COLLAGENASE FROM SMOOTH WEAKFISH: EXTRACTION, PARTIAL PURIFICATION, CHARACTERIZATION AND COLLAGEN SPECIFICITY TEST FOR INDUSTRIAL APPLICATION

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ABSTRACT

Fish processing residues are rich sources of biomolecules with industrial potential, such as enzymes with collagenolytic properties applied in the pharmaceutical, textile and leather sectors. Here, collagenolytic serine proteases were partially purified from the waste (viscera) of smooth weakfish Cynoscion leiarchus and characterized for the purpose of obtaining a value-added product from fisheries resources. The higher activity of the enzyme (72.5 U mL⁻¹) was verified in optimal temperature and pH of 55°C and 8.0, respectively. The enzyme was stable in wide ranges of temperature (25–60°C) and pH (6.5 to 11.5). The ions Ca²⁺ and Mg²⁺ increased the protease activity, whilst Pb²⁺, Al³⁺ and Cu²⁺ had an inhibitory effect, as observed in the presence of Benzamidine and TLCK (inhibitors of serine proteases). Hydrolysis was detected after 48 hours, when the enzyme and bovine collagen type I were incubated together. Thus, digestive viscera of C. leiarchus is suggested as an alternative source of enzymes capable of cleaving type I collagen, with similar biochemical properties to bacterial collagenases commonly employed in industrial processes, reducing costs, adding value to the fisheries product and minimizing the environmental impact caused by its waste.

Key words: byproducts; collagenolytic protease; Cynoscion leiarchus; smooth weakfish; wastes.

COLAGENASE DE PESCADA BRANCA: EXTRAÇÃO, PURIFICAÇÃO PARCIAL, CARACTERIZAÇÃO E TESTE DE ESPECIFICIDADE AO COLÁGENO PARA APLICAÇÃO INDUSTRIAL

RESUMO

Resíduos do processamento do pescado são fontes ricas em biomoléculas com potencial industrial, como as enzimas com propriedades colagenolíticas empregadas nos segmentos farmacêuticos, têxteis e de couro. No presente trabalho, serino-proteases colagenolíticas dos resíduos (vísceras) do processamento de pescada-branca, Cynoscion leiarchus, foram parcialmente purificadas e caracterizadas, visando à obtenção de um produto de valor agregado, maximizando o aproveitamento de recursos pesqueiros. A atividade da melhor etapa de extração foi 72,5 U mL⁻¹, com temperatura e pH ótimos de 55°C e 8,0, respectivamente. A enzima manteve-se estável em faixas amplas de temperatura (25–60 °C) e pH (6,5-11,5). Os ions Ca²⁺ e Mg²⁺ aumentaram a atividade proteolítica, ao passo que Pb²⁺, Al³⁺ e Cu²⁺ inibiram essa atividade assim como os inibidores de serino-proteases (Benzamidina e TLCK). A hidrólise foi detectada após 48h de incubação com colágeno bovino tipo I. Assim, sugere-se vísceras digestivas de C. leiarchus como fonte alternativa para o fornecimento de enzimas com capacidade de clivar o colágeno do tipo I e com propriedades bioquímicas semelhantes às colagenases bacterianas já empregadas nas etapas de processamento industrial, como forma de redução de custo, agregação de valor ao produto pesqueiro e contribuindo para minimizar o impacto ambiental deste tipo de resíduo.

Palavras-chave: subprodutos; protease colagenolítica; Cynoscion leiarchus; pescada branca; resíduos.

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INTRODUCTION

According to the Food and Agriculture Organization of the United Nations (FAO), in 2012, the global production of fish for human consumption was estimated to be approximately 136.2 million tons; which is responsible for providing 20% of animal protein to 2.9 billion people and expected to be increased to 151.77 million in 2030 (FAO, 2013; 2014). In Brazilian aquaculture, the importance of the smooth weakfish (Cynoscion leiarchus) is due to the significance of the capture industry, representing around 44.6% of coastal fish production (MPA, 2013).

Increase in consumption implies in a higher product demand, resulting in increased generation of waste (byproducts of processing) such as digestive viscera, fins, head and tail (BEZERRA et al., 2006; FAO, 2013). Efficient utilization of byproducts has direct impact on the economy and environmental pollution of the country (JAYATHILAKAN et al., 2012). The use of proteases and other esterases such as pepsin, gastricsin, trypsin, chymotrypsin, elastase, carboxypeptidase and collagenase from viscera of fish brings an advantage over commercial enzymes, since the production costs can be greatly reduced (BEZERRA et al., 2006; KIM and MENDS, 2006; KLOMKLAO, 2008).

Solid residues from fish processing are potential sources of biomolecules with diverse biotechnological applications, mainly for providing enzymes with physicochemical properties similar to those used in industry (BEZERRA et al., 2005). Collagenases (EC 3.4.24.7) constitute a group of enzymes that hydrolyze the peptide bonds of various types of collagen, the most abundant protein in mammals, under physiological conditions of pH and temperature both in vivo and in vitro (DABOOR et al., 2010; HAYET et al., 2011; SOUCHET and LAPLANTE, 2011). They are highly specific for native or denatured collagen, showing no activity to any other protein. If the collagen is denatured it becomes susceptible to hydrolysis by other proteases (TERUEL, 1997). Collagenolytic enzymes are relevant for their biotechnological potential use in medicine, food industry, cosmetics, leather processing, and obtaining bioactive collagen peptides (DABOOR et al., 2010; KANTH et al., 2008; LIMA et al., 2013; SARAN et al., 2013).

Physicochemical characterization of enzymes is an important tool that produces new data on the operation conditions of these biocatalysts. Such information is crucial for industrial purposes since this sector requires reduction of costs and optimization of results. Fish waste proteases are promising biomolecules presenting the advantage of higher activities at extreme temperatures required by some industrial processes (FRANÇA et al., 2016).

In this context, the present study aimed to obtain proteases capable to cleave the collagen in order to maximize the processing waste of smooth weakfish Cynoscion leiarchus, besides to characterize biochemically the extracted collagenolytic enzyme for future biotechnological applications.

METHODS

Materials

Azo dye-impregnated collagen (azocoll), azocasein, bovine serum albumin (BSA), Na-benzoyl-DL-arginine-p-nitroanilide (BApNA), Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), bovine serum albumin (BSA), Tris (hydroxymethyl) aminomethane and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Glycine was acquired from Amersham Biosciences. HCl was obtained from Merck. Samples were centrifuged in a SorvallÒ Superspeed Centrifuge RC-6 (Thermo Fisher Scientific, Waltham, MA, USA). The spectrophotometer used was Bio-Rad Smartspec™ 3000 and the microplate reader was Bio-Rad xMark™ (Bio-Rad Laboratories, Hercules, CA, USA). Software MicroCal Origin Version 8.0 (MicroCal, Northampton, MA, USA).

Fish Waste

Viscera wastes of smooth weakfish C. leiarchus were obtained from the fishermen association of Ponta Verde, Maceió, Alagoas, Brazil. The viscera were kindly provided after evisceration process. Samples of 500 g intestine and 50 g muscle were collected separately, packaged in plastic containers, kept on ice and transported to the Laboratory of Enzymology, Center of Biological Sciences, Department of Biochemistry, Federal University of Pernambuco, Recife, Pernambuco, Brazil, where they were stored at -27°C for further processing.
Extraction of collagenolytic enzymes

The collagenolytic enzyme was extracted from smooth weakfish processing waste according to modifications of TERUEL and SIMPSON (1995) method. The ratio waste (intestine and muscle) to extraction buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl₂) was 1:3 (w/v). The extraction method followed systematic processes (Steps I, II, III and IV) for later analysis, as described in the flowchart of Figure 1. The supernatant fraction with the highest total protein, specific and volumetric activity was used as a crude extract (CE) for the tests of physicochemical characterization of collagenolytic enzyme and sensitivity to collagen test. After selecting the best processing step, the crude extract (100 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.

Figure 1. Schematic diagram for collagenase extraction from processing waste (intestine and muscle) of smooth weakfish Cynoscion leiarchus.

Protein determination

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

Activity of trypsin and chymotrypsin

Enzyme activity was measured using BApNA and Suc-Phe-p-Nan prepared with DMSO, as substrate specific for trypsin and chymotrypsin, respectively, with a final concentration of 8 mM. The substrate (30 μL) was incubated in microplate wells with the CE (30 μL) and 140 μL of 50 mM Tris-HCl pH 7.5, containing 5 mM CaCl₂. The release of p-nitroaniline was measured as an increase in absorbance at 405 nm, performed in quadruplicate. Controls were performed without crude extract. One unit of enzyme activity was considered as the amount of enzyme able to produce 1 μmol of p-nitroaniline per minute (SOUZA et al., 2007).
Determination of collagenolytic properties

The collagenolytic properties of the crude extract of intestine was determined according to modifications in the methodology of ADIGÜZEL et al. (2009), using azocoll as substrate. A reaction mixture, containing 5 mg of azocoll, 500 μL of 50 mM Tris-HCl (pH 7.5) with 5 mM CaCl2 and 500 μL of crude extract, was incubated at 55°C for 30 minutes, under stirring. Thereafter, 200 μL of 40% trichloroacetic acid (TCA) 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 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.

Physicochemical properties of the collagenolytic enzyme

Optimum temperature and thermal stability

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 90°C. For optimal temperature, the assays were carried out by incubating the crude extract in a water bath. To test thermal stability, the enzyme was incubated in a water bath for 1 hour and the activity was performed as described before. The highest enzymatic activity observed for the enzyme in different temperatures was defined as 100% (KIM et al., 2002).

Optimum pH and stability

These experiments were carried out 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 CaCl2. The influence of pH on enzyme stability was determined by incubating the enzyme with the mentioned buffer solutions, at a ratio of 1:1 for 1 hour at 25°C. The activity was performed as described before. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100% (KIM et al., 2002).

Effect of metal ions

The effect of metal ions on the enzyme activity was investigated by adding metal ions (K+, Na+, Hg²⁺, Pb²⁺, Cu²⁺, Cd²⁺, Zn²⁺, Ba²⁺, Mg²⁺, Ca²⁺ and Al³⁺) 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. Controls were performed with the absence of metal ions. The results were expressed as percentage of the control activity (PARK et al., 2002).

Effect of Inhibitors

The sensitivity of the collagenolytic enzymes to inhibitors was evaluated: phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-p-tosyl-L-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; N-tosyl-L-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent. The final concentration of each inhibitor was 8 mM. Each ion was incubated for 1 hour at a ratio of 1:1, and then the activity was performed as described above. Control activity was performed with the absence of inhibitors. The results were expressed as percentage of the control activity (PARK et al., 2002).

Hydrolytic capacity assay with collagen

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 a slight modification. A reaction mixture, which contained 5 mg of collagen, 1 mL of 50 mM Tris-HCl (pH 7.5 with 5 mM CaCl2) 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. A buffer (50 mM Tris-HCl, pH 7.5) that contained 5 mM CaCl2 was used instead of an enzyme solution.
as the reference. The concentration of hydrolyzed-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–Wilks 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).

Table 1. Enzymatic activity of alkaline digestive proteases in enzymatic extracts of Cynoscion leiarchus.

<table>
<thead>
<tr>
<th>Processing</th>
<th>Organ</th>
<th>Serino activity¹ (U mg⁻¹)</th>
<th>Serino activity² (U mg⁻¹)</th>
<th>Total protein (µg mL⁻¹)</th>
<th>Collagenolytic Activity³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Enzyme activity (U mL⁻¹)</td>
</tr>
<tr>
<td>Step I</td>
<td>Intestine</td>
<td>1.01 ± 0.07</td>
<td>0.48 ± 0.23</td>
<td>1931.37</td>
<td>54.77 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.0 ± 0.13</td>
<td>0.82 ± 0.45</td>
<td>2754.90</td>
<td>8.0 ± 0.01</td>
</tr>
<tr>
<td>Step II</td>
<td>Intestine</td>
<td>0.83 ± 0.08</td>
<td>0.40 ± 0.24</td>
<td>3882.35</td>
<td>56.02 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.17 ± 0.11</td>
<td>0.81 ± 0.16</td>
<td>3039.21</td>
<td>16.5 ± 0.11</td>
</tr>
<tr>
<td>Step III</td>
<td>Intestine</td>
<td>0.79 ± 0.19</td>
<td>0.61 ± 0.18</td>
<td>3294.11</td>
<td>72.5 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.11 ± 0.10</td>
<td>1.04 ± 0.15</td>
<td>3970.58</td>
<td>39.02 ± 0.58</td>
</tr>
<tr>
<td>Step IV</td>
<td>Intestine</td>
<td>1.35 ± 0.02</td>
<td>0.63 ± 0.06</td>
<td>3372.54</td>
<td>56.07 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>1.28 ± 0.08</td>
<td>1.51 ± 0.01</td>
<td>4147.05</td>
<td>16.32 ± 0.01</td>
</tr>
</tbody>
</table>

¹Using Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), trypsin substrate.
²Using Succinyl-DL-phenylalanine-p-nitroanilide (Suc-Phe-p-Nan), chymotrypsin substrate.
³Using Assaying proteinases with azocoll, collagenase substrate.

RESULTS

Stage of extraction and collagenolytic activity

The results are summarized emphasizing the extraction/selection method of proteases resistant to high temperatures. The fraction with the highest collagenolytic activity was detected in step III, with an increase of 32% in relation to step I, as seen in Table 1, which shows the enzyme activity of the crude extract obtained from intestinal waste of smooth weakfish C. leiarchus. After setting that fraction, the selected crude extract was heated as a mean for the selection of heat-resistant proteases. The volumetric and specific collagenolytic activities of the pre-heated crude extract, during 30 min of assay at 45°C, reached 84.8 ± 0.02 U mL⁻¹ and 25.74 ± 0.01 U mg⁻¹, respectively, an increase of 17% of both activities when compared to the unheated crude extract.

Temperature and pH assay of the collagenolytic enzymes

The enzyme activity presented optimum temperature at 55°C (Figure 2A), showing thermostability in the range from 25 to 60°C (Figure 2B) with a significant loss of activity at temperatures above 70°C and being completely inactivated at 75°C. The enzyme activity presented highest value at pH 8.0 (Figure 2C) and were stable at pH range from 6.5 to 11.5 (Figure 2D).

Collagenase from smooth weakfish: extraction, partial...

**Figure 2.** Effect of temperature and thermal stability on the activity of collagenolytic serine protease of smooth weakfish *Cynoscion leiarchus*: (A) Optimum temperature in a range of 25–90°C; (B) Thermal stability after 30 minutes incubation at a temperature in the range of 25–90°C. Effect of pH and stability at different pH on the activity of collagenolytic serine protease of *C. leiarchus*: (C) Optimal pH using different buffers in the pH range from 4.5 to 12.0; (D) pH stability after incubation for 1 hour in the pH range 4.0 to 12.0.

*Sensitivity of the collagenolytic enzyme to metal ions and inhibitors*

The effects of metal ions and inhibitors on the enzyme activity are summarized in Table 2. The samples exposed to ions Zn$^{2+}$, Na$^+$, Ca$^{2+}$ and Mg$^{2+}$ showed no significant difference of activity (p < 0.05) compared to the control group (100%). In contrast, all the other ions showed a significant statistical difference (p < 0.05). The presence of Ca$^{2+}$ ions slightly increased the activity of the protease while Pb$^{2+}$, Al$^{3+}$, Cu$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$ were potential inhibitors, similarly as observed for the inhibitors of serine proteases (PMSF, Benzamidine and TLCK), when a significant inhibition difference (p<0.05) on the activity was verified.

**Collagen specificity test**

The collagenolytic potential of the enzyme is showed in Figure 3. A linear growth of the activity was detected after 24 hours of incubation of the protease in the presence of bovine type I collagen, not previously hydrolyzed.

**Figure 3.** Activity of proteinase with collagenolytic properties extracted from digestive viscera of smooth weakfish *Cynoscion leiarchus* against collagen type I in various incubation times (12-24-36-48 hours) at 37°C. Data represent the mean of four replicates.
Table 2. Effect of ions and inhibitors on the activity of collagenolytic enzyme from Cynoscion leiarchus.

<table>
<thead>
<tr>
<th>Ions and Inhibitors</th>
<th>Collagenolytic activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal ions (1 mM)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.0^a</td>
</tr>
<tr>
<td>Cd^{2+}</td>
<td>66.23^b</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>64.49^b</td>
</tr>
<tr>
<td>K^+</td>
<td>88.15^b</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>93.18^a</td>
</tr>
<tr>
<td>Al^{3+}</td>
<td>63.21^b</td>
</tr>
<tr>
<td>Hg^{2+}</td>
<td>64.92^b</td>
</tr>
<tr>
<td>Na^+</td>
<td>93.91^a</td>
</tr>
<tr>
<td>Pb^{2+}</td>
<td>60.57^b</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>105.52^a</td>
</tr>
<tr>
<td>Ba^{2+}</td>
<td>75.68^b</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>100.39^a</td>
</tr>
<tr>
<td>Inhibitors (8 mM)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.0^a</td>
</tr>
<tr>
<td>PMSF</td>
<td>61.67^b</td>
</tr>
<tr>
<td>TPCK</td>
<td>89.58^b</td>
</tr>
<tr>
<td>TLCK</td>
<td>26.84^b</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>23.73^b</td>
</tr>
<tr>
<td>EDTA</td>
<td>38.11^b</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>58.38^b</td>
</tr>
</tbody>
</table>

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)

**DISCUSSION**

Solid waste from fish processing has been a subject of research for providing a significant amount of biologically active molecules (BEZERRA et al., 2006; OLIVEIRA et al., 2017a). Among these, enzymes deserve special attention because of their application in several biotechnological processes (OLIVEIRA et al., 2017b). Thus, enzymes which have the ability to cleave native collagen and/or such residues are extremely desirable from the industrial point of view as a way of maximizing production profits and minimizing environmental damage. According to the results shown here, the crude extract of smooth weakfish C. leiarchus, after the successive stages of extraction, may be suggested as potential source of collagenolytic enzymes for application in several stages of leather processing due to their similar properties to commercial enzymes, operating as a non-toxic biocatalyst, improving dyeing characteristics and contributing to an ecologically viable process. The steps of extraction and characterization of the enzyme were the basis for establishing their physicochemical characteristics, determining their optimal conditions of temperature and pH, their sensitivity to metal ions and inhibitors.

*Investigation of collagenase in the viscera*

For the characterization and hydrolysis of native and commercial collagen, only the intestinal viscera of smooth weakfish C. leiarchus treated as disposable waste were used, thereby highlighting the potential of the species used to provide enzymes with ability to degrade the collagen structure and to endure industrial processes. Internal viscera, such as the intestine (or portions) and pyloric caeca when present have been reported as a source of enzymes with collagenolytic properties from the crude extract of tuna Thunnus thynnus (16.5 U mg^{-1}) (BYUN et al., 2002), filefish Novodon modestus (114.15 U mg^{-1}) (KIM et al., 2002) and mackerel Scomber japonicus (16.5 U mg^{-1}) (PARK et al., 2002). Muscle of fish species as a source of collagenases have also been reported by TERUEL and SIMPSON (1995) for winter flounder Pseudopleuronectes americanus (3.82 U mg^{-1}) and WU et al. (2010a) for sea bream Pagrus major.

YOSHINAKA et al. (1978) investigated the site of production of collagenase in different organs that comprise the digestive system (liver, stomach, intestine) of different species of fish, such as sardine Sardinops melanosticta (34.3 U mg^{-1} intestine), rainbow trout Salmo gairdnerii (2.8 and 36.8 U mg^{-1} liver and intestine, respectively), gimbuna Carassius Carassius langsdorffi (4.8 and 7.2 U mg^{-1} intestine anterior and posterior, respectively), common carp Cyprinus carpio (10.4 U mg^{-1} intestine posterior), catfish Parasilurus asotus (1.4 and 3.1 U mg^{-1} liver and intestine, respectively), eel Anguilla japonica (1.1 and 39.9 U mg^{-1} stomach and intestine, respectively), yellowtail Seriola quinqueradiata (0.7 U mg^{-1} pyloric caeca), bluefin tuna Thunnus thynnus, umazurahagi Novodon modestus and shotted halibut Eopsetta grigorjewi (5.2, 66.3 and 82.8 U mg^{-1} stomach, intestine and pyloric caeca, respectively). The authors noted that portions of the intestine, pyloric caecum, mesentery, pancreas and adipose tissue are rich in viscera collagenases.
The activity often found in the intestine of pancreatic collagenase was obtained from the intestinal mucosa plus pancreatic tissue around the intestine, since it is very difficult to completely separate the bowel contents from the surrounding tissues. Feeding habits affect the optimal pH conditions for the degradation of collagen in the intestinal tract, since some fish species exhibit collagenolytic proteases which are stable under neutral to alkaline pH conditions (ranging from 6 to 8), as described by DABOOR et al. (2010).

**Temperature and pH assay of the collagenolytic enzyme**

The optimum temperature depends on the type of enzyme, the analyzed tissue and the source species, and has been reported for collagenolytic enzymes in the range of 30°C and 60°C (KIM et al., 2002; PARK et al., 2002; DABOOR et al., 2012; WU et al., 2010a; HAYET et al., 2011; SUPHATHARAPRAT EEP et al., 2011; BAEHAKI et al., 2012; LIMA et al., 2013). Similar results to the present study were described for winter flounder *P. americanus* (TERUEL and SIMPSON 1995), filefish species *N. modestrus* (KIM et al., 2002), tuna *T. thymus* (BYUN et al., 2002) and mackerel *S. japonicus* (PARK et al., 2002).

HAYET et al. (2011) reported an optimum temperature of 60°C for sardinelle (*Sardinella aurita*), with decreased activity at 70°C, whilst KRISTJÁMSSON et al. (1995) observed that a temperature of 50°C was beneficial to enzymes obtained from Atlantic cod (*Gadus morhua*), although a decrease of 50% of its activity was verified, as a consequence of increasing the temperature by 5°C. WU et al. (2010a) found, for an enzyme obtained from sea bream (*P. major*), an optimum temperature of 40°C, similar to that reported by MURADO et al., (2009), when they studied rayfish (*Raja clavata*). DABOOR et al. (2012) found an optimal activity of 35°C using a mixture of haddock, herring, ground fish and flounder; and at the same time a temperature of 30°C was reported by ROY et al. (1996) when studied greenshore crab (*Carcinus maenas*). SOVIK and RUSTAD (2006) verified a decrease in the enzyme activity in temperatures below 35°C in the guts of prey Brosme brosme and Molva molva, while MUKHERJEE et al. (2009), studying sponge (*Rhopaloeides odorabile*), observed an optimal enzyme activity at 30°C. Enzymes with collagenolytic properties from microbial sources are often objects of investigation, having been reported by WU et al. (2010b) when they studied bacterial strains of *Bacillus pumilus* and determined an optimal enzyme activity at 45°C, showing that more than 50% of residual activity was lost after 5 min of incubation at 70°C or 10 min at 60°C. SUPHATHARAPRAT EEP et al. (2011) observed for collagenolytic proteases of *Bacillus cereus* and *Klebsiella pneumoniae* optimum activity at 45°C and 40°C, respectively.

The optimum pH values presented in this study are in conformity to those reported for other fish species, such as: *N. modestrus* filefish (KIM et al., 2002), sea bream *P. major* (WU et al., 2010a) and sardinelle *S. aurita* (HAYET et al., 2011). TERUEL and SIMPSON (1995), BYUN et al. (2002), PARK et al. (2002), SOUCHET and LAPLANTE (2011) and DABOOR et al. (2012) reported an optimum pH of 7.5 to enzymes obtained from the aquatic species: winter flounder (*P. americanus*), tuna (*T. thymus*), Mackerel (*S. japonicus*), snow crab (*Chionoecetes opilio*) and fish waste, respectively. KRISTJÁMSSON et al. (1995), ROY et al. (1996) and BAEHAKI et al. (2012) found an optimum pH of 7.0 for enzymes from Atlantic cod (*G. morhua*), greenshore crab (*C. maenas*) and *Bacillus licheniformis*, respectively. MUKHERJEE et al. (2009) observed that pH 5.0 improved the activity of enzymes from species of sponge (*R. odorabile*). The stability of pH has also been reported for aquatic species such as fish, crustaceans, and sponges, and optimal values were found oscillating between <5.0 and >10.0 (TURKIEWIC et al., 1991; KRISTJÁMSSON et al., 1995; TERUEL and SIMPSON, 1995; MURADO et al., 2009; WU et al., 2010a; WU et al., 2010b; HAYET et al., 2011; SOUCHET and LAPLANTE, 2011; SUPHATHARAPRAT EEP et al., 2011; BAEHAKI et al., 2012).

The results described herein for temperature and pH conform to the needs required by industrial sectors for application of collagenolytic enzyme properties in their segments, in special enzymes with water prolonged heat resistance and a broad range of activity at different pH conditions. These factors are conditions for the use of collagenase, since these enzymes are secreted aszymogens and require modifications in their structure (active conformation) to exert a practical activity (hydrolysis of collagen), which is maximized by the control of physical and chemical parameters (DABOOR et al., 2012).
Sensitivity of the collagenolytic enzymes to metal ions and inhibitors

In relation to the susceptibility to metal ions, the data obtained from our study corroborate those reported by KIM et al. (2002) and WU et al. (2010a) which observed increased activity of collagenolytic proteases of filefish N. modestus and sea bream P. major when exposed to the ions Ca$^{2+}$ and Mg$^{2+}$, respectively. HAYET et al. (2011) reported for sardinelle S. aurita increased activity in the presence of Ca$^{2+}$, but with marked reduction upon exposure to Mg$^{2+}$. ROY et al. (1996), BYUN et al. (2002), PARK et al. (2002), KIM et al. (2002) and WU et al. (2010a) also observed increased activity of collagenolytic proteases from greenshore crab (C. maenas), tuna (T. thymus), mackerel (S. japonicus), filefish (N. modestus) and sea bream P. major in the presence of the ions Ca$^{2+}$ and Mg$^{2+}$. BYUN et al. (2002) reported increased activity of enzymes exposed to the ions Pb$^{2+}$, Ba$^{2+}$ and Na+, and decreased activity in the presence of Cu$^{2+}$, Hg$^{2+}$, Cd$^{2+}$, K+, and Zn$^{2+}$; whilst WU et al. (2010a) detected increased activity upon exposure to Zn$^{2+}$, Ba$^{2+}$ and K+, and marked reduction (55.2%) of the activity when exposed to the ion Pb$^{2+}$. The ions Hg$^{2+}$ and Zn$^{2+}$ were also reason for reduced activity in collagenolytic proteases according to this last study. These data are essential for better application of collagenolytic proteases in industrial processing steps, since they provide information for their control and handling, aiming to increase their catalytic efficiency and minimize the interaction with potential inhibitors, as for example, Pb$^{2+}$ ions shown in Table 2.

The study of enzyme inhibitors provides information about enzyme mechanisms and the data presented in this study may be a valuable tool for better handling and use of proteases obtained from the waste of processed smooth weakfish. The studies of KIM et al. (2002) and HAYET et al. (2011) describe the employment of a specific trypsin inhibitor, TLCK. KRISTJÁMSSON et al. (1995) reported strong inhibition of collagenolytic protease from Atlantic cod Gadus morhua exposed to the specific inhibitor of chymotrypsin, TPCK, in contrast to the reports by BYUN et al. (2002) which studied enzymes from tuna T. thymus. Taking into account such properties, these proteases probably belong to a class of serine collagenase. The loss of enzyme activity upon exposure to EDTA has been observed in tuna species such as tuna T. thymus (BYUN et al., 2002) and sardinelle S. aurita (HAYET et al., 2011), although no inhibition was observed for mackerel S. japonicus (PARK et al., 2002). WU et al. (2010b) and DABOOR et al. (2012) also reported loss of activity upon exposure to EDTA. The reduction of the enzyme activity when subjected to β-Mercaptoethanol, suggest that the structure of the enzyme presents disulfide bonds as expected for serine proteases. The results of this present study also demonstrate an opposite action of enzyme activators and inhibitors, similar to those required in industrial processes, since the determination of the class of collagenolytic enzymes (belonging to serine or metalloproteinases) defines their conditions for application. The inactivation of the enzyme, partially or totally, by inhibitors, as suggested for DABOOR et al. (2012), is due to interactions between ionic or molecular inhibitors, specific to each class of enzyme.

Hydrolysis of collagen by the collagenolytic enzyme

Hydrolysis of type I collagen has been detected by using collagenolytic proteases produced by various aquatic organisms, such as for the species of fish described by: KRISTJÁMSSON et al. (1995) for Atlantic cod (G. morhua); TERUEL and SIMPSON, (1995) for winter flounder (P. americanus); BYUN et al. (2002) for tuna (T. thymus); PARK et al. (2002) for mackerel (S. japonicus); KIM et al. (2002) for filefish (N. modestus); HERREIRO-HERNANDEZ et al. (2002) for iced cod (G. morhua); HAYET et al. (2011) for sardinelle (S. aurita); and ROY et al. (1996) for greenshore crab (C. maenas). In our studies, the intestinal protease of smooth weakfish was able to cleave the collagen type I tested, as shown in Figure 3. Type I collagen has been extracted from the skin, bones, fins and scales of fish and other animals from freshwater and marine environments, such as squid, jellyfish and starfish. It is the most abundant type, being widely distributed in the body as structures classically referred to as collagen fibrils that form bones, dentin, tendons, capsules bodies, corneal and dermal blood vessels. These structures are known to play an important role in the morphogenesis and cell metabolism of new tissue, providing mechanical and biochemical properties (MYLLYHARJU and KIVIRIKKO, 2004; SÖDERHÅLL et al., 2007; CHUNG and UITTO, 2010; DABOOR et al., 2010; FERREIRA et al., 2012; MAKAREEVA and LEIKIN, 2014).

TURKIEWIC et al. (1991) isolated a serine
proteinase from *Euphausia superba* with collagenolytic properties, which was able to hydrolyze collagens from Achilles tendon type I and release fibrils of calf skin collagen, under conditions that do not denature the substrates. KRISTJÁMSSON et al. (1995) reported cleavage of native collagen types I, III, IV and V for marine species of fish in Atlantic cod (*G. morhua*). BYUN et al. (2002) found an intestinal serine protease with collagenolytic properties and preference for cleavage in the following decreasing order: collagen type V > collagen type II > type I collagen > type III collagen. The abundance of type V is low, but it is found to be associated with types I and II, bone and cartilage, and other tissues, with important participation in the function of tensile strength (DABOOR et al., 2010). LIMA et al. (2013) observed that the cleavage of type I collagen by collagenase produced by *Penicillium aurantiogriseum*, was similar to those already described with aquatic organisms (PARK et al., 2002; KIM et al., 2002; DABOOR et al., 2012). According to HERREIRO-HERNANDEZ et al. (2002), once one collagenolytic enzyme works degrading particular collagen types, it is likely that other proteases may follow the process.

CONCLUSIONS

This work presents a method for obtaining a collagenolytic enzyme extracted from the waste (viscera) of *C. leiarchus*, properly characterized to select similar properties to commercial enzymes to reach industrial requirements for their application. It is suggested that the enzymes in question are hydrolases susceptible to the main protease inhibitors. Moreover, their physicochemical characteristics, resistance to high temperatures and optimum activity at alkaline pH, as well as the activation by Ca$^{2+}$, indicate that they are serine proteases capable of cleaving type I collagen. All the features presented by the enzymes are interesting for biotechnological applications, such as their use in the leather processing steps and in the textile industry, since in these sectors there is no need to obtain collagenases with a high degree of purification, making the waste of *C. leiarchus* a cost-effective alternative source, providing affordable processing and adding value to the final product.

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