Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Carangoides bartholomaei (Cuvier, 1833) stomach: a source of aspartic proteases for industrial and biotechnological applications

Estômago de *Carangoides bartholomaei* (Cuvier, 1833): uma fonte de proteases aspárticas para aplicações industriais e biotecnológicas

M. K. S. Silva[a,b*], T. A. Silva[a,b], J. A. F. Silva[a,b], L. D. A. Costa[a], M. L. E. Leal[a], R. S. Bezerra[c], H. M. S. Costa[a,b] and A. C. V. Freitas-Júnior[a,b]

[a] *Universidade Federal da Paraíba – UFPB, Centro de Ciências Exatas e da Natureza, Departamento de Biologia Molecular, Laboratório de Biomoléculas de Organismos Aquáticos, João Pessoa, PB, Brasil*

[b] *Universidade Federal da Paraíba – UFPB, Centro de Ciências Exatas e da Natureza, Programa de Pós-graduação em Biologia Celular e Molecular, João Pessoa, PB, Brasil*

[c] *Universidade Federal de Pernambuco – UFPE, Centro de Biociências, Departamento de Bioquímica, Laboratório de Enzimologia, Recife, PE, Brasil*

Abstract

The viscera and other residues from fish processing are commonly discarded by the fishing industry. These by-products can be a source of digestive enzymes with industrial and biotechnological potential. In this study, we aimed at the extraction, characterization, and application of acidic proteases from the stomach of *Carangoides bartholomaei* (Cuvier, 1833). A crude extract from the stomachs was obtained and submitted to a partial purification process by salting-out, which obtained a Purified Extract (PE) with a specific proteolytic activity of 54.0 U·mg^{-1}. A purification of 1.9 fold and a yield of 41% were obtained. The PE presents two isoforms of acidic proteases and a maximum proteolytic activity at 45 °C and pH 2.0. The PE acidic proteolytic activity was stable in the pH range of 1.5 to 7.0 and temperature from 25 °C to 50 °C. Purified Extract kept 35% of its proteolytic activity at the presence of NaCl 15% (m/v) but was totally inhibited by pepstatin A. Purified Extract aspartic proteases presented high activity in the presence of heavy metals such as Cd^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+}, and Cu^{2+}. The utilization of PE as an enzymatic addictive in the collagen extraction from Nile tilapia scales has doubled the process yield. The results indicate the potential of these aspartic proteases for industrial and biotechnological applications.

Keywords: Carangidae, fish pepsin, scale collagen, waste recovery, guarajuba, industrial proteases.

Resumo

As vísceras e outros resíduos do processamento de peixes são geralmente descartados pela indústria pesqueira. Esses resíduos podem ser uma fonte de enzimas digestivas com potencial industrial e biotecnológico. Neste estudo, objetivamos a extração, caracterização e aplicação de proteases aspárticas do estômago de *Carangoides bartholomaei* (Cuvier, 1833). Um extrato bruto do estômago foi obtido e submetido a um processo de purificação parcial, que obteve um Extrato Purificado (EP) com uma atividade proteolítica específica de 54,0 U·mg^{-1}. Foi obtida uma purificação de 1,9 vezes e um rendimento de 41%. O EP apresenta duas isoformas de proteases ácidas e atividade proteolítica máxima a 45 °C e pH 2,0. A atividade proteolítica do EP foi estável na faixa de pH de 1,5 a 7,0 e temperatura de 25 °C a 50 °C. O EP manteve 35% de sua atividade proteolítica na presença de NaCl a 15% (m/v), mas foi totalmente inibida pela pepstatina A. As proteases ácidas do EP apresentaram alta atividade na presença de metais pesados como o Cd^{2+}, Hg^{2+}, Pb^{2+}, Al^{3+} e Cu^{2+}. A utilização de EP como aditivo enzimático na extração de colágeno a partir de escamas de tilápia do Nilo dobrou o rendimento do processo. Os resultados indicam um potencial dessas proteases para aplicações industriais e biotecnológicas

Palavras-chave: Carangidae, pepsina de peixe, colágeno de escama, aproveitamento de resíduos, guarajuba, enzimas digestórias.

1. Introduction

Fish are foods that need some processing, not only because they are very perishable, but also because it is required by modern consumers. The by-products of this process (scales, viscera, bones, skin, fins, etc.) are important environmental contaminants that most of the time are discarded *in natura* in the environment (Freitas-Júnior and Bezerra, 2015). The large national production of fish, when processed, generates thousands of tons of by-products.

*e-mail: mari.anykarla@hotmail.com
Received: February 25, 2020 – Accepted: August 2, 2020

Among the fish that are produced, processed and marketed in the Northeast of Brazil, are the *Carangoides bartholomaei* (Cuvier, 1833) and *Oreochromis niloticus* (Linnaeus, 1758).

The *C. bartholomaei*, popularly known as yellow jack or guarajuba, lives in small groups, in subtropical and temperate marine waters. This species belongs to the family Carangidae and plays important ecological and economic roles (Duarte et al., 2017). The yellow jack is captured throughout the northeast coast of Brazil through artisanal fishing, presenting itself as a fishing resource for the region's economy (Santos, 2012). According to the latest data published by the Brazilian government, the *C. bartholomaei* national production was approximately 1,649 t in the year 2011 (Brasil, 2011).

Nile tilapia (*O. niloticus*) is the second most important group of farmed fish (Wang et al., 2018a). According to the United Nations Food and Agriculture Organization (FAO, 2018) report, more than 135 countries grow Nile tilapia, the second best-selling species. Intensive cultivation of Nile tilapia has steadily increased, especially in Asia, making it one of the most popular fish in terms of consumption (Roslan et al., 2014). China has an approximate production of Nile tilapia of 158 million tons per year, being the main producer country of Nile tilapia (Wang et al., 2018b). In 2016, Brazil was the largest producer of Nile tilapia in Latin America, with a production of approximately 240 thousand tons (El-Sayed, 2019).

In fish viscera, a wide variety of biomolecules of industrial interest can be found, such as proteins, enzymes, polysaccharides, amino acids, and lipids (Guo et al., 2018). Among these biomolecules, proteases stand out because they are the enzymes most used by industry. However, most of these commercial enzymes are bacteria and fungi proteases (Bougatef, 2013). Other proteases, such as pepsin are obtained commercially from mammalian tissues such as cattle and pigs (Acevedo Gomez et al., 2018).

Several authors have extracted and characterized pepsin-like aspartic proteases from the stomach of fish (Nalinanon et al., 2008; Vannabun et al., 2014; Mazumder et al., 2018; Acevedo Gomez et al., 2018). These fish pepsins have been obtained and tested with excellent results in the preparation of protein hydrolysates, fish collagen extraction and gelatin production (Bougatef et al., 2008; Nalinanon et al., 2010).

Collagen and gelatin obtained from fish waste are considered a possible alternative to mammalian collagen in pharmaceutical and food applications, due to the low risk of zoonosis transmission and not having religious restrictions (Villamil et al., 2017; Liu et al., 2019). Collagen extracted from fish scales has been valued for not having skin characteristics, such as strong odor and high fat content (El-Rashidy et al., 2015). Skin and scale collagen obtained from the fish Nile tilapia has been widely reported, for it is one of the most popular freshwater fish, easily accessible and with a wide consumer market, being responsible for a large amount of all fish grown through aquaculture worldwide (El-Rashidy et al., 2015).

Therefore, the aim of this work was to evaluate the use of *C. bartholomaei* stomach as a source of acid proteases for application in extraction of *O. niloticus* scales collagen.

2. Materials and Methods

2.1. Samples and extraction of proteases

The viscera of *C. bartholomaei* and the skins with scales of *O. niloticus* were donated by fishmongers in the metropolitan region of the João Pessoa city, Paraíba, and taken to Laboratório de Biomoléculas de Organismos Aquáticos (BiOAQUA) from the Universidade Federal da Paraíba, where the stomachs and scales were separated, weighed and stored at -20 °C. The stomachs were homogenized with 0.15 M NaCl solution, in a proportion of 1:10 (g/mL) and centrifuged for 20 minutes, at 4 °C, at 10,000 *g*. The collected supernatant, crude extract (CE), was stored at -20 °C until its later use.

2.2. Enzyme activity assays

The acid proteolytic activity of CE was determined according to Pavlisko et al. (1997), using as a substrate a 2% (w/v) hemoglobin solution in 0.03 M HCl. The assay was performed in triplicate and the result was determined in a microplate reader Multiskan GO (Thermo®). One unit of enzymatic activity was determined to be the amount of enzyme capable of promoting a change in absorbance at 280 nm of 0.1 per minute of reaction. The quantification of total soluble proteins was determined using the method of Bradford (1976), using bovine serum albumin as a standard.

2.3. Activation of zymogens

The activation of zymogens, such as pepsinogen, present in the CE, was performed by adding 6 M HCl until the CE reached pH 2.0. After reaching pH 2.0, aliquots were incubated under different conditions of temperature (25 and 4 °C) and time (30, 60 and 90 min). After incubation, the aliquots were centrifuged and the supernatants used in enzyme activity assays, in which it was determined that the best activation and preservation condition would be at room temperature for 60 minutes. Therefore, this parameter was used to activate the acid proteases zymogens present in CE.

2.4. Crude extract partial purification

The CE was submitted to a salting-out process by adding ammonium sulfate to the extract until saturation of 60%. The mixture was incubated at 4 °C for 120 min and centrifuged at 8,000 g at 4 °C for 20 min. After centrifugation, the precipitate was collected, solubilized in 0.15 M NaCl solution (pH 2.0) and dialyzed with the same solution for 24 h at 4 °C. The 0-60% dialyzed fraction was stored and called the Purified Extract (PE).

2.5. Zymogram

The number of acidic proteases present in PE was determined by zymogram using the methodology described by García-Carreño et al. (1993), with modifications, in which after the Polyacrylamide Gel Electrophoresis (PAGE) run, the gel was washed in distilled H_2O and incubated in 0.03 M HCl for 5 min at 4 °C. Then the gel was incubated with a 2% hemoglobin solution in 0.03 M HCl for 90 min at 37 °C.

After incubation, the gel was washed in distilled H_2O and stained with Comassie blue R-250 solution.

2.6. Physicochemical characterization of PE acidic proteases.

The effects of pH and temperature on the acidic proteases present in PE were evaluated using the methodology described by Acevedo Gomez et al. (2018). The optimal temperature, enzyme activity tests were carried out at different temperatures, in the temperature range of 25 °C to 70 °C. The assessment of thermal stability was performed by incubating PE aliquots for 30 minutes in the temperature range aforementioned, and enzyme activity tests were performed. The determination of the optimum pH and pH stability (30 min of incubation) was carried out in the pH range of 1.5 to 7.0, by use of 0.2 M glycine-HCl (pH 1.5 to 2.5) and citrate phosphate (pH 3.0 to 7.0) buffers.

2.7. Effect of NaCl

The effect of NaCl on the activity of PE acidic proteases was evaluated by incubating for 30 min, at 25 °C, aliquots of PE, in the proportion of 1:1 (v/v), with NaCl solution in different concentrations (5, 10 and 15%; m/v) (w/v). Then the residual proteolytic activity was determined at 37 °C and for 10 minutes, using 2% hemoglobin as a substrate, following the method described by Klomklao et al. (2007) with minor modifications.

2.8. Chemical agents effect

The effect of presence of some chemicals on PE acidic proteases was determined using the methodology described by Acevedo Gomez et al. (2018). The effect of metal ions presence on the enzyme activity was evaluated by incubating an aliquot of PE in a 20 mM ion solution, in the proportion of 1:1, for 30 minutes, at 25 °C. Then, enzyme activity tests were performed using the mixture with the ions (Al^{3+}), (Cu^{2+}), (Mg^{2+}), (Pb^{2+}), (Mn^{2+}), (Ca^{2+}), (Na^{+}), (Hg^{2+}) and (Cd^{2+}). The effect of the aspartic protease inhibitor pepstatin A on the proteolytic activity of PE was evaluated by incubating the enzyme, in the proportion of 1:1 (v/v) with a solution of pepstatin A (2 µg/mL) in methanol for 30 min, at 25 °C. After incubation, an enzyme activity assay was performed using the mixture. The effect of the reducing agent DTT (dithiothreitol) (1 mM) on the enzymatic activity of PE was evaluated as in the previous assay.

2.9. Collagen extraction from Nile tilapia scales

The collagen extraction process was carried out using the methodology described by Liu et al. (2015) with modifications. The scales of *O. niloticus* were treated with 0.2 M NaOH solution, using a ratio of 1:5 (m/v) for 24 h at 4 °C, under continuous stirring. After the previous step, the scales were demineralized using 0.5 M EDTA solution pH 7.5 for 48 h, at 4 °C under continuous stirring.

For the Acid Soluble Collagen (ASC) obtaining, the pre-treated scale was added in 0.5 M acetic acid, 1:10 (w/v) and stirred continuously for 72 hours at 4 °C. The mixture was then centrifuged for 60 min at 10,000 g. The supernatant was collected and subjected to precipitation by adding NaCl to a concentration of 2.6 M, for 12 h, at 4 °C. The mixture was centrifuged at 10,000 g for 60 min. The precipitate was collected and solubilized in 0.5 M acetic acid. The solution obtained was lyophilized and the dry mass of collagen was weighed.

For Pepsin Soluble Collagen (PSC) obtaining, the pre-treated scale was added in 0.5 M acetic acid in the proportion of 1:10 (m/v) and the enzyme was added in the proportion of 20 U/g of scale. The mixture was subjected to continuous stirring for 72 h at 4 °C. The mixture was centrifuged at 10,000 *g*, at 4 °C, for 60 minutes. The same steps described for the extraction of ASC were performed.

The extraction process yield was calculated according to the Formula 1:

$$Yield\,(\%) = (mC \times 100) / mE \quad (1)$$

mC = mass (g) of lyophilized extracted collagen; mE = mass (g) of lyophilized scale used in the extraction.

2.10. Statistical analysis

All analyzes were performed in triplicate (n = 3), and analyzed statistically by ANOVA and Tukey's test, when recommended. The program used was OriginPro® 8 and all tests were performed with a significance level of $p<0.05$.

3. Results and Discussion

3.1. Crude extract partial purification

Crude extract obtained from *C. bartholomaei* stomach showed specific activity of 54.0 U·mg^{-1}. By means of salting-out, a 1.9-fold purification rate was obtained with a 40.7% yield (as shown in Table 1). The use of a simple step to refine the stomach crude extract can contribute to the economic viability of these proteases application in industrial and biotechnological processes that do not require enzymes with a high purity degree, such as in collagen extraction process. Similarly, Acevedo Gomez et al. (2018) through the same process report a yield of 45.1% and a purification 1.8-fold purification rate for aspartic proteases from stomach crude extract of fish *Prochilodus lineatus* (Valenciennes, 1837).

3.2. Zymogram

In the PE zymogram (Figure 1) it is possible to observe the presence of two acidic proteases. Due to the fact that pepsin-like enzymes are the main acidic proteases in the stomach of fishes, and the results observed with the specific inhibitor, it can be suggested that PE presents two acidic proteases, possibly pepsin-like enzymes. Bkhairia et al. (2016) report for the stomach crude extract of *Liza aurata* (Risso, 1810) the presence of one acidic protease in the zymogram. However, other authors when purifying pepsins from fish observed more than two pepsins in different fish species (Zhou et al., 2007; Wu et al., 2009; Cao et al., 2011).

Table 1. Purification table of acidic digestive proteases from the crude extract of *Carangoides bartholomaei* stomach.

Process steps	Total activity (U)*	Total protein (mg)*	Specific activity (U·mg^{-1})	Purification (folds)	Yield (%)
Crude Extract	52,224 ± 3.20	972.2 ± 76.5	54.0	1	100
Purified Extract	21,254 ± 1.14	213.8 ± 15.2	99.4	1.9	40.7

*Parameters established for the process yield using 1L of crude extract.

Figure 1. Zymogram of PE. Arrows indicate the presence of proteolytic bands.

3.3. Effect of pH and temperature over the PE acidic proteases

The PE showed maximum proteolytic activity at pH 2.0 (see Figure 2A). However, it was observed that in the range of 1.5 to 4.5 the fraction showed more than 60% of its maximum proteolytic activity. PE presented maximum activity at a temperature of 45 °C. Nevertheless, a high activity could be observed in the temperature range of 25 to 50 °C (see Figure 2B).

Fish pepsins present maximum activity in the pH range of 1.5 to 3.5, becoming completely inactive after pH 7.0 (Klomklao et al., 2010; Acevedo Gomez et al., 2018). The maximum PE proteolytic activity was observed at pH 2.0. Such data is interesting, as it can result in a more versatile industrial application, especially in processes that use weak acids, such as collagen extraction. Similar results were observed by Acevedo Gomez et al. (2018) and Candiotto et al. (2017), who also observed maximum proteolytic activity at pH 2.0 when characterizing acidic proteases present in the stomach of the fishes *P. lineatus* and *Paralichthys orbignyanus* (Valenciennes, 1839), respectively. The same result were reported in the studies of Bougatef et al. (2008) and Nalinanon et al. (2010), for pepsins of the fish *Mustelus mustelus* (Linnaeus, 1758) and *Thunnus alalunga* (Bonnaterre, 1788), respectively.

The optimum temperature for the acidic proteases present in PE was 45 °C, similarly to the observed for pepsins in the stomach of the fish *Coryphaenoides pectoralis* (Gilbert, 1852) (Klomklao et al., 2007) and *P. lineatus* aspartic proteases (Acevedo Gomez et al., 2018). Approximated results were found by Khaled et al. (2011), who reported characterizing a pepsin from the fish *Sardinella aurita* (Valenciennes, 1837) with optimum temperature of 40 °C. Candiotto et al. (2017) report a 50 °C optimum temperature for the acidic proteases of *P. orbignyanus* stomach. Zhou et al. (2007) characterized four pepsins from the stomach of the fish *Sparus latus* (Houttuyn, 1782) and found the optimum temperature to be 50 °C for pepsins II, III and IV and 45 °C for pepsin I. Miura et al. (2015) reported the optimum temperatures of 50 and 40 °C, for two pepsins from *Micropterus salmoides* (Lacepede, 1802).

3.4. pH and temperature stability

The PE acidic proteases were stable in the pH range of 1.5 to 7.0 (see Figure 2C) and remained stable in the temperature range of 25 to 50 °C after an incubation period of 30 minutes. At temperatures above 55 °C, the enzymes were denatured (see Figure 2D). Zhao et al. (2011) reported fish and mammal pepsins to be stable in the pH range of 1.5 to 6.0, but denatured at pH 7.0. However, the acidic proteases of the PE were stable up to pH 7.0.

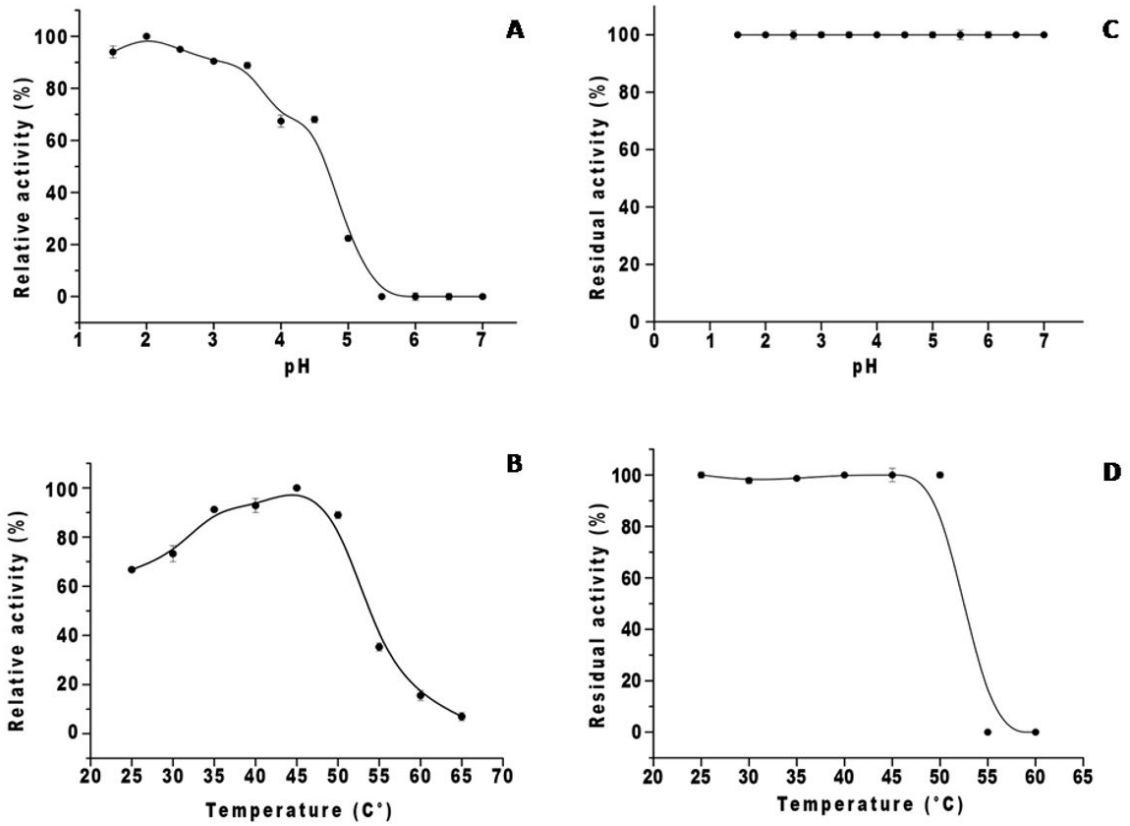

Figure 2. pH and temperature effect on PE acidic proteases. (A) optimal pH; (B) optimal temperature; (C) pH stability; (D) temperature stability.

This may be related to the ability of *C. bartholomaei* acidic proteases to return to their native conformation after the conformational change, caused by changes in charge at pHs far from their optimum pH. However, some studies with fish acidic proteases have shown that, although the enzymes were not stable after being exposed to pH 7.0, they still retained a high proteolytic activity of 80% (Klomklao et al., 2007), 75% (Acevedo Gomez et al., 2018), and 50% (Nalinanon et al., 2010). Nonetheless, there are reports in which fish acidic proteases were found to be stable at pH 7.0 (Castillo-Yanez et al., 2004; Bkhairia et al., 2016).

The PE proteolytic activity remained stable in the temperature range of 25 °C to 50 °C and maintained 100% of its residual activity at 50 °C. This behavior of the digestive proteases of *C. bartholomaei* approximates to the result reported for porcine pepsin in Acevedo Gomez et al. (2018). Such characteristic is important, as it demonstrates the potential for using these enzymes to replace commercial porcine pepsin in processes that require heating, like those from the food industry. Similar results were reported for the proteases from the fish *T. alalunga* with stability up to 50 °C and for two *C. pectoralis* pepsins that were stable up to 40 and 50 °C, but showed residual activity of 10 and 55%, respectively (Klomklao et al., 2007; Nalinanon et al., 2010).

3.5 NaCl effect on PE acidic proteases activity

The effect of NaCl on acidic proteases activity was tested at different concentrations. Pepsin activity decreased as NaCl concentrations increased. It presented 75%, 40% and 35% of residual activity in concentrations of 5%, 10% and 15%, NaCl (m/v), respectively (see Figure 3). Acevedo Gomez et al. (2018) in their results on the effect of NaCl on aspartic proteases from *P. lineatus*, found that the concentration of 5% decreased the enzymatic activity by 20%. This result was similar to *C. bartholomaei* PE, in which a 25% decrease in activity was observed. Several authors have also observed a decrease in activity due to increasing NaCl concentration for both acidic and alkaline fish proteases (Klomklao et al., 2007; Khaled et al., 2011; Freitas-Junior et al., 2012; Vannabun et al., 2014; Acevedo Gomez et al., 2018).

The decrease in enzyme activity may be due to the salting out effect. It is possible that the highest concentration of NaCl competed with the enzyme in water binding which results in a stronger protein-protein interaction that leads to precipitation (Klomklao et al., 2007; Khaled et al., 2011).

3.6. Effect of some chemicals

The proteolytic activity of PE was completely inhibited by pepstatin A. Dithiothreitol (DTT) did not significantly inhibit the proteolytic activity of PE. All ions tested

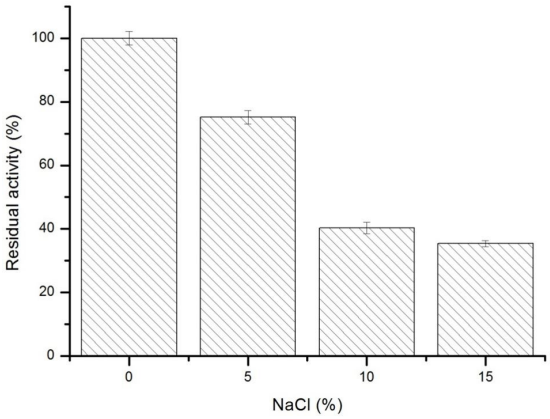

Figure 3. Effect of NaCl on PE acidic proteases.

promoted a slight inhibition in the proteolytic activity of PE in the tested concentration (as shown in Table 2). Since the proteolytic activity of PE was completely inhibited by pepstatin A, and taking into consideration the effects of DTT and the tested ions on PE, it can be suggested that the acidic proteases present in it are probably pepsin-like aspartic proteases. Similar enzyme inhibition by pepstatin A have been reported by other authors for pepsins and digestive aspartic proteases from other fish species (Klomklao et al., 2007; Nalinanon et al., 2010; Khaled et al., 2011; Acevedo Gomez et al., 2018). The DTT effect may suggest the absence of disulfide bonds in the structure of the aspartic proteases from the stomach of *C. bartholomaei*. Similar result was described by Khaled et al. (2011) on the fish *S. aurita*, in which the reducing agent b-mercaptoethanol also did not significantly inhibit the enzymatic activity of acid proteases.

All the tested ions inhibited the PE proteolytic activity. However, PE retained more than 70% of proteolytic activity in the presence of these metals. An aspect that draws attention is that, besides being inhibited by Ca^{2+}, a known enzyme activator, these aspartic proteases presented high activity in the presence of heavy metals such as Hg^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}. Klomklao et al. (2007) reports an activation of two pepsins of *C. pectoralis* by Ca^{2+}, Mg^{2+} and Co^{2+}. Acevedo Gomez et al. (2018) report the same activation effect by Ca^{2+} and Mg^{2+} for aspartic proteases from *P. lineatus*. On the other hand, Ca^{2+} and Na^{+} not promoted effect on *T. alalunga* pepsin. The aspartic protease from *S. aurita* was not affected by Ca^{2+}, Na^{+} and K^{+} ions, but presented a high inhibition by Hg^{2+} (60%), Mg^{2+} (50%) and Cu^{2+} (50%) ions (Khaled et al., 2011).

3.7. Collagen extraction from tilapia scales

The collagen extraction yields were 0.93% for the extraction in acid medium (ASC), 3.0% for the extraction with commercial porcine pepsin (PSC/S) and 1.86% when using PE (PSC/CB) (see Figure 4). Porcine pepsin and PE extraction presented an approximately 3 and 2 folds greater yield, respectively, than ASC non-enzymatic extraction. The difference in yield found between different enzymatic sources (commercial porcine pepsin and PE) is

Table 2. Effect of chemicals on the proteolytic activity of PE.

Chemical agents	Residual Activity (%)	Inhibition (%)
Control	$100 \pm 0.0^{a*}$	0.00
Pepstatin A	0.00 ± 0.8^{b}	100
DTT	96.6 ± 0.9^{c}	3.46
Cu^{2+}	74.8 ± 1.8^{d}	25.20
Mn^{2+}	76.5 ± 2.0^{e}	23.50
Al^{3+}	78.2 ± 0.8^{f}	21.80
Hg^{2+}	79.0 ± 1.1^{g}	21.00
K^{2+}	80.5 ± 1.8^{h}	19.50
Pb^{2+}	81.5 ± 2.7^{i}	18.50
Mg^{2+}	82.2 ± 2.4^{j}	17.8
Cd^{2+}	82.5 ± 2.6^{k}	17.50
Ca^{2+}	83.8 ± 2.8^{l}	16.20
Zn^{2+}	89.9 ± 1.2^{m}	10.10

*Different superscript letters mean statistical differences in relation to the control according to the Tukey's test ($p<0.05$).

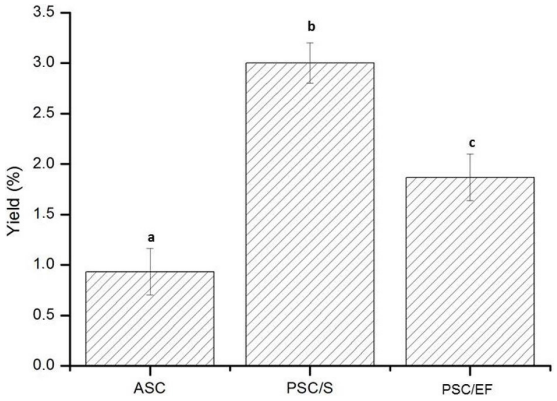

Figure 4. Yield of Nile tilapia collagen extraction using different methods. ASC (Acid Soluble Collagen), PSC/S (Pepsin Soluble Collagen obtained using porcine commercial pepsin) and PSC/PE (Pepsin Soluble Collagen obtained using PE). Different superscript letters mean statistical differences between extraction methods ($p<0.05$).

because they do not have the same ability to cleave the telopeptide present in the tilapia scale collagen (Acevedo Gomez et al., 2018). Despite having a lower yield than porcine pepsin, the low cost for obtaining PE and its doubling the collagen extraction yield may contribute to the economic viability of its industrial use.

Ahmed et al. (2019) extracted collagen from the scale of the *Thunnus obesus* (Lowe, 1839) and, similarly to our results, obtained the highest yield, 4.6%, with the use of PSC/S, whereas acid extraction (ASC) yielded 0.05%. Zhang et al. (2010) extracted collagen from the scale of *Hypophthalmichthys molitrix* (Valenciennes, 1844) for 72h and obtained a yield of 0.86% for (ASC), and 2.32% for (PSC/S).

The results obtained in this work showed that the stomach of *C. bartholomaei* is a low-cost source for obtaining digestive aspartic proteases. Through these by-products

it was possible to obtain a partial purified extract (PE) containing two isoforms of aspartic proteases, probably of the pepsin-like enzymes, with high proteolytic activity. The physical-chemical and fish collagen extraction assays indicate that the PE has potential for industrial and biotechnological applications and may, under certain conditions, be a substitute for porcine pepsin.

Acknowledgements

M.K.S. Silva is a postgraduate student at the Cellular and Molecular Biology Program at UFPB and had the support of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through scholarship. H.M.S. Costa is a postdoctoral research at the Cellular and Molecular Biology Program at UFPB and had the support of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) through scholarship. The authors would like to thank the fisherman and fishmonger Francisco de Assis and the fish market Shalom for donating fish processing residues. This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP).

References

ACEVEDO GOMEZ, A.V., GOMEZ, G., CHAMORRO, E., BUSTILLO, S. and LEIVA, L.C., 2018. Digestive aspartic proteases from sábalo (*Prochilodus lineatus*): characterization and application for collagen extraction. *Food Chemistry*, vol. 269, pp. 610-617. http://dx.doi.org/10.1016/j.foodchem.2018.07.043. PMid:30100479.

AHMED, R., HAQ, M. and CHUN, B., 2019. Characterization of marine derived collagen extracted from the by-products of bigeye tuna (*Thunnus obesus*). *International Journal of Biological Macromolecules*, vol. 135, pp. 668-676. http://dx.doi.org/10.1016/j.ijbiomac.2019.05.213. PMid:31154039.

BKHAIRIA, I., MHAMDI, S., JRIDI, M. and NASRI, M., 2016. New acidic proteases from *Liza aurata* viscera: characterization and application in gelatin production. *International Journal of Biological Macromolecules*, vol. 92, pp. 533-542. http://dx.doi.org/10.1016/j.ijbiomac.2016.07.063. PMid:27451025.

BOUGATEF, A., 2013. Trypsins from fish processing waste: characteristics and biotechnological applications – comprehensive review. *Journal of Cleaner Production*, vol. 57, pp. 257-265. http://dx.doi.org/10.1016/j.jclepro.2013.06.005.

BOUGATEF, A., BALTI, R., ZAIED, S.B., SOUISSI, N. and NASRI, M., 2008. Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): purification, characterization and amino acid terminal sequences. *Food Chemistry*, vol. 107, no. 2, pp. 777-784. http://dx.doi.org/10.1016/j.foodchem.2007.08.077.

BRADFORD, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3. PMid:942051.

BRASIL. Ministério da Pesca e Aquicultura – MPA, 2011. *Boletim estatístico da pesca e aquicultura*. Brasília: MPA, 60 p.

CANDIOTTO, F.B., FREITAS-JÚNIOR, A.C.V., NERI, R.C.A., BEZERRA, R.S., RODRIGUES, R.V., SAMPAIO, L.A. and TESSER, M.B., 2017. Characterization of digestive enzymes from captive Brazilian flounder *Paralichthys orbignyanus*. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 78, no. 2, pp. 281-288. http://dx.doi.org/10.1590/1519-6984.06616. PMid:28832833.

CAO, M.-J., CHEN, W.-Q., DU, C.-H., YOSHIDA, A., LAN, W.-G., LIU, G.-M. and SU, W.-J., 2011. Pepsinogens and pepsins from Japanese seabass (*Lateolabrax japonicus*). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, vol. 158, no. 4, pp. 259-265. http://dx.doi.org/10.1016/j.cbpb.2010.12.003. PMid:21167955.

CASTILLO-YAÑEZ, F.J., PACHECO-AGUILAR, R., GARCÍA-CARREÑO, F.L. and NAVARRETE-DEL TORO, M.A., 2004. Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. *Food Chemistry*, vol. 85, no. 3, pp. 343-350. http://dx.doi.org/10.1016/j.foodchem.2003.07.008.

EL-RASHIDY, A.A., GAD, A., ABU-HUSSEIN, A.E.-H.G., HABIB, S.I., BADR, N.A. and HASHEM, A.A., 2015. Chemical and biological evaluation of Egyptian Nile Tilapia (*Oreochromis niloticas*) fish scale collagen. *International Journal of Biological Macromolecules*, vol. 79, pp. 618-626. http://dx.doi.org/10.1016/j.ijbiomac.2015.05.019. PMid:26026980.

EL-SAYED, A.-F., 2019. Tilapia trade and marketing. In: A.-F. EL-SAYED, ed. *Tilapia culture*. 2nd ed. London: Academic Press, pp. 261-274.

FAO 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome: FAO Fisheries and Aquaculture Department, 210 p.

FREITAS-JÚNIOR, A.C.V. and BEZERRA, R.S., 2015 [viewed 3 January 2019]. Aproveitamento Integral do Pescado: novos horizontes para o fortalecimento da cadeia produtiva. *Panorama da Aquicultura* [online], vol. 2, pp. 48-53. Available from: https://panoramadaaquicultura.com.br

FREITAS-JÚNIOR, A.C.V., COSTA, H.M.S., ICIMOTO, M.Y., HIRATA, I.Y., MARCONDES, M., CARVALHO JUNIOR, L.B., OLIVEIRA, V. and BEZERRA, R.S., 2012. Giant Amazonian fish pirarucu (*Arapaima gigas*): its viscera as a source of thermostable trypsin. *Food Chemistry*, vol. 133, no. 4, pp. 1596-1602. http://dx.doi.org/10.1016/j.foodchem.2012.02.056.

GARCÍA-CARREÑO, F.L., DIMES, L.E. and HAARD, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Analytical Biochemistry*, vol. 214, no. 1, pp. 65-69. http://dx.doi.org/10.1006/abio.1993.1457. PMid:8250256.

GUO, Y., HUANG, W., WU, Y., QI, X. and MAO, X., 2018. Application of a low-voltage direct-current electric field for lipid extraction from squid viscera. *Journal of Cleaner Production*, vol. 205, pp. 610-618. http://dx.doi.org/10.1016/j.jclepro.2018.08.339.

KHALED, H.B., GHORBEL-BELLAAJ, O., HMIDET, N., JELLOULI, K., ALI, N.E.-H., GHORBEL, S. and NASRI, M., 2011. A novel aspartic protease from the viscera of Sardinelle (*Sardinella aurita*): purification and characterization. *Food Chemistry*, vol. 128, no. 4, pp. 847-853. http://dx.doi.org/10.1016/j.foodchem.2011.03.104.

KLOMKLAO, S., BENJAKUL, S. and KISHIMURA, H., 2010. Proteinases in hybrid catfish viscera: characterization and effect of extraction media. *Journal of Food Biochemistry*, vol. 34, no. 4, pp. 711-729. http://dx.doi.org/10.1111/j.1745-4514.2009.00310.x.

KLOMKLAO, S., KISHIMURA, H., YABE, M. and BENJAKUL, S., 2007. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, vol. 147, no. 4, pp. 682-689. http://dx.doi.org/10.1016/j.cbpb.2007.04.008. PMid:17493857.

LIU, D., ZHANG, X., LI, T., YANG, H., ZHANG, H., REGENSTEIN, J.M. and ZHOU, P., 2015. Extraction and characterization of acid- and pepsin-soluble collagens from the scales, skins and swim-bladders of grass carp (*Ctenopharyngodon idella*). *Food Bioscience*, vol. 9, pp. 68-74. http://dx.doi.org/10.1016/j.fbio.2014.12.004.

LIU, W., ZHANG, Y., CUI, N. and WANG, T., 2019. Extraction and characterization of pepsin-solubilized collagen from snakehead (*Channa argus*) skin: effects of hydrogen peroxide pretreatments and pepsin hydrolysis strategies. *Process Biochemistry*, vol. 76, pp. 194-202. http://dx.doi.org/10.1016/j.procbio.2018.10.017.

MAZUMDER, S.K., DAS, S.K., RAHIM, S.M. and GHAFFAR, M.A., 2018. Temperature and diet effect on the pepsin enzyme activities, digestive somatic index and relative gut length of Malabar blood snapper (*Lutjanus malabaricus*). *Aquaculture Reports*, vol. 9, pp. 1-9. http://dx.doi.org/10.1016/j.aqrep.2017.11.003.

NALINANON, S., BENJAKUL, S. and KISHIMURA, H., 2010. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). *Food Chemistry*, vol. 121, no. 1, pp. 49-55. http://dx.doi.org/10.1016/j.foodchem.2009.11.089.

NALINANON, S., BENJAKUL, S., VISESSANGUAN, W. and KISHIMURA, H., 2008. Tuna pepsin: characteristics and its use for collagen extraction from the skin of threadfinbream (*Nemipterus spp.*). *Journal of Food Science*, vol. 73, no. 5, pp. C413-C419. http://dx.doi.org/10.1111/j.1750-3841.2008.00777.x. PMid:18576987.

PAVLISKO, A., RIAL, A., VECCHI, S. and COPPES, Z., 1997. Properties of pepsin and trypsin isolated from the digestive tract of *Parona signata* "palometa. *Journal of Food Biochemistry*, vol. 21, no. 3, pp. 289-308. http://dx.doi.org/10.1111/j.1745-4514.1997.tb00210.x.

ROSLAN, J., YUNOS, K.F.M., ABDULLAH, N. and KAMAL, S.M.M., 2014. Characterization of fish protein hydrolysate from Tilapia (Oreochromis niloticus) by-product. *Agriculture and Agricultural Science Procedia*, vol. 2, pp. 312-319. http://dx.doi.org/10.1016/j.aaspro.2014.11.044.

SANTOS, M.N.S., 2012. *Reprodução e alimentação da guarajuba Carangoides bartholomaei (Cuvier, 1833) (Perciformes: Carangidae) na plataforma continental de Pernambuco, Brasil.* Recife: Universidade Federal de Pernambuco, 46 p. Dissertação de Mestrado em Biologia Animal.

VANNABUN, A., KETNAWA, S., PHONGTHAI, S., BENJAKUL, S. and RAWDKUEN, S., 2014. Characterization of acid and alkaline proteases from viscera of farmed giant catfish. *Food Bioscience*, vol. 6, pp. 9-16. http://dx.doi.org/10.1016/j.fbio.2014.01.001.

VILLAMIL, O., VÁQUIRO, H. and SOLANILLA, J.F., 2017. Fish viscera protein hydrolysates: Production, potential applications and functional and bioactive properties. *Food Chemistry*, vol. 224, pp. 160-171. http://dx.doi.org/10.1016/j.foodchem.2016.12.057. PMid:28159251.

WANG, L., JIANG, Y., WANG, X., ZHOU, J., CUI, H., XU, W., HE, Y., MA, H. and GAO, R., 2018a. Effect of oral administration of collagen hydrolysates from Nile tilapia on the chronologically aged skin. *Journal of Functional Foods*, vol. 44, pp. 112-117. http://dx.doi.org/10.1016/j.jff.2018.03.005.

WANG, Z.-C., YAN, Y., SU, P., ZHAO, M.-M., XIA, N. and CHEN, D.-W., 2018b. Treatments of tilapia (*Oreochromis niloticus*) using nitric oxide for quality improvement: establishing a potential method for large-scale processing of farmed fish. *Nitric Oxide*, vol. 77, pp. 19-25. http://dx.doi.org/10.1016/j.niox.2018.04.003. PMid:29635033.

WU, T., SUN, L.-C., DU, C.-H., CAI, Q.-F., ZHANG, Q.-B., SU, W.-J. and CAO, M.-J., 2009. Identification of pepsinogens and pepsins from the stomach of European eel (*Anguilla anguilla*). *Food Chemistry*, vol. 115, no. 1, pp. 137-142. http://dx.doi.org/10.1016/j.foodchem.2008.11.077.

ZHANG, J., DUAN, R., YE, C. and KONNO, K., 2010. Isolation and characterization of collagens from scale of silver carp (*hypophthalmichthys molitrix*). *Journal of Food Biochemistry*, vol. 34, no. 6, pp. 1343-1354. http://dx.doi.org/10.1111/j.1745-4514.2010.00439.x.

ZHOU, Q., FU, X.-P., ZHANG, L.-J., SU, W.-J. and CAO, M.-J., 2007. Purification and characterization of sea bream (*Sparus latus* Houttuyn) pepsinogens and pepsins. *Food Chemistry*, vol. 103, no. 3, pp. 795-801. http://dx.doi.org/10.1016/j.foodchem.2006.09.021.

DUARTE, M.R., TUBINO, R.A., MONTEIRO-NETO, C., MARTINS, R.R.M., VIEIRA, F.C., ANDRADE-TUBINO, M.F. and SILVA, E.P., 2017. Genetic and morphometric evidence that the jacks (Carangidae) fished off the coast of Rio de Janeiro (Brazil) comprise four different species. *Biochemical Systematics and Ecology*, vol. 71, pp. 78-86. http://dx.doi.org/10.1016/j.bse.2017.01.013.

MIURA, Y., KAGEYAMA, T. and MORIYAMA, A., 2015. Pepsinogens and pepsins from largemouth bass, *Micropterus salmoides*: purification and characterization with special reference to high proteolytic activities of bass enzymes. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, vol. 183, pp. 42-48. http://dx.doi.org/10.1016/j.cbpb.2015.01.001 PMid:25608034.

ZHAO, L., M BUDGE, S., E GHALY, A. and S BROOKS, M., 2011. Extraction, purification and characterization of fish pepsin: a critical review. *Journal of Food Processing & Technology*, vol. 2, no. 6, pp. 126-139. http://dx.doi.org/10.4172/2157-7110.1000126.