *et al.* (2002) the soluble peptides can act as a substrate, competing with the protein to be hydrolyzed or partially hydrolyzed by the enzyme, this may have occurred when the DCB was hydrolyzed with Alcalase and Flavourzyme enzymes (Table 3), due to this, higher content of free tyrosine occurred compared with the degree of hydrolysis.

Conclusion

The different enzymes tested showed capability to hydrolyze both MSM proteins as well as the DCB, and the DCB had higher HD. The higher affinity of the enzyme for the substrate was noted when the enzyme Protamex was used, where it had higher HD. From the above results, it can also be concluded that although the enzyme Protamex presented higher degree of hydrolysis, the same cannot be said about the percentage of free tyrosine, which means that the two results are not directly related.

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