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Study of a highly alkaline protease extracted from digestive tract of sea cucumber (*Stichopus japonicus*)

Xue-yan Fu [a], Chang-hu Xue [a,b,*], Ben-chun Miao [c], Zhao-jie Li [a], Wen-ge Yang [b], Dong-feng Wang [a]

[a] *Department of Food Science and Technology, Ocean University of China, No. 5, Yu Shan Road, Qingdao, Shandong Province 266003, PR China*
[b] *Institute of Food Technology, Ningbo University, Ningbo, Zhejiang Province 315211, PR China*
[c] *Department of Pharmacology, Marine Drug and Food Institute, Ocean University of China, Qingdao, Shandong Province 266003, PR China*

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Abstract

Digestive proteases from the digestive tract of sea cucumber (*Stichopus japonicus*) have been characterized. There exist acidic proteases with optimum activities at pH 2.0 and 5.0, and alkaline proteases with optimal activities at pH 8.0 and 13.5, in the digestive tract of sea cucumber. The result of SDS–PAGE with or without protease inhibitors at a series of pH ranges confirmed the existence of at least three proteases with molecular weights of 20.6, 39.1 and 114.1 kDa. The high alkaline protease (HAP), with an optimum pH of 13.5, was purified using a combination of ion exchange and gel filtration chromatography. The purified HAP was then characterized in detail. The results showed that the molecular weight of the HAP is 20.6 kDa, and that it has relatively good thermal and pH stability with an optimum pH of 13.5 and optimum temperature of 37 °C. Therefore, it could be well applied in many fields to meet special needs, especially under extremely alkaline conditions. According to the inhibition profiles obtained with numerous specific protease inhibitors and the renaturing effect of Cu^{2+} on the EDTA-denatured HAP, the purified HAP was defined to be a metallo-protease containing Cu^{2+} with serine protease-like activity.

Keywords: Sea cucumber (*Stichopus japonicus*); Proteases; Purification; HAP; Characterization

1. Introduction

The abilities of marine species to utilize ingested nutrients depend on the activities of digestive enzymes that exist in their digestive tract (Alarcon, Diaz, Moyano, & Abellan, 1998; Clark, MacDonald, & Stark, 1985). The rate of digestion and absorption of essential amino acids during proteolysis could be determined with proper knowledge of functional activities of proteases (Eshel, Lindner, Smirnoff, Newton, & Harpaz, 1993). In recent years a number of studies have been conducted to characterize digestive enzymes of aquatic organisms, which are useful both to ascertain the maximum periods for their storage to avoid autolysis, and to develop practical applications for digestive proteases in physiology, biochemistry and food science (García-Carreño, 1992; Haard, 1992; Jiang, Moody, & Chen, 1991). In aquaculture, this may help the selection of feed ingredients (Lan & Pan, 1993).

Proteases have been reported to be responsible for different forms of spoilages, such as autolysis (Nishimura, Kawamura, Matoba, & Yonezawa, 1983) and mushiness (Linder, Angel, Weinberg, & Granit, 1988). Sea cucumber (*Stichopus japonicus*) is one of the important cultured aquatic species with strong digestive and autolysis capabilities, however, little information about the digestive enzymes from sea cucumber have been

* Corresponding author. Tel./fax: +86 532 203 2468.
E-mail address: xuech@mail.ouc.edu.cn (C.-h. Xue).

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known until now. In the present work, we investigated and purified the digestive proteases that exist in the digestive tract of sea cucumber and characterized the high alkaline protease (HAP) in detail.

2. Materials and methods

2.1. Reagents

Benzoyl-DL-arginin-*p*-nitroanilide (BApNA), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), trypsin inhibitor (TI), ethylenediamine tetra acetate (EDTA), *N*α-tosyl-L-lysine chloromethyl ketone (TLCK), *N*α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), casein, azocasein, and molecular mass protein standards were purchased from Sigma, St. Louis, MO, USA. Q-Sepharose FF, Sephacryl S-300 were purchased from Pharmacia LBK Biotechnology, Uppsala, Sweden. Sea cucumbers (100–120 g) were provided by a local culture farm (Qingdao, Shandong Province, China), in May.

2.2. Preparation of crude enzyme extract

The sea cucumbers were starved for approximately 12–24 h prior to sampling and subsequently killed and dissected immediately. The digestive tracts were separated, and rinsed with cold distilled water after removing the tissue contents. The tissues were homogenized in cold 50 mM Tris–HCl buffer (pH 7.5) and the homogenate was then centrifuged at 10,000*g* for 30 min. All of the operations were carried out at 4 °C. The supernatant containing the enzymes was stored at −80 °C before analysis. The protein content of the enzyme extract was measured following the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.3. Proteolytic activity

The effect of pH on the proteolytic activities of the crude enzyme extract was determined based on the azocasein hydrolysis assay. A series of buffers were used for different pH conditions: 0.1 M KCl–HCl (pH 1.5–2.5), 0.2 M Gly–HCl (pH 3.0–4.0), 0.2 M phosphate buffer (pH 5.0–6.0), 0.1 M Tris–HCl (pH 7.0–9.0) and 0.1 M Gly–NaOH (pH 10.0–14.0) (Glass, MacDonald, Moran, & Stark, 1989; Munilla-Moran & Rey, 1996). Briefly, 0.2 mL protease extract was added to 0.8 mL of the above buffers containing 2 g L^{-1} azocasein, followed by an incubation at 37 °C for 1 h. The reaction was stopped by the addition of 0.5 mL 12% trichloroacetic acid (TCA). After centrifugation, 1 mL of supernatant was mixed with 0.1 mL of 10 M NaOH and the optical density was determined at 450 nm. One unit of activity was defined as an increase of 0.01 optical density after 30 min incubation at 37 °C. The blank used for this assay was prepared by incubating a mixture of the crude enzyme extract, corresponding buffer and distilled water for 1 h at 37 °C, followed by the addition of TCA and azocasein in turn.

2.4. Electrophoresis assay

Protease composition was studied after separation by substrate-SDS–PAGE (García-Carreño, 1992). Briefly, the enzyme extract was incubated together with various protease inhibitors including EDTA, PMSF, SBTI and TI for 1 h at 25 °C prior to electrophoresis. Then electrophoresis was conducted at 4 °C and the molecular weight lane was cut apart and stained. The remaining gel was immersed in 30 g L^{-1} casein solution at 4 °C for 1 h and then divided into two parts and incubated at pH 8.0 and 13.5, respectively, for another 1 h at 37 °C, to allow proteases in the gel to digest the casein, followed by staining with 0.1% Coomassie brilliant blue R-250 and distaining with methanol–acetic acid–water (1:1:8, v/v). Molecular weight markers of 14.4–97.4 kDa were used for molecular weight determination.

2.5. Purification of the high alkaline protease

2.5.1. Q-Sepharose FF ion-exchange chromatography

Briefly, the crude enzyme was applied to a Q-Sepharose FF column (1.5 × 20 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM Ca^{2+} and firstly washed with the loading buffer. Then the bound proteases were eluted in turn with Tris buffers containing 0.25, 0.5 and 1.0 M NaCl at a flow rate of 90 mL h^{-1}, monitoring at 280 nm. The fractions containing protease with an optimal pH of 13.5 were pooled and concentrated.

2.5.2. Gel filtration chromatography with Sephacryl S-300

The concentrated fractions were applied to a Sephacryl S-300 column (1.5 × 150 cm), equilibrated, and eluted with the same loading buffer mentioned above. The fractions containing the HAP were measured, collected, pooled and concentrated as described above.

2.5.3. Second Q-Sepharose FF ion-exchange chromatography

The concentrated enzyme was applied to a Q-Sepharose FF column (1.0 × 6 cm) previously equilibrated with the same loading buffer mentioned above. After washing with loading buffer, the bound protease was eluted with a linear gradient of 0–1 M NaCl in Tris buffer with a flow rate of 90 mL h^{-1}. The fractions containing the HAP were pooled and dialyzed against 50 mM