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Characterization of acid and alkaline proteases from viscera of farmed giant catfish

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ABSTRACT

This work aimed to characterize acid and alkaline proteases extracted from farmed giant catfish (*Pangasianodon gigas*) viscera by using the aqueous two-phase system (ATPS). Determinations of optimum pH and pH stability, optimum temperature and thermal stability, salt stability and hydrolysis activities against bovine muscle protein and gelatin were performed. ATPS consisting of 20% polyethylene glycol (PEG1500)–15% $MgSO_4$ or 15% PEG2000–15% $NH_3C_6H_5O_7$ was used for acid and alkaline proteases extraction. The optimum pH and temperature for acid protease was 3.0 and 40 °C, while alkaline protease was 9.0 and 60 °C. High pH stability of the enzymes was found in the ranges of 1.0–5.0 and 8.0–12.0 for acid and alkaline proteases, respectively. About 40 and 60% activities reduction of acid and alkaline proteases were observed when incubated at 90 °C for 30 min. In addition, 0.5% NaCl addition decreased >50% of total enzyme activities. Hydrolytic activities of the acid and alkaline proteases against bovine muscle protein and gelatin were in the concentration dependent manner as clearly indicated by SDS-PAGE. The results showed that the acid and alkaline proteases obtained from farmed giant catfish viscera could be useful for food protein hydrolysate production.

1. Introduction

The value of fishery processing by-products, specifically as a source of enzymes, can be increased by using such starting materials in different disciplines, such as medicine, cosmetics, food, wastewater treatment, and pharmaceutical industries. Digestive enzymes of marine fish have been studied widely, but information on freshwater fish enzymes is limited. The most important proteolytic enzymes in the viscera of fish and aquatic invertebrates are aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase and elastase) (Simpson, 2000). Proteases comprise the class of enzymes most used worldwide, accounting for 60% of the world's total enzyme production (Gupta, Beg, & Larenz, 2002). Some proteases have been explored as food processing aids and as reducers of stick-water viscosity in fishmeal processing (Castillo-Yanez, Pavheco-Aguilar, Garcia-Carreno, & Navarrete-Del Toro, 2004). Recently, there has been increasing demand for proteolytic enzymes in the pharmaceutical and food biotechnology industries.

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Chiang Rai has one of the highest farmed freshwater fish production capacities in Thailand. Giant catfish is the chief species that is captured locally. Most farm-raised giant catfish are sold raw to restaurants, and in the near future, fish farmers hope to export its meat to other countries, especially in Asia and Europe. With an increase in fish processing, a large amount of internal organs and by-product will be generated. During the filleting of this fish, 50–65% of the body is discarded as processing by-products, which are environmental pollutants. Utilization of these by-products could make more profit to the producer.

There have been many reports about the isolation of proteolytic enzymes from fish viscera by various separation techniques, especially various types column chromatography. Most of those operations are time consuming, difficult to scale up, involve expensive reagents, and require technical skill. Partitioning in an aqueous two-phase system (ATPS) or three-phase partitioning (TPP) have shown to be powerful for separating and partial purifying the proteases from farmed giant catfish viscera (Rawdkuen, Vanabun, & Benjakul, 2012; Ketnawa, Benjakul, Ling, Martinez-Alvarez, & Rawdkuen, 2013). ATPS and TPP constitute a favorable method to separate and purify mixtures of proteins; it is fast and economical; and processes are easy to implement (Farruggia, Porfiri, Pico, & Romanini, 2011; Rawdkuen et al., 2012). Biochemical characteristics of the proteases from farmed giant catfish have been studied inadequately, in spite of being the important fishery product of Chiang Rai. Therefore, this study aimed to characterize the isolated acid and alkaline proteases from the viscera of farmed giant catfish.

2. Materials and methods

2.1. Materials

L-Cysteine, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (βME), Coomassie Brilliant Blue G-250, and casien were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N,N,N′,N′-tetramethyl ethylene diamine (TEMED) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid (TCA), hydrochloric acid, tris-(hydroxymethyl)-aminomethane, and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany).

Alive farmed giant catfish (25–30 kg/fish) were catched from the artificial pond of Jarun farm, Phan District, Chiang Rai Province, Thailand. Then they were killed by putting in ice box before subjected to eviscerate by hand. Viscera of farmed giant catfish were collected, put in plastic bag and covered with ice and then transported to the laboratory at Mae Fah Luang University, Chiang Rai within 60 min.

2.2. Preparation of crude enzyme extract

Crude enzyme extract was prepared according to the method of Castillo-Yanez et al. (2004) with a slight modification. Viscera from farmed giant catfish were homogenized for 2 min with extraction buffers (10 mM Citrate/HCl pH 3.0 for acid protease or 10 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ for alkaline protease in the ratio of 1:5 (w/v). The mixture was then centrifuged at 10,000*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was collected and used as "crude enzyme extract" with the specific activity of 0.60 and 392.91 units/mg proteins for acid and alkaline proteases, respectively.

2.3. Aqueous two-phase system

The ATPS was prepared in 10-ml centrifuge tubes according to the method of Nalinanoon, Benjakul, Visessanguan, and Kishimura (2009). Acid protease was extracted by using ATPS consisting of 20% polyethylene glycol (PEG1500)–15% $MgSO_4$, while alkaline protease was extracted by using the system consist of 15% PEG2000–15% $NH_3C_6H_5O_7$. Fifty percentage of crude enzyme extract was used in the ATPS. Distilled water was used to adjust the system to obtain the final weight of 8 g. The mixture was mixed thoroughly for 3 min using vortex mixer. Phase separation was achieved by centrifugation at 2000*g* for 10 min at 4 °C. The top phase was carefully separated using a Pasteur pipette, collected and then dialysed against 100 volumes of distilled water for 12 h before used for further experiments. The specific activity of the top phase of enzyme enriched fraction was 1.29 and 1767.23 units/mg proteins with the purity of 2.16 and 4.35-fold for acid and alkaline proteases, respectively.

2.4. Proteolytic activity determinations

Acid protease activity against acid-denatured bovine hemoglobin was determined at pH 3.0 and 37 °C, according to the method of Wu et al. (2009). Briefly, 50 μl of appropriately diluted enzyme sample was mixed with 350 μl of 0.25 M HCl-sodium acetate buffer (pH 3.0), 100 μl of 2.0% acid-denatured bovine hemoglobin was then added to the mixture to initiate the reaction. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 500 μl of 8.0% TCA and then centrifuged at 8000 g for 10 min. The absorbance of the supernatant was measured at 280 nm using a spectrophotometer. One unit of enzymatic activity was defined as the amount of acid protease that catalyzes an increase of absorbance of 1.0 unit per minute at 280 nm under the activity assay conditions.

Alkaline protease activity was determined by using casein as a substrate according to the method of Rawdkuen, Chaiwut, Pintathong, and Benjakul (2010) with a slight modification. An alkaline protease sample of 500 μl was mixed with 500 μl of 2% (w/v) casein in 0.1 M Tris–HCl (pH 8.0). The reaction was started by incubation the mixture at 37 °C for 10 min. The reaction was stopped by adding 500 μl of 5% TCA. After centrifugation at 10,000*g* for 10 min, the absorption of the soluble peptides in supernatant was measured at 280 nm. One of caseinolytic activity units is defined as the amount of enzymes needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition.