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Evaluation of properties of protein recovered from fish muscles by acid solubilization process

[1*]Freitas, I. R., [2]Cortez-Vega, W. R. and [1]Prentice, C.

[1]*Laboratory of Food Technology, School of Chemistry and Food, Federal University of Rio Grande, Rio Grande - RS – Brazil*
[2]*Faculty of Engineering, Federal University of Grande Dourados, Dourados - MS - Brazil*

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Abstract

The aim of this research was to isolate of whitemouth croaker and argentine anchovy protein by acid solubilization process and isoelectric precipitation and evaluate and physicochemical and functional properties of this product. Proximate composition, color, texture, solubility, water and oil holding capacities were determined. The results showed high lipid reduction and high protein content. The lowest process yield was 86.81% and the highest whiteness was 73.63 for the protein recovered from croaker muscle. Both samples showed a tendency towards green and yellow. The values obtained for cutting force and cutting work for the croaker muscle sample were 21:53 N and 62.67 N.s, respectively. The solubilization of the recovered protein was studied in the pH range of 3, 5, 7, 9 and 11, with the maximum solubility occurring at pH 3 and 11 while minimum solubility occurred at pH 5 for the species under study. The water holding capacity was lowest at pH 5. The highest oil holding capacity observed for the anchovy muscle protein was 3.99 ml oil.g^{-1} protein. The process of acid solubilization can be an alternative in harnessing fish that are usually used for production of animal feed or discarded in the environment, they may be used to produce products intended for human consumption.

Introduction

With the increase of the population there is a need the development of processing strategies to maximize the recovery of functional and nutritious fish muscle proteins from low-value/underutilized species (Nolsoe and Undeland, 2009). Some of the aquatic species are not utilized for human consumption for various reasons. Unexploited in Brazil, the croaker *(Micropogonias furnieri)* is a very common demersal fish species in the southern coastal zone. However, despite the wide availability of this raw material, this species reaches the market at a low price in relation to other regional species, especially those of smaller sizes (Badolato *et al*., 1994) such as anchovy *(Engraulis anchoita)*, a pelagic fish found in South western Atlantic. One of the reasons why this raw material is discarded or processed as a product of low commercial value is due to its dark meat, susceptible to oxidation and after taste (Thiansilakul *et al*., 2007).

The recovered proteins are used in value added seafood products destined for human consumption. They are important due to their economic viability besides adding value to products from the aquatic food industry. The fish proteins isolation process involves the solubilization of a dispersed form of the fish tissue in either an acidic (pH ≤ 3.5) or in an alkaline (pH ≥ 10.5) aqueous solution (Taskaya *et al*., 2009). At these low or high pH values, the net protein charge leads to the repulsion of protein chains and their solubilization. The protein in the aqueous solution is separated from solids (insoluble proteins, skin, bones, and scales) and neutral lipids by centrifugation (Batista *et al*., 2007).

The specific properties of the recovered proteins facilitate their adequate implementation contributing to a better use of this technology. A good solubility is necessary for applications in food, while oil holding capacity is of great importance in the formulation of foods, being able to influence the order of addition of dry ingredients into the mixture (Ferreira *et al*., 2013). The water holding capacity is very useful in the manufacture of meat products, preventing the loss of water in the cooking process, which usually improves the texture of foods (Centenaro *et al*., 2009).

The gelation of fish protein is the most important step in the formation of the desired texture in many products. Functional properties like gel strength, which may be affected by various physical conditions as well as protein concentration, temperature and time settings are also vital (Luo *et al*., 2008). The

*Corresponding author.
Email: *irfreitas@yahoo.com.br*
Tel: +55 (53) 3233-6969; Fax: +55 (53) 3233-8745

aim of this study was to obtain protein recovered from whitemouth croaker *(Micropogonias furnieri)* and argentine anchovy *(Engraulis anchoita)* fish muscles by acid solubilization and protein isoelectric precipitation process and to evaluate the physicochemical and functional properties.

