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RECOVERY OF FIBRINOLYTIC AND COLLAGENOLYTIC ENZYMES FROM FISH AND SHRIMP BYPRODUCTS: POTENTIAL SOURCE FOR BIOMEDICAL APPLICATIONS

Vagne de Melo Oliveira[1,2]
Caio Rodrigo Dias Assis[2]
Jéssica Costa Silva[1]
Quesia Jemima Silva[1]
Ranilson de Souza Bezerra[2]
Ana Lúcia Figueiredo Porto[1]

[1]Universidade Federal Rural de Pernambuco – UFRPE, Laboratório de Tecnologia de Produtos Bioativos – LABTECBIO, Departamento de Morfologia e Fisiologia Animal – DMFA, Av. Dom Manoel de Medeiros, s/n, CEP 52171-900, Recife, PE, Brasil. E-mail: vagne_melo@hotmail.com (corresponding author).

[2]Universidade Federal de Pernambuco – UFPE, Laboratório de Enzimologia – LABENZ, Departamento de Bioquímica, Campus Universitário, s/n, CEP 50670-901, Recife, PE, Brasil.

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ABSTRACT

Fish and shrimp industries generate a significant amount of by-products. These by-products can be used for the extraction of enzymes of biomedical interest, such as fibrinolytic and collagenolytic. Thus, this work aimed to perform a screening of fish and shrimp byproducts as sources of enzymes with fibrinolytic and collagenolytic activities and to characterize the biochemical properties of crude extracts with collagenolytic activity from *Cichla ocellaris* residues. Fibrinolytic enzymes were recovered with activities between 5.51 ± 0.02 $U.mL^{-1}$ (*Caranx crysos*) and 56.16 ± 0.42 $U.mL^{-1}$ (*Litopenaeus vannamei*), while collagenolytic enzymes were detected in a range between 6.79 ± 0.00 $U.mg^{-1}$ (*Trachurus lathami*) and 94.35 ± 0.02 $U.mg^{-1}$ (*C. ocellaris*). After collagenolytic screening, the selected species was *C. ocellaris*, being subjected to a preheating, which culminated with an increase of enzymatic activity of 35.07% (up to 127.44 ± 0.09 $U.mg^{-1}$). The optimal collagenolytic activity recovered from *C. ocellaris* byproducts was 55 °C (thermostable between 25 and 60 °C) and 7.5 (stable between 6.5 and 11.5) for temperature and pH evaluations, respectively. The kinetic parameters were determined, obtaining K_m of 5.92 mM and V_{max} of 294.40 $U.mg^{-1}$. The recovered enzyme was sensitive to the Cu^{2+}, Hg^2 and Pb^{2+} ions, being partially inhibited by phenylmethylsulphonyl fluoride (PMSF), N-p-tosyl-L-lysin chloromethyl ketone (TLCK) and Benzamidine. Furthermore, it was able to cleave native type I collagen, the most important type for industry. Thus, the recovery of biomolecules, besides offering to the industry an alternative source of active molecules, contributes to the reduction of the environmental impact, adding value to the fish product and providing a new source of income.

Key words: byproducts; collagenase; thrombolytic; byproducts.

RECUPERAÇÃO DE ENZIMAS FIBRINOLÍTICAS E COLAGENOLITÍCAS DE RESÍDUOS DE PEIXES E CAMARÕES: FONTE POTENCIAL PARA APLICAÇÕES BIOMÉDICAS

RESUMO

As indústrias de peixe e camarão geram uma quantidade significativa de subprodutos. Estes subprodutos podem ser utilizados para a extração de enzimas de interesse biomédico, como fibrinolítica e o colagenolítica. Assim, este trabalho teve como objetivo realizar uma triagem de subprodutos de peixes e camarões como fontes de enzimas com atividade fibrinolítica e colagenolítica e caracterizar as propriedades bioquímicas do extrato bruto com atividade colagenolítica a partir de resíduos de *Cichla ocellaris*. Enzimas fibrinolíticas foram recuperadas com atividades entre 5,51 ± 0,02 $U.mL^{-1}$ (*Caranx crysos*) e 56,16 ± 0,42 $U.mL^{-1}$ (*Litopenaeus vannamei*), enquanto enzimas colagenolitícas foram detectadas numa variação entre 6,79 ± 0,00 $U.mg^{-1}$ (*Trachurus lathami*) e 94,35 ± 0,02 $U.mg^{-1}$ (*C. ocellaris*). Após a triagem colagenolítica, a espécie selecionada foi a *C. ocellaris*, sendo submetida a um pré-aquecimento, o que culminou com um aumento de atividade enzimática de 35.07% (127.44 ± 0.09 $U.mg^{-1}$). A atividade colagenolítica ótima recuperada de resíduos de *C. ocellaris* foi a 55°C (termoestável entre 25 a 60°) e 7,5 (estável entre 6,5 a 11,5) para avaliações de temperatura e de pH, respectivamente. Os parâmetros cinéticos foram determinados, obtendo-se K_m de 5,92 mM e V_{max} de 294,40 $U.mg^{-1}$. A enzima recuperada foi sensível aos íons de Cu^{2+}, Hg^{2+} e Pb^{2+}, sendo parcialmente inibida por Fluoreto de Fenilmetilsulfonil (PMSF), N-α-tosil-L-lisina clorometil cetona (TLCK) e Benzamidina. Ainda, foi capaz de clivar o colágeno nativo tipo I, o tipo mais desejável pela indústria. Assim, a recuperação de biomoléculas, além de oferecer à indústria uma fonte alternativa de moléculas ativas, contribui para a redução do impacto ambiental, agregando valor ao produto pesqueiro e proporcionando nova fonte de renda.

Palavras-chave: subprodutos; colagenase; trombolítica; resíduo.

INTRODUCTION

World fish production has increased substantially to meet consumer demand. Thereby, the fish processing industries have generated significant quantities of organic material from daily processing waste (Daboor et al., 2010; Leite et al., 2016), with potential for industrial use after the accomplishment of bioprocesses of enzymatic recovery. For every 1 kg of fish, about 60 to 80% of this biomass is discarded by the fishing industry as devoid of commercial value, leading to environmental pollution from inadequate disposal. This discarded material includes head, tail, fins, scales and internal viscera, and of this latter, deserves attention the digestive ones because they are sources of several biomolecules of industrial interest such as proteins (Bezerra et al., 2006; Daboor et al., 2012; Freitas-Junior et al., 2012; Oliveira et al., 2017a).

The process of obtaining a commercial product from the fish byproducts goes through a succession of steps, often at a high cost, from obtaining the byproducts to the identification of a protein, passing by sequential processes such as isolation and characterization to define features and applicability of the protein (Oliveira et al., 2017a, 2017b, 2017c, 2017d). Among these, proteases received notoriety because of their widespread commercial use (Bezerra et al., 2006). Studies on the isolation and characterization of proteases from unusual species have demonstrated the enormous potential of these byproducts as sustainable sources. Among the several proteases, fibrinolytic and collagenolytic enzymes gain special attention due to their physiological properties, the first to act as thrombolytic agents (Chandrashekar et al., 2018; Guedes et al., 2018), while the second to assist in healing processes (Alipour et al., 2017).

The thrombosis caused by fibrin accumulation hinders blood flow, promoting the development of cardiovascular diseases. The role of fibrinolytic enzymes is to degrade clots of fibrin formed in blood vessels (Chandrashekar et al., 2018; Guedes et al., 2018). In animals, these clots may cause jugular obstruction which, in turn, may lead to edema of the laryngeal edemax, resulting in airway obstructions (Barbosa et al., 2009). Furthermore, systemic fibrinolytic therapy currently available for human treatment is associated with low efficacy and many side effects (Cicha, 2015). Linked to this is the high costs of current therapies, that have led researches to look for alternative sources of fibrinolytic enzymes, such as those recovered from fish waste byproducts (Oliveira et al., 2017d).

Collagenolytic proteases are valuable enzymes in the veterinary clinic, assisting in the treatment of tendinitis in horses (Watts et al., 2012) and osteoarthritis in dogs (Chu et al., 2002; Hayashi et al., 2009). Also, in cases of uterine fibroids (Jayes et al., 2016), for the isolation of pancreatic islets in studies on the molecular mechanism underlying the disappearance of β-cells in diabetic cats (Zini et al., 2009) and in the treatment of canine histiocytic sarcoma (Fayyad et al., 2018). In fish processing, it acts on the muscle, helping in the steps of skin removal, collagen extraction and production of bioactive peptides for industrial application (Suphatharaprateep et al., 2011; Oliveira et al., 2017a), many of which are of interest to the areas of nutrition and animal clinic due to their antioxidant and antimicrobial activities (Lima et al., 2014). Thus, this work aimed to perform a screening of fish and shrimp byproducts as sources of proteinases with fibrinolytic and collagenolytic activities and to characterize the biochemical properties of crude extracts with collagenolytic activity from *Cichla ocellaris* residues.

MATERIAL AND METHODS

Fish byproducts: extraction of collagenolytic/ fibrinolytic enzymes

Samples of intestine (20 g) per species were collected separately, washed in distilled water to remove dirt, packaged in plastic containers, kept on ice and transported in an average time of 6 hours to the Laboratório de Produtos Bioativos, Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Recife, Pernambuco. The processed fish byproducts used in this work is described in Table 1, as well as the origin of each material. The extraction of the enzymes from each species was carried out following the methodology described by Oliveira et al. (2017a), through successive stages of maceration and homogenization of the collected material. The ratio between tissue and extraction buffer 50 mM Tris-HCl (Tris - Sigma, St. Louis, MO, USA; HCl – Merck, Darmstadt, Germany) pH 7.5 containing 5 mM $CaCl_2$ was 1:5 (w/v). The intestinal viscera were homogenized in mechanical homogenizer (IKA RW 20D S32, Guangzhou, China) for 5 minutes at 10,000-12,000 rpm (4 °C). The obtained homogenate was centrifuged at 12,000 x *g* (Sorvall® Superspeed Centrifuge RC-6, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 4 °C. The material was stored until further processing. The supernatant fraction with the highest total protein, specific and volumetric collagenolytic activities was used as the crude extract (CE) for physicochemical characterization and collagen type I test. After selecting the best processing step, the crude extract (50 mL) was incubated at 55 °C for 30 min and centrifuged at 10,000 x *g* for 10 min at 4 °C. The material is stored at - 25 °C for further processing. The preparation of the protein hydrolyzate from the shrimp species was performed following the methodology described by Cahu et al. (2012).

Protein determination

The protein concentration of all tissue extracts was determined by the method of bicinchoninic acid (BCA) according to Smith et al. (1985).

Fibrinolytic assay

The species used in the investigation/prospection of fibrinolytic enzymes are described in Table 1. The fibrinolytic activity was determined using a spectrophotometric method: First, 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 5 min. Then, 0.1 mL of a 20 $U.mL^{-1}$ thrombin solution (Sigma-Aldrich, St. Louis, MO, USA) was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation

Table 1. Fish and shrimp processing byproducts used in the screening and recovery of collagenolytic and fibrinolytic enzymes.

Scientific name	Common name	Species	Enzymatic activity	
			Collagenolytic	Fibrinolytic
*Rachycentron canadum**	Cobia	Fish	X	X
*Caranx crysos**	Blue runner	Fish	X	X
*Lutjanus synagris**	Lane snapper	Fish	X	X
*Scomberomorus mackerel**	Mackerel	Fish	X	X
*Parachromis managuensis***	Jaguar cichlid	Fish	X	X
*Colossoma macropomum***	Tambaqui	Fish	X	ND
*Oreochromis niloticus***	Nile tilapia	Fish	X	X
*Pseudoplatystoma corruscans****	Surubim	Fish	X	X
*Cichla ocellaris****	Peacock bass	Fish	X	X
*Cynoscion leiarchus**	Smooth weakfish	Fish	ND	X
*Centropomus undecimalis*****	Common snook	Fish	ND	X
*Mugil liza*****	Lebranche mullet	Fish	ND	X
*Sparisoma axillare*****	Parrotfish	Fish	ND	X
*Eucinostomus gula*****	Jenny mojarra	Fish	ND	X
*Anisotremus virginicus*****	Porkfish	Fish	ND	X
*Astronotus ocellatus***	Oscar	Fish	X	X
*Pomatomus saltatrix******	Bluefish/anchovy	Fish	X	ND
*Trachurus lathami******	Rough scad	Fish	X	ND
*Litopenaeus vannamei*******	gray shrimp	Shrimp	X	X

ND - not determined. *Were kindly provided by the fishing village of Ponta Verde, Maceió, Alagoas, Brazil. **Were kindly provided by fishing and aquaculture base of the Federal Rural University of Pernambuco, Recife, Pernambuco, Brazil. ***were obtained from the community of fishermen from the town of Petrolândia, Pernambuco, Brazil. ****The intestinal viscera of the species used came from artisanal/autonomous fishermen in the city of Recife and Olinda, Pernambuco, Brazil, and were gently given after evisceration by the fisherman himself. ***** Fishermen's colony of Pina, Recife, Pernambuco, Brazil. ****** Kindly provided by the company Noronha Pescados, Recife, Pernambuco, Brazil.

continued at 37 °C. At 60 min, 0.7 mL of 0.2 M trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) was added, and mixed. The reaction mixture was centrifuged at 15,000 × g for 10 min. Then, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit) was defined as the increase of 0.01 in absorbance per minute at 275 nm (Wang et al., 2011; Oliveira et al., 2017d). The tests were performed in triplicate.

Collagenolytic assay

The collagenolytic properties of the crude extract was determined according to Oliveira et al. (2017a), using Azo dye-impregnated collagen (azocoll) as substrate (Sigma, St. Louis, MO, USA). A reaction mixture, containing 5 mg of azocoll, 500 µL of 50 mM Tris-HCl (pH 7.5) with 5 mM $CaCl_2$ and 500 µL of crude extract, was incubated at 55 °C for 30 minutes, under stirring. Thereafter, 200 µL of 40% trichloroacetic acid was added to stop the reaction. After 10 minutes, the samples were centrifuged at 10,000 x *g* for 10 minutes at 4 °C. The sample reading was performed using a spectrophotometer (Bio-Rad Smartspec™ 3000, Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 595 nm. One enzyme unit (U) was defined as the amount of enzyme required to increase the absorbance in 0.01 at 595 nm. The assays were performed in quadruplicate.

Biochemical and kinetic properties of the selected collagenolytic enzyme

Michaelis–Menten kinetics (K_m and V_{max})

The substrates used in the kinetic tests were azocoll at increasing concentrations (1.0 - 20.0 $mg.mL^{-1}$) in 50 mM Tris–HCl pH 7.5 buffer at 55 °C for 30 min as described in the previous section. The reactions were performed in quadruplicate.

Optimum temperature and thermal stability

The effect of temperature on collagenolytic activity was investigated in various temperatures at pH 7.5. The enzyme in extraction buffer was assayed for 1 hour in a range from 25 to 90 °C, and the remaining collagenolytic activity was then measured as described before. Control activity represented 100% and sample activities were expressed in relation to control. The thermal stability was measured in the same way of optimum temperature after incubation of the enzyme in the range from 25 to 90 °C

for 1 hour and after 15 min equilibration at room temperature (Oliveira et al., 2017a).

Optimum pH and stability

These assays were carried out in different pH using the buffers: 0.5 M citrate–phosphate (pH 4.0-7.0), 0.1 M Tris–HCl (pH 7.5-8.5) and 0.1 M glycine-NaOH (pH 9.0-12.0), containing 5 mM $CaC1_2$. The reaction mixtures were incubated for 1 hour at 37 °C and the enzyme activities were measured as described before. The influence of pH on enzyme stability was determined by incubating the enzyme with various buffer solutions, at a ratio of 1:1 for 1 hour at 37 °C and returning to the optimum pH (Oliveira et al., 2017a).

Sensitivity to metal ions and inhibitors

The effect of metal ions on the enzyme activity was investigated by adding metal ions (K^+, Na^+, Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+} and Al^{3+}) to the reaction mixture. The final concentration of each metal ion was 1 mM. Each ion was incubated for 1 hour at a ratio of 1:1, and then the activity was performed as described before. The sensitivity to inhibitors was performed by incubating the crude extract (30µL) was incubated for 1 hour at 25 °C with protease inhibitors (30 µL, 8 mM): phenylmethylsulphonyl fluoride (PMSF); N-p-tosyl-L-lysin chloromethyl ketone (TLCK); benzamidine; N-tosyl-L-phenylalaninechloromethyl ketone (TPCK); ethylenediamine tetra-acetic acid (EDTA); and β-mercaptoethanol. Each ion was incubated for 1 hour at a ratio of 1:1, and then the activity was assayed as described above. The results were expressed as percentage of the control activity (Oliveira et al., 2017a).

Collagen type I test

The measure of the digestion of native collagen Type I was performed according to the method of Moore and Stein (1954) and Park et al. (2002) with slight modifications by Oliveira et al. (2017a). A reaction mixture, which contained 5 mg of collagen type I, 1 mL of 50 mM Tris-HCl (pH 7.5 with 5 mM $CaCl_2$) and 0.1 mL of the enzyme solution, was incubated at 37 °C for 12, 24, 36 and 48 hours. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at 1,800 (×) g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100 °C for 20 min, and then cooled to room temperature. Subsequently, the mixture was diluted with 5 mL of 50% 1-propanol for absorption measurement at 570 nm. The concentration of hydrolyzate-amino acids was determined using a standard curve of L-leucine. One unit (U) of enzyme activity is defined as the amount of enzyme that is required for the hydrolysis of 1 mmol of substrate per h.

Statistical analysis

All values are presented as means ± standard deviations. Data were statistically analyzed for normal distribution by Shapiro–Wilk and Kolmogorov-Smirnov tests and 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% ($p < 0.05$). Figures created with MicroCal® Origin® Version 8.0 (MicroCal, Northampton, MA, USA).

RESULTS

Fish byproducts: recovery of fibrinolytic activity

Here, some of the selected fish processing byproducts used in the initial screening of this study presented ability to cleave fibrin aggregates at several rates ($U.mL^{-1}$): blue runner *C. crysos* (5.73 ± 0.01), lane snapper *L. synagris* (5.51 ± 0.02), cobia *R. canadum* (13.29 ± 0.06), mackerel *S. mackerel* (24.12 ± 0.03), jaguar cichlid *P. managuensis* (28.55 ± 0.07), tambaqui *C. macropomum* (35.09 ± 0.13), surubim *P. corruscans* (16.93 ± 0.11), smooth weakfish *C. leiarchus* (22.78 ± 0.01), peacock bass *C. ocellaris* (22.53 ± 0.60), common snook *C. undecimalis* (12.15 ± 0.05), lebranche mullet *M. liza* (23.50 ± 0.07), Nile tilapia *O. niloticus* (37.53 ± 0.03), parrotfish *S. axillare* (39.50 ± 0.01), jenny mojarra *E. gula* (15.6 ± 0.43), porkfish *A. virginicus* (38.79 ± 0.01), oscar *A. ocellatus* (29.52 ± 0.00) and gray shrimp hydrolyzate *Litopenaeus vannamei* (56.16 ± 0.42).

Fish byproducts: recovery of collagenolytic activity

Collagenolytic activity varied according to the species of fish tested, and it was detected, in ascending order ($U.mg^{-1}$): rough scad *T. lathami* (6.79 ± 0.00), surubim *P. corruscans* (15.95 ± 0.07), jaguar cichlid *P. managuensis* (31.98 ± 0.01), lane snapper *Lutjanus synagris* (35.18 ± 0.07), mackerel *S. mackerel* (46.88 ± 0.03), cobia *R. canadum* (52.13 ± 0.11), Nile tilapia *O. niloticus* (58.27 ± 0.19), tambaqui *C. macropomum* cecum (66.68 ± 0.04), Oscar *A. ocellatus* (68.42 ± 0.11), blue runner *C. crysos* (70.54 ± 0.09), tambaqui *C. macropomum* intestine (73.49 ± 0.00), bluefish *Pomatomus saltatrix* (82.24 ± 0.02) and peacock bass *C. ocellaris* (94.35 ± 0.02). The *L. vannamei* hydrolyzate had the lowest collagenolytic activity (3.29 ± 0.00 $U.mg^{-1}$) when compared to the enzymes extracted from the gut of the fish species. After screening, the crude intestinal extract of peacock bass *C. ocellaris* had its activity increased by 35.07% after preheating, to the specific activity of 127.44 ± 0.09 $U.mg^{-1}$.

Kinetic properties (K_m and V_{max}) of the collagenolytic enzyme

The Michaelis-Menten constant (K_m) for the crude extract of peacock bass *C. ocellaris* was 5.92 mM and maximum velocity rate (V_{max}) was 294.40 $U.mg^{-1}$.

Optimum temperature and pH and their stabilities of the collagenolytic enzyme

The optimum temperature of the enzyme activity was 55 °C with marked reduction above 65 °C and total loss of activity at 75 °C (Figure 1A). The thermal stability was observed between 25 °C and 60 °C (Figure 1b).

The optimum pH of the enzyme was 7.5 (Figure 2) and it was stable in pH between 6.5 and 11.5 (Figure 2).

Sensitivity of the collagenolytic enzyme to metal ions and inhibitors

The data obtained in the sensitivity tests for ions and inhibitors are described in Table 2. The samples exposed to ions K^+, Ca^{2+}, Ba^+ and Mg^{2+} showed no significant difference of activity ($p < 0.05$) compared to the control group (100%).

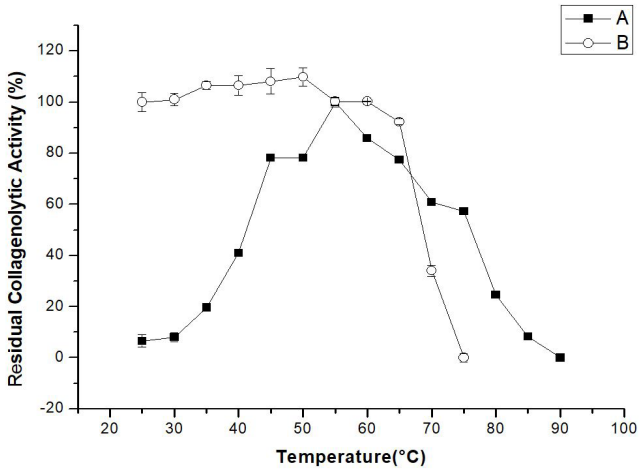

Figure 1. Effect of temperature and thermal stability of the activity of protease with collagenolytic properties extracted from intestinal byproducts of peacock bass *Cichla ocellaris*. (**A**) Optimum temperature in a range of 25-90°C. (**B**) Thermal stability after 1 hour of incubation in the temperature range of 25-90°C. Data represented as mean ± SD.

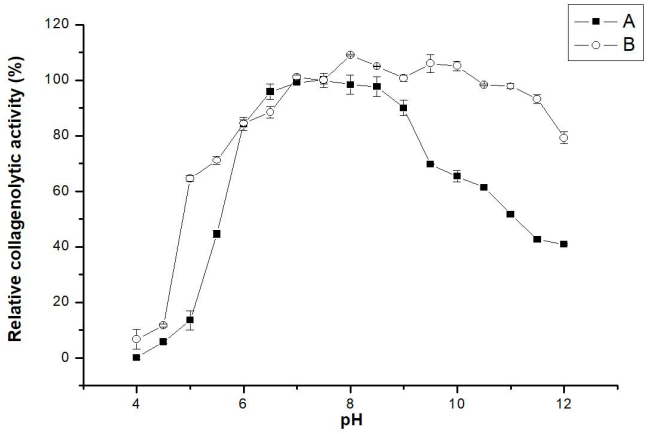

Figure 2. Effect of pH and pH stability of the activity of protease with collagenolytic properties extracted from intestinal byproducts of peacock bass *Cichla ocellaris*. (**A**) Optimal pH, using different buffers in the pH range from 4.5 to 12.0. (**B**) pH stability after incubation for 1 hour in the pH range from 4.5 to 12.0. Data represented as mean ± SD.

In contrast, all the other ions showed a significant statistical difference ($p < 0.05$). The collagenolytic enzyme was partially inhibited by ions Cu^{2+}, Zn^{2+}, Al^{3+} and Na^{2+}, while Pb^{2+} and Hg^{2+} were strong inhibitors, similarly as observed for the inhibitors of serine proteases and trypsin-like enzymes (PMSF, Benzamidine and TLCK), when a significant inhibition difference ($p < 0.05$) on the activity was verified.

Values are means ± SD. Data was significantly drawn from a normally distributed population by Shapiro–Wilk and Kolmogorov-Smirnov tests and presented homogeneity of variances according to Levene's test. Means with different lowercase superscript letters differ significantly by Tukey's test ($p < 0.05$) ($n = 4$).

Collagen type I test

The collagenolytic enzyme extracted from *C. ocellaris* presented ability to cleave collagen type I reaching the highest hydrolysis degree after 48 hours. The collagen cleavage by these proteases showed a marked increase from 36 to 48h (Figure 3).

Table 2. Effect of ions and inhibitors on the activity of collagenolytic enzyme from peacock bass *Cichla ocellaris*.

Ions and Inhibitors	**Collagenolytic activity (%)**
Metal ions (1 mM)	
Control	100.0 ± 0.12[a]
Cd^{2+}	67.29 ± 0.02[b]
Cu^{2+}	52.12 ± 0.04[b]
K^+	101.54 ± 0.03[a]
Zn^{2+}	72.18 ± 0.01[b]
Al^{3+}	71.06 ± 0.01[b]
Hg^{2+}	40.53 ± 0.00[b]
Na^+	68.07 ± 0.03[b]
Pb^{2+}	46.28 ± 0.01[b]
Ca^{2+}	100.31± 0.06[a]
Ba^{2+}	89.13 ± 0.03[a]
Mg^{2+}	107.53 ± 0.04[a]
Inhibitors (8 mM)	
Control	100.0 ± 0.03[a]
PMSF	38.23 ± 0.03 [b]
TPCK	74.60 ± 0.03[b]
TLCK	18.14 ± 0.03[b]
Benzamidine	22.90 ± 0.02[b]
EDTA	48.79 ± 0.00[b]
β-Mercaptoethanol	84.89 ± 0.01[b]

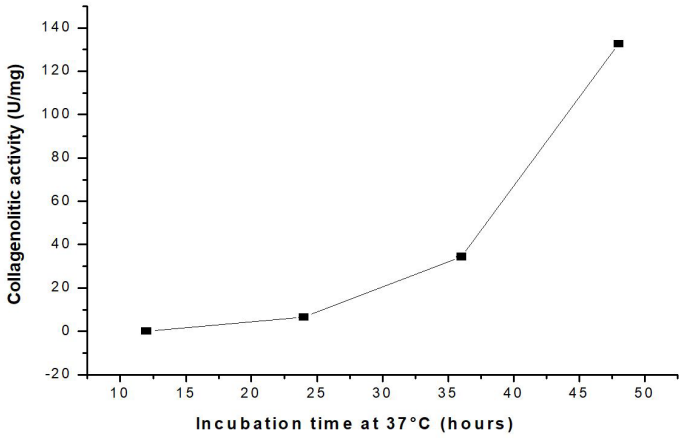

Figure 3. Peacock bass *Cichla ocellaris* collagenolytic protease activity towards bovine Achilles tendon collagen type I at different incubation times (12, 24, 36 and 48 hours). Data represent the mean of four replicates.

DISCUSSION

After screening using simple technological processes, enzymes with fibrinolytic and collagenolytic activity of fish byproducts were recovered (Table 1). Fibrinolytic assays were promising in all tested fish and shrimp species. Then, the collagenolytic activities of the tested species were measured and peacock bass *C. ocellaris* presented better collagenolytic activity. As a result, it was selected to go through biochemical characterization stages, determining its optimal conditions, and testing for type I collagen hydrolysis.

Recovery of fibrinolytic enzyme activity

The fibrinolytic activity recovered from the byproducts of the species described in table 1 eminently suggest the potential of aquatic organisms as an alternative source of thrombolytic agents, corroborating with that described by Oliveira et al. (2017d) when they recovered the fibrinolytic enzyme (26.70 ± 0.05 $U.mL^{-1}$) of intestinal viscera of greater amberjack *S. dumerili*. Here, intestinal byproducts of tambaqui *C. macropomum* (35.09 ± 0.13 $U.mL^{-1}$), porkfish *A. virginicus* (38.79 ± 0.01 $U.mL^{-1}$), parrotfish *S. axillare* (39.50 ± 0.01 $U.mL^{-1}$), and the shrimp hydrolyzate *Litopenaeus vannamei* (56.16 ± 0.42 $U.mL^{-1}$). Because the use as thrombolytic agents in cardiac valves (Karakurt and Başbuğ, 2015), in cancer therapy (Amiral and Seghatchian, 2017), in diabetic retinopathy (Behl et al., 2017) it is necessary that the new sources of fibrinolytic proteases pass by a rigorous process of purification, besides pharmacological and toxicological tests to guarantee the safety and the clinical viability (Cicha, 2015). However, the data obtained in this research serve as preliminary results for further research, in order to develop this aspect of the use of fish byproducts in thrombolytic therapy.

Recovery of the collagenolytic enzyme

Digestive viscera byproducts from fish species (tropical and neotropical) has been reported as a potential source in the recovery of enzymes with collagenolytic properties, such as those described for proteases extracted from the intestine (and its portions) and/or from the pyloric cecum (when present) of winter flounder *Pseudopleuronectes americanus* (3.82 $U.mg^{-1}$) (TerueL and Simpson, 1995), tuna *Thunnus thynnus* (16.5 $U.mg^{-1}$) (Byun et al., 2002), filefish *Novoden modestrus* (114.15 $U.mg^{-1}$) (Kim et al., 2002), mackerel *Scomber japonicus* (16.5 $U.mg^{-1}$) (Park et al., 2002), smooth weakfsh *C. leiarchus* (72.0 $U.mL^{-1}$) (Oliveira et al., 2017a) and greater amberjack *S. dumerili* (42.44 $U.mg^{-1}$) (Oliveira et al., 2017d). Recovery of collagenolytic enzymes have also been described from aquatic invertebrates such as antarctic krill *Euphausia superba* (Turkiewicz et al., 1991) and muscle of fish species such as red sea bream *Pagrus major* (Wu et al., 2010). Here, the results found for the species tested confirmed the potential of the promising sources of collagenase, especially for *C. ocellaris* species (94.35 $U.mg^{-1}$), being chosen to partially define their biochemical properties. It is noteworthy that, after the species was defined, the crude extract underwent a preheating with the purpose of eliminating proteases sensitive to high temperatures (Freitas-Junior et al., 2012; Oliveira et al., 2017a), increasing the viability of using the enzyme collagenolytic, observing an increase in its activity increased by 35.07% after preheating (127.44 $U.mg^{-1}$).

Kinetic properties (K_m and $V_{máx}$) of the collagenolytic enzyme

Collagenase enzymes, as specific enzymes for the collagen substrate, have been isolated and characterized from both microbial cells and animal tissues (Daboor et al., 2010). Kinetic properties with fish byproducts were recorded to date using different types of substrates, such as the Type I collagen of tuna *T. thymus* (3.82 mM and 851.5 $U.mg^{-1}$) (Byun et al., 2002), mackerel *S. japonicus* (1.1 mM and 2.34 $U.mg^{-1}$) (Park et al., 2002), the use of BocLeu-Lys-Arg-MCA from the skeletal muscle of sea bream *P. major* (3.58 mM) (Wu et al., 2010) and the use of SAPNA as a substrate in the species sardinelle *Sardinella aurita* (0.033 mM) (Hayet et al., 2011), assuming it to be a chymotrypsin-like collagenase. The K_m is used to assess the affinity of the enzyme for the substrate and the results showed that collagenolytic enzyme from *C. ocellaris* have affinity for azocoll. The kinetic parameters provide data about efficacy of the enzyme, adding more information to the industrial processing (Park et al., 2002). However, no results were found in studies that employ azocoll as substrate in fish species regarding kinetic parameters for more precise comparisons. Furthermore, possible interferences must be taken into account, since the filtration and centrifugation steps do not completely eliminate debris or binding agents, with more specific bioprocesses being necessary according to the industrial need.

Optimum temperature and pH and stability study of the collagenolytic enzymes

Temperature is a limiting factor for less sensitive proteases and also can works as an activator of proteases that convert procolagenases. Collagenolytic enzymes recovered from fish byproducts have shown activity in a range of temperatures, depending on the tissue and species from which they were isolated (Daboor et al., 2010) and the range reported so far is between temperatures 20 °C and 60 °C (Daboor et al., 2010, 2012; Wu et al., 2010; Hayet et al., 2011; Sriket et al., 2011; Oliveira et al., 2017a, 2017d). Thus, similar results of optimal activity at 55 °C (Figure 1A) were reported for collagenases recovered from mackerel species *S. japonicas* (Park et al., 2002) and smooth weakfsh *C. leiarchus (*Oliveira et al., 2017a). Sriket et al. (2011) reported optimal activity at 60 °C from hepatopancreas of fresh water prawn Macrobrachium rosenbergii. Oliveira et al. (2017a) reported that the collagenolytic enzyme recovered from smooth weakfsh *C. leiarchus* intestine presented thermostability in the range of 25 to 60 °C (Figure 1B), results similar to found for *C. ocellaris*.

Results of activities at pH 7.5 (Figure 2A), similar to those found for *C. ocellaris*, were reported for digestive and muscular byproducts of fish and invertebrates, as described by Teruel and Simpson (1995), Byun et al. (2002), Park et al. (2002), Souchet and Laplante (2011) and Daboor et al. (2012) for the aquatic species winter flounder *P. americanus*, tuna *T. thymus*, mackerel *S. japonicus*, snow crab *C. opilio* and mixed viscera of different fish species, respectively, differing from Kim et al. (2002) for filefish *N. modestrus*, Wu et al. (2010) for sea bream *P. major*, Hayet et al. (2011) for sardinelle *S. aurita* and Oliveira et al. (2017a) for smooth weakfsh *C. leiarchus* which reported optimum activity at pH 8.0. Also, were reported for hepatopancreas of fresh water prawn optimum activity at pH 7.0 (Sriket et al., 2011). Furthermore, Mukherjee et al. (2009) reported optimum activity at pH 5.0 for the enzyme recovered from sponge *Rhopaloeides odorabile*, while Murado et al. (2009) found a pH of 6.0 to rayfish *Raja clavata*; and Kristjánsson et al. (1995) and Roy et al. (1996) found a pH of 7.0 for Atlantic cod *G. morhua* and greenshore crab *C. maenas*, respectively.

Enzymes with collagenolytic properties recovered from aquatic organisms (fish, crustaceans, sponges) generally exhibit stability over a broad pH range (5.0 to 10) (Mukherjee et al., 2009; Murado et al., 2009; Daboor et al., 2010; Hayet et al., 2011; Souchet and Laplante, 2011; Oliveira et al., 2017a, 2017d). In the present work, enzymatic activity in a wide range of pH conditions have been detected and remained stable between 6.5 and 11.5, as seen in Figure 2B, results that are similar to those described by Oliveira et al. (2017a) for the species smooth weakfsh *C. leiarchus.* Industrially, it is fundamental to control the temperature and the pH, since they directly influence the enzymatic activity, influencing the productive process (Daboor et al., 2010, 2012; Oliveira et al., 2017a, 2017d). Here, from the results obtained for *C. ocellaris*, it was possible to recover from intestinal viscera a collagenolytic enzyme resistant to a wide range of temperature and pH, as well as its oscillations.

Sensitivity of collagenolytic enzyme to ions and inhibitors

Metal ions are often required as cofactors for the conversion of collagenase from its zymogenic form to active molecule (Daboor et al., 2012; Oliveira et al., 2017d). In this sense, the use of activators and inhibitors is essential for regulation of the enzyme action in the industrial processing. The data obtained in this work for the collagenolytic enzyme of *C. ocellaris* in relation to the sensitivity of metallic ions are in agreement with those found for other neotropical fish species (Table 2), such as that described by Oliveira et al. (2017a), in which the authors observed a marked reduction in the activity of a collagenolytic serine protease extracted from weakfsh *C. leiarchus* when subjected to Cd^{2+}, Cu^{2+}, Al^{3+}, Pb^{2+} and Hg^{2+}. Byun et al. (2002) observed a marked reduction in the activity of a collagenolytic serine protease extracted from tuna *T. thymus* when subjected to Cd^{2+}, Hg^{2+} and Zn^{2+} and exposed to increasing Ca^{2+} and Mg^{2+}. Analyzing collagenolytic recovered byproducts from sea bream *P. major*, Wu et al. (2010) observed a complete inhibition when in presence of Cu^{2+}, Cd^{2+} and Zn^{2+}. As mentioned above, the information about the enzymatic sensitivity to metal ions is essential for the optimization of its catalytic efficiency in the industrial segment, allowing the control and the handling of its activity, besides minimizing possible interactions with potential inhibitors (Daboor et al., 2010, 2012; Oliveira et al., 2017a, 2017d), such as those described in table 2 for Hg^{2+}, Pb^{2+} and Cu^{2+}.

Results similar to collagenolytic enzyme recovered from crude extract of *C. ocellaris* exposure to inhibitors (Table 2) have been described for other fish species, such as for weakfish *C. leiarchus* (Oliveira et al., 2017a). Also, similar effects of the inhibitors TLCK and PMSF (suggesting collagenolytic enzymes with trypsin-like characteristics) were reported by Kim et al. (2002), Byun et al. (2002), Park et al. (2002), Hayet et al. (2011), Souchet and Laplante (2011) and Sriket et al. (2011) for collagenolytic proteases from fish byproducts from filefish *N. modestrus*, tuna *T. thymus*, mackerel *S. japonicas,* sardinelle *S. aurita*, snow crab *C. opilio* and water prawn *M. rosenbergii*, respectively. The assay with inhibitors of metalloproteinases (EDTA) detected reduced enzyme activity. Marked loss of activity when subjected to EDTA has been reported for tuna *T. thymus* (Byun et al., 2002) and sardinelle *S. aurita* (Hayet et al., 2011) and no inhibition for mackerel *S. japonicus* (Park et al., 2002). Here, a chelation process may have occurred, leading to a reduction in enzyme activity, probably because it is a crude extract.

However, other collagenolytic enzymes recovered from aquatic organism byproducts showed a marked reduction in collagenolytic activity, such as described by Kim et al. (2002) for filefish *N. modestrus*, Souchet and Laplante (2011) for snow crab *C. opilio*, Daboor et al. (2012) for fish byproducts and Oliveira et al. (2017a) for intestinal viscera of weakfsh *C. leiarchus.* There was also reduced activity when subjected the enzyme to β-mercaptoethanol, indicating that the structure of the enzyme presents disulfide bonds, such as that reported by Oliveira et al. (2017a) for weakfsh *C. leiarchus*.

Type I collagen test

Type I collagen is the most abundant and searched by industrial and biomedical segments (Oliveira et al., 2017b, 2017c; Chen et al., 2018), since the dermis of the skin is composed of compressed, long, organized and predominantly collagen bundles of this type (Lima-Junior et al., 2017). Cleavage of interstitial collagens occurs by a limited number of proteases (Amar et al., 2017). Here, the enzyme recovered from *C. ocellaris* intestinal byproducts was able to cleave type I collagen from the bovine tendon (Figure 3). Furthermore, it is likely that other proteases (also trypsin-like, chymotrypsin-like and metalloprotease, for example) may have aided in the cleavage process of type I collagen by the *C. ocellaris* enzyme, since intestinal fish byproducts is a rich and potential source of a number of protein biomolecules, as described by Hernandez-Herreiro et al. (2003) and Oliveira et al. (2017a). Similar results of type I collagen cleavage have been reported for proteases recovered from byproducts of fish species and marine invertebrates, such as antarctic krill *Euphausia superba* (Turkiewicz et al., 1991), Atlantic cod *G. morhua* (Kristjánsson et al., 1995), winter flounder *P. americanus* (Teruel and Simpson, 1995), greenshore crab *C. maenas* (Roy et al., 1996), tuna *T. thymus* (Byun et al., 2002), mackerel *S. japonicus* (Park et al., 2002), filefish *N. modestrus* (Kim et al., 2002), iced cod *Gadus morhua* (Hernandez-Herrero et al., 2003), sardinelle *S. aurita* (Hayet et al., 2011), a mixture of haddock, herring, ground fish and flounder (Daboor et al., 2012) and weakfish *C. leiarchus* (Oliveira et al., 2017a).

CONCLUSIONS

In this work, it was possible to recover enzymes with fibrinolytic and collagenolytic properties from byproducts discarded by the fishing industry and colonies of artisanal fishermen. The data obtained with the fibrinolytic action are promising. All the byproducts tested presented significant results, mainly the results of the shrimp hydrolyzate *L. vannamei*. Also, proteases with collagenolytic activity were recovered. Byproducts of peacock bass *C. ocellaris* were those that met the best selection parameters and, therefore, their biochemical properties were determined. In this sense, it was possible to verify that its physicochemical characteristics (resistance to high temperatures for long periods of time, stability in wide range of pH, besides the control and handling by exposing to metallic ions) and kinetics are consistent with the collagenases employed in several stages in the industrial segments; in addition to being able to cleave the native type I collagen, the most abundant and found in skin human and fish species, suggesting its use in the treatment of wounds and burns after undergoing purification processes and toxicological and antiallergic tests.

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