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Trypsin from viscera of vermiculated sailfin catfish, *Pterygoplichthys disjunctivus*, Weber, 1991: Its purification and characterization

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ABSTRACT

Pterygoplichthys disjunctivus viscera trypsin was purified by fractionation with ammonium sulphate, gel filtration, affinity and ion exchange chromatography (DEAE-Sepharose). Trypsin molecular weight was approximately 27.5 kDa according to SDS–PAGE, shown a single band in zymography. It exhibited maximal activity at pH 9.5 and 40 °C, using *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate. Enzyme was effectively inhibited by phenyl methyl sulphonyl fluoride (PMSF) (100%), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (85.4%), benzamidine (80.2%), and soybean trypsin inhibitor (75.6%) and partially inhibited by *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (10.3%), ethylendiamine-tetraacetic acid (EDTA) (8.7%) and pepstatin A (1.2%). Enzyme activity was slightly affected by metal ions ($Fe^{2+} > Hg^{2+} > Mn^{2+} > K^{+} > Mg^{2+} > Li^{+} > Cu^{2+}$). Trypsin activity decreased continuously as NaCl concentration increased (0–30%). K_m and k_{cat} values were 0.13 mM and 1.46 s^{-1}, respectively. Results suggest the enzyme have a potential application where room processing temperatures (25–35 °C) or high salt (30%) concentration are needed, such as in fish sauce production.

1. Introduction

The search for proteases from different sources has increased in the last years with an estimation of nearly 50% of total industrial enzyme sales consisting of proteases. In order to offer a variety of proteases, particularly those with unique properties, new sources of proteolytic enzymes have been studied, including proteases from fish, especially from fish viscera (Souza, Amaral, Espíritu Santo, Carvalho, & Bezerra, 2007). Although fish proteases are basically similar to their mammalian counterparts, differences in structural and catalytic properties have been reported (Fong, Chan, & Lau, 1998). Fish proteases have shown higher catalytic activities over a wide range of pH and temperature conditions (Shahidi & Kamil, 2001) at relatively low concentrations (Haard, 1998). As an example, industrial applications of serine proteinases for detergent, food, pharmaceutical, leather and silk industries have been conducted (Klomklao, Benjakul, Visessanguan, Simpson, & Kishimura, 2005). Therefore, studies describing enzymes isolated from these animals represent the first step to evaluate their potential for technological application. In fact, experiments at laboratory level are essential for future production at industrial scale (Silva et al., 2011).

In the digestive tract of fish, one of the main peptidases is trypsin (EC 3.4.21.4) known as the serine endoprotease that hydrolyze peptide bonds at the carboxylic end of the amino acid residues arginine (R) and lysine (K). Trypsins play major roles in biological process including digestion, activation of zymogens chymotrypsin and other enzymes of (Klomklao, Kishimura, Nonami, & Benjakul, 2009).

Trypsin have been isolated and characterized from several fish species such as spotted goatfish (*Pseudupeneus maculatus*) (Souza et al., 2007), brownstripe red snapper (*Lutjanus vitta*) (Khantaphant & Benjakul, 2010), hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) (Klomklao, Benjakul, Kishimura, & Chaijan, 2011), sardinelle (*Sardinella aurita*) (Khaled et al., 2011), silver mojarra (*Diapterus rhombeus*) (Silva et al., 2011) and zebra blenny (*Salaria basilisca*) (Ktari et al., 2012).

One interesting fish that has been studied for the fore purpose at our laboratory is the vermiculated sailfin catfish (*Pterygoplichthys disjunctivus*), classified within the loricariidae family. Being native from the Amazonia Basin in Brazil and Bolivia (Gibbs, Shields, Lock, Talmadge, & Farrell, 2008), it has successfully invaded several inland waters around the world. It was introduced in Mexico either as an ornamental fish or as a fish tank cleaner; however, somehow species made it through the wild, now invading most of the inland waters south, central and parts of the northwest of Mexico. Interestingly, its viscera correspond to approximately 10% of the total body weight (Ramirez-Suarez JC, pers. Comm.) *versus* 5% of most fish species.

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Although nowadays is considered a waste by the fisherman in Mexico, it has the potential to become an important fishing resource due to the easiness to proliferate in water bodies around the world, being its consumption the only way to control its population; thus, studies about its possible use must be conducted. Previous work at our laboratory has shown a partial characterization of alkaline proteases from viscera of vermiculated sailfin catfish (*P. disjunctivus*, Weber, 1991) (Villalba-Villalba et al., 2011). Hence, the aim of the present study was to purify and characterize trypsin from vermiculated sailfin catfish (*P. disjunctivus*) viscera, thus generating basic information about this by-product.

2. Materials and methods

2.1. Fish samples

Vermiculated sailfin catfish specimens were obtained from the Adolfo Lopez Mateos dam also commonly known as "El Infiernillo", located at the boundary of the Mexican states of Michoacan and Guerrero (18°52′–18°15′ North and 101°54′–102°55′ West). Samples were cryogenically frozen *in situ* with liquid N_2, placed between layers of CO_2 and transported by airplane, to the CIAD Seafood Products Quality and Biochemistry Laboratory located in Hermosillo, Sonora, Mexico.

2.2. Preparation of crude enzyme extract

At the laboratory, thawed vermiculated sailfin catfish specimens were dissected, their viscera (intestines and pyloric caeca) removed and immediately frozen, keeping them at -80 °C until further analysis. Viscera (100 g) were homogenized at 20,000*g* with 200 mL of 50 mM Tris–HCl buffer (pH 7.5), with 10 mM $CaCl_2$ and 0.5 M NaCl for 2 min and centrifuged at 18,000*g* for 30 min at 2–4 °C. After centrifugation, most lipids (upper layer) were removed manually, and then the supernatant was filtered through gauze for further lipid removal. Finally, supernatant (enzyme extract) was frozen and kept at −80 °C until further analysis (Heu, Kim, & Pyeun, 1995; Whitaker, 1994).

2.3. Enzyme purification

Crude extract was fractionated with ammonium sulphate at 30% and 70% saturation according to Castillo-Yañez, Pacheco-Aguilar, Garcia-Carreño, Toro, and Lopez (2005). Each time sample was centrifuged at 20,000*g* for 20 min at 4 °C. Precipitate was dissolved in 20 mL of 50 mM Tris–HCl buffer (pH 7.5) with 10 mM $CaCl_2$ (buffer A), loaded into a 1.6 cm × 120 cm G-75 Sephadex gel filtration chromatography column (Amersham Pharmacia Biotech, Uppsala, Sweden), eluted at 0.3 mL/min and equilibrated with buffer A. Then, fractions with trypsin activity were pooled and loaded into a 1.1 cm × 15 cm Benzamidine-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) equilibrated with same buffer. Retained fractions were eluted by changing the mobile phase pH from 7.5 to 3.0, using a 50 mM Gly-HCl (pH 3.0) buffer. Fractions eluted were combined and dialyzed against 6 L of 20 mM Tris–HCl, pH 7.5 (buffer B) (Castillo-Yañez et al., 2005; Cohen, Gertler, & Birk, 1981; Simpson & Haard, 1987). Dialyzed fractions with trypsin activity were then loaded into a 1.6 × 10 cm DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated with buffer B. Unabsorbed protein was washed with same buffer, and the column was eluted with a linear gradient of NaCl from 0 to 0.4 M. Fractions revealing trypsin activity were pooled for further analysis. In all chromatographic purification steps, 5 mL of each fraction were collected.

2.4. Protein concentration determination

Protein concentration in all purification steps was determined according to Bradford (1976), using bovine serum albumin as standard.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), native-PAGE and zymography

SDS–PAGE was carried according to the method of Laemmli (1970) using 4 and 14% stacking and separating gels, respectively. For protein band analysis, a volume of enzyme extract was mixed with two volumes of sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue), heated at 95 °C for 4 min, cooled immediately, and loaded into the gel. After electrophoresis, gels were stained with 0.25% Coomasie brilliant blue R-250 (CBB) in 40% methanol, 10% acetic acid or silver staining depending of purification process (protein concentration). Bovine serum albumin (66 kDa), ovoalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14.2 kDa) were used as molecular weight markers. Zymography was performed according to the procedure of Laemmli (1970) except that samples were not heated and no reducing agents were added. After electrophoresis, the gel was soaked for 30 min in 1.25% casein (as substrate) in 50 mM Tris–HCl at pH 8.0 at 4 °C. Then, gel was immersed in same solution at 37 °C for 60 min and then soaked in trichloroacetic acid for 30 min to stop the reaction, washed in distilled water, fixed and stained with 0.05% Coomasie Blue solution finally destained with 40% methanol and 10% acetic acid.

2.6. Specific activity

Trypsin amidase activity was evaluated according to Erlanger, Kokowski, and Cohen (1961) using 10 μL of enzyme extract was combined with 990 μL *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) solution (1 mM BAPNA dissolved in 50 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$ buffer) at 25 °C. Production of *p*-nitroaniline was measured by monitoring the increment in absorbance at 410 nm (A_{410}) every 30 s for 10 min. BAPNA hydrolysis units (U) were calculated with the following equation: $U = A_{410}/\text{min} \times 1000 \times 1/8800 \times \text{mg enzyme}$, where $8800\ M^{-1}\ cm^{-1}$ is the *p*-nitroanilide molar extinction coefficient at 410 nm. Esterase activity was evaluated according to Humel (1959) using 1 mM of *N*-α-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) (50 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$ buffer) as substrate. Briefly, 10 μL of enzyme solution were mixed with 990 μL of TAME at 25 °C. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm (A_{247}) every 30 s for 10 min. TAME units (U) were calculated with the following equation: $U = A_{247}/\text{min} \times 1000 \times 1/540 \times \text{mg enzyme}$, where $540\ M^{-1}\ cm^{-1}$ is the *p*-tosyl-arginine molar extinction coefficient at 247 nm. Controls were performed without enzyme and substrate solution.

2.7. Optimum pH and temperature

The effect of pH on trypsin activity was evaluated using a universal buffer from pH 4 to 12 at 25 °C for 15 min (Stauffer, 1989). In order to study the effect of temperature over enzyme activity, extract was incubated at 15, 20, 30, 40, 50, 60 and 70 °C for 15 min in 50 mM Tris–HCl buffer under optimal pH. Residual activity was measured using BAPNA 1 mM (dissolved in 50 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$ buffer) as substrate at 25 °C. Percentage of enzyme activity was estimated considering 100% the highest activity detected in this assay.