Materials and Methods

Recovered protein

The raw material used was the Whitemouth croaker *(Micropogonias furnieri)* muscle provided by Pescal S.A. fish industry located in the city of Rio Grande - Rio Grande do Sul, Brazil and anchovy (Engraulis anchoita) captured off the coast of Rio Grande do Sul on cruises conducted by the "South Atlantic Oceanographic Vessel", owned by the Federal University of Rio Grande – FURG, Brazil. The croaker and anchovy were transported in coolers with ice to the Laboratory of Food Technology, Federal University of Rio Grande - FURG where they were cleaned with chlorinated water 2 $g.L^{-1}$, filleted and stored frozen at (-18°C) until use.

The process of acid solubilization was performed as described by Kristinsson *et al.* (2005) with slight modifications. The fillets were minced and then homogenized (IKA Model RW 20DZM.n) in a ratio of 1:9 (w/v) with distilled water at 4°C for 60 s. The protein solubilization was performed at 3-4°C in temperature controlled by an ultrathermostatic bath (Quimis, model 214 D2) for 20 minutes under constant stirring with a propeller shaft stirrer (IKA, RW 20DZM.n model). 1N HCl acid solution was used for protein extraction at pH 3.0 for 20 min then centrifuged (SIGMA 6-15 Model) at 9000 × g for 20 min. Soluble proteins were subjected to isoelectric precipitation (pH 5.5) with the addition of 1N NaOH solution. The second centrifugation was performed at 9000 x g for 20 min, where the precipitate, referred to as recovered protein, was mixed with cryoprotectants (0.3% polyphosphate, 4% sorbitol and 4% sucrose).

Proximate composition

Moisture, crude protein and crude fat content were determined according to the methods described by AOAC (2000). Moisture was determined by the oven drying method at 105°C and total protein content by the Kjeldahl method. Total lipids were evaluated by the Soxhlet method and ashes by calcination at 550°C.

Lipid reduction

Lipid reduction was calculated from the difference between the total lipids in the raw material (dry basis) and the total lipids present in the recovered protein (dry basis) (Kristinsson and Liang, 2006).

Process Yield

Process yield was calculated from the ratio between the amount of recovered protein obtained at the end and the amount of raw material (muscle) used in each process (Rawdkuen *et al.*, 2009).

Color

Color was determined by using a Minolta Colorimeter model CR-400 (Minolta Camera Co. Ltd., Osaka, Japan) and CIELAB system to measure the degree of Luminosity (L^*) redness (a^*) and yellowness (b^*). Whiteness was calculated according to Rawdkuen *et al*., (2009).

$$\text{Whiteness} = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Gel Preparation

To determine the texture, a gel was prepared from fish protein concentrate according Luo *et al*. (2008) with modifications. 100 g of a properly thawed sample was weighed and mixed with 1% NaCl. After mixing, the samples were placed in cylindrical trays of 4 cm height and 2 cm in diameter and subjected to heat treatment for 20 minutes in a 90 °C water bath (Quimis model Q 215-2). After treatment, the cylindrical trays were immediately submitted to cooling (4-7 °C) for 20 minutes, and stored under refrigeration for 24 hours.

Texture

Texture analysis of the gel was carried out using a texture analyzer Model TA-XT2 plus (Stable Micro Systems, Surrey, England). Analogously, samples with 40 mm height and 20 mm diameter were submitted to the cutting/shearing test using a knife blade, at a test speed of 2.00 mm/g and a distance of 10 mm. The cutting strength (N) is correlated to the firmness of the sample and the work of shear (N.s) indicated the total energy (work) required to shear (SMS, 2000). For the measurement of gel strength, a sample with 25 mm height and 20 mm diameter was used with a spherical probe of 5 mm diameter, speed of 1 mm/sec, penetration speed of 1.1 mm/sec, post-test speed 10.0 mm/sec and a distance of 15 mm.

