RESEARCH ARTICLE OPEN ACCESS

Alkaline Extraction of Cobia (*Rachycentroncanadum*) Proteins: Physicochemical characteristics, functional and thermal properties

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Abstract
Cobia (*Rachycentroncanadum*) is an important emerging species in Brazilian mariculture. The aim of the study was to obtain and characterize a protein isolate from cobia muscle using chemical extraction process by alkaline solubilization and isoelectric precipitation of proteins. The extraction yield was 98.17g/100g protein on a dry basis. The highest solubility and water holding capacity (WHC) of cobiaprotein isolate (CPI) was obtained at pH 11and 21.9mL of water per gram of protein. The electrophoretic profiles revealed masses characteristic of myofibrillar proteins (myosin and actin). The main peaks identified by qualitative spectroscopy analysis of the infrared spectrawere characteristic of peptide bonds such as amide I and amide II. The highest fusion and degradation points of CPI were 259.1°C and 378°C, respectively. The results showed that the CPI has great biotechnological value in various industrial areas that require a product of high protein value.
Keywords:fish, isolated protein,meat, muscle, protein.

I. Introduction

When aiming at obtaining functional foods that have significant market potential, the development of products whose main ingredient is isolated and recovered protein is a viable option. Raw fish protein isolates are sources of nutrients and therefore have potential use in the development of food products intended for human consumption (SHAHIDI and BARROW, 2008).

Protein isolates derived from fish muscle are a concentrated source of complete muscle protein (myosin and actin) of high nutritional quality (CANDELA, LOPEZ and KOHEN, 2011). Fish protein isolates (FPI) can be obtained by chemical solubilization and isoelectric precipitation of the protein either from residues or the whole fish. FPIs resulting from alkaline extraction have higher nutritional qualities as well as higher essential amino acid (EAA) content compared to FPIs resulting from acidic extractions (CHEN, TOU, and JACZYNSKI, 2009).

Cobia (*Rachycentroncanadum*) is widely found in the tropics and sub-tropical waters. This species has emerging potential in marine aquaculture, due to advantages such as rapid growth, generalresistance in captivity and high nutritional value (HOLTA, FAULKA, and SCHWARZ, 2007). However, no data was found in literature on the extraction, recovery and characterization of cobia muscle proteins. Therefore, the objective of this study was to obtain a cobia protein isolate (CPI)and characterize it forphysicochemical, structural and functional characteristics.

II. Materials and methods

Cobia (*Rachycentroncanadum*) was used for protein extraction. The specimens of approximately 4 kgwere supplied by NearShore breeding siteinAngra dos Reis on the coast of Rio de Janeiro, Brazil. The fish were transported alive to the Aquaculture Marine Station, Federal University of Rio Grande (FURG), Rio Grande / RS.

2.1. Extraction of cobia proteins

The extraction of proteins from cobia muscle was performed using alkaline solubilization and isoelectric protein precipitation process (adapted byKRISTINSSON 2005).

After slaughter,the fish was cleaned and gutted at the Laboratory of Food Technology.Soon after, it was processed in a meat separator (High Tech, HT250 Santa Catarina, Brazil) to remove bones and skin. This first process generated a biomass that was homogenized with distilled water in the ratio of 1:9 (w/v) in a propeller shaft stirrer (713 D, Fisatom, São Paulo, Brazil). The whole process was carried out at a constant temperature of 4°C. The resulting solution was homogenized for 20 min with 1M sodium hydroxide (NaOH) until pH 10.8, centrifugation was then performed at 9,000 rpm for 20 min (Hitachi,

High - Speed Refrigerated Centrifuge CR 22 GIII). The precipitate and supernatant (neutral lipids) from the first centrifugation were discarded while the soluble proteins were subjected to precipitation with 1 M hydrochloric acid (HCl) to reach the previously studied isoelectric pH (5.8) of proteins. Soon after, another centrifugation was performed at 9,000 rpm for 20 min. The supernatant was discarded and the precipitate frozen in an ultrafreezer (Indrel, Paraná, Brazil) and later lyophilized (Liotop, L108, Sao Paulo).

2.2.Chemical proximal composition

Centesimal composition of the CPI was performed according to the official method (AOAC, 2005). All analyses were performed in triplicate.

2.3. Functional properties

2.3.1. Solubility

The solubility of the cobia protein isolate (CPI) was determined according to the method adapted from Chalamaiah et al. (2010) and Tadpitchayangkoon et al. (2010). The soluble protein content in the supernatant was determined by Folin-Ciocalteaumethod according to Lowry et al. (1951).

2.3.2.Water retention Capacity (WRC)

Water retention capacityof cobia protein isolate was determined according to the adapted method of Regenstein et al. (1984). Proteins soluble in the supernatant were quantified by the Bradford (1976) method and subtracted from the total protein in the original sample.

2.4.Molecular weight distribution (SDS-PAGE)

The determination of protein fractions was performed by molecular mass according to the method described by Laemmli (1970).

2.5.Infrared spectroscopy

The spectra of the sample were determined using a qualitative method by infrared spectroscopy according to ASTM E 1252 method, the spectra were recorded in an absorption band 4000-500 cm^{-1} 32 scans and resolution at 4 cm^{-1} (Spectrum 1000, Perkin Elmer).

2.6.Thermal Analysis

2.6.1.Differential Scanning Calorimetry

Differential Scanning calorimetry analysis was used to determine the denaturation temperature of proteins present in the CPI using the method of determining Transition Temperatures and Enthalpies of Fusion and Crystallization of Polymers by Differential Scanning Calorimetry according to ASTM E1269-01. Variation in the process temperature ranged from 20°C to 300°C at a rate of 10°C/min (DSC Q20-TA Instruments).

2.6.2.Thermogravimetric

The thermal degradation and the degree of impurity of the CPI, in inert atmosphere, were checked by thermogravimetric analysis using the ASTM D3850-12. A sample of approximately 5 mg was heated from 25 to 500°C with an increase of 10°C/min, with molecular nitrogen (N_2) as the drag gas.

III. Results and discussion

3.1.Proximal chemical composition

The results of the proximal chemical composition of the cobia muscle and the cobia protein isolate (CPI) obtained are shown in Table 1.

Component	Wetbasis		Dry basis	
	Cobiamuscle	CPI	Cobia muscle	CPI
Proteins(g/100g)	16.80 ± 0.80	86.60 ± 2.40	50.45 ± 0.80	98.63 ± 0.05
Lipids (g/100g)	25.50 ± 0.04	0.40 ± 0.03	76.57 ± 0.04	0.45 ± 0.03
Ash (g/100g)	0.90 ± 0.10	0.80 ± 0.10	2.70 ± 0.10	0.91 ± 0.10
Moisture (g/100g)	66.70 ± 1.20	12.20 ± 0.10	-	-

Table 1 - Mean values for the proximal chemical composition of the muscle cobia (M)and thecobia protein isolate (CPI)

Values are means ± standard deviation of triplicates

Observing the Table 1, it is seen that the CPI had a yield of 98g/100g of protein on a dry basis. The results of the proximal chemical composition of the CPI show that the alkaline extraction method also promoted a reduction in lipid content (98%).

According toKristinsson (2005), the reduction of lipids is because these components are separated in the centrifugation, associated with the solubilization temperature of 4°C which contributes to the separation of fat during centrifugation. The decrease in the lipid content in the CPI can significantly

contribute to reduce the lipid oxidation, increasing the stability of the product.

Marquez, Mira and Neves (2004) showed that a high concentration of ash may be derived from the accumulation of sodium chloride used in the protein extraction process. According toKristinsson and Rasco (2000), the ash content is normally greater than 3g/100g in protein isolates, however, this was not observed in the studied protein isolate (0.85 g/100g).

3.2. Functional Properties

3.2.1. Solubility and water retention capacity (WRC)

The solubility decreased at pH 5.0 and 7.0, probably,the proteins presented hydrophobic interactions, thereby promoting protein-protein bonds, resulting in decreased solubility, while an increase of the protein solubility was observed in pH 3.0, 9.0 and 11.0, since the ionic interactions promoted protein-water bonds. The solubility of proteins is a thermodynamic manifestation of the balance between protein-protein and protein-solvent interactions. The main interactions that influence the solubility of proteins are of hydrophobic and ionic nature (DAMODARAN, 2010). The low solubility observed near the isoelectric pH (5.8) of CPI is mainly due to the lack of electrostatic repulsion, leading to aggregation and precipitation through hydrophobic interactions of proteins. In pH 9.0 and 11.0 higher solubility of the proteins (82.57% and 100% respectively) was observed, this is because these pH values are distant from the protein isoelectric point (pH 5.8),thus occurring the effect of varying ionic balance of proteins as a function of pH (MEINKE et al.1972). However, it is worth pointing outthatlyophilization also played a decisive role, since heat denaturation changes the solubility profile in function of the protein pH.

With regard to the WRC values of the CPI,greaterwater retentions by proteins were observed in extreme pH values(3.0 and 11.0), with 21.9 and 19.1 $mL.g^{-1}$, respectively. In the present study, the pH factor influenced the ability of proteins to bind to water molecules becausethe valuesof pH 3.0 and 11.0 are distant from the isoelectric pH of the CPI where there is predominance of charges of the same sign causing repulsion and distancing between molecules, leaving more space to be filled by water molecules, thereby increasing the WRC.

This phenomenon occurs because at pH valuesbelow 5.0 and above 7.0, the water molecules are combined with the polar groups of the protein and WRC tends to increase. An opposite phenomenon occurs in the valuesnear the isoelectric pH (pH 5.0 and 7.0) the proteins are less hydrated because there is increased protein-protein interaction resulting in minimal interaction with water molecules (PACHECO and SGARBIERI, 2005).

3.2.2. Determination of the molecular mass of the CPI through gel electrophoresis (SDS-PAGE)

The Fig. 1 shows the bands and their respective molecular weights of the CPI samples. Samples with concentration of 1 mg/mL (1, 2, and 3) and samples 11, 22 and 33 with dilutions of 2.10^{-2}, 1.10^{-2}, 5.10^{-3} mg/mL, respectively, were mixed with 5µLbuffer, and run in gel containing SDS (10 mL/100 mL). The markers used were Page Ruler Prestained Protein Ladder (M1) and Color Burst (M2).

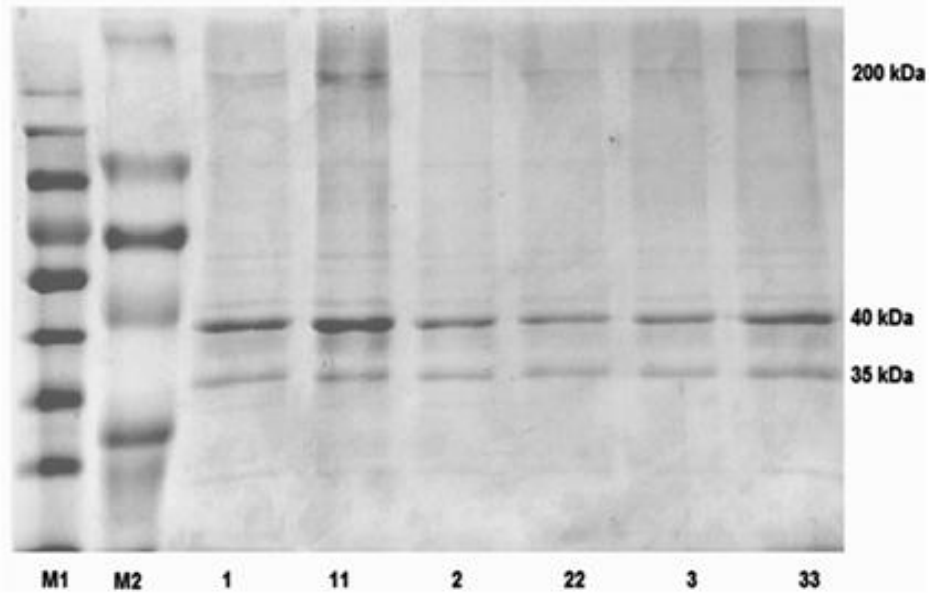

Fig.1. separation by electrophoresis with standard protein markers (M1) and (M2).samples (1), (2) and (3) 1 $mg.mL^{-1}$ of cobia protein isolate (CPI), and samples (11) $2.10^{-2}mg.mL^{-1}$ CPI, (22) 1.10^{-2} $mg.mL^{-1}$ CPI and (33) 5.10^{-3} $mg.mL^{-1}$ CPI

The identified bands are characteristic of the myosin type of myofibrillar proteins (200 kDa), actin (35 kDa) and β-tropomyosin (40 kDa), confirming the removal of sarcoplasmic proteins during the isolation method.

The protein band of 200 kDa molecular mass observed in this study is the mainmyofibrillarprotein present in fish muscle. This protein consists of two polypeptides, a heavy chain of 200 kDa and a light chain (40 kDa) (KRISTINSSON, 2001). Li et al. (2014) observed similar molecular masses of myosin, actin and tropomyosin in croaker muscle (*Pseudosciaenacrocea*).

Assis et al. (2012), working with croaker protein isolate identified proteins of molecular weight near 220 kDa (myosin) and some bands between 20 and 50 kDa, representing the β-Tropomyosin and troponin protein fractions. Tongnuanchan et al. (2011) reported that myosin chains and high molecular weight troponin are the dominant proteins muscle in the electrophoretic profile of Nile Tilapia.

The myofibrillar proteins are responsible for the gelling properties, water retention and emulsification. These proteins have important applications in the food industry, since they are responsible for the formation of gels, which give structure and stability to various foods.

The sarcoplasmic proteins have as a main characteristic the ability to adhere to the myofibrillar proteins, preventing the formation of a gel of high elasticity, low viscosity, low water retention capacity and low capacity to absorb flavors and colorants. Artharn et al. (2008) studied the effect of the proportion of myofibrillar and sarcoplasmic proteins on the properties of films prepared with mackerel muscle proteins and reported that the increase in the content of sarcoplasmic proteins reduced tensile strength and increased water vapor permeability.

3.2.3.Infrared spectroscopy analysis

The absorption spectra show that variations in the structure of the CPI sample are distinguished by the location of the absorption peak in the wavelength of the characteristic wave and by the energy absorption intensity (Fig.2).

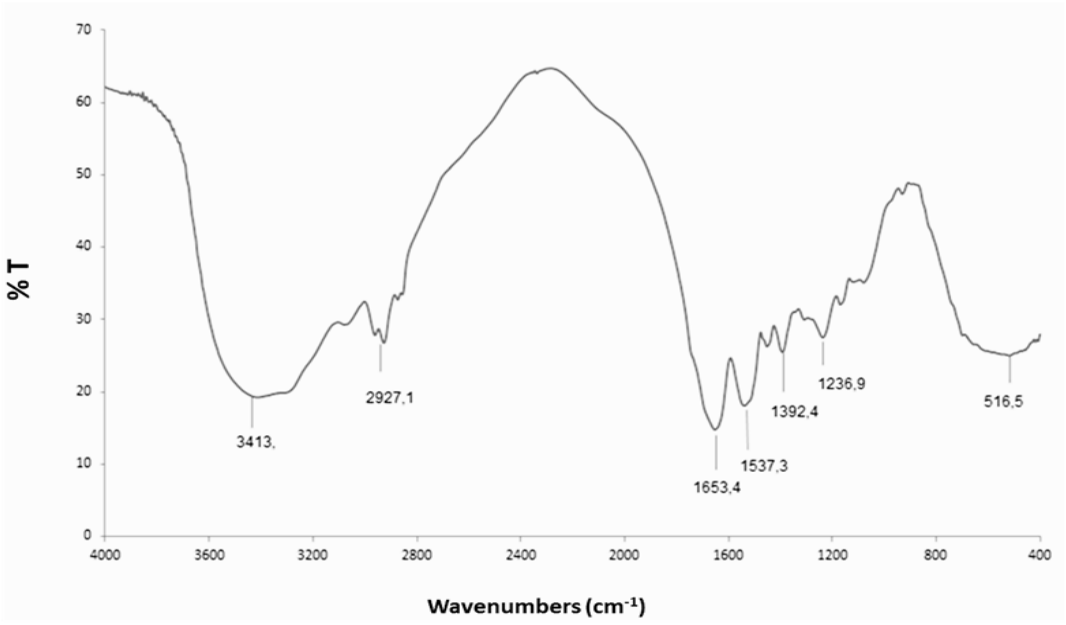

Fig.2. spectroscopy of the infrared (FTIR) of cobia protein isolate (CPI)

The main peaks showed absorptions of amino group (NH_2) and hydroxyl OH at wavelengths between 3600-3100 cm^{-1} derived from peptide bonds that seem to overlap carboxyl groups of amino acids, as well as others not fully identified. Because at wavelength 3413.2 cm^{-1}axial stretching of O-H and N-H present in proteins is observed, as reported by Araújo(2001), where in 3413.2 $cm^{-1}$the first harmonic C=O stretches may be identified. The peak at region 2927.1 cm^{-1} is related to the C-H axial deformation of aliphatic groups (CH_3 and CH_2) that can be of amide B (Oujifard, 2013). Bands 1653.4 cm^{-1}comprise of the stretching vibration of C=O, typical of amide I (Böcker et al.,2007 andOujifard et al., 2013) with α-helical type structures, indicating compact structures (Ju and Kilara, 1998), which were similar to those reported previously by Bertram, Kohler, Böcker, Ofstad and Andersen (2006), whereas wavelength 1537.3 cm^{-1} is characteristic of Amide II with angular deformation of NH_3groups (Ramos, 2013). The

regions between 1392.4 $cm^{-1}$and 1236.9 cm^{-1} indicate symmetrical angular deformation and vibration of C-H bonds, with symmetric stretching without changing the dipole moment of the molecule, characteristics of amide III. Nagarajan et al. (2012) obtained Amide I, II, III at wavelengths 1632 cm^{-1}, 1541 cm^{-1} and 1236 cm^{-1}, respectively, in agreement with the spectra found in this work. Ahmad and Benjakul (2011) and Muyonga et al. (2004) found similar spectra in fish protein isolate in the range of 1800 and 600 cm^{-1}, characteristic of amide I, II and III. With respect to the absorption peak of 516.5 cm^{-1}, angular deformation of N-H and C-N was observed (SILVERSTEIN et al. 2005).

3.2.4. Analysis of Differential Scanning Calorimetry (DSC)

The Fig. 3 shows the glass transition temperature (T_g) and the endothermic events that are related to the bond breaking processes, fusion of polymerization and volatilization of the polymer. From Fig. 4 it can be seen that the transition from the glassy state to an amorphous state begins at a temperature of 81.2°C, therefore, a process accompanied by variation of heat capacity of the sample.

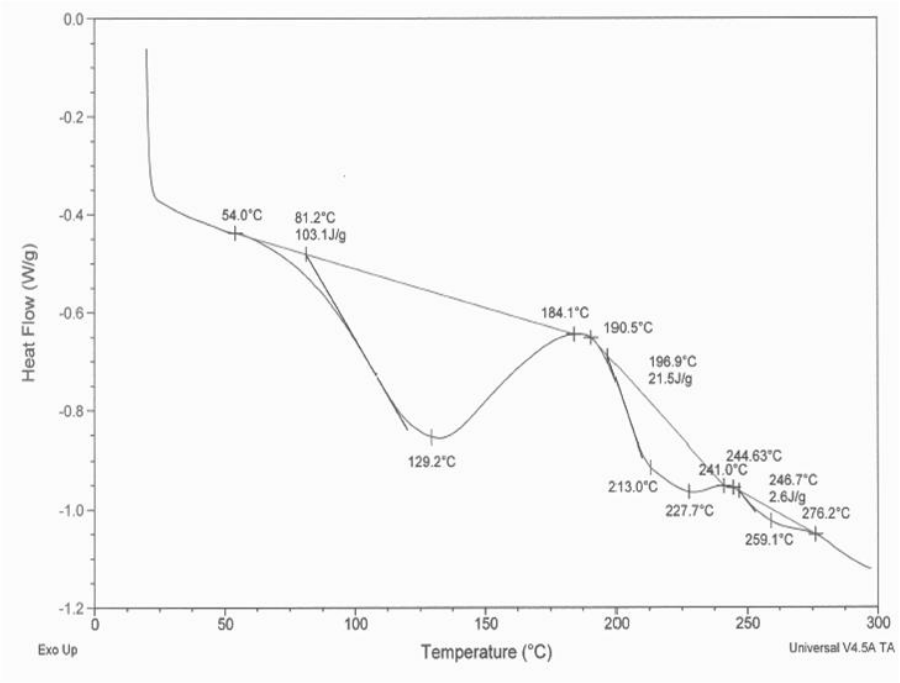

Fig.3. determination of transition temperatures and fusion enthalpies of cobia protein isolate (CPI) by differential scanning calorimetry (DSC)

Endothermic events at 129.2, 227.7 and 259.1°C melting processes characteristic of semi-crystalline polymers. Due to the size distribution of the crystalline regions present in macromolecules such as proteins, fusion of a semi-crystalline polymer always occurs in a temperature range rather than a point itself. The smaller crystals fuses first, soon after, the temperature at which crystallinity disappears completely (final crystal fusion) is considered the fusion point of the polymer (259.1°C) and corresponds to the maximum fusion peak in the DSC curve.

The CPI results show higher transition temperatures when compared with protein species studied by Monterrey-Quintero et al. (2000) and Dergez et al. (2006) who found transition temperatures of the protein fractions as 55.6, 71.1, 53.3 and 57.8 and 65°C, respectively.

Park and Lanier (2000), studying a tilapia species (*Oreochromisaureus*) determined that myosin denatures at 58.3°C and actin at 78.6°C, demonstrating greater thermal stability of the proteins present in CPI.

These phenomena mentioned are still difficult to explain, but must be consequences of the destruction of certain interactions between the myofibrils, which made these proteins more thermally stable. However, with the DSC analysis it was possible to determine the degree of crystallinity of the sample (88.43%), suggesting a semicrystallinetype compound of higher thermal resistance.

3.2.5.Thermogravimetric Analysis (TGA)

The Fig. 4show the thermal behavior and initial and final temperatures of thermal degradation and the loss of mass variation (Δw) of the CPI.The first degradationof mass of the CPI was observed between 31.5°C to 210°C with loss of 10.23% of the original mass, possibly associated with the evaporation of water present in the sample. The second degradation mass 210°C to 340°C was observed a reduction of 38.23% which is probably due to decomposition of the protein fractions of lower molecular weight. From 340°C is greater degradation of protein fractions with a reduction of 32%.

The results of the thermal analysis suggest proteins characteristic of the CPI endothermic reactions, and are stabilized as the temperature increases (CASEY and HUGHES, 2004). This thermal stability of the IPB proteins is attributed to the waste composition of hydrophobic amino acids which tend more stable than the more hydrophilic amino acids present in proteins (PRIMING and WALKER, 1998).

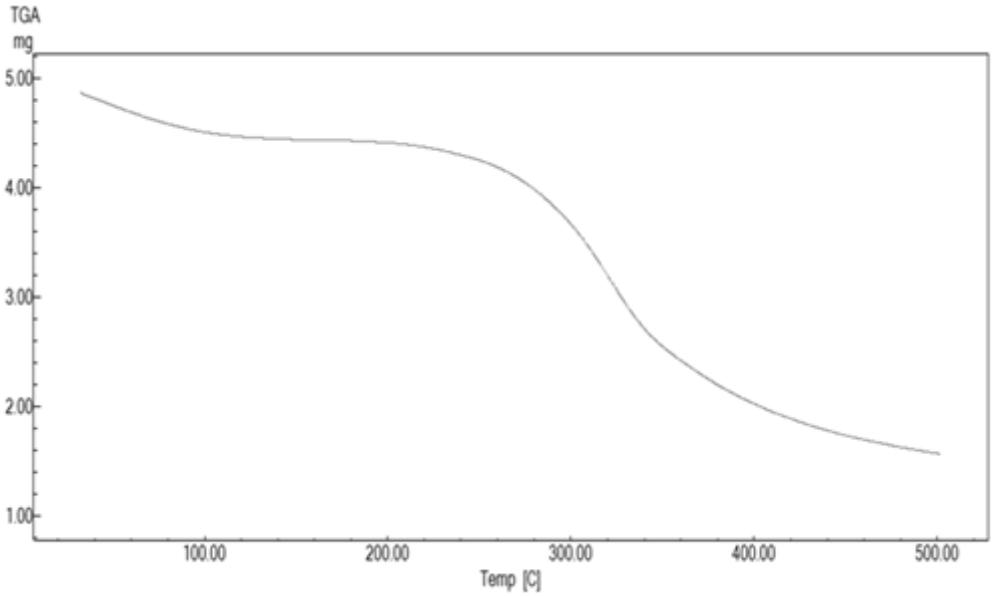

Fig.4. thermogravimetricanalysis of cobia protein isolate (CPI)

IV. Conclusion

Protein recovery was successfully accomplished by alkaline solubilization and its subsequent lyophilization led to obtaining recovered cobia proteins with high protein concentration (98 g/100g), basically actin and myosin, as well as the high lipid reduction.

The studied characteristics of the obtained CPI showed that it is a thermally stable (259 °C) protein product of high functionality that can be used as raw material in the production of gels and emulsions of high added value.

Acknowledgment

We acknowledge the financial support of the Coordination for the Improvement of Higher Education Personnel (CAPES) of Brazil.

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