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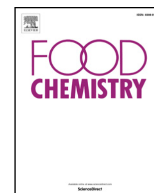

Digestive aspartic proteases from sábalo (*Prochilodus lineatus*): Characterization and application for collagen extraction

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A R T I C L E I N F O

Keywords:
Acidic protease
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Chemical compounds studied in this article:
Acetic acid (PubChem CID: 176)
Butyl alcohol (PubChem CID: 263)
Hydrochloric acid (PubChem CID: 313)
Glycine (PubChem CID: 750)
Methanol (PubChem CID: 887)
Phenylmethylsulfonyl fluoride (PubChem CID: 4784)
Sodium chloride (PubChem CID: 5234)
Ethylenediaminetetraacetic acid (PubChem CID: 6049)
Sodium hydroxide (PubChem CID: 14798)
Sodium dodecyl sulfate (PubChem CID: 3423265)

A B S T R A C T

Acid proteases from sábalo stomach mucosa were recovered using salting-out procedure. This single step produced an enzyme extract purified 1.8-fold over the crude extract with a recovery of 45.1% of its initial proteolytic activity. Sábalo proteases exhibited the highest activity at 45 °C-pH 2.0, showed pH stability between 2.0 and 5.0 and retained more than 70% of its activity after incubation at pH 7.0 for 2 h. Fish extract was unstable at temperatures greater than 45 °C. Its activity was inhibited by pepstatin A but not by PMSF, while EDTA and SDS showed partial inhibitory effects. Presence of $CaCl_2$ and $MgCl_2$ increased the proteolytic activity, while increasing concentrations of NaCl strongly decreased it. In addition, compared to the acid extraction method, the use of sábalo enzymatic extract increased 1.7 times the yield of collagen extraction.

1. Introduction

Prochilodus spp. (Characiformes, Prochilodontidae) are large migratory detritivores freshwater fishes with substantial economic and ecological importance. They are widely distributed throughout South America (except for Ecuador) and support important fisheries in many parts of the continent (Santana & Freitas, 2013).

The species of interest in this work, *Prochilodus lineatus* (sábalo), represents about 60% of the total fish biomass of Paraná-Paraguay basin and is of commercial interest (Della Rosa, Roux, Sánchez, Ortiz, & Domitrovic, 2014). The Paraná basin includes territorial portions of Brazil, Paraguay, Argentina and Uruguay, with a total area of 1.5 million square kilometres.

During fish processing, between 20 and 60% of starting raw material become solid waste and by-products including viscera, skin, bone and scale (Ferraro et al., 2010). Their final disposal may create environmental pollution issues. Thus, fish waste frequently is utilized for silage production, visceral protein hydrolysates and many other purposes (Murthy et al, 2017). However, these unwanted materials are likely to be sources of various novel and valuable biomolecules. In that sense, a huge range of proteolytic enzymes, including pepsin, could be extracted from fish viscera as well as collagen and gelatine might be isolated from skin, bones and connective tissue (Liu, Liang, Regenstein, & Zhou, 2012). Proteases occupy a central role in the enzyme industry and among them, pepsin is the main digestive proteolytic enzyme (El-Beltagy, El-Adawy, Rahma, & El-Bedawey, 2004). Despite commercial pepsin is generally obtained from porcine gastric mucosa, pepsins from different fish species have been purified and characterized (Wu et al., 2009). Increasing interest in pepsins from this source is due to its unique properties: ease thermal denaturation, high molecular activity at

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low temperature and ability to catalyse hydrolysis of native proteins (Haard, 1992). Pepsinogen and pepsin from freshwater and marine fish species have been extensively studied such as the antarctic rock cod (*Trematomus bernacchii*) (Brier et al., 2007) and pectoral rattail (*Coryphaenoides pectoralis*) (Klomklao, Kishimura, Yabe, & Benjakul, 2007). However, there is scant information about tropical fish digestives enzymes (Castillo-Yañez, Pacheco-Aguilar, Garcia-Carreño, de Navarrete-Del Toro, & de los A., 2004).

Collagen and collagen-derived products are currently used in food, pharmaceutical and cosmetic industries (Liu et al., 2015). Although it is commonly isolated from mammalian tissues, collagen could be obtained from fish skin. In recent years, collagen from several freshwater fishes, such as silver carp (*Hypophthalmichthys molitrix*) (Zhang, Duan, Tian, & Konno, 2009), bighead carp (*Hypophthalmichthys nobilis*) (Liu et al., 2012) and grass carp (*Ctenopharyngodon idella*) (Liu et al., 2015), has been extracted and characterized.

The use of fish waste as source of proteolytic enzymes and collagen is an emerging field of research. In Argentina previous works only included alkaline proteases studies from *Engraulis anchoita* (Lamas, Yaennes, & Massa, 2017).

As far as *Prochilodus lineatus* research is concerned, its role as indicator of contamination caused by anthropogenic activity (Palermo, Risso, Simonato, & Martinez, 2015); its migratory behaviour (Capeleti & Petrere, 2006) and its relevance on artisanal fishery (Payne & Harvey, 1989) have been extensively studied. However, there is no published research on their endogenous proteases or the use of the skin of this species as a source of collagen.

According to the statistical data from the Ministry of Agribusiness, Argentina exported 17,034 tons of processed sábalo in 2016 (MAGyP, 2017) and a large amount of its processing by-products were discard as wastes. Thus, the aim of this study was to describe a process for the recovery of acid proteases from the stomach of sábalo, characterize the enzyme extract and evaluate its ability to extract collagen against acid and porcine pepsin collagen solubilisation. In addition, results were compared with those obtained with commercial porcine pepsin under the same conditions.

2. Materials and methods

2.1. Chemicals

Pepsin from porcine gastric mucosa (lyophilized powder, ≥2500 units/mg protein), hemoglobin from bovine blood (substrate powder), bovine serum albumin, pepstatin A, *trans*-4-hydroxy-l-proline, 4-(dimethylamino)benzaldehyde, chloramine-t trihydrate, phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Aldrich (St. Louis, MO). Acrylamide, N,N′-methylenebisacrylamide, Protein Molecular Weight Markers (LMW-SDS marker kit) N, N, N′, N′-tetramethylethylenediamine (TEMED), Sodium dodecyl sulfate (SDS), tris (hydroxymethyl)aminomethane, glycine were purchase from GE Healthcare Life Sciences™. Ethylenediaminetetraacetic acid (EDTA) and trichloroacetic acid were purchased from Merck. Acetic acid, hydrochloric acid, sodium hydroxide and methanol butyl alcohol were purchased from Cicarelli Reagents S.A. (Santa Fé, Argentina). All reagents used in this study were analytical grade.

2.2. Samples

Sábalo (*Prochilodus lineatus)* viscera were provided by the Northeast Institute of Ichthyology (INICNE) of Northeast National University (UNNE) Corrientes, Argentina. Animal protocols were in accordance with the ethical principles of animal experimentation and approved by protocol N° 0019/14-2011-02204 of the Ethics and Biosafety Committee of UNNE.

Stomachs were packed in polyethylene bags and stored at -20 °C until use. The maximum time period of storage for any sample was 2 weeks. Sábalo fishes used for collagen extraction were captured a day before the extraction and transported in ice to the laboratory. Skin was manually separated, and residues of adhering tissues were removed. Separate skin was cut into small pieces (approximately 0.5 cm × 0.5 cm) and stored at −20 °C in polyethylene bags until used.

2.2.1. Preparation of crude extract

Frozen stomachs were thawed using running water (25–28 °C) until the core temperature reached −2 to 0 °C. Stomach mucosas were extracted, minced and homogenized manually by adding 0.1 M Glycine-HCl buffer pH 2.0 in a ratio of 1:5 w/v mucosa/buffer. The homogenate was sonicated 15 min and then centrifuged at 10,000*g* for 15 min to remove tissue debris. Supernatant was collected, used as “crude extract” and conserved at −20 °C until use. The maximum storage period was 3 months.

2.3. Protease recovery

Crude extract was mixed with ammonium sulfate (0–60% saturation) and the precipitate was collected by centrifugation at 8000*g* for 1 h and dissolved in a minimal volume of 0.1 M Glycine-HCl buffer pH 2.0. The resultant solution was dialyzed against the same buffer for 48 h. The dialysate was lyophilized and referred to as “enzyme extract”.

2.4. Protein determination

Protein concentration was measured by Bradford method (1976), using bovine serum albumin as a standard. Briefly, 5 µL of sample were mixed with 0.245 mL of Bradford reagent and after 10 min incubation the absorbance was measured at 545 nm. Glycine-HCl buffer, pH 2.0, was used as reagent blank.

2.5. Assay of proteolytic activity

Proteolytic activity was measured by the method described by Anson and Mirsky (1932) with slight modifications. To initiate the reaction, 100 µL of enzyme extract or porcine pepsin 0.5 mg/mL were added to the assay mixture containing 200 µL of hemoglobin (2.5% in 0.1 M glycine-HCl at pH 2.0). After incubation at 37 °C for 30 min, reaction was stopped by addition of 400 µL of 5% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 10,000*g* for 15 min and the absorbance of the supernatant was measured at 280 nm. Assays were run in triplicate including appropriate blanks. One unit of enzyme activity was defined as the amount of protein that resulted in an increase of absorbance at 280 nm, under the assay conditions above described (Zhou, Fu, Zhang, Su, & Cao, 2007).

2.6. Effects of pH and temperature on the enzyme activity

The effect of pH on enzymatic activity of the extract or porcine pepsin was studied over a pH range of 1.0–8.0, according to the method previously described (item 2.5). The used buffers were 0.1 M HCl-KCl (pH 1.0), 0.1 M glycine-HCl (pH 2.0–3.0), 0.1 M acetate (pH 5.0), and 20 mM Tris-HCl (pH 7.0–8.0). Effect of temperature on pepsin activity was examined over the range of 0–80 °C. Samples and substrates were pre-incubated for 5 min at the defined temperature before the reaction.

2.7. Effects of pH and temperature on the enzyme stability

For pH stability measurement, the enzyme extract was incubated at room temperature for 120 min in different buffers and then the residual proteolytic activity was determined under standard assay conditions. The buffer systems used were the same as described above.

Thermal stability of the enzyme extract was determined by incubating the samples 120 min at 0, 4, 20, 37, 45, 60 and 80 °C at pH 2.0 prior to quantify its proteolytic activity. The non-heated enzyme extract

was considered as control (100%).

2.8. Effects of some chemicals

Sodium dodecyl sulfate (SDS), $MgCl_2$, $CaCl_2$, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) or pepstatin A (200 µL) were mixed with the enzyme extract or porcine pepsin (200 µL). Final concentrations were 0, 1, 5 mM for $MgCl_2$ and $CaCl_2$; 0, 0.1, 0.005% (w/v) for SDS; 1 mM for PMSF, 2 mM for EDTA and 10 µM for pepstatin A. Mixtures were kept at room temperature for 30 min before measuring its remaining proteolytic activity.

2.9. Effect of NaCl

The effect of NaCl on the protease activity of the enzyme extract and the porcine pepsin was studied following the method described by Klomklao, Kishimura and Benjakul (2008) with minor modifications. Samples were mixed with NaCl to obtain final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v) and pre-incubated at room temperature for 120 min. The residual activity was determined at 37 °C and pH 2.0 for 30 min using hemoglobin as substrate.

2.10. Extraction of collagen from the skin of sábalo

The skin pre-treatment and the collagen extraction were performed according to the method of Liu et al. (2015) with some modifications. All the procedures were carried out at 4 °C with the solutions under continuous stirring.

2.10.1. Approximate composition of sábalo skin

The approximate composition of sábalo skin including moisture, crude fat, ash, and crude protein was quantified following the methodology of the Association of Official Analytical Chemistry AOAC (1990). Hydroxyproline content was determined using the procedure described by Reddy and Enwemeka (1996). Collagen content was calculated assuming 12.5% of collagen is hydroxyproline.

2.10.2. Skin pre-treatment

An alkaline pre-treatment was done to remove non-collagenous proteins and pigments. Thus, sábalo skin was soaked in 0.1 M NaOH using a sample/alkaline solution ratio of 1:10 (w/v) for 72 h. The solution was changed every 12 h. Afterwards, samples were washed with cold distilled water until the pH became neutral. Residual fat was removed using 10% (v/v) butyl alcohol with a sample/solution ratio of 1:30 (w/v) for 48 h and changing it every 12 h. Thereafter, skin was thoroughly washed with 5 volumes of cold distilled water.

2.10.3. Extraction of acid soluble collagen (ASC)

The pre-treated skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:15 (w/v) for 72 h. The mixture was then centrifuged for 1 h at 10,000*g* and the supernatant salted-out by adding NaCl to a final concentration of 2.3 M. After 12 h, the resultant precipitate was collected by centrifugation at 10,000*g* for 30 min, re-dissolved in 0.5 M acetic acid and dialyzed against cold distilled water for 24 h.

2.10.4. Extraction of porcine pepsin soluble collagen (PSC) and enzyme extract soluble collagen (ESC)

The pretreated skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:15 (w/v) and porcine pepsin (PSC) or enzyme extract (ESC) (of 10 U/g skin) was added. The mixture was continuously stirred for 72 h, centrifuged for 1 h at 10,000*g* and the supernatant salted-out by adding NaCl to a final concentration of 2.3 M. After 12 h, the resultant precipitate was collected by centrifugation at 10,000*g* for 30 min, re-dissolved in 0.5 M acetic acid and dialyzed against cold distilled water for 24 h.

2.11. Collagen purity and extraction yield

Collagen purity and its protein pattern were determined by SDS-PAGE technique described in item 2.12. Hydroxyproline amount on the collagen extracted was quantified according to the methodology described by Reddy and Enwemeka (1996). Collagen content was calculated assuming 12.5% of collagen is hydroxyproline. The extraction yields of ASC, PSC and ESC were calculated according to the ratio between obtained collagen and the weight of starting material.

2.12. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under non-reducing conditions according to the method described by Laemmli (1970), using a 4% stacking gel and a 10% (collagen study) or 14% (proteases analysis) acrylamide resolving gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie Blue R-250 according to the method described by Chaijan, Panpipat, and Sottawat (2010).

2.13. Statistical analysis

Data represent the mean ± standard deviation (SD) of at least three replications. Statistical significance was tested by one-way ANOVA and Tukey (HSD) and p-values inferior to 0.01 were considered significant.

3. Results and discussion

3.1. Protease recovery

Crude extract obtained from sábalo stomach mucosa exhibited proteolytic activity using acid hemoglobin as substrate with a specific activity of 43.4 U/mg.

Subsequently, the salting-out procedure produced an enzyme extract purified 1.8-fold over the crude extract. This single step allowed a recovery of 45.1% of the initial proteolytic activity. The acquired enzyme extract showed a specific activity of 77.8 U/mg of protein using hemoglobin as substrate.

According with these results, enzyme extract SDS-PAGE (Fig. 1) showed a reduction of bands with respect to the crude sample, principally those with molecular weight between 14.4 and 20.1 kDa. Salt precipitation increased protein amount of around 45 kDa. Many pepsins isolated from fishes have their molecular weight in this range (Zhao, Budge, Ghaly, & Brooks, 2011).

Thus, with the application of a simple purification step it is possible to obtain an extract rich in acid proteases that could be suitable for diverse uses (*e.g.* industrial).

3.2. Influence of pH and temperature on enzymatic activity

The pH activity curves of enzyme extract and porcine pepsin are shown in Fig. 2a. The optimum pH value for both samples was 2.0, and they lost equally their enzymatic activities at pH 5.0. However, a differential behaviour was observed at pH minors than 2.0 where porcine pepsin was more active (100%) in comparison with the enzyme extract (65.2%). Also, at the pH range between 2.0 and 5.0, porcine pepsin activity decline in a very significant way compared with the fish extract, which preserved more than 70% of its enzymatic activity.

Enzyme extract optimum pH was in concordance with those obtained from the stomach of several tunas species (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008), smooth hound (Bougatef, Balti, Zaied, Souissi, & Nasri, 2008), african coelacanth (Tanji et al., 2007) and antarctic rock cod (Brier et al., 2007). Fish pepsins are generally stable at low pH, became susceptible at neutral and alkaline values and their optimum falls in the range of 2.0–4.0 (Castillo-Yañez et al., 2004). This decrease in activity could be explained by enzyme conformational

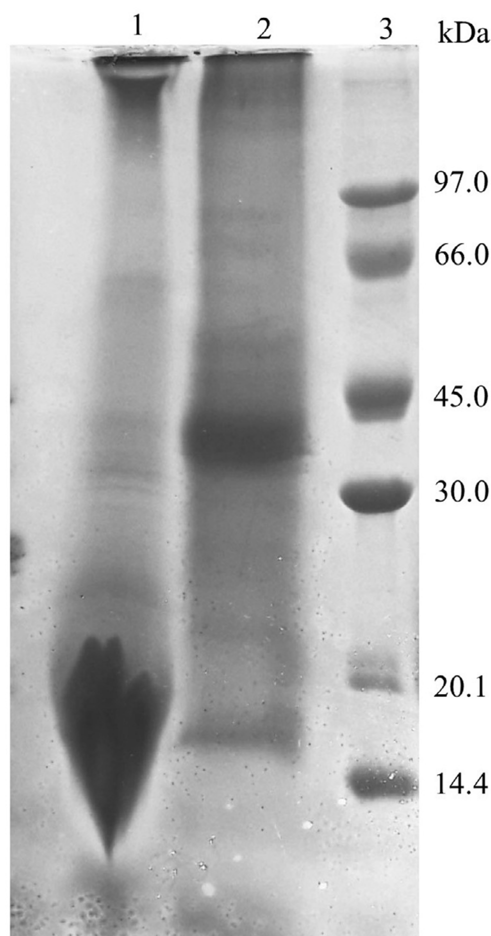

Fig. 1. SDS-polyacrylamide gel electrophoresis (PAGE). Line 1: crude extract; line 2: enzyme extract; line 3: standard protein markers.

changes under harsh condition (Nalinanon, Benjakul, & Kishimura, 2010).

Temperature profiles of enzyme extract and porcine pepsin are illustrated in Fig. 2b. The optimum temperatures were 45 and 60 °C for enzyme extract and porcine pepsin, respectively. Under 37 °C both samples exhibited similar behaviour, a gradually decrease, maintaining 50% of its activity at 0 °C.

Sábalo enzyme extract was unstable at high temperatures, above 45 °C its proteolytic activity was reduced drastically. At 80 °C, while enzyme extract was not active, porcine pepsin conserved a 40% of its maximal activity.

The optimum temperature of fish pepsins ranges from 30 to 55 °C (Zhao et al., 2011). In this sense, the optimal temperature for sábalo enzyme extract here determined, was comparable to other previously reported such as pectoral rattail (Klomklao et al., 2007), sea bream –P-I-(Zhou et al., 2007), mandarin fish - P-II and P-III(b)- (Zhou et al., 2008) and monterey sardine (Castillo-Yañez et al., 2004).

3.3. Effects of pH and temperature on the stability

In the pH range 1.0–5.0, enzyme extract and porcine pepsin were highly stable after being incubated for 2 h at room temperature. However, with increasing pH values their activities declined (Fig. 2c).

Despite this similarity, enzyme extract was more stable at neutral and alkaline pH values than commercial pepsin. Thus, enzyme extract retained more than 70% at pH 7.0 and about 26% at pH 8.0 of its original activity, while porcine pepsin totally lost its activity at pH values superior than 7.0. Similar results have been reported for bolti fish acidic proteases (El-Beltagy et al., 2004) and for true sardine endogenous proteases (Klomklao et al., 2008). However, many acid proteases from different fish species have exhibited a marked decrease on their activity for pH above 5.0, such as sardinella (Khaled et al., 2011), albacore tuna (Nalinanon et al., 2010), smooth hound (Bougatef et al., 2008), monterey sardine (Castillo-Yañez et al., 2004). Difference in enzyme stability at a specific pH among different fish species could be due to the net charge of the enzyme at that pH (Klomklao et al., 2008).

Sábalo acid proteases were fully active after 2 h incubation at temperatures below 37 °C (Fig. 2d), at higher temperatures (above 37 °C) showed a pronounced loss of activity. Porcine pepsin exhibited a higher thermostability than enzyme extract, retained 86.1% of its maximal activity at 60 °C. After being incubated at 80 °C both samples were completely inactivated, due to their thermal denaturation (Nalinanon et al., 2010).

Thermostability reported for other fishes indicate enzymes of this source are generally stable up to 45–55 °C (Castillo-Yañez et al., 2004; El-Beltagy et al., 2004; Klomklao et al., 2008; Nalinanon et al., 2010). The behaviour exhibited by sábalo stomach proteases was in agreement with results demonstrated for pectoral rattail pepsins (A and B) (Klomklao et al., 2007), sea bream pepsin I (Zhou et al., 2007) and mandarin fish pepsins I and IV (Zhou et al., 2008).

3.4. Effects of some chemicals

The effects of some inhibitors and chemicals on enzyme extract and porcine pepsin were investigated and results are showed in Table 1.

Divalent cations Ca^{+2} and Mg^{+2} stimulate the activity of several fish pepsins (Klomklao et al., 2008, 2007). In the present work, an increase of ~10% was observed when ions were added to the reaction. Results are parallel to those reported for acidic proteases from bolti fish, true sardine and pectoral rattail (El-Beltagy et al., 2004; Klomklao et al., 2008, 2007). However, Nalinanon et al. (2008, 2010) evidenced that the presence of $CaCl_2$ and $MgCl_2$ had no significant effect on the proteolytic activity of pepsins of some tuna species.

Pepstatin A is well known to be an inhibitor of aspartic proteases, including pepsin (Brier et al., 2007). It is a *hexa*-peptide that binds and blocks the enzyme active site cleft (Nalinanon et al., 2010). This specific inhibitor practically inhibited the proteolytic activity exhibited by the fish stomach extract at a concentration of 10 μM. Porcine pepsin, used as positive control sample, showed a strong inhibition of its activity. These results confirm that the proteolytic activity detected in sábalo stomach extract is due to the presence of pepsin type acid proteases. Similar results were reported for numerous purified fish pepsins which combined with pepstatin A abolished their activity (Zhao et al., 2011).

Ethylenediamine tetraacetic acid (EDTA) caused a partial inhibition on enzyme extract decreasing its activity on 22%, however, porcine pepsin activity was almost unchanged. This metalloprotease inhibitor showed similar results with true sardine endogenous proteinases (Klomklao et al., 2008) and turbot fish stomach homogenate (Munilla-Morán & Saborido-Rey, 1996). Nevertheless, EDTA has no remarkable effect on other fish pepsins, such as pectoral rattail pepsin A and B (Klomklao et al., 2007), albacore tuna pepsin (Nalinanon et al., 2010) and european eel (Wu et al., 2009), which activities dropped approximately 4–12%. It is believed that EDTA reduces enzyme activity by affecting the structure of enzyme or the active site conformation (Klomklao et al., 2007).

Thus, according to the results here obtained and those with divalent cations above described, it is possible that pepsin-like proteases present on sábalo stomach extract may increase their activity in presence of metal ions.

Phenylmethylsulphonyl fluoride (PMSF) had no inhibitory effect on the activity of enzyme extract or porcine pepsin. Results are in agreement with those reported for other purified pepsins like smooth hound pepsin (Bougatef et al., 2008), mandarin fish pepsin (Zhou et al., 2008), european eel pepsin (Wu et al., 2009), and confirmed the absence of

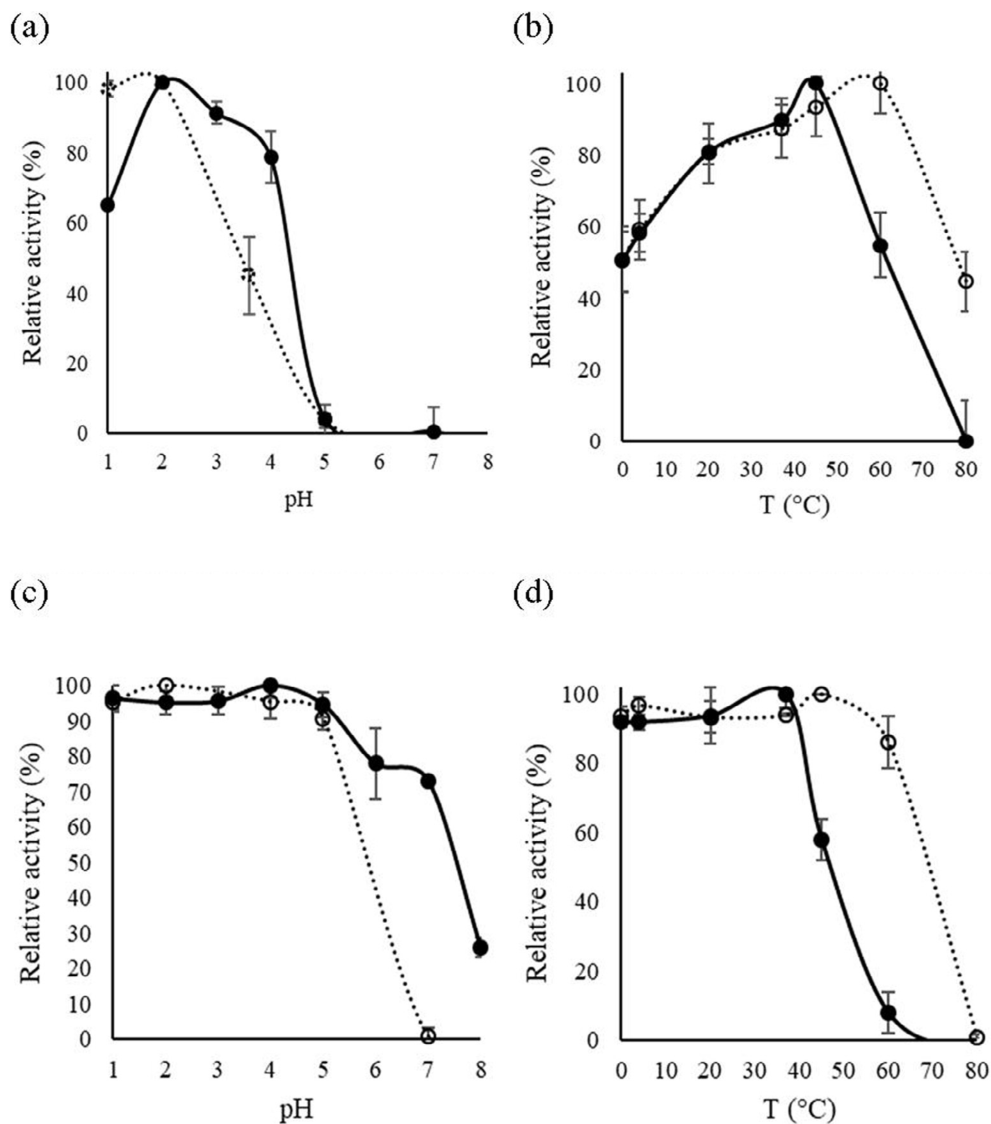

Fig. 2. Effect of pH (a) and temperature (b) on proteolytic activity of enzyme extract (●) and porcine pepsin (○) for haemoglobin hydrolisis. Effect of pH (c) and temperature (d) on stability of enzyme extract (●) and porcine pepsin (○). Bars represent the standard deviation (n = 3).

Table 1
Effect of some chemicals on the activity of enzyme extract and porcine pepsin.

Chemicals[b]	Concentration	Relative activity[a]	
		Enzyme extract	Porcine pepsin
Control		100	100
$MgCl_2$	1 mM	108 ± 4	110 ± 4
	5 mM	109 ± 3	111 ± 2
$CaCl_2$	1 mM	108 ± 3	106 ± 2
	5 mM	107 ± 2	111 ± 2
SDS	0.005% (w/v)	74 ± 3	93 ± 3
	0.1% (w/v)	56 ± 6	81 ± 1
PMSF	1 mM	100 ± 0	100 ± 0
EDTA	2 mM	78 ± 6	96 ± 2
Pepstatin A	10 µM	2 ± 0	ND

[a] Values are mean ± standard deviation, from triplicate determinations.
[b] SDS: sodium dodecyl sulphate. PMSF: Phenyl methyl sulphonyl fluoride. EDTA: Ethylene diamine tetraacetic acid. ND: not detectable.

serine proteases in the enzyme extract.

Sodium dodecyl sulfate (SDS) at 0.05–0.10% (w/v) had a partial inhibitory effect on enzyme extract and porcine pepsin. Enzyme extract activity decreased up to 56.39% after being incubated with 0.1% SDS. Previous works indicated that albacore tuna, skipjack tuna, tongol tuna were strongly inhibited by SDS (Nalinanon et al., 2010, 2008). Sábalo acid proteases exhibited a higher resistance to SDS presence than tuna proteases. The loss of the enzymatic activity could be the consequence of possible interactions between the surfactant and the enzyme in dissolution media (Guzman, Marques, Olivera, & Stippler, 2016).

3.5. Effect of NaCl

The effect of NaCl at different concentrations on protease activity in enzyme extract and porcine pepsin are depicted in Fig. 3. Proteolytic activity from both samples continuously decreased with increasing NaCl concentrations. However, the decrease in porcine pepsin activity was not as marked as that of the enzyme extract. After incubation with 5% of NaCl, the activity of pepsin extract and porcine enzyme decreased by 20% and 9%, respectively. Also, with the highest salt concentration assayed, the residual proteolytic activity reached to 9.12% for enzyme extract and 15.31% for porcine pepsin. Enzyme extract results are in line with those described by Klomklao et al. (2008) and Khaled et al. (2011) for other fish proteases activities in response to NaCl.

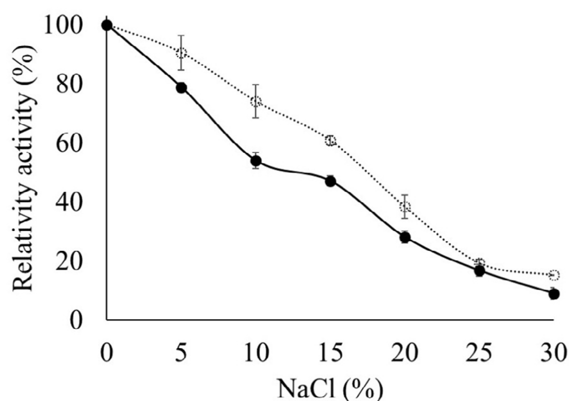

Fig. 3. Effect of NaCl on proteolytic activity of enzyme extract (●) and porcine pepsin (○). Bars represent the standard deviation from triplicate determination.

3.6. Sábalo skin proximate composition

The approximate analyses of sábalo skin revealed amounts of high moisture 52.5 ± 0.9% and ash 5.5 ± 0.1%, on wet bases. These results are coincident with those reported for other skins of freshwater fish as bighead carp (Liu et al., 2012) and nile tilapia (Songchotikunpan, Tattiyakul, & Supaphol, 2008). Also, sábalo skin showed a higher proportion of fat than protein (12.82% and 28.12%), contrary to what is seen in the skin of freshwater and marine fishes. Other noteworthy result was that hydroxyproline content, 3.48 mg per gram of skin, was lower than those reported for other species such as carp, cod and silver carp (Zhang et al., 2009). The occurrence of hydroxyproline is thought to be confine almost exclusively to the connective tissue collagen, consequently it quantification allows the direct measurement of collagen content in tissue (Reddy & Enwemeka, 1996).

3.7. Collagen purity and extraction yield

Collagen extracted by the three different solubilization conditions was subjected to SDS-PAGE in order to verify its purity and define its type. Electrophoresis patterns of ASC, PSC and ESC from the skin of sábalo, under non-reducing conditions, are shown in Fig. 4. Collagens obtained from different extraction methods were composed of α1 and α2 chains, their dimmer (β chains), and small amounts of γ components. Protein patterns of isolated ASC, PSC and ESC were similar to those of collagen type I. Results were in concordance with collagens isolated from the skin of other fish species such as, bigeye snapper (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007), channel catfish (Liu, Li, & Gou, 2007) and grass carp (Liu et al., 2015).

Yields for collagen extraction with ASC, PSC and ESC are shown in Table 2. Skin collagen recovery obtained were 14.92%, 17.7% and 25.29% for ASC, PSC and ESC respectively. Extraction procedures with the aid of enzyme (porcine pepsin or enzyme extract) improved the process in comparison with the acid solubilization method. Sábalo enzyme extract exhibited the highest efficiency in collagen extraction compared with porcine pepsin and acid solubilization ($p < 0.01$). Similar results were reported by other authors when pepsin from porcine stomach or from other fish species was added to enhance collagen extraction of fish skin (Ahmad & Benjakul, 2010; Nalinanon et al., 2007). The difference on the collagen yield extraction due to the addition of pepsin from different sources, suggests that both enzymes do not have the same ability to cleavage of telopeptide of collagen from sábalo skin. According to pH effect on both enzymes, sábalo stomach extract presented a higher proteolytic activity than porcine pepsin at pH 3.0 where the extraction process was carried on. Thus, sábalo enzyme extract might be a suitable low-cost additive to enhance the yield of collagen extraction.

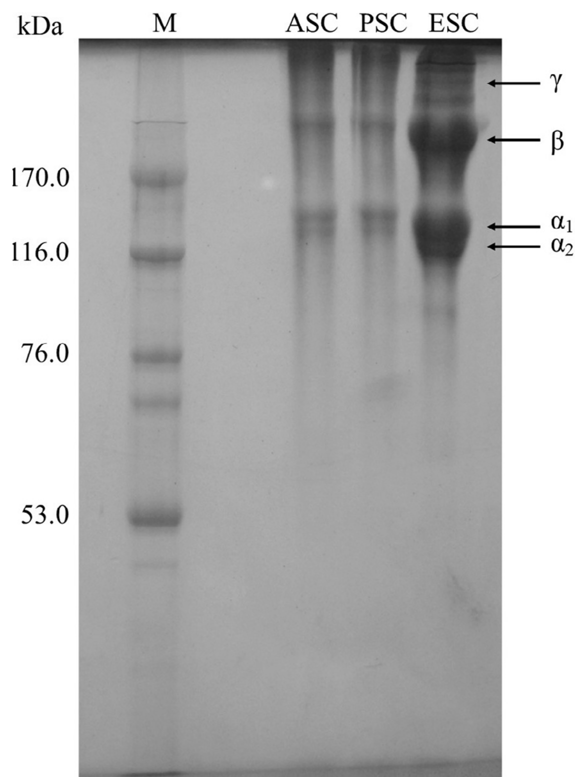

Fig. 4. SDS-PAGE patterns of collagens from the skin of sábalo. M: standard protein markers; ASC: acid-solubilised collagen; PSC: porcine pepsin-solubilised collagen; ESC: enzyme extract-solubilised collagen.

Considering sábalo economic impact in South America fisheries (Della Rosa et al., 2014), the use of its skin for collagen extraction using a stomach enzyme extract from the same fish is an attractive option for the region, despite the fact that sábalo skin has a low amount of collagen.

4. Conclusion

The present study demonstrates that a single step, the salting-out method, produced an enzyme extract purified 1.8-fold over the crude extract with a recovery of 45.1% of its initial proteolytic activity. Moreover, the physicochemical characteristics here analyzed for this pepsin stomach extract (particularly the easy thermal inactivation and the retention of the proteolytic activity at non-highly acidic medium conditions) highlights the possibility for its industrial use. Additionally, the use of this enzymatic extract in collagen extraction improves its yield and leads to a double exploitation of sábalo fishery discards using not only viscera as protease source but also the skin to extract collagen.

Conflict of interest statement

I declare, on behalf of all co-authors, that there is no conflict of interest.

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Table 2
Yield of collagen extracted from the skin of sábalo on wet basis.

Sample[A]	Hydroxyproline (mg/g skin)	Collagen[B] (mg/g skin)	Yield[C](%)	Reference
ASC	0.53 ± 0.06[a]	4.21 ± 0.49[a]	14.92 ± 1.7[a]	This work
ASC*			4.19 ± 0.4	Ahmad and Benjakul (2010)
ASC*			5.31 ± 0.11	Nalinanon et al. (2007)
PSC	0.62 ± 0.09[b]	4.99 ± 0.75[b]	17.7 ± 1.44[b]	This work
PSC***			13.0 ± 0.15	Nalinanon et al. (2007)
PSC+			7.56 ± 0.4	Ahmad and Benjakul (2010)
ESC	0. 89 ± 0.05[c]	7.13 ± 0.4[c]	25.29 ± 1.44[c]	This work
BSP**			19.8 ± 0.12	Nalinanon et al. (2007)
APSC++			8.48 ± 0.3	Ahmad and Benjakul (2010)
YPSC++			8.40 ± 0.3	Ahmad and Benjakul (2010)

Average ± standard deviation, from triplicate determinations.
[a,b,c]Different superscripts in the same column indicate the significant differences ($p < 0.01$).
[A] ASC: collagen extracted with acid for 72 h; PSC: collagen extracted with acid containing porcine pepsin (10 U/g skin) for 72 h; ESC: collagen extracted with acid containing enzyme extract (10 U/g skin) for 72 h.
[B] The conversion factor from hydroxyproline to collagen was 8 (Reddy & Enwemeka, 1996).
[C] Yield was calculated on based on collagen content in different extracts (mg) in comparison with the wet skin (g).
* 48 h of extraction.
** 24 h of acid extraction follow by extraction using bigeye snapper pepsin (BSP) 20 kU/g of defatted skin for 48 h.
*** 24 h of acid extraction follow by extraction using porcine pepsin (PSC) 20 kU/g of defatted skin for 48 h.
+ 48 h of acid extraction with the addition of porcine pepsin 20 kU/g of defatted skin.
++ 48 h of acid extraction with the addition of albacore tuna (APSC) or yellowfin tuna stomach extract (YPSC) 20 kU/g of defatted skin.

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