Functional properties

Solubility

Solubility was determined according to the method proposed by Chalamaiah *et al*. (2010) and

Tadpitchayangkoon *et al.* (2010) with modifications. The amount of soluble protein in the supernatant was determined by the Folin-Ciocalteu method according to Lowry *et al.* (1951). The solubility of the protein was calculated according to the following equation:

$$S(\%) = \frac{\text{Protein content in the supernatant}}{\text{Total protein in the sample}} \times 100$$

For the calculation of protein in the supernatant, the albumin standard curve was used.

Determination of the water holding capacity (WHC)

WHC was determined according to the method of Regenstein *et al*. (1984), with modifications. Soluble proteins in the supernatant were quantified by the method defined by Bradford (1976), and deducted from the total protein of the original sample. The WHC was determined as follows.

$$\text{WHC}\ (g.g^{-1}) = \frac{\text{Amount of water retained}}{\text{Original protein mass}} \times 100$$

Determination of the oil holding capacity (OHC)

OHC was determined According to the method described by Fonkwe and Singh (1996). The data was obtained by using the equation below:

$$\text{OHC}\ (ml.g^{-1}) = \frac{\text{Oil retained}}{\text{Protein mass}} \times 100$$

Statistical Analysis

The experiments were conducted in triplicate. Results were expressed as mean and standard deviation values. The results were evaluated using analysis t-Student test, with the significance level of 5%, using Statistica 7.0 software.

Results and Discussion

Proximate composition

Results for proximate composition, lipid reduction and process yield are shown in Table 1. Through the analysis of t- Student test no significant difference ($p > 0.05$) was verified on the percentage of protein (dry basis) between the croaker and anchovy muscles, being above 96%. The results were similar to those of Fontana *et al.* (2009) for croaker muscle concentrate obtained by the acid process. The anchovy muscle protein concentrate presented much higher protein content than that found by Batista *et al*., (2007) using sardine muscle. The process of acid solubilization was effective in the recovery of proteins.

The moisture in the analyzed concentrates was lower than that found in carp concentrates by Taskaya *et al.* (2009), who obtained values greater than 89.00% with solubilization at pH 2.0 Marmon *et al*. (2009) analyzed a high amount of moisture in the concentrates of Baltic herring (89.3%). The separation system used in this study was sufficient to remove water after the isoelectric precipitation of the protein.

The reduction in ash content is attributed to the efficiency of the depulper and the removal of soluble minerals in the wash water. The concentrate of croaker muscle showed higher ash content (1.08%) when compared to the concentrate of anchovy muscle (0.87%), probably during the separation of soluble from insoluble proteins any insoluble fraction present may have been mixed with soluble one resulting in higher amount of ash. However, Cortez-Vega *et al.*, (2013) obtained 1.32% ash in the protein isolate from Whitemouth croaker *(Micropogonias furnieri)* solubilized at pH 11.2.

According to Tadpitchayangkoon and Yongsawatdigul (2009) and Fontana *et al.* (2009), high ash concentration is a result of the accumulation of NaCl due to pH adjustment during the protein concentrate extraction process. This ash content can be minimized with the neutralization of the concentrate after isoelectric precipitation. Although the concentrates under this study were not neutralized, the results were less than 5.83% for protein concentrate (pH 2) obtained by Taskaya *et al.* (2009).

The lipid content was reduced when compared to the raw material, this because the vast majority of lipids were removed during the first centrifugation together with the insoluble fractions. These components are separated by difference in density and solubility during centrifugation (Kristinsson *et al.*, 2005). Lipid reduction for concentrates obtained from muscles do not differ statistically among themselves, these were higher than those reported by Rawdkuen *et al*., (2009), who found a lipid reduction of 85.2% for tilapia muscle concentrate and by Kristinsson *et al*., (2005) who obtained lipid reduction of 85.4% for catfish concentrate. Batista *et al*., (2007) obtained a lipid reduction of 95.3% for sardine concentrates, results similar to those of concentrates obtained in this study.

Between croaker and anchovy muscles, the lowest yield was of the croaker concentrate (86.8%). For the acid solubilization process Kristinsson and Demir (2003) found yields of 71.5%, 73.6%, 81.2% and 78.7% for the catfish, mackerel, croaker and mullet species respectively. The pH values were statistically similar due to the fact that the pH of precipitation of the protein for protein concentrates was 5.5

Table 1. Proximate composition in dry basis, lipid reduction and process yield of the proteins recovered by acid solubilization

Protein recovered	Protein (%)	Moisture* (%)	Lipids (%)	Ashes (%)	Lipid reduction (%)	Process yield (%)	pH
MA (HCl/NaOH)	97.18 ± 0.86[a]	75.10 ± 0.60[b]	0.61 ± 0.15[a]	0.87 ± 0.01[b]	95.31 ± 0.56[a]	98.48 ± 0.88[a]	5.56 ± 0.01[a]
MC(HCl/NaOH)	96.78 ± 0.57[a]	79.88 ± 0.22[a]	0.37 ± 0.01[b]	1.08 ± 0.47[a]	95.12 ± 0.20[a]	86.81 ± 0.98[b]	5.53 ± 0.01[a]

Averages of three determinations (n=3) ± standard deviation. * Wet basis. The same letters in the same column do not differ by t-Student test p > 0.05. MA- Anchovy muscle; MC- Withemouth croaker muscle.

Table 2. Values of L*, a*, b*, whiteness and texture of the proteins recovered by acid solubilization process

Protein recovered	L*	a*	B*	Whiteness	Cutting strength (N)	Work of shear (N.s)	Gel strength (g.cm)
MA(HCl/NaOH)	70.35±0.99[c]	-1.14±0.40[c]	5.05±1.58[c]	69.87±1.22[c]	1.407±0.21[c]	5.22±0.68[c]	595.63±55.66[c]
MC(HCl/NaOH)	74.34±0.62[b]	-2.57±0.36[d]	5.42 ±0.65[c]	73.63±0.62[b]	21.53±0.70[a]	62.67±0.99[a]	12497.14±301.15[a]

Averages of three determinations (n = 3) ± standard deviation. The same letters in the same column do not differ by t-Student test p > 0.05. MA - Anchovy muscle; MC - Withemouth croaker muscle

Color

Table 2 shows the color parameters of the protein concentrates, where it can be seen that the lightness values are significantly different ($p<0.05$). The largest value for lightness (L) was obtained from croaker muscle protein, with a greater tendency to white color and consequently showed higher whiteness, which shows higher removal of myoglobin (Rawdkuen *et al.*, 2009). The values of Chroma a^* for the concentrates under study showed tendency to green. In the chromaticity coordinate b^*, muscle proteins tended to yellow. According to Kristinsson and Hultin (2004) the high value of Chroma b^*, presented at acidic pH (pH 1.5-3), is because hemoglobin unfolds, oxidizes and forms aggregates when it is adjusted to pH 5.5 and hence is easily precipitated during centrifugation. This generally results in lower L^* value and lower whiteness. A similar result was reported by Chaijan *et al.*, (2006) for the gel prepared from mackerel muscle.

Texture

The data of cutting force, cutting work and gel strength are shown in Table 2. The gels that showed the best quality were those obtained from croaker species, since the protein recovered from this species showed higher cutting force, as a result, had greater cutting work statistically differing significantly ($p<0.05$) from the gels from anchovy muscle. Chaijan *et al*. (2006), evaluating the gel forming ability of the sardine protein noted that the difference in this ability may result from differences in protein integrity and the bond formed during the heat treatment.

The acid process showed good gels for protein species of cod, mackerel (Hultin and Kelleher, 1999), tilapia (Kristinsson and Liang, 2006), who found a greater gel strength in croaker gels obtained by the acid process followed by the alkali and finally the traditional surimi gels. These authors showed that the ability to form good protein isolate gels by acid solubilization depends on the species, on the methods used and the time the sample is exposed to the solubilising pH, which may cause differences in results.

Functional properties

The results for solubility are shown in Table 3. By applying ANOVA and Tukey's test between the tests it can be seen that for the analyzed pH's the tests were statistically different ($p<0.05$). The lowest solubility was found at pH 5 for all tests and the lowest value was obtained for the Whitemouth croaker muscle isolate (1.45%) and higher solubility were found at extreme pHs, especially in pH 11 followed by pH 3.

Yongsawatdigul and Park (2004) evaluated the solubility of rockfish muscle isolates, where the

Table 3. Solubility, WHC and OHC values presented by fish proteins recovered by the process of acid solubilization

Protein recovered	Solubility (%)					Oil Holding Capacity – OHC (mL.g^{-1})
	pH					
	3	5	7	9	11	
MA - (HCl/NaOH)	75.98 ± 0.58^{a}	6.00 ± 0.02^{a}	12.69 ± 0.06^{a}	31.2 ± 0.04^{b}	99.75 ± 1.39^{a}	3.99 ± 0.20^{a}
MC - (HCl/NaOH)	58.06 ± 0.03^{b}	1.45 ± 0.01^{d}	3.58 ± 0.02^{b}	37.15 ± 0.02^{a}	98.06 ± 0.01^{a}	3.23 ± 0.38^{a}
	Water Holding Capacity – WHC (g.g^{-1})					
MA - (HCl/NaOH)	12.44 ± 0.28^{a}	2.77 ± 0.12^{a}	4.28 ± 0.20^{a}	11.57 ± 1.08^{a}	16.21 ± 0.01^{a}	
MC - (HCl/NaOH)	13.29 ± 0.54^{b}	3.43 ± 0.26^{a}	5.22 ± 0.78^{a}	8.91 ± 0.13^{b}	12.46 ± 0.17^{b}	

Averages of three determinations (n = 3) ± standard deviation. The same letters in the same column do not differ by t-Student test p > 0.05.MA - Anchovy muscle; MC - Withemouth croaker muscle.

results suggest that the solubilization at pH 2.5 and 11 induced denaturation and aggregation of both myofibrillar and sarcoplasmic proteins. The same was reported by Batista *et al.*, 2007, where the solubility of sardine protein isolate in pH 7 was less than 10% for the solubilization processes used, indicating the denaturation and aggregation of proteins. Freitas *et al*. (2014) observed that the lowest solubility values of the Whitemouth croaker were obtained at pH 5, due to the fact that proteins commonly exhibit minimum solubility at their isoelectric point (pI).

It can be observed that at pH 5 there was low water retention capacity for all tests, followed by neutral pH as shown in Table 3. Mireles Dewitt *et al.* (2007) found WHC of 1.15 g/g of protein in catfish gels obtained by acid solubilization process, these authors report that acidification can expose many hydrophobic domains of these proteins. When the proteins are recovered and subsequently precipitated they do not return to its native conformation and this may have occurred with the products obtained in this work.

The highest water holding capacity was obtained at pH 11, with anchovy muscle standing out with a protein of 16.21 g.g^{-1} and at pH 3 obtaining 13.29 g.g^{-1} for sea bass protein muscle. The low OHC presented by protein isolates obtained by acidic solubilization may be due to the low amount of lipids that are present in the initial sample. Comparing the OHC of croaker muscle protein isolate obtained by acidic solubilization and OHC of the raw material, Martins *et al.* (2009) observed that the acid process resulted in an increase of 102% over the processed muscle.

Conclusion

The protein recovered from the anchovy muscle showed higher process yield and better solubility. While that from the croaker muscle showed better whiteness and texture properties. Therefore, the process of acid solubilization can be an alternative to the harnessing of fish.

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