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Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (*Oreochromis niloticus* L.)

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ABSTRACT

Trypsin from intestinal extracts of Nile tilapia (*Oreochromis niloticus* L.) was characterised. Three-step purification – by ammonium sulphate precipitation, Sephadex G-100, and Q Sepharose – was applied to isolate trypsin, and resulted in 3.77% recovery with a 5.34-fold increase in specific activity. At least 6 isoforms of trypsin were found in different ages. Only one major trypsin isozyme was isolated with high purity, as assessed by SDS-PAGE and native-PAGE zymogram, appearing as a single band of approximately 22.39 kDa protein. The purified trypsin was stable, with activity over a wide pH range of 6.0–11.0 and an optimal temperature of approximately 55–60 °C. The relative activity of the purified enzyme was dramatically increased in the presence of commercially used detergents, alkylbenzene sulphonate or alcohol ethoxylate, at 1% (v/v). The observed Michaelis–Menten constant (K_m) and catalytic constant (K_{cat}) of the purified trypsin for BAPNA were 0.16 mM and 23.8 s^{-1}, respectively. The catalytic efficiency (K_{cat}/K_m) was 238 $s^{-1}\,mM^{-1}$.

1. Introduction

Nile tilapia (*Oreochromis niloticus* L.) is an important economic fish species in Thailand. The processing of fish generates a large amount of waste. Different applications, in using the by-products from this by product, have been developed to overcome the pollution problems. The majority of fish waste consists of viscera, which are a potential source of many digestive enzymes, such as trypsin, pepsin, chymotrypsin, collagenase and elastase. In the stomach, the majority of proteolytic enzymes display high activity at pH 2.0–4.0, while the alkaline proteases in the intestine are highly active over a pH range of 8.0–10.0 (Bezerra et al., 2005; El Hadj Ali, Hmidet, Bougatef, Nasri, & Nasri, 2009; Supannapong et al., 2008). Acidic proteases showed lower enzymatic activity than did alkaline proteases when using the same substrate, such as casein (Rungruangsak & Utne, 1981; Torrissen, 1984) or azocasein.

Trypsin is a serine protease, which is produced as an inactive precursor. It has a function in the hydrolysis of target proteins at the amino acids arginine and lysine. Many trypsins have been isolated from the viscera of different fish species, such as tilapia (Bezerra et al., 2005; El-Shemy & Levin, 1997; Wang et al., 2010), smooth hound (Bougatef et al., 2010), skipjack tuna (Klomklao, Kishimura, Nonami, & Benjakul, 2009), grey triggerfish (Jellouli et al., 2009), Pacific cod and saffron cod (Fuchise et al., 2009), and the Amazonian fish tambaqui (Marcuschi et al., 2010). Trypsin has various industrial applications, especially in food industries, due to its high stability and activity under harsh conditions, such as in the presence of surfactants and oxidative agents. Although the habitat temperature of cultivated fish seems to correlate with

Abbreviations: SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; K_m, Michaelis–Menten constant; K_{cat}, catalytic constant; K_{cat}/K_m, catalytic efficiency; TAME, *N*-*p*-tosyl-L-arginine methyl ester hydrochloride; BAPNA, benzoyl-DL-arginine-*p*-nitroanilide; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; V_{max}, maximum velocity.

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a particular optimal temperature and heat stability (Kishimura, Klomklao, Benjakul, & Chun, 2008), the optimal enzyme temperature and heat stability could be much higher than the habitat temperature (Rungruangsak, 2007). However, trypsin from tropical fish would be applicable for food industries.

One of the most important processes for predicting fish growth is the efficiency of the digestive enzyme trypsin and the activity ratio of trypsin to chymotrypsin, which influences the conversion of feed to nutrients for utilisation (Rungruangsak, 2007; Rungruangsak, Moss, Andresen, Berg, & Waagbø, 2006; Rungruangsak et al., 2009; Sunde, Taranger, & Rungruangsak, 2001). Therefore, trypsin is the key enzyme for food utilisation and growth. In the market, most of selling tilapia has been captured at age 6th months. A lot of waste products from fish viscera is discarded every day. The implications in this study may increase the value of waste products from the market. We used fish intestine at 6th months for purification. Furthermore, characterisation of trypsin isoforms at each age was also performed.

2. Materials and methods

2.1. Experimental fish

Eggs of Nile tilapia (*O. niloticus* L.) were incubated in a hatching funnel. At 7 day post-hatching, the fish were sex-reversed to males by feeding with shrimp feed (at least 45% protein) containing 17-α methyltestosterone, until one month old. After that, the fish were reared in intensive net cages until 6 months old at Khamphaengsaen Fisheries Research Station, Faculty of Fisheries, Kasetsart University, Nakhon Pathom province. They were fed with feeds containing mainly from plants with protein levels of 35%, 30% and 24–26%, during 2–3 months, for 4–5 months and 6–10 months, respectively. For sample collection, pooled samples of intestine were collected at 6 months. The fish were kept on ice while the intestines were dissected and briefly cleaned with deionized water. Pooled samples of intestine were collected at 1 month, and at 3–7 months. The intestinal samples were immediately placed in liquid nitrogen for transportation, and stored at −80 °C until used.

2.2. Preparation of crude enzyme extracts

The pooled samples of intestine were briefly thawed on ice. Each pooled sample was homogenised at a ratio of 1:50 (w/v) in 50 mM Tris–HCl buffer, pH 8.0, containing 1 mM $CaCl_2$, referred to as starting buffer (SB). The homogenates were centrifuged at 10,000*g* for 30 min at 4 °C to remove the tissue debris. The supernatants were kept at −80 °C until used; these were referred to as the "crude enzyme extracts."

2.3. Polyacrylamide gel electrophoresis (PAGE)

Protein samples were prepared for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) by mixing each crude enzyme extract at a ratio of 1:1 (v/v) with the sample buffer (0.5 M Tris–HCl buffer, pH 6.8, containing 10% SDS, 30% glycerol, and 10% β-mercaptoethanol), and subsequently boiling for 10 min. The mixed samples were cooled on ice. Then 15 μl were loaded onto a gel made of 4% stacking and 12% separating gels, and subjected to electrophoresis using a Mini-PROTEAN II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the composite gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 40% ethanol and 10% acetic acid for 30 min. The gel was then destained with 40% ethanol and 10% acetic acid. The molecular weights of particular proteins were estimated using protein standards (Fermentas, USA) as markers.

2.4. Zymography of trypsin

Zymography of trypsin was performed on native-PAGE according to the substrate gel electrophoresis method modified by Garcia-Carreño, Dimes and Haard (1993). Briefly, the native-PAGE was performed using 12% separating gels in a manner similar to described above, except that the samples were mixed with sample buffer without the reducing agent β-mercaptoethanol, and were not heated. After electrophoresis, the gels were submerged in 100 ml of 5 M *N*-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) substrate buffer (in 50 mM Tris–HCl buffer, pH 8, containing 1 mM $CaCl_2$) for 30 min at 37 °C, with gentle agitation. A clear zone on the white background of the gel indicated the presence of protease activity of trypsin.

2.5. Purification of trypsin

The crude enzyme extract from the pooled intestines of 6-month-old tilapia was subjected to ammonium sulphate, at 20–60% saturation, to fractionate particular proteins. The precipitated proteins in the 40–60% saturation were collected by centrifugation at 10,000*g* for 30 min at 4 °C. The pellet was dissolved in a minimal volume of SB and dialysed against SB overnight, with gentle stirring at 4 °C. The protein dialysate was then applied onto a Sephadex G-100 column (1 × 140 cm, Fluka, Switzerland), pre-equilibrated with approximately two column volumes of SB. The column was eluted with the same buffer at a flow rate of 0.1 ml min^{-1}, and fractions of 1 ml were collected. Protein concentration was determined by the Lowry method, and trypsin activity was measured using benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; Sigma). The fractions containing trypsin activity were pooled, and then chromatographed using a Q Sepharose column (0.8 × 5 cm), pre-equilibrated with SB at a flow rate of 0.5 ml min^{-1}. The column was washed with SB until the absorbance at 280 nm (A_{280}) reached baseline, and was then eluted with linear gradient using 0.0–1.0 M NaCl in SB. Fractions of 1 ml were collected; those with trypsin activity against BAPNA were pooled, stored at −20 °C, and used for further study.

2.6. Determinations of protein concentration and trypsin activity

Protein concentration was measured by the Lowry method, using bovine serum albumin (BSA) as standard. Protease activity of trypsin was assayed by a method modified from Rungruangsak (2007), based on Erlanger, Kokowsky, and Cohen (1961), using initial enzyme reaction rate and optimal temperature of Nile tilapia trypsin activity. The enzyme solution (10 μl) was mixed with 50 μl of preheated 1.25 mM BAPNA in 50 mM Tris–HCl buffer, pH 8.0; the initial enzyme reaction rate of 1 min at 50 °C was measured at 410 nm. The production of *p*-nitroaniline was calculated by monitoring the increment in absorbance at 410 nm, which was the difference between the sample and the blank (ΔA_{410}).

$$\text{Trypsin activity (unit)} = \frac{(\Delta A_{410}\text{min}^{-1}) \times \text{ml of total volume} \times 1000}{8800 \times \text{ml of enzyme solution}}$$

$$\text{Trypsin specific activity (unit/mg)} = \frac{\text{Enzyme activity}}{\text{Protein concentration}(\text{mgml}^{-1})}$$

The molar extinction coefficient of *p*-nitroaniline is 8800 M^{-1} cm^{-1}. One unit (U) of enzyme activity corresponds to 1 nmol *p*-nitroaniline released per min at 37 °C.