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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-