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Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*)

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Abstract

Trypsin-like enzyme was partially purified from the intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*) by a simple three steps procedure: heat treatment, ammonium sulphate precipitation and Sephadex G-75 filtration. The enzymes from the intestine and pyloric caeca were 96- and 57.7-fold purified with yield values of 68.1% and 26.1%, respectively. The pyloric caeca enzyme collected from the Sephadex G-75 filtration showed a single band in SDS–PAGE (24.5 kDa). Both enzymes presented identical optima pH (9.0) and temperature (55 °C). After incubation at 45 °C for 30 min, enzymes obtained from intestine remained fully activity while a loss of activity (10%) of enzyme extracted from pyloric caeca was registered. Michaelis constant was not significantly different for trypsin-like enzyme from pyloric caeca (1.82 ± 0.19 mM) and that from the intestine (1.94 ± 0.45 mM) acting on benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). Finally, their activities were inhibited by the following ions in decreasing order: $Al^{3+} > Zn^{2+} > Hg^{2+} = Cu^{2+} > Cd^{2+}$. The effects of Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, K^{1+}, Li^{1+} and Co^{2+} showed to be less intensive. The similarities between them provide basis for the proposition of obtaining an attractive protease preparation from the tons of intestine and pyloric caeca, that are usually discarded, from this fish which is an important species exported by North-eastern Brazilian fishery industry.

Keywords: Enzyme; Protease; Trypsin; Tropical fish; Spotted goatfish; *Pseudupeneus maculatus*

1. Introduction

There is a large diversity of aquatic species in the tropical zones. Fishes are ectothermic animals, and have many morphological and physiological adaptations, presenting different food habits and characteristics of the digestive tracts. It is reflected in the digestive enzyme activities and their properties (Alencar et al., 2003; Bezerra et al., 2001, 2005; Bezerra, Santos, Lino, Vieira, & Carvalho, 2000; Cohen, Gertler, & Birk, 1981; El-Shemy & Levin, 1997; Guizani, Rolle, Marshall, & Wei, 1991).

Spotted goatfish is a marine fish living in the coastal waters. It presents a typical carnivore digestive tract composed of stomach followed by pyloric caeca, which in turns precedes a very short intestine (from 0.8- to 1.0-fold standard length). This developed pyloric caeca is probably responsible for a higher amount of alkaline proteases encountered in this aquatic animal (Alencar et al., 2003).

Proteases represent an important class of industrial enzymes, accounting for about 50% of the total sale of enzymes in the world. There are many studies on new sources of proteolytic enzymes, and proteases from fish have been frequently brought to focus. These proteins are present in fish viscera, an important foodstuff industry processing waste, usually discarded, that could be employed as an alternative source of bioactive molecules for biotechnological applications (De Vecchi & Coppes, 1996; Haard, 1992).

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The occurrence of digestive alkaline proteases in intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*) has been recently reported (Alencar et al., 2003). Evidences supported by specific substrate and inhibitors indicate that a trypsin-like enzyme is present in the pyloric caeca of this animal. On the other hand large amounts of viscera are produced by processing of this fish, which are actually discarded. In Northeast Brazil, amounts of about 1400 tons of spotted goatfish have been caught in 2004 yielding as waste an amount of about 140 tons of viscera per year. It is the second most important exported item in the Pernambuco state fishery industry, Northeast Brazil. Therefore, these wastes could represent an alternative source of biomolecules, such as proteases.

This enzyme occupies a key role in foodstuff industry. It can be employed in cheese ripening and flavouring, protein hydrolysate production (high quality aminoacids and peptides containing preparations), soya sauce production, meat flavouring and tenderising. These proteins are also used to recover protein from fish carcasses that would otherwise go to waste after filleting (Haard, 1992; Mackie, 1982).

The recovery of proteolytic enzymes from fish's viscera is an alternative to minimize the economical and ecological onus of this processing waste. However, the high cost of purification could be a limiting problem to propose fish's proteases as far their commercial application is concerned.

A simple and low cost method to purify fish thermostable alkaline proteolytic enzymes has been developed in our laboratory based on heat treatment, ammonium sulphate precipitation and Sephadex G-75 filtration (Bezerra et al., 2001, 2005). Trypsins from tambaqui (*Colossoma macropomum*) and Nile tilapia (*Oreochromis niloticus*) have been successfully purified following this protocol. Here, this procedure was again used to purify trypsin-like enzyme from spotted goatfish intestine and pyloric caeca. Moreover, in this paper, some physico-chemical and kinetics properties of these partially purified preparations were investigated as well as the effect of metal ions on their activities.

2. Materials and methods

2.1. Material

Empresa de Armazenagem Frigorífica Ltda (EMPAF) kindly donated the specimens of spotted goatfish. Azocasein and benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were acquired from Sigma Chemical Com. and all others reagents used were of analytical grade.

2.2. Enzyme extraction

Fish intestine and pyloric caeca were dissected, cautiously cleaned with deionised water. Then the extracted tissues were homogenized in 0.9% (w/v) NaCl (40 mg of tissue/mL) by using a tissue homogeniser. Then the homogenate was centrifuged at 7000*g* for 10 min at 4 °C. The supernatant (crude extract) was used for further purification steps.

2.3. Enzyme purification

The trypsin-like enzyme was purified from the intestine and pyloric caeca by three steps procedure (Bezerra et al., 2001). Crude extract (50 ml) was incubated at 45 °C for 30 min and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was then collected and fractionated with ammonium sulphate during 1 h at 4 °C to obtain the fractions of saturation from 0% to 40% and from 40% to 80%. The 40–80% fraction precipitate was dissolved in 50 ml of 0.2 M Tris–HCl, pH 7.2, and dialysed against this buffer for 24 h at 4 °C. Afterwards the dialysed enzyme was applied (10 ml) on a Sephadex G-75 column (1.2 × 42 cm) which was eluted with 0.9% (w/v) NaCl at a flow rate of 20 ml/h. The second peak was pooled and used throughout the enzyme characterisation.

2.4. Enzyme assays

Proteolytic activity (Alencar et al., 2003): in a micro centrifuge tube (quadruplicates) 1% (w/v) azocasein (100 μl), prepared in 0.2 M Tris–HCl, pH 7.2, was incubated with the crude extract (60 μl) for 60 min at 25 °C. Then, 480 μl of 10% (w/v) trichloroacetic acid (TCA) were added to stop the reaction. After 15 min, centrifugation was carried out for 5 min at 8000*g*. The supernatant (320 μl) was added to 1 M NaOH (560 μl) and the absorbance of this mixture was measured at 440 nm (Varian/UV–Vis 634-S) against a blank similarly prepared except that 0.15 M NaCl replaced the crude extract sample. Previous experiment showed that for the first 60 min the reaction carried out under the conditions described above follows first order kinetics. One unit (U) of enzymatic activity was defined as the amount of enzyme capable to produce a 0.001 change in absorbance per minute.

Trypsin activity (Alencar et al., 2003): 30 μl of 4 mM BAPNA, prepared in dimethylsulphoxide (DMSO), was incubated in the well microtiter plate with the enzyme (30 μl) and 0.2 M Tris–HCl buffer (140 μl) pH 8.0. The release of *p*-nitroaniline was followed by the increase in absorbance at 405 nm in a microtiter plate reader (Bio-Rad 550). Controls were performed without enzyme and substrate solution.

2.5. Measurement of protein

The protein content was estimated by measuring sample absorbance at 280 and 260 nm and using the following equation: $[\text{protein}]\ \text{mg/mL} = A_{280\ \text{nm}} \times 1.5 - A_{260\ \text{nm}} \times 0.75$ (Warburg & Christian, 1941).

2.6. Electrophoresis SDS–PAGE and zymogram

Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli (1970), using a 4% (w/v) stacking gel and a 12.5% (w/v) separating gel. The gels were stained for protein overnight in 0.01% (w/

v) Coomasie Brilliant Blue R250. The background of the gel was destained in 10% (v/v) acetic acid. The molecular weight of the spotted goatfish trypsin-like enzyme was estimated using the protein standards (Sigma) bovine albumin (66 kDa), ovalbumin (46 kDa), glyceraldehyde 3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29 kDa), trypsinogen (24.0 kDa) and α-lactalbumin (14.2 kDa). The proteolytic activity of the bands (zymogram) was demonstrated according to García-Carreño, Dimes, and Haard (1993). Briefly, the enzymes preparations were submitted to electrophoresis as above except that neither reducing agents nor pre-boiling treatment were used before loading them onto the gels. After the SDS–PAGE, the gels were immersed in 2,5% (100 ml) Triton X-100 in 100 mM Tris–HCl, pH 8.0, for 30 min at 4 °C under mild mixing to remove the SDS. The Triton X-100 was removed by washing the gels three times with 100 ml of 100 mM Tris–HCl buffer, pH 8.0. Then, the SDS and Triton X-100 free gels were incubated with 50 ml of 3% (w/v) casein in 50 mM Tris–HCl buffer, pH 8.0, for 30 min at 4 °C. Afterwards, the temperature was raised to 25 °C and kept for 90 min to allow the digestion of the casein by the active fractions. Finally, the gels were stained with 0.01% (w/v) Coomasie Brilliant Blue R250 as above described.

2.7. Physico-chemical properties

The influences of temperature and pH on the trypsin activity of the enzyme preparations were studied as follows: the purified extract was assayed (quadruplicates) as described above at temperatures ranging from 25 to 65 °C and pH values from 6.0 to 10.5 (Tris–HCl buffer).

The thermal stability of the enzymes was determined by assaying (quadruplicates) its activity (25 °C) after pre-incubation for 30 min at temperatures ranging from 30 to 60 °C followed by 25 °C equilibrium.

2.8. Effect of metal ions

Samples of the purified enzymes (30 μl) were added in a 96-well microtiter plate with 3 mM solution (70 μl) of $AlCl_3$, $BaCl_2$, $CaCl_2$, $CdSO_4$, $CoCl_2$, $CuSO_4$, $HgCl_2$, KCl, LiCl, $MgCl_2$, $MnCl_2$, and $ZnSO_4$. After 30 min of incubation, Tris–HCl buffer (70 μl), pH 8.0, and 4 mM BAPNA (30 μl) were added. The *p*-nitroaniline produced was measured in a microplate reader at 405 nm after 30 min of reaction.

2.9. Kinetic parameters

BAPNA prepared in DMSO was used as substrate (final concentration from 0.1 to 1 mM), in a total volume of 170 μl, at pH 8 (0.2 M Tris–HCl) in a 96-well microtiter plate. The reaction (quadruplicates) was started by addition of 30 μl of purified enzyme solution (420 μg protein/mL) and *p*-nitroaniline release followed at 405 nm by using a microtiter plate reader (Bio-Rad 550). The blanks were similarly prepared without enzyme. The reaction rates were fitted to a Michaelis–Menten kinetics using Enzfitter Software Version 1.05 (Elsevier-Biosoft).

3. Results and discussion

Table 1 summarizes the three-step procedure used to purify the alkaline proteases from the spotted goatfish viscera. The enzymatic preparations from the intestine and pyloric caeca were 96-fold and 57.7-fold purified, respectively, and yields of 68.1% and 26.1% were achieved, respectively.

The results from the Sephadex G-75 filtration for both enzymes are shown in Fig. 1. The enzymatic activity was coincident with the second protein peak for the intestine dialysed ammonium sulphate precipitate, whereas two peaks with activities were found for the pyloric caeca. In this work, the second peak (higher specific enzymatic activity) was selected for the characterisation of the enzyme from the pyloric caeca. Similar chromatogram profiles were obtained for tambaqui and Nile tilapia, representing a reproducible protocol for purification of tropical fish trypsin (Bezerra et al., 2001, 2005). Although the heat treatment does not enhance the purification performance its inclusion has been shown to improve the ammonium sulphate precipitation. Probably, the protein hydrolysis catalysed by the fish thermostable proteases for 30 min at 40 °C

Table 1
Purification of trypsin-like enzyme from spotted goatfish intestine and pyloric caeca

Organ and step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Intestine					
Crude extract	203.0	184.9	0.911	100.0	1
Heat treatment	148.3	174.1	1.174	94.1	1.3
Ammonium sulphate precipitation	5.7	79.2	13.895	42.8	15.3
Sephadex G-75 filtration	1.44	126.0	87.500	68.1	96.0
Pyloric caeca					
Crude extract	331.2	432.7	1.307	100.0	1
Heat treatment	271.8	399.0	1.468	92.2	1.1
Ammonium sulphate precipitation	13.3	134.3	10.098	31.0	7.7
Sephadex G-75 filtration	1.5	113.1	75.400	26.1	57.7

Proteins and enzymatic activities were established, respectively, according to Warburg and Christian (1941) and Alencar et al. (2003) using azocasein as substrate.

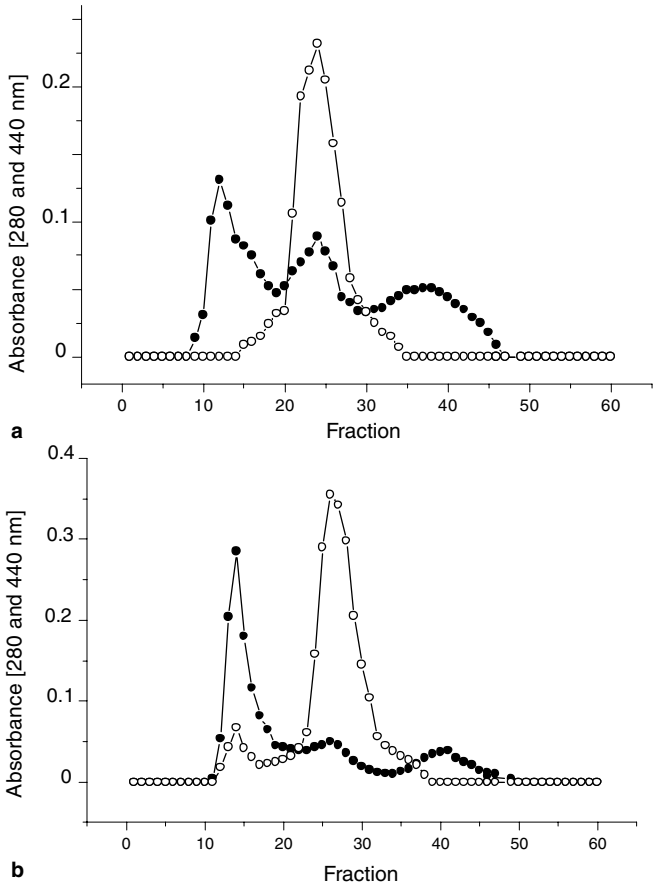

Fig. 1. Chromatogram of the sephadex G-75 filtration of dialysed ammonium sulphate precipitate obtained from the spotted goatfish intestine (a) and pyloric caeca (b). The protein eluted was monitored at 280 nm (•) and the azocasein hydrolysis catalysed by each fraction was followed at 440 nm (○).

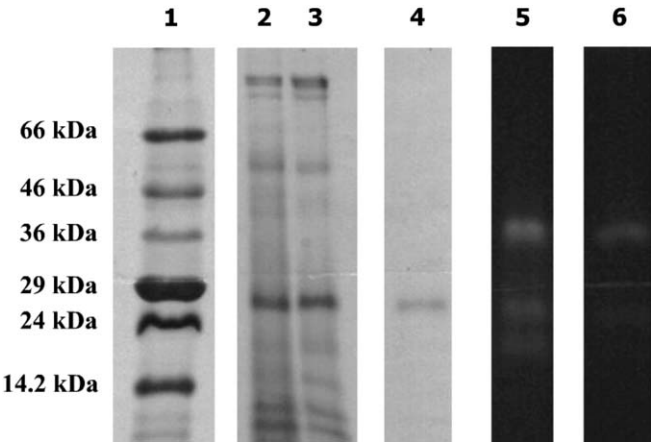

Fig. 2. SDS–PAGE and zymogram of spotted goatfish intestine and pyloric caeca alkaline proteases. Pattern of stained bands produced by standard proteins (lane 1), intestine crude extract (lane 2), pyloric caeca crude extract (lane 3) and pyloric caeca fraction collected by Sephadex G-75 filtration (lane 4) during SDS–PAGE and zymogram specific to proteases from intestine (lane 5) and pyloric caeca (lane 6). Protein standards (Sigma) were bovine albumin (66 kDa), ovalbumin (46 kDa), glyceraldehyde 3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29 kDa), trypsinogen (24.0 kDa) and α-lactalbumin (14.2 kDa).

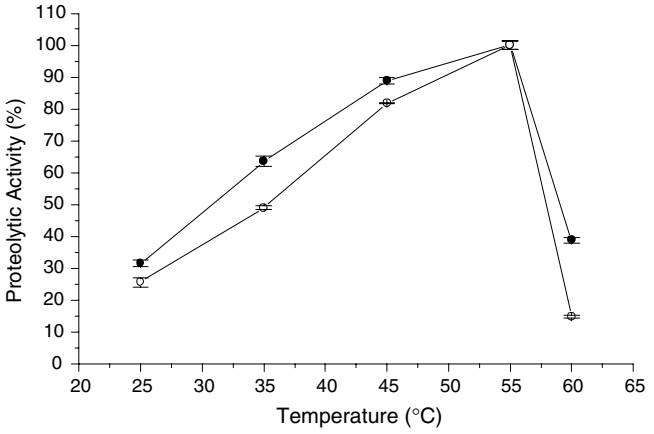

Fig. 3. Temperature effect on the trypsin-like enzyme of spotted goat fish pyloric caeca (•) and intestine (○). The activities were assayed (quadruplicates) by incubating the enzyme (60 μl) with 1% azocasein prepared in 0.2 M Tris–HCl buffer pH 8.0 (100 μl) at indicated temperatures and the absorbance of this mixture was measured at 440 nm. The highest activity for the pyloric caeca enzyme was considered 100%.

produces soluble smaller peptides that are not precipitated under 0–40% and 40–80% ammonium sulphate. This is a simple and low cost method when compared with other protocols that includes several chromatographic steps using expensive stationary phases.

The second protein peak collected from the pyloric caeca by Sephadex G-75 filtration showed a single band in SDS–PAGE (Fig. 2, lane 4). This fraction was also able to hydrolyse casein (Fig. 2, lane 6). The molecular weight of this band was estimated to be 24.5 kDa. This value is similar to that reported for trypsin-like enzyme from intestine of Nile tilapia (Bezerra et al., 2001). This apparent molecular weight is inside the fish trypsin range from 22.5 to 31.4 kDa (Kolodziejska & Sikorski, 1996). The second protein peak collected from the intestine by Sephadex G-75 also presented a single band (not shown) and was capable to hydrolyse casein (Fig. 2, lane 5).

The optimum temperature and pH for both enzymes were 52 °C and 9.0, respectively (Figs. 3 and 4). Furthermore, the intestine and pyloric caeca enzymes showed similar thermal stability, namely, the activity was almost unaltered after incubated at 45 °C for 30 min (Fig. 5). Similar values have been reported for tropical fishes, for example: hybrid tilapia *T. mossambica/aurea* (El-Shemy & Levin, 1997); mullet *Mugil cephalus* (Guizani et al., 1991); tambaqui *C. macropomum* (Bezerra et al., 2001). This pH value is common to fish trypsin, whereas the optimum temperature and thermal stability are slightly higher than those usually reported for trypsin from other fishes (De Vecchi & Coppes, 1996).

The Michaelis–Menten constant for the trypsin-like enzyme from the intestine and pyloric caeca were 1.94 ± 0.45 and 1.82 ± 0.19 mM, respectively. This difference of K_m was not statistically significant. These values are higher than those reported for other tropical fishes: common carp, *Cyprinus carpio* – 0.039 mM (Cohen et al.,

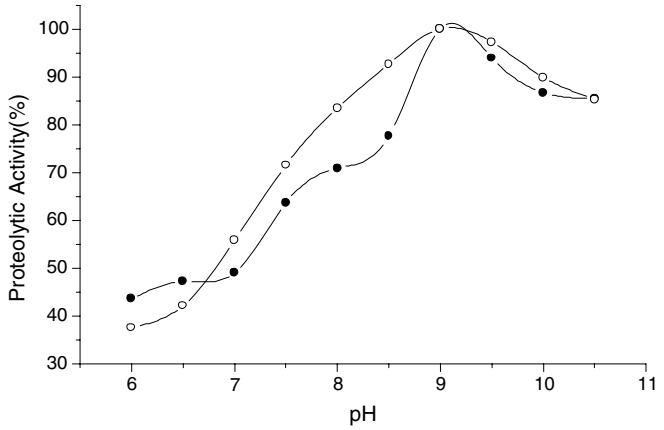

Fig. 4. pH effect on the trypsin-like enzyme of spotted goat fish goat fish pyloric caeca (•) and intestine (○). The activities were assayed (quadruplicates) by incubating the enzyme (60 μl) with 1% azocasein (100 μl) prepared in 0.2 M Tris–HCl buffer at indicated pH and the absorbance of this mixture was measured at 440 nm. The highest activity for the pyloric caeca enzyme was considered 100%.

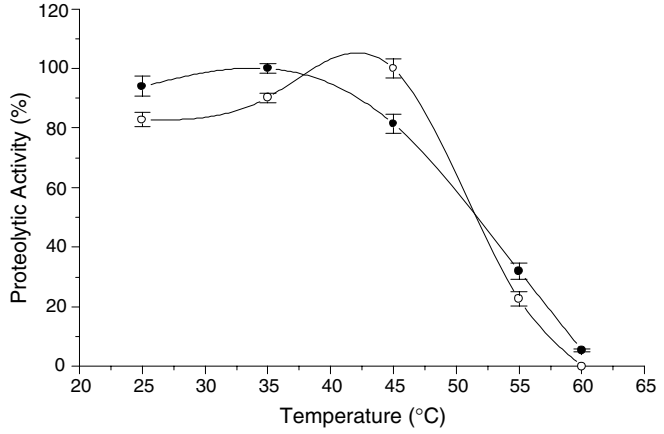

Fig. 5. Thermal stability of the trypsin-like enzyme of spotted goat fish pyloric caeca (•) and intestine (○). Aliquots of the enzyme were incubated at indicated temperatures for 30 min. After 25 °C equilibration the activities were assayed (quadruplicates) by incubating the enzyme (60 μl) with 1% azocasein prepared in 0.2 M Tris–HCl buffer pH 8.0 (100 μl) at 25 °C and the absorbance of this mixture was measured at 440 nm. The highest activity for the pyloric caeca enzyme was considered 100%.

1981); Mullet, *Mugil cephalus* – 0.490 mM (Guizani et al., 1991) and Nile tilapia, *Oreochromis niloticus* – 0.755 mM (Bezerra et al., 2005).

The effects of the ions on the trypsin-like enzyme from both intestine and pyloric caeca tissues are shown in Table 2. As described for other tropical fish proteases, these enzymes demonstrated to be sensitive to ions (Cohen et al., 1981), particularly, Al^{3+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and Cd^{2+}. These ions also inhibited the trypsin-like enzyme obtained from Nile tilapia intestines (Bezerra et al., 2005). However, the effects of Al^{3+}, Zn^{2+}, Hg^{2+} on the spotted goatfish enzyme from the pyloric caeca (respectively, 2.51%, 25.06% and 29.12%) and intestine (respectively, 10.18%, 19.26% and 29.51%) showed to be more

Table 2
Ions effect on the trypsin-like enzyme of spotted goatfish pyloric caeca and intestine

Ion (1 mM)	Intestine	Pyloric caeca
	Residual activity ± SD (%)	Residual activity ± SD (%)
Al^{3+}	10.18 ± 3.54[a]	2.51 ± 1.29
Zn^{2+}	19.26 ± 3.23[a]	25.06 ± 1.04
Hg^{2+}	29.51 ± 4.00	29.12 ± 1.68
Cu^{2+}	42.47 ± 2.57[a]	30.80 ± 0.97
Cd^{2+}	49.43 ± 4.57[a]	46.85 ± 1.93
Co^{2+}	63.73 ± 3.93[a]	86.80 ± 1.94
Ba^{2+}	69.12 ± 3.25[a]	91.51 ± 5.10
Mn^{2+}	72.82 ± 3.93[a]	82.42 ± 1.94
K^{+}	78.81 ± 2.45[a]	91.64 ± 2.45
Ca^{+}	74.95 ± 3.10[a]	93.44 ± 0.84
Li^{+}	74.62 ± 4.39[a]	95.57 ± 2.58
Mg^{2+}	89.70 ± 2.91	86.29 ± 1.80

Samples (quadruplicates) of the purified enzyme (30 μl) were added to the metal ions 3 mM solutions (70 μl). After 30 min of incubation, 0.2 M Tris–HCl pH 8.0 buffer (70 μl) and 4 mM BAPNA (30 μl) were added. The *p*-nitroaniline produced was spectrophotometrically measured at 405 nm after 30 min of reaction.

[a] Statistically different from pyloric caeca residual activity.

severe than those registered for the Nile tilapia enzyme (respectively, 39.9%, 38.4% and 73.4%). The influence of heavy metals or other inhibitory compounds on trypsin activity has been employed as a means to detect xenobiotics in a solution containing commercially available trypsin (Šafařik et al., 2002). The inhibition displayed by tested ions may well render the purified enzyme to be used in those methods of detection.

The inhibition effects by Ca^{2+}, Co^{2+}, Li^{1+}, K^{1+}, Mn^{2+}, Mg^{2+} and Ba^{2+} on the spotted goatfish enzymes were less intensive than those displayed by the ions above mentioned. Furthermore, the effects of Co^{2+}, Ba^{2+}, Mn^{2+}, K^{1+} and Li^{1+} on the pyloric caeca enzyme were lower than those observed for the intestine enzyme. On the contrary, the effects of Al^{3+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} were higher on the pyloric caeca enzyme than on the intestine trypsin. There were no statistically significant differences ($p > 0.05$) between the effects of Hg^{2+}, and Mg^{2+} on both enzymes (Table 2). These discrepant ion effects on the enzymes probably reflect structural differences between them despite they share some properties (K_m, optimal pH and temperatures).

Although it is known that calcium is required for trypsin activity, especially in mammalians, the same was not observed for this tropical fish trypsin. Similar results regarding calcium effect on alkaline proteases from aquatic animals have been reported by other research communications (Bezerra et al., 2005; Kishimura & Hayashi, 2002; Saborowski, Sahling, Toro, Walter, & García-carreño, 2004).

All this information emerged in this contribution indicate that these trypsin-like enzymes present in the spotted goatfish intestine and pyloric caeca have very similar characteristics. Therefore, these viscera can be homogenised together and the purification according to the present

protocol would yield a preparation presenting similar properties.

The trypsin-like from spotted goatfish have shown properties alike other trypsins from tropical fishes (tambaqui and tilapia) and commercial trypsins. These features can add aggregated value to the fishery industry waste that is presently discarded in the environment without treatment. Also, it could be used in the foodstuff industry applications, such as: protein hydrolysates and fish sauce production; and shrimp waste recovery (Gildberg, 2001; Gildberg & Stenberg, 2001; Haard, 1992; Mackie, 1982).

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References

Alencar, R. B., Biondi, M. M., Paiva, P. M. G., Vieira, V. L. A., Carvalho, L. B., Jr., & Bezerra, R. S. (2003). Alkaline proteases from digestive tract of four tropical fishes. *Brazilian Journal of Food Technology, 6*(2), 279–284.

Bezerra, R. S., Lins, E. J. F., Alencar, R. B., Paiva, P. M. G., Chaves, M. E. C., Coelho, L. C. B. B., et al. (2005). Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). *Process Biochemistry, 40*(5), 1829–1834.

Bezerra, R. S., Santos, J. F., Lino, M. A. S., Vieira, V. L. A., & Carvalho, L. B. Jr., (2000). Characterization of stomach and pyloric caeca proteinases of tambaqui (*Colossoma macropomum*). *Journal of Food Biochemistry, 24*(3), 189–199.

Bezerra, R. S., Santos, J. F., Paiva, P. M. G., Correia, M. T. S., Coelho, L. C. B. B., Vieira, V. L. A., et al. (2001). Partial purification and characterization of a thermostable trypsin from pyloric caeca of tambaqui (*Colossoma macropomum*). *Journal of Food Biochemistry, 25*(3), 199–210.

Cohen, T., Gertler, A., & Birk, Y. (1981). Pancreatic proteolytic enzymes from carp *Cyprinus carpio* – II. Kinetic properties and inhibition studies of trypsin, chymotrypsin and elastase. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 69*(3), 647–653.

De Vecchi, S., & Coppes, Z. (1996). De Marine fish digestive proteases – relevance to food industry and the south-west Atlantic region – a review. *Journal of Food Biochemistry, 20*(3), 193–214.

El-Shemy, M. G., & Levin, R. E. (1997). Characterization of affinity-purified trypsin from hybrid tilapia (Tilapia nilotica/aurea). *Journal of Food Biochemistry, 21*, 163–175.

García-Carreño, F. L., Dimes, L. E., & Haard, N. F. (1993). Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinases inhibitors. *Analytical Biochemistry, 214*(1), 65–69.

Gildberg, A. (2001). Utilisation of male Artic capelin and Atlantic cod intestines for fish sauce production – evaluation of fermentation conditions. *Bioresource Technology, 76*(2), 119–123.

Gildberg, A., & Stenberg, E. (2001). A new process for advanced utilization of shrimp waste. *Process Biochemistry, 36*(8–9), 809–812.

Guizani, N., Rolle, R. S., Marshall, M. R., & Wei, C. I. (1991). Isolation, purification and characterisation of a trypsin from the pyloric caeca of mullet (*Mugil cephalus*). *Comparative Biochemistry Physiology, 98B*(4), 517–521.

Haard, N. F. (1992). A review of proteolytic enzymes from marine organisms and their application in the food industry. *Journal of Aquatic Food Product Technology, 1*(1), 17–35.

Kishimura, H., & Hayashi, K. (2002). Isolation and characteristics of trypsin from pyloric caeca of the starfish *Asterina pectinifera*. *Comparative Biochemistry and Physiology B Biochemistry and Molecular Biology, 132*(2), 485–490.

Kolodziejska, I., & Sikorski, Z. E. (1996). The digestive proteases of marine fish and invertebrates. *Bulletin of Sea Fisheries Institute, 137*(1), 51–56.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Mackie, I. M. (1982). Fish protein hydrolysates. *Process Biochemistry, 17*(1), 26–31.

Saborowski, R., Sahling, G., Toro, M. A., Walter, I., & García-carreño, F. L. (2004). Navarete del Stability and effects of organic solvents on endopetidades from the gastric fluid of the marine crab *Cancer pagurus*. *Journal of Molecular Catalysis B: Enzymatic, 30*(3–4), 109–118.

Šafařik, I., Ptáeková, L., Koneracká, M., Šafaoiková, M., Timko, M., & Kopeanský, P. (2002). Determination of selected xenobiotics with ferrofluid-modified trypsin. *Biotechnology Letters, 24*(5), 355–358.

Warburg, O., & Christian, W. (1941). Isolierung und Kristallisation des Garunges Ferments enolasc. *Biochemioche Zeitschrift, 310*, 384–421.