Extraction and Purification of Protease from Silver Catfish (*Pangasius sutchi*) Viscera

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ABSTRACT

Protease is the enzyme which can be extracted from plant, animal, and microorganism. About 60% enzymes widely used nowadays are proteases. The objectives of this study were to determine the proteolytic activity and molecular weight distribution of protease extracted from Pangasius sutchi silver catfish viscera. Crude protease was extracted in 10 mM Tris-HCL buffer at pH 8.0. Subsequently, the protease was partially purified in stages beginning from ammonium sulphate precipitation (60%) followed by dialysis and gel filtration chromatography. Fractions collected from gel filtration chromatography was freeze-dried and analysed for optimum temperature and pH. Results showed that the viscera contain 13.79% protein. The protease total activity was 344.08 U with specific activity of 31.92 U/mg, purification fold 3.5 and 63.16% percent recovery. The protease proteolytic activity was optimum at 60 °C and pH 6. This study indicated that purification steps involving ammonium sulphate precipitation at 60% saturation, gel filtration chromatography and freeze drying led to the production of Pangasius sutchi visceral protease with a relatively high proteolytic activity.

Keywords: *Pangasius sutchi, silver catfish, viscera, protease, ammonium sulphate precipitation*

INTRODUCTION

Proteases are enzymes that break down the peptide bonds of protein. They are categorized into serine proteases, cystein proteases, aspartic proteases, and metalloproteases based on their catalytic mechanism [1]. Protease can be extracted from plant, animal, and microorganism. Approximately 60% enzymes used currently are proteases [2]. Despite of that fact, proteases are not commonly extracted from marine and aquatic counterpart [3].

Proteases have been utilized in various physiological and commercial fields. Alkaline protease has been used in several of industrial applications including foods, detergents and phamaceuticals [2]. It can also be used as recovery silver in x-ray film [4]. It was also reported that protease can be used as food processing aids by decreasing the stick-water viscosity during the production of fish meal [5]. Thirty percent of the original fish raw materials regarded as wastes have been removed and discarded during industrial processing for instance in canning process [6]. Fish waste comprises of viscera, scales, fins and frame bones [7]. Some initiatives have been taken to produce powdered fish flour from fish waste for animal feed purposes to reduce waste that could contribute to environmental pollution [6]. Fish viscera are rich in enzyme that can be extracted such as protease, lipase and amylase [8]. These enzymes give benefits in biotechnology related industries as source of digestive enzymes [9]. Digestive proteases are responsible for rapid abdominal degradation, especially acidic proteases from the stomach and alkaline proteases from the pyloric caeca, pancreas, and intestine [5]. This evidence makes fish viscera a possible source of proteolytic enzyme for industrial purposes. This study was carried out to determine the characteristics of protease extracted and purified from *Pangasius sutchi* silver catfish viscera.

MATERIALS AND METