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Digestive acid protease from zebra blenny (*Salaria basilisca*): Characteristics and application in gelatin extraction

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ABSTRACT

The present study reports on the characterization and evaluation of a crude acidic protease from the viscera of zebra blenny (*Salaria basilisca*) for use in gelatin extraction. Using hemoglobin, zymogram analysis revealed the presence of at least one clear band. The crude acid protease was noted to be optimally active at pH 3.0 and 50 °C and highly stable over a pH range of 2.0 to 7.0. The enzymatic extract lost about 87% of its activity after incubation with pepstatin A for 30 min at 4 °C. The acidic protease from the viscera of zebra blenny was noted to be effective in the extraction of gelatin from the skin of zebra blenny, with an extraction yield of 14.65% based on the wet weight of zebra blenny skin. The extracted zebra blenny skin gelatin (ZBSG) was characterized based on its chemical composition, polypeptide pattern, gel strength, textural parameters, and functional properties. ZBSG had high protein (90.6%) and low ash (3.1%) and fat (0.6%) contents. It contained α_1 and α_2-chains as the major constituents and determined as belonging to type I. The bloom strength of solidified gelatin was 151.3 g. The findings from Fourier Transformed Infrared (FT-IR) spectroscopy suggested the presence of helical arrangements of ZBSG. The latter showed excellent concentration-dependent functional properties. While emulsion activity index (EAI) and emulsion stability index (ESI) decreased, foam expansion (FE) and foam stability (FS) increased as the concentration of gelatin increased. Overall, zebra blenny endogenous acid protease could open new promising opportunities for the extraction of gelatin.

1. Introduction

Fish viscera have wide biotechnological potential as a rich source of digestive enzymes, especially proteases. The biological diversity of fish species provides a wide array of enzymes with unique properties. They have the advantages for the applications in food industry since their temperature and other characteristics differ from homologous proteases from warm-blooded animals. Therefore, digestive proteases can be isolated as a value-added product from fish viscera and used as the processing aids in food industries to maximize the utilization of marine resources (Gildberg, Simpson, & Haard, 2000). Proteases are classified as acid, neutral and alkaline proteases. Acid proteases dependent on the presence of aspartic (E.C. 3.4.23) residue in catalytic site are characterized by pH optima in the acid range and specific inhibition by pepstatin (Davies, 1990; Vishwanatha, Rao, & Singh, 2009). On the basis of their different molecular characteristics and tissue cellular localisation, aspartic proteinases of vertebrates have been classified as cathepsin D, cathepsin E, gastricsin, pepsin, chymosin and renin. In addition, other types of aspartic proteinases have been isolated from invertebrates (Kay et al., 1996), plants (Brodelius, Cordeiro, & Pais, 1995) and a number of microbial sources (Hill & Phylip, 1997; Toogood, Prescott, & Daniel, 1995). Acid proteases are widely applied in the hydrolysis of proteins. They are used in collagen extraction (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005) and in gelatin extraction (Jridi et al., 2013) as a rennet substitute (Aehle, 2007).

Due to strict restrictions with regard to the consumption of gelatin from certain origins, including the religious prohibition of consuming gelatin from porcine and bovine sources in Muslim, Jew and Hindu countries, there has been a continuous search for alternative natural sources of gelatin production.

Fish gelatin is generally produced by an acid treatment process, with clear dominance of acetic-acid extractions. Acid treatment has been used to disrupt acid-labile cross-links with negligible peptide bond hydrolysis and amino acid degradation (Slade & Levine, 1987). Since collagen cross-links are stable to thermal and acid treatment (Galea, Dalrymple, Kuypers, & Blakeley, 2000), a low yield of the resulting gelatin is generally obtained. To improve the yield of extraction, some proteases may be carefully employed. Pepsin has been reported to cleave peptides in the telopeptide region of native collagen, thus the yield of partially cleaved collagen can be increased (Jongjareonrak et al., 2005).

Recently, skin gelatins from commercially important species involving those used for domestic consumption in Tunisia, such as cuttlefish (*Sepia officinalis*) (Jridi et al., 2013), have been extracted using an acid protease-aided process.

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Zebra blenny is a very common benthic fish abundant in the Gulf of Gabes. It is brownish green in color and reaches a maximum length of 18 cm. *Salaria basilisca* is relatively important in the fish catches of Tunisia and is used for human consumption. In our previous findings, we have reported the evaluation of digestive alkaline proteases from *S. basilisca* as detergent additive and in shrimp waste deproteinization (Ktari, Ben Khaled, Nasri, et al., 2012; Ktari, Ben Khaled, Younes, et al., 2012). Furthermore, we have demonstrated that zebra blenny protein hydrolysates obtained by treatment with crude enzymes from the same species, sardinelle (*Sardinella aurita*) and smooth hound (*Mustelus mustelus*) significantly attenuated hyperglycemia and hyperlipidemia in alloxan-induced diabetic rats (Ktari et al., 2013) and exhibited a good antioxidant activity (Ktari, Jridi, Bkhairia, et al., 2012).

In the present paper, we describe the extraction and characterization of acid crude protease from zebra blenny and its application in zebra blenny gelatin production process. The physicochemical characteristics as well as the functional properties of the extracted gelatin were also evaluated.

2. Materials and methods

2.1. Materials

Zebra blenny (*S. basilisca*) with an average total length of 16.3 ± 1.1 cm and a weight of 37.6 ± 5.9 g was freshly purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. After the fish was washed with water, their viscera were separated. The skin was then removed and scraped and cut into small pieces. Samples were then stored in sealed plastic bags at −20 °C until they were used for enzyme and gelatin extraction.

2.2. Preparation of zebra blenny crude acid protease (ZBCAP)

Viscera from *S. basilisca* (100 g) were washed with distilled water, and then homogenized for 60 s with 200 ml of extraction buffer (10 mM glycine–HCl, pH 2.0) using Moulinex® R62 homogenizer. The homogenate was centrifuged at 8500 ×*g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and used as the crude protease extract. Proteolytic activity was assayed using hemoglobin as a substrate by the method of Anson (1938) after incubation at pH 3.0 and 50 °C for 15 min. One unit of acidic protease activity against hemoglobin was defined as the amount of enzyme that catalyzed an increase of one unit per minute in the absorbance at 280 nm under the assay conditions.

2.3. Properties of the enzyme

2.3.1. Optimum pH and pH stability

The optimum pH of the crude acid protease was studied over a pH range of 1.0–7.0 using hemoglobin as a substrate at 50 °C. For the measurement of pH stability, the crude enzyme preparation was incubated for 1 h at 4 °C in different buffers, and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM glycine–HCl buffer, pH 1.0–3.0; 100 mM acetate buffer, pH 4.0–6.0; and 100 mM Tris–HCl buffer, pH 7.0.

2.3.2. Effect of temperature on activity and stability

To investigate the effect of temperature, the activity was tested at different temperatures from 30 to 70 °C in 100 mM glycine–HCl buffer, pH 3.0, using hemoglobin as a substrate. Thermal stability was examined by incubating the enzyme preparation for 60 min at different temperatures (30 to 60 °C). Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 3.0 and 50 °C. The non-heated crude enzyme was taken as 100%.

2.3.3. Effects of metal ions on the acidic protease activity

The influence of various metal ions at a concentration of 5 mM on enzyme activity was investigated by adding the monovalent (Na^+ or K^+) or divalent (Mg^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+} or Mn^{2+}) metal ions to the reaction mixture. The activity of the crude enzyme without any metallic ions was considered as 100%.

2.3.4. Effects of enzyme inhibitors on the acidic protease activity

The effects of enzyme inhibitors (5 mM) on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, pepstatin A and ethylene-diaminetetraacetic acid (EDTA). The crude acid enzyme extract was preincubated with each inhibitor for 30 min at 4 °C, and then the remaining protease activity was tested using hemoglobin as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as control.

2.4. Extraction of gelatin from zebra blenny skin

Gelatin was extracted from the zebra blenny skin according to a slightly modified method of Nalinanon, Benjakul, Visesssanguan, and Kishimura (2008). The skins were washed and soaked in 0.05 M NaOH at a skin/solution ratio of 1:5 (w/v) for 1 h at 4 °C. They were then washed with tap water until a neutral pH was obtained for the wash water. The alkaline treated skins were then soaked in 100 mM glycine–HCl buffer pH 2.0 with a solid/solvent ratio of 1:10 (w/v) and submitted to limited hydrolysis with ZBCAP at a level of 15 units/g of skin. The mixture was stirred for 18 h at 4 °C. The pH of the mixture was then raised to 7.0, using 10 M NaOH, and stirred gently for 1 h at 4 °C. The enzyme-treated skins were then incubated for 8 h at 50 °C with continuous stirring to extract gelatin from the skin. The mixture was centrifuged at 10,000 ×*g* for 30 min to remove insoluble material. The supernatant was collected and freeze dried (ModulyoD Freeze dryer, Thermo Fisher, USA). The powder obtained, designated as zebra blenny skin gelatin (ZBSG), was stored at −20 °C until further use. The gelatin extraction yield was calculated using the following formula:

$$\text{Yield}(\text{g}/100\ \text{g}) = \frac{\text{Weight of freeze dried gelatin (g)}}{\text{Wet weight of fresh skin (g)}} \times 100.$$

2.5. Proximate analysis of gelatin

The moisture, protein, lipid, and ash contents of the gelatins extracted from zebra blenny skin were determined in triplicate as described in AOAC (2000).

2.6. Determination of color

Color was evaluated using a ColorFlex spectrocolorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) and reported as L*, a* and b* values, where L* refers to the measure of lightness, a* to the chromatic scale from green to red, and b* to the chromatic scale from blue to yellow (Jamilah & Harvinder, 2002).

2.7. Determination of bloom strength of gelatin gel

The gel strength of ZBSG was determined according to the method of Gomez-Guillen et al. (2002) using 6.67% gels (w/v) prepared by mixing the dry gelatin in distilled water at 60 °C for 30 min and cooling down the solution in a refrigerator at 7 °C (maturation temperature) for 16–18 h. The gel strength of gelatin gel was determined at 7 °C using a Model TA-TX2 texture analyzer with a 5 kN load cell equipped with a 1.27 cm diameter flat-faced cylindrical Teflon plunger. The dimensions of the sample were 3.8 cm in diameter and 2.7 cm in height. Gel strength was expressed as maximum force (in grams), required for the plunger to press the gel by 4 mm depression at a rate of 0.5 mm/s. The measurement was performed in triplicate.