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# Purification and partial characterisation of a trypsin from the processing waste of the silver mojarra (*Diapterus rhombeus*)

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ABSTRACT

An alkaline peptidase was purified from the viscera of the silver mojarra (*Diapterus rhombeus*) in a three-step process: heat treatment, ammonium sulphate fractionation and molecular exclusion chromatography (Sephadex® G-75), with final specific activity 86-fold higher than the enzyme extract and yield of 22.1%. The purified enzyme had an estimated molecular mass of 26.5 kDa and $NH_2$-terminal amino acid sequence IVGGYECTMHSEAHE. Higher enzyme activity was observed at pH 8.5 and between 50 and 55 °C. The enzyme was completely inactivated after 30 min at 55 °C and it was significantly more stable at alkaline pH. $K_m$, $K_{cat}$ and $K_{cat} \cdot K_m^{-1}$ values, using BApNA as substrate, were 0.266 mM, 0.93 $s^{-1}$ and 3.48 $mM^{-1}$ $s^{-1}$, respectively. Enzyme activity increased in the presence of the ions (1 mM) $K^+$, $Li^+$ and $Ca^{2+}$, but was inhibited by $Fe^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Al^{3+}$, $Hg^{2+}$, $Zn^{2+}$ and $Pb^{2+}$ as well as by the trypsin inhibitors TLCK and benzamidine.



## 1. Introduction

The Brazilian coast has a large diversity of fish species, of which approximately 130 have some commercial value. Fish are usually processed before their commercialisation, thus generating large amounts of waste, which is usually discarded in the environment without any previous treatment, causing serious pollution problems. According to Bezerra et al. (2005), fish viscera are rich in peptidases, which are enzymes that occur naturally in all organisms and are involved in a variety of physiological and biotechnological processes. Due to the diverse feeding habits of fish in general, differences in characteristics and composition of their enzymes are expected. Therefore, studies describing enzymes isolated from these animals represent the first step to evaluate their potential for technological application. In fact, to save time and money, experiments at laboratory conditions are essential for future production in industrial scale.

Peptidases are amongst the most important groups of commercial enzymes, representing up to 60% of enzymes marketed in the world. In the digestive tract of fish, one of the main peptidases is trypsin (EC 3.4.21.4), a serinopeptidase that cleaves peptide bonds at the carboxy end of the amino acid residues arginine and lysine. This enzyme plays a key role in the digestion of dietary proteins and is also responsible for the activation of trypsinogen and other zymogens (Polgár, 2005).

Recently, many studies have reported the use of common and simple chromatographic procedures on the purification of trypsin isoforms from various fish species, such as *Colossoma macropomum* (Bezerra et al., 2001; Marcuschi et al., 2010), *Oreochromis niloticus* (Bezerra et al., 2005), *Gadus macrocephalus* (Fuchise et al., 2009), *Theragra chalcogramma* (Kishimura, Klomklao, Benjakul, & Chun, 2008) and *Katswonus pelanis* (Klomklao, Kishimura, Nonami, & Benjakul, 2009). These protocols proved to be efficient in purifying fish trypsins in a few steps, and are of relative low cost, being easily adapted to industrial scale and affording between 1 and 3 g of purified trypsin per 1 kg of wet waste. These studies also emphasise features in these enzymes that enable their use in industrial processes, with applications as additives for washing powder (Espósito et al., 2009), food processing (Shahidi & Kamil, 2001) and pharmacology (Jónsdóttir, Bjarnason, & Gudmundsdóttir, 2004).

The silver mojarra (*Diapterus rhombeus*) is a marine finfish from the northeastern Brazilian coast, of economic and ecological importance that can be used to extract proteases for biotechnological applications. This fish belongs to the family Gerreidae and is

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found in coastal estuaries throughout the tropical waters of the Atlantic Ocean (Austin, 1973). According to the Brazilian Environmental Agency IBAMA (2008), 2080 tons of mojarras were captured in northeastern Brazil in 2006, which generated an estimated annual discharge of about 100 tons of viscera. Therefore, the investigation into enzymes present in this type of byproduct may help optimise the use of these resources, by adding value to these industrial segments.

The aim of the present study was to purify a trypsin from the digestive tract of the silver mojarra and characterise its physical and biochemical properties, such as the effect of temperature, pH, ions, inhibitors, substrate concentration and $NH_2$-terminal amino acid sequence.

## 2. Materials and methods

### 2.1. Samples

Specimens of *D. rhombeus* were obtained from a fishing community in Itapissuma, Pernambuco, Brazil. Fish were packed in ice and transported to the laboratory. Average weight and length was 350 ± 20 g and 28 ± 2 cm, respectively. The intestine and pyloric caeca of ten fish (about 30 g) were removed and stored in a freezer at −25 °C until analysis.

### 2.2. Enzyme extract

Fish intestines and pyloric caeca were mixed together and homogenised at a concentration of 40 mg $ml^{-1}$ (w.$v^{-1}$) of tissue in a solution of 0.01 M Tris–HCl, pH 8.0, with 0.9% NaCl, using a tissue homogeniser (Bondine Electric Company, Chicago, IL) at 300 rpm for 60 s. The homogenate was then centrifuged (Herolab Unicen MR Centrifuge, Germany) at 10,000*g* for 25 min at 4 °C for the removal of insoluble particles. The supernatant (enzyme extract) was collected and stored in a freezer at −25 °C for subsequent use in the purification steps.

### 2.3. Enzyme assay and protein determination

Enzyme activity was measured using BApNA (Nα-benzoyl-L-arginine-p-nitroanilide) prepared with dimethylsulphoxide (DMSO), as substrate specific for trypsin. The assay was carried out by mixing 30 μl of sample with 140 μl of 0.1 M Tris–HCl, pH 8.0 and 30 μl of 8 mM BApNA (final concentration of 1.2 mM) for 10 min at 25 °C. The formation of p-nitroaniline (product) was measured at 405 nm with a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). A blank control was prepared by replacing sample with 0.1 M Tris–HCl, pH 8.0 (Souza, Amaral, Santo, Carvalho, & Bezerra, 2007). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolysing 1 μmol of BApNA per min under the established conditions, using a molar coefficient of 9100 $mM^{-1}$ $cm^{-1}$. The protein content was obtained by measuring the absorbance of the samples at 260 nm and 280 nm based on the method proposed by Warburg and Christian (1941), using the following equation: [protein] mg $ml^{-1} = A_{280} \times 1.5 - A_{260} \times 0.75$.

### 2.4. Enzyme purification

For each purification step, trypsin activity was assayed using BApNA as substrate. The parameters used were: degree of purification (specific activity rate between the purification step sample and enzyme extract) and yield (total activity rate between the purification step sample and enzyme extract). The enzyme extract was placed in a water bath at 45 °C for 30 min and then placed on ice for rapid cooling. This material was centrifuged at 10,000*g* for 25 min at 4 °C. The precipitate was discarded and the supernatant (heated enzyme extract) was collected. Precipitation was then performed with ammonium sulphate, yielding fractions of 0–30%, 30–60% and 60–90% salt saturation. The salt was slowly added to the extract under agitation. After the total dissolution of the salt, the extract was kept at 4 °C for 4 h. Each salt saturation fraction was centrifuged at 10,000*g* for 25 min at 4 °C and the precipitate was resuspended with 38.5 ml of 0.1 M Tris–HCl, pH 8.0. The fraction with the greatest specific activity for trypsin was applied to a Sephadex® G-75 gel filtration column. Maintaining a flow of 20 ml $h^{-1}$, aliquots of 2 ml were collected and subsequently analysed for protein content and specific enzyme activity (Bezerra et al., 2001).

### 2.5. SDS–PAGE

The samples were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE), following the method described by Laemmli (1970), using a 4% concentration gel and 15% separation gel. SDS–PAGE was conducted at 11 mA using a vertical electrophoresis system (Vertical Electrophoresis System, Bio-Rad Laboratories, Inc.). The molecular mass of the purified protein band was estimated by comparison with a molecular mass standard (Amersham Biosciences, UK) containing myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), transferrin (80 kDa), bovine serum albumin (66 kDa), glutamate dihydrogenase (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21 kDa).

### 2.6. Optimum pH and stability

These experiments were carried out using different buffer solutions: 0.1 M citrate–phosphate (pH from 4.0 to 7.5), 0.1 M Tris–HCl (pH from 7.2 to 9.0) and 0.1 M glycine-NaOH (pH from 8.6 to 11.0). Optimum pH was determined by mixing 30 μl of the purified enzyme with 140 μl of buffer solutions, then adding 30 μl of substrate (8 mM BApNA, generating a final concentration of 1.2 mM) for 10 min at 25 °C. The influence of pH on enzyme stability was determined by incubating the purified enzyme with various buffer solutions, at a ratio of 1:1 for 30 min at 25 °C. Then, 30 μl aliquots were withdrawn and used to assess the residual activity of the enzyme at optimum pH presented by peptidase, using 8 mM BApNA as substrate. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100%.

### 2.7. Optimum temperature and thermal stability

The effect of temperature on the purified enzyme activity and stability was evaluated at temperatures ranging from 25 to 80 °C. For optimal temperature, the assay was carried out by incubating the samples with the substrate, 8 mM BApNA, in a water bath. To test thermal stability, the enzyme was incubated in a water bath for 30 min and the remaining activity was then measured at 25 °C, using the method previously described for BApNA.

### 2.8. Inhibitor effect on trypsin activity

The inhibition tests were performed using the methodology adapted by Bezerra et al. (2005). A 30 μl sample of the purified enzyme was incubated in microplates for 30 min with 30 μl of different peptidase inhibitors whilst maintaining a final concentration of 2 mM. The inhibitors used in this test were ethylene diamine tetraacetic acid – EDTA (metallopeptidase inhibitor), β-mercaptoethanol (reducing agent), phenylmethylsulphonyl fluoride – PMSF (serine peptidases inhibitor), benzamidine (trypsin inhibitor), tosyl lysine chloromethyl ketone – TLCK (trypsin inhibitor) and tosyl phenylal-

anyl chloromethyl ketone – TPCK (chymotrypsin inhibitor). After incubation, 110 μl of buffer 0.1 M Tris–HCl and 30 μl of BApNA were then added. After 10 min, the absorbance reading was performed in microplate reader (BioRad xMarktm) at a wavelength of 405 nm.

### 2.9. Metal ions effect on trypsin activity

Aliquots of 30 μl of the purified enzyme were incubated with 30 μl of various metals ($AlCl_3$, $BaCl_2$, $CaCl_2$, $CdCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, KCl, LiCl, $MnCl_2$, PbCl2, $ZnCl_2$) for 30 min in microplates with final concentration of 1 mM. Next, 110 μl of 0.1 M Tris–HCl, pH 8.0, and 30 μl of the substrate BApNA were added. After 10 min of reaction, enzyme activity was measured in a microplate reader at 405 nm.

### 2.10. Michaelis–Menten kinetic assay

The substrate used in the kinetic test was BApNA (final concentration from 0 to 4.8 mM), prepared with DMSO. The reaction was performed in triplicate in microplates and consisted of a mixture of a 30 μl solution of purified enzyme (109 μg protein $ml^{-1}$) with 140 μl of 0.1 M Tris–HCl, pH 8.0 and 30 μl of substrate. The release of the product (p-nitroaniline) was monitored by a microplate reader at 405 nm. The activity values (U $s^{-1}$) obtained for each substrate concentration were plotted on a graph and the Michaelis–Menten asymptotic kinetic parameters ($V_{max}$ and $K_m$) were calculated using the MicrocalTM OriginTM program version 6.0 (Software Inc., USA).

### 2.11. Sequencing of $NH_2$-terminal region

The purified trypsin was sequenced at the Biochemistry Laboratory of the Escola Paulista de Medicina, Universidade Federal de São Paulo (Brazil). The $NH_2$-terminal amino acid sequence was obtained through Edman degradation using a PPSQ-23 sequencer (Shimadzu, Tokyo, Japan). The $NH_2$-terminal amino acid sequence obtained for the present study was aligned with other's sequences using the software BioEdit Sequence Alignment Editor (Hall, 1999).

### 2.12. Statistical analysis

All data was analysed using one-way analysis of variance (ANOVA) complemented with Tukey's test. Differences were reported as statistically significant when $p < 0.05$. The statistical program used was MicrocalTM OriginTM version 8.0 (Software, Inc., US).

## 3. Results and discussion

A trypsin from the pyloric caeca and intestine of the silver mojarra (*D. rhombeus*) was isolated through a three-step purification process ($n = 3$). The specific activity in the initial enzyme extract was 1.81 ± 0.3 mU $mg^{-1}$, whilst total activity was 916.10 ± 81.3 mU. The first step (heat treatment) resulted in a slight increase in the specific activity, generating a purification factor of 1.2 ± 0.2-fold and a yield of 113.4 ± 12.5%. In the second step (ammonium sulphate fractionation), the fraction with greatest specific activity was 30–60% of salt saturation, in which it was observed a 5.6 ± 3.1-fold increase was observed, with a yield of 36.2 ± 7.6%. Following gel-filtration chromatography (Sephadex® G-75), the degree of purification was 86.8 ± 7.7-fold higher than the enzyme extract, yielding 22.1 ± 6.4%. The chromatography pool revealed only one band in the SDS–PAGE with an estimated molecular mass of 26.5 kDa (Fig. 1). The literature reports that the molecular mass of fish trypsins usually varies between 24 kDa and 28 kDa (Castillo-Yáñes, Pacheco-Aguiar, García-Carreño, & Toro, 2005; Fuchise et al., 2009; Heu, Kim, & Pyeun, 1995; Klomklao, Benjakul, Visessanguan, Khishimura, & Simpsom, 2007).

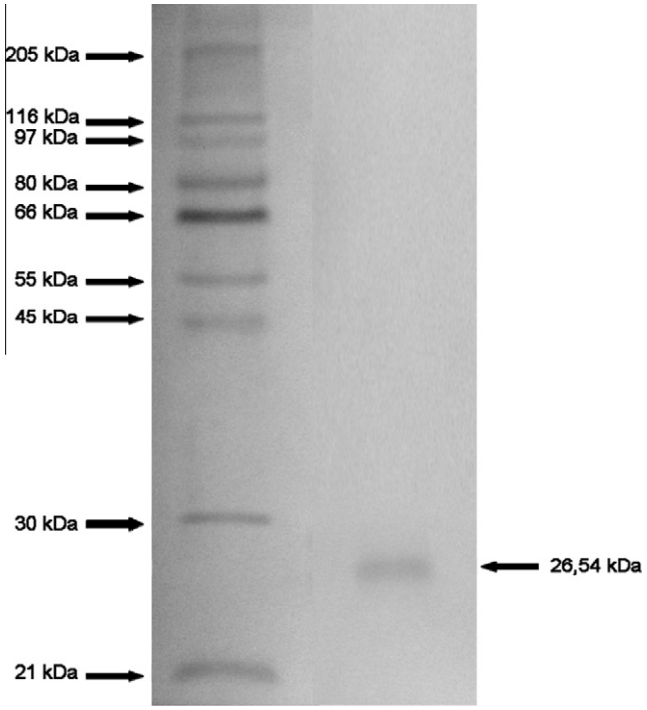


**Fig. 1.** Polyacrylamide gel electrophoresis (SDS–PAGE) of the trypsin purified from the viscera of silver mojarra (*D. rhombeus*); lane 1: standard protein markers of different molecular mass; lane 2: purified trypsin.

This same protocol has been successfully used in the purification of other trypsins from tropical fish (Bezerra et al., 2001, 2005; Souza et al., 2007). Bezerra et al. (2001) reported the importance of the heat treatment in the purification of a trypsin from *C. macropomum*. Despite the low purification factor obtained in this stage, heating eliminates thermolabile proteins and promotes the hydrolysis of the thermostable contaminating proteins. This property improves the performance in the subsequent stages of ammonium sulphate fractionation and gel-filtration chromatography.

After purification, the physical and chemical characteristics of the trypsin isolated from the digestive tract of *D. rhombeus* were evaluated. Assays to define the optimal pH revealed greater enzyme activity in the range of alkaline pH (7.5–11.0), with peak activity at 8.5 (Fig. 2A). These results found for *D. rhombeus* are common amongst digestive enzymes from fish, as reported for *T. chalcogramma* (Kishimura et al., 2008) and *O. niloticus* (Bezerra et al., 2005), but lower than those found in *P. saltatrrix* (Klomklao et al., 2007). The effects of pH on the stability of *D. rhombeus* trypsin are shown in Fig. 2B. The enzyme exhibited stability in an alkaline pH range, maintaining over 85% of its optimum activity between pH 8.5 and 11.0, whereas from 35% to 65% of the residual activity was maintained at pH from 4.5 to 8.0. However, only 10% of the residual activity was observed at pH 4.0. Changes in pH may affect both the substrate and enzyme by changing the charge distribution and conformation of the molecules (Klomklao et al., 2006). Most enzymes undergo irreversible denaturation in a very acid or alkaline solution, resulting in a loss of activity.

The optimal temperature of the purified enzyme (Fig. 2C) was between 50 and 55 °C. A sharp decrease in activity was found at temperatures above 60 °C and negligible activity was observed at 85 °C. The loss of activity was presumably caused by thermal denaturation of the purified enzyme. Similar results are reported for *Mugil cephalus* (Guizani, Rolle, Marshall, & Wei, 1991) and

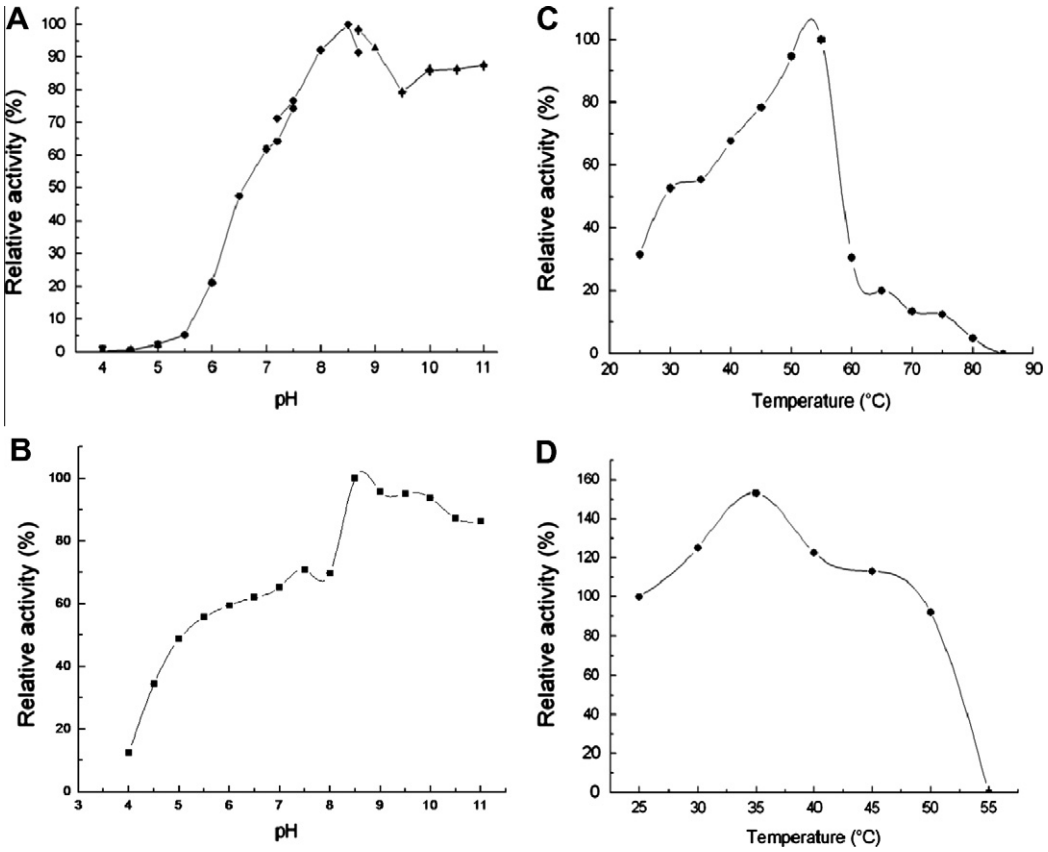


**Fig. 2.** Effect of pH and temperature on activity of the trypsin purified from silver mojarra (*D. rhombeus*), using BApNA as substrate; (A) trypsin activity in different buffers: citrate–phosphate (■, pH 4.0–7.5), Tris–HCl (○, pH 7.2–8.5) and glycine-NaOH (▲, pH 8.7–11.0); (B) trypsin stability after incubation for 30 min at 25 °C in different pH (from 4.0 to 11); (C) trypsin activity in temperatures ranging from 25 to 85 °C; (D) trypsin stability after incubation for 30 min in different temperatures (from 25 to 85 °C). Values are shown as mean ± SD of triplicates of three purified extracts.

*S. s. caerulea* (Castillo-Yáñes et al., 2005), both with an optimal temperature of 50 °C, and for *C. macropomum*, with an optimal temperature of 60 °C. The high optimal temperature may be due to the fact that *D. rhombeus* lives in warm waters, whereas most species analysed thus far live in cold waters. With regard to thermostability, the trypsin from the fish cited proved also to be sensitive to temperatures above 45 °C, which is similar to the results found in the present study (Fig. 2D). Kishimura et al. (2008) reported a direct correlation between the temperature of the habitat and the thermal stability of fish trypsin.

The effects of metal ions (1 mM) on the activity of trypsin from *D. rhombeus* are displayed in Table 1. Enzyme activity was higher than the control (100%) when incubated in the presence of $K^+$ (34%), $Li^+$ (46%) and $Ca^{2+}$ (83%). Calcium was shown to be a positive effector for *D. rhombeus* trypsin. In fact, this ion is known as a classic activator for mammal trypsins. However, Bezerra et al. (2005) and Souza et al. (2007) found that trypsin from the Nile tilapia and spotted goatfish were inhibited by calcium. These results suggest that there are differences in calcium dependence amongst the trypsins from mammal and some fish. The activity of trypsin from the Nile tilapia and spotted goatfish was also inhibited in the presence of $Mn^{2+}$ and $Ba^{2+}$, but trypsin isolated from the species analysed in the present study exhibited no traces of enzyme inhibition with these ions. $Fe^{2+}$, $Cd^{2+}$, $Cu^{2+}$ and $Al^{3+}$ decreased enzyme activity by about 20–35%, whereas $Hg^{2+}$ and $Zn^{2+}$ inhibited trypsin activity by 53% and 71%, respectively. However, these inhibition values are less expressive than those described for the spotted goatfish. In the presence of $Pb^{2+}$, there was total inactivation of the trypsin purified from *D. rhombeus*. Ions such as $Cd^{2+}$ and $Hg^{2+}$

**Table 1**
Effects of metal ions and inhibitors on activity of trypsin from silver mojarra (*D. rhombeus*).

| Ions/inhibitors | Residual activity (%) |
|---|---|
| *Control | [a]100.00 |
| *[Ions] 1 mM* | |
| $Ca^{2+}$ | [b]183.13 ± 0.75 |
| $Li^+$ | [c]146.18 ± 14.32 |
| $K^+$ | [c]134.46 ± 3.77 |
| $Ba^{2+}$ | [a]108.35 ± 4.52 |
| $Mn^{2+}$ | [a]101.24 ± 4.52 |
| $Fe^{2+}$ | [d]79.57 ± 8.53 |
| $Cd^{2+}$ | [d]78.69 ± 0.75 |
| $Cu^{2+}$ | [e]69.27 ± 0.00 |
| $Al^{3+}$ | [e]66.96 ± 0.75 |
| $Hg^{2+}$ | [f]46.89 ± 4.52 |
| $Zn^{2+}$ | [f]28.77 ± 13.41 |
| $Pb^{2+}$ | [g]0.00 ± 0.00 |
| *[Inhibitor] 2 mM* | |
| PMSF | [d]77.40 ± 7.37 |
| PMSF (4 mM) | [f]32.64 ± 3.03 |
| TPCK | [a]103.64 ± 13.03 |
| TLCK | [g]0.00 ± 0.00 |
| Benzamidine | [f]25.01 ± 0.47 |
| EDTA | [de]78.51 ± 11.09 |
| 2-Mercaptoethanol | [e]64.61 ± 1.87 |

Different superscript letters represent statistical differences ($p < 0.05$) of residual activity of purified trypsin incubated with various metal ions and inhibitors solutions.
* Residual activity without any ion or inhibitor.

are known to act on sulfhydryl residues in proteins (Aranishi et al., 1998) and, according to Bezerra et al. (2005), inhibition caused by these metal ions suggests the importance of sulfhydryl residues to the catalytic action of this peptidase. This relevance was also reinforced by the inhibition (approximately 35%) of the *D. rhombeus* trypsin activity by 2-mercaptoethanol. Moreover, the influence of metals ions or other inhibitory compounds over trypsin activity has been employed as a means to detect xenobiotics in a solution containing commercially available trypsin (Šafařik et al., 2002).

The influence of some synthetic inhibitors on the activity of the enzyme purified from the viscera of the *D. rhombeus* is displayed in Table 1. The trypsin from *D. rhombeus* was completely inhibited in the presence of TLCK. Similar results are reported for the Nile tilapia (Bezerra et al., 2005), bluefish (Klomklao et al., 2007) and yellowfin tuna (Klomklao et al., 2006). TLCK is a well-known trypsin-specific inhibitor, inactivating only trypsin-like enzymes by forming a covalent bond with the histidine residue from the catalytic site and then blocking the substrate-binding portion at the active centre (Jeong, Wei, Preston, & Marshall, 2000). The purified enzyme from *D. rhombeus* was also inhibited by 75% by benzamidine (a synthetic trypsin inhibitor), 36% by 2-mercaptoetanol, 22.8% and 71.36% by 2 mM and 4 mM PMSF, respectively (a serine proteinase inhibitor) and 21.5% by EDTA. TPCK (a typical chymotrypsin inhibitor) had no effect on the activity of the purified enzyme. The pattern of action of these inhibitors was characteristic of those reported for trypsins, thereby supporting the identity of this purified enzyme as trypsin.

Kinetics parameters of BApNA hydrolysis rates were examined in the present study (Table 2). Michaelis constant ($K_m$) indicate the affinity of the enzyme to the substrate, $K_{cat}$ indicates molecular catalytic constant and $K_{cat} \cdot K_m^{-1}$ indicates its catalytic efficiency. $K_m$, $K_{cat}$ and $K_{cat} \cdot K_m^{-1}$ values for the trypsin-like enzyme from *D. rhombeus* were 0.266 mM, 0.93 $s^{-1}$ and 3.48, respectively. This $K_m$ value is lower than that reported for trypsin from *Priacanthus macrachanhtus* (Hau & Benjakul, 2006), *O. niloticus* (Bezerra et al., 2005), *Salmo salar* (Outzen, Berglund, Smalas, & Willassen, 1996), bovine (Asgeirsson, Fox, & Bjarnason, 1989) and swine (Outzen et al., 1996) and higher than that reported for *S. s. caerulea* (Castillo-Yáñes et al., 2005) and *E. japonica* (Heu et al., 1995). This result indicates the considerable affinity of the purified enzyme from *D. rhombeus* to the BApNA substrate. The catalytic constant ($K_{cat}$) of the trypsin purified in the present study was higher than the value reported for *G. morhua* (Asgeirsson et al., 1989) and *S. salar* (Outzen et al., 1996). A higher molecular activity ($K_{cat}$) denotes a greater amount of substrate molecules that are converted into product by a single enzyme, thus indicating that the enzyme purified in the present study is as highly active as the other fish trypsin. The catalytic efficiency ($K_{cat} \cdot K_m^{-1}$) results reveal that the trypsin purified in the present study is able to hydrolyse a classic trypsin synthetic substrate more efficiently than the trypsin from bovine (Asgeirsson et al., 1989), swine (Outzen et al., 1996), *P. macracanthus* (Hau & Benjakul, 2006) and *S. salar* (Outzen et al., 1996), but less efficiently than that from *E. japonica* (Heu et al., 1995), *S. officinalis* (Balti, Barkia, Bougatef, Ktari, & Nasri, 2009) and *G. morhua* (Asgeirsson et al., 1989).

The 15 $NH_2$-terminal amino acids residues in *D. rhombeus* trypsin were IVGGYECTMHSEAHE. This $NH_2$-terminal amino acid sequence was compared to that of other vertebrates (Fig. 3). According to Cao et al. (2000), the first seven $NH_2$-terminal amino acid residues (IVGGYEC) and the residues between positions 15 and 19 (QVSLN) are generally conserved in vertebrate trypsins. In mammals, however, the glutamic acid (E) in position 6 is replaced by threonine (T)

**Table 2**
Kinetic parameters for trypsin from silver mojarra (*D. rhombeus*) using BApNA as substrate.

| Species | Parameters | | | References |
|---|---|---|---|---|
| | $K_m$ (mM) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) | |
| *D. rhombeus* | 0.266 | 0.93 | 3.48 | Present study |
| *E. japonica* | 0.049 | 1.55 | 31.00 | Heu et al. (1995) |
| *S. officinalis* | 0.064 | 2.32 | 36.25 | Balti et al. (2009) |
| *P. macracanthus* | 0.312 | 1.06 | 3.40 | Hau and Benjakul (2006) |
| *G. morhua* | 0.102 | 0.70 | 6.80 | Asgeirsson et al. (1989) |
| Bovine | 0.650 | 2.00 | 3.10 | Asgeirsson et al. (1989) |
| Swine | 0.820 | 1.55 | 1.89 | Outzen et al. (1996) |
| *S. salar* | 0.300 | 0.80 | 2.67 | Outzen et al. (1996) |
| *O. niloticus* | 0.772 | – | – | Bezerra et al. (2005) |

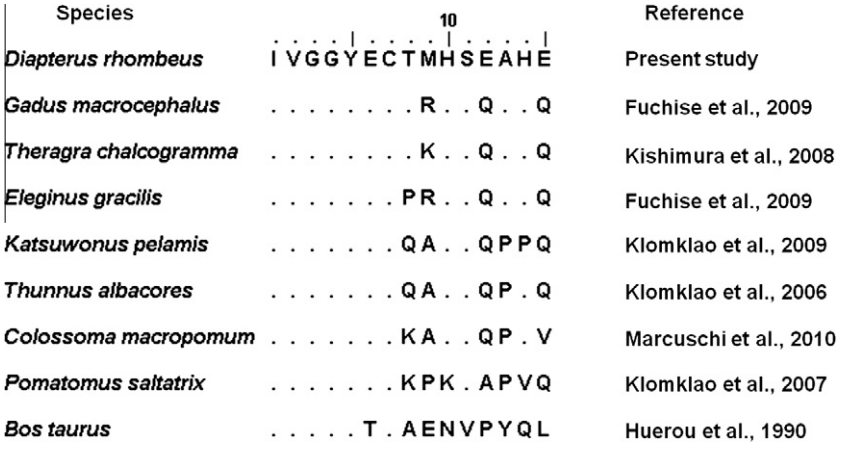


**Fig. 3.** $NH_2$-terminal amino acid residues alignment of trypsin from silver mojarra (*D. rhombeus*) with other fish trypsin and bovine trypsin; dots represent the same amino acid residues as the main sequence (present study) and letters indicate amino acids that are different. The alignment was performed with the software BioEdit (Hall, 1999).

(Huerou, Wicker, Guilloteau, Toullec, & Puigserver, 1990). As can be seen in Fig. 3, this $NH_2$-terminal amino acid sequence from *D. rhombeus* exhibited high homology and revealed similarity to that of *G. macrocephalus* (Fuchise et al., 2009), *Theragra chalcograma* (Kishimura et al., 2008) and *Eleginus gracilis* (Fuchise et al., 2009).

The results of the present study suggest that the peptidase purified from *D. rhombeus* is a trypsin. Because of its high activity and stability at pH from 8.5 to 11, this enzyme has good potential to be used as an additive in commercial detergent formulations, which demonstrates the feasibility of using waste from *D. rhombeus* as a source of biomolecules of biotechnological interest. Enzymes from fish viscera contribute toward sustainable development by utilising byproducts from waste that are usually discarded.

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## References

Aranishi, F., Watanabe, T., Osatomi, K., Cao, M., Hara, K., & Ishihara, T. (1998). Purification and characterization of thermostable dipeptidase from carp intestine. *Journal of Marine Biotechnology, 6*, 116–123.

Asgeirsson, B., Fox, J. W., & Bjarnason, J. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *European Journal of Biochemistry, 180*, 85–94.

Austin, H. M. (1973). Northern range extension of the rhomboid mojarra, *Diapterus rhombeus* Cuvier and Valencienes (Gerreidae). *Chesapeaker Science, 3*, 222.

Balti, R., Barkia, A., Bougatef, A., Ktari, N., & Nasri, M. (2009). A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterization. *Food Chemistry, 113*, 146–154.

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.

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*, 1829–1834.

Cao, M. J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K., & Ishihara, T. (2000). Purification and characterization of two anionic trypsins from the hepatopancreas of carp. *Fisheries Science, 66*, 1172–1179.

Castillo-Yáñes, F. J., Pacheco-Aguiar, R., García-Carreño, F. L., & Toro, M. A. N. (2005). Isolation and characterization of trypsin from pyloric caeca of Monterrey sardine *Sardinops sagax caerulea*. *Comparative Biochemistry Physiology, Part B, 140*, 91–98.

Espósito, T. S., Amaral, I. P. G., Buarque, D. S., Oliveira, G. B., Carvalho, L. B., Jr., & Bezerra, R. S. (2009). Fish processing waste as a source of alkaline proteases for laundry detergent. *Food Chemistry, 112*, 125–130.

Fuchise, T., Kishimura, H., Sekizaki, H., Nonami, Y., Kanno, G., Klomklao, S., et al. (2009). Purification and characteristics of trypsins from cold-zone fish, Pacific cod (*Gadus macrocephalus*) and saffron cod (*Eleginus gracilis*). *Food Chemistry, 116*(3), 611–616.

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

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hau, P. V., & Benjakul, S. (2006). Purification and characterization of trypsin from caeca of bigeye snapper (*Pricanthus macracanthus*). *Journal of Food Biochemistry, 30*, 478–495.

Heu, M. S., Kim, H. R., & Pyeun, J. H. (1995). Comparison of trypsin and chymotrypsin from víscera of anchovy (*Engraulis japonica*). *Comparative Biochemistry Physiology, Part B, 112*, 557–568.

Huerou, L., Wicker, C., Guilloteau, P., Toullec, R., & Puigserver, A. (1990). Isolation and nucleotide sequence of cDNA clone for bovine pancreatic anionic trypsinogen structural identity within the trypsin family. *European Journal of Biochemistry, 193*, 767–773.

IBAMA (2008). Estatística da pesca 2006 Brasil: Grandes regiões e unidades da federação. Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. Brasília-DF.

Jeong, Y., Wei, C. I., Preston, J. F., & Marshall, M. R. (2000). Purification and characterization of proteases from hepatopacreas of crawfish (*Procambarus clarkii*). *Comparative Biochemistry Physiology, Part B, 24*, 311–332.

Jónsdóttir, G., Bjarnason, J. B., & Gudmundsdóttir, Á. (2004). Recombinant cold-adapted trypsin I from Atlantic cod: Expression, purification, and identification. *Protein Expression and Purification, 33*, 110–122.

Kishimura, H., Klomklao, S., Benjakul, S., & Chun, B. S. (2008). Characteristics of trypsin from ceca of walleye pollock (*Theragra chalcogramma*). *Food Chemistry, 106*, 194–199.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpsom, B. K., & Saeki, H. (2006). Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. *Comparative Biochemistry Physiology, Part B, 144*, 47–56.

Klomklao, S., Benjakul, S., Visessanguan, W., Khishimura, H., & Simpsom, B. K. (2007). Trypsin from the pyloric ceca of bluefish (*Pomatomus saltatrix*). *Comparative Biochemistry Physiology, Part B, 148*, 382–389.

Klomklao, S., Kishimura, H., Nonami, Y., & Benjakul, S. (2009). Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*). *Food Chemistry, 115*, 155–162.

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

Marcuschi, M., Espósito, T. S., Machado, M. F. M., Hirata, I. Y., Machado, M. F. M., Silva, M. V., et al. (2010). Purification, characterization and substrate specificity of a trypsin from the Amazonian fish tambaqui (*Colossoma macropomum*). *Biochemical and Biophysical Research Communications, 396*, 667–673.

Outzen, H., Berglund, G. I., Smalas, A. O., & Willassen, N. P. (1996). Temperature and pH sensitivity of trypsins from Atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin. *Comparative Biochemistry and Physiology, 115B*, 33–45.

Polgár, L. (2005). The catalytic triad of serine peptidases. *Cellular and Molecular Life Sciences, 62*, 2161–2172.

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.

Shahidi, F., & Kamil, Y. V. A. J. (2001). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science and Technology, 12*, 435–464.

Souza, A. A. G., Amaral, I. P. G., Santo, A. R. E., Carvalho, L. B., Jr., & Bezerra, R. S. (2007). Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*). *Food Chemistry, 100*, 1429–1434.

Warburg, O., & Christian, W. (1941). Isolierung und kristallisation des garungs ferments enolasc. *Biochemical Zeitschrift, 310*, 384–421.