COLLAGEN OF PEACOCK BASS SKIN AS POTENTIAL SOURCE FOR THE PHARMACEUTICAL INDUSTRY IN THE PRODUCTION OF PEPTIDES

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ABSTRACT - Pepsin-soluble collagen (PSC) from peacock bass (Cichla ocellaris) was extracted (with a yield of 2.9%), isolated and physicochemically characterized. Its structure was found to be composed of two different α chains (α1 and α2), dimmers and trimmers (β and γ chain, respectively). Moreover, PSC showed high absorption in the near UV spectrum (211 nm), indicating that the protein is type I collagen, and high solubility rates in different NaCl concentrations (from 0 to 3%) and acid pH media. Our results indicate the feasibility of obtaining PSC from C. ocellaris skin as well as its potential for applications in food and pharmaceutical products.

Keywords: collagen; skin; waste.

RESUMO - Colágeno solúvel em pepsina (PSC) de peacock bass (Cichla ocellaris) foi extraído (com um rendimento de 2.9%), isolado e físico-quimicamente caracterizado. Sua estrutura foi composta por duas cadeias α (α1 e α2), dimmers e trimmers (cadeia β e γ, respectivamente). Além disso, o PSC mostrou alta absorção no espectro UV próximo (211 nm), indicando que a proteína é colágeno tipo I e altas taxas de solubilidade em diferentes concentrações de NaCl (de 0 a 3%) e meio de pH ácido. Nossos resultados indicam a viabilidade de obter PSC de pele de C. ocellaris, bem como o seu potencial para aplicações em alimentos e produtos farmacêuticos.

Palavras-chave: colágeno; pele; resíduos.
1. INTRODUCTION

Fish processing wastes are potential sources of biologically active, functional molecules of interest to agro-industry (FERRARO et al., 2016). According to the former Brazilian Ministry of Fisheries and Aquaculture (MPA) (MPA, 2011), Brazil has an annual fish production of about 1.4 million tons (9,304.400 t corresponding to peacock bass Cichla ocellaris), which makes it among the twenty major fish producers in the world. Peacock bass C. ocellaris is a common carnivorous species from the Amazon basin, mainly used for food and sport purposes but not commercially fished.

Collagen, a fibrous protein, is the main component of connective tissues in mammals. It was evolutionarily adapted to perform different functions, especially structural (CHUNG and UITTO, 2010). Relevant biomaterial properties as low melting point, biocompatibility, high porosity, ease of combination with other materials, abundance and inexpensively, make collagen desired for tissue engineering, at the same time that its simple processing, low antigenicity and high absorption by the body make it suitable for medical applications (SUBHAN et al., 2015; FERRARO et al., 2016). Collagen from viscera such as bovine and porcine skin and bone have been widely used. However, diseases transmitted through the residues of these species have led to the discovery of alternative sources of this protein. Fish collagen has similar physicochemical properties to collagen of mammals, besides commercial advantages such as: 1) reduced risk of disease transmission, transmitted spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) due to the large evolutionary distance between fish and humans; 2) absence of religious obstacles; 3) high-yield recovery and 4) low level of toxicity (WANG et al., 2014).

The hydrolysis of fish collagen produces peptide fragments with medical utilities and several biological activities including: antihypertensive, antioxidant, antimicrobial, anticoagulant, antidiabetic, anticarcinogenic, immunostimulatory, hypocholesterolemic, appetite suppressant, among others. For these properties they have been widely applied in medical/aesthetic procedures such as ulcerative processes, replacement and/or regeneration of tissue (vessels, bone, cartilage, skin, blood, trachea, esophagus and others) (SUBHAN et al., 2015; FERRARO et al., 2016), as well as in the food and beverage industry. Thus, this present study aims to isolate the collagen from the C. ocellaris skin, to determine its physicochemical properties and to obtain collagen hydrolysis products using a collagenase from intestinal residues of the same fish specimens.

2. MATERIAL AND METHODS

2. Isolation of collagen

Adult specimens of C. ocellaris obtained from a fishermen’s village in the city of Petrolândia, Pernambuco, Brazil. The prepared skin samples were mixed with 0.2 M NaOH at a skin/alkali solution ratio of 1:10 (w/v), to remove non-collagenous proteins. The mixture was continuously stirred for 3 h at 4°C and the alkali solution was changed every 30 min. The treated skin was then washed with cold distilled water until a neutral or faintly basic pH of the wash water was reached, with further addition of butyl alcohol 10% (w/v) to remove fats. The mixture was then stirred for 6 h at 4°C, and the skin was washed (as previously described) and clarified by the addition of hydrogen peroxide 10% (w/v) (SINGH et al., 2011). PSC was prepared based on the methods of NAGAI and SUZUKI (2000), with some modifications.

3. Determination of total proteins

Protein content was determined as described by Smith et al (1985), using bovine serum albumin as standard.
4. Properties of collagen

4.1 UV absorption spectrum
UV absorption spectra were carried out according to Nalinanon et al (2011).

4.2 Collagen solubility test
The solubility of collagen was determined based on the methods of Montero et al (1991) with modification.

4.3 Effect of NaCl on the collagen solubility
Solutions of collagen and NaCl (in 0.5 M acetic acid) were mixed to give different final concentrations of 0, 1, 2, 3, 4, 5 and 6% (w/v) in a final volume of 0.5 mL. The mixture was stirred continuously at 4°C for 60 min and further centrifuged at 20,000 x g for 60 min at 4°C. The protein content in the supernatant was measured and the relative solubility was calculated as previously described.

4.4 Effect of pH on the collagen solubility
The collagen solutions (0.8 mL) were transferred to centrifuge tube and had their pH adjusted (by using 6 M NaOH or HCl solutions) to obtain different pH values ranging from 1 to 12. The solution had its final volume adjusted to 1 mL by adding deionized water (with the same pH value) and was centrifuged at 20,000 g for 60 min at 4°C. For all the samples, the protein content in the supernatant was measured and related to the one found in the solution with the highest solubility.

4.5 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)
SDS–PAGE was performed following the method of Laemmli (1970). After electrophoresis, the gel was stained with 0.05 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and 5 % (v/v) acetic acid and destained with 30 % (v/v) methanol and 10 % (v/v) acetic acid.

5. Collagenase extraction and Hydrolysis of collagen by collagenase
Intestinal collagenase was extracted from digestive waste of peacock bass C. ocellaris specimens. The waste-processing was based on the methods of Oliveira et al. (2017). The protein concentration was determined. The digestion of peacock bass C. ocellaris native collagen was analyzed based on the methods of Moore and Stein (1954) and Oliveira et al. (2017).

6. Statistical analysis
Data are presented as means ± standard deviations, and were statistically analyzed for normal distribution by Shapiro–Wilk and Kolmogorov–Smirnov tests and for homogeneity of variances by Levene’s test. One-way analysis of variance (ANOVA) followed by Tukey’s test was used for normally distributed data, whereas Kruskal–Wallis ANOVA test would be used in case of non-normally distributed data. Differences between groups were accepted as significant at a confidence level of 95% (p< 0.05).

3. RESULTS AND DISCUSSIONS

3.1 Yield of the extracted PSC
The collagen isolated from peacock bass C. ocellaris was obtained with a yield of 2.9%
(calculated based on dry weight of skin), which is higher than the reported for collagen extracted from blacktip shark *Carcharhinus limbatus*, 1.04% (KITTIPHATTANABAWON et al., 2010), bighead carp *Hypophthalmichthys nobilis*, 2.7% (LIU et al., 2012) and skipjack tuna *Katsuwonus pelamis*, 2.47% (DI et al., 2014) for cartilage (acid-soluble collagen, ASC), scale (PSC) and spine (ASC), respectively. The yield found in this work can be attributed to the low content of intermolecular crosslinks, which increases the collagen solubility (HICKMAN et al., 2000). PSC was more viable than the ASC, since the cross-linked molecules at the telopeptide region were more susceptible to hydrolysis, allowing a more efficient extraction (KRISHNAMOORTHI et al., 2017). It is known that the collagen yield varies depending on the type of extraction, fish species or tissue, biological conditions and concentration of pepsin (LIU et al., 2012).

### 3.2 UV-vis spectra

Proteins have generally a maximum UV absorption at 280 nm (HUANG et al., 2011), whilst collagen from peacock bass *C. ocellaris* showed higher rate of activity at 211 nm, probably due the presence of the intact telopeptide domain. Non-detection of absorbance in the 240 to 280 nm amplitude suggests that the collagen alone does not contain aromatic amino acids, such as tyrosine and phenylalanine. Thus, the protein in question is type I collagen. The results are similar to those reported for red drum fish *Sciaenops ocellatus* (CHEN et al., 2016). Previous studies, using type I collagen from the skin of fish species by the PSC method, reported absorptions greater than 220, as for example for collagen from cobia *Rachycentron canadum*, 221 nm (ZENG et al., 2012), balloon fish *Diodon holocanthus*, 230 nm (HUANG et al., 2011) and ornate threadfin bream *Nemipterus hexodon*, 230 nm (NALINANON et al. 2011).

### 3.3 Solubility of collagen

#### 3.3.1 Effect of NaCl concentration on collagen solubility

The PSC remained more than 90% soluble in a NaCl gradient from 0 to 3%, with high precipitation rates in concentrations above 4%. Similar results were reported in studies of the species skipjack tuna *K. pelamis* (DI et al., 2014) in which collagen lost its solubility when the NaCl concentration exceeded 2% (w/v). A “salting out” effect is supposed to decrease the collagen solubility, an effect which occurs at relatively high NaCl concentrations (LIU et al., 2012). A decrease on the solubility was also observed when the concentration of PSC was higher than 4%, as for example for skin of balloon fish *D. holocanthus* (HUANG et al., 2011) and bighead carp *H. nobilis* (LIU et al., 2012). The PSC method affects the solubility of the collagen due to the partial hydrolysis of high molecular weight (MW) cross-linked molecules by pepsin (SINGH et al., 2011; JONGJAREONRAK et al., 2005).

#### 3.3.2 Effect of the pH on the collagen solubility

Collagen extracted from peacock bass *C. ocellaris* skin presented similar solubility [high solubility in acidic pH (2 to 6)] to other fish species, as for example from the skin, scaling, bone and bladder of bighead carp *H. nobilis* (LIU et al. 2012), although collagens are normally solubilized in the pH range from 1 to 4 (HUANG et al., 2011; SINGH et al., 2011; CHEN et al., 2016).

A slightly increase in solubility was also found at pH 8 and 10, similar to that reported for collagen extracted from skipjack tuna *K. pelamis* spine and skull by the PSC method. That would be explained by a repulsive effect of collagen molecules at pH above its isoelectric point (pI), since the decrease in solubility can be caused by a reduction in the amount of molecular charges (DI et al., 2014; CHEN et al., 2016).
2.4 SDS–PAGE and optical densitometry analysis

This experiment was performed in order to investigate the degree of extraction/hydrolysis provided by the methodology used in the present study and compare with other works. Here, the SDS-PAGE profile showed that the target products (α1 and α2) were obtained.

2.5 Industrial potential of collagenase extraction and native collagen hydrolysis

The collagenolytic enzyme extracted was able to cleave native collagen in 24 hours of incubation. An exponential profile of collagen hydrolysis was verified, justified by the fact that since one collagenolytic enzyme degrades the trapped structure in the collagen matrix, other proteases would accelerate the process by finding their specific site of action after the release of collagen (HERREIRO-HERNANDEZ et al., 2003). Collagenase from other fish species were already reported as efficient to hydrolyze the Achilles tendon type I collagen, as described by Herreiro-Hernandez et al (2003) and Oliveira et al (2017) and proving the catalytic potential of enzymes from viscera of ced cod Gadus morhua and smooth weakfish C. leiarchus, as well as the efficiency of collagen extracted from the fish skin in the production of biologically active peptides of industrial interest.

4. CONCLUSIONS

Pepsin-soluble method was efficient in extracting collagen from the skin of peacock bass C. ocellaris. The PSC was soluble at acidic pH and low NaCl concentrations, relevant conditions for the preservation of its biochemical characteristics. Based on: 1) the SDS-PAGE and optical densitometry analyzes of the monomeric subunits and crosslinks; 2) the telopeptide and helical structures detected in the UV absorption spectra; the extracted material can be characterized as type I collagen, which highlights its importance for commercial applications. The extracted collagen was cleaved by collagenase being therefore potentially useful in the production of biologically active collagen-peptides for food, biomedical and pharmaceutical sectors.

5. REFERENCES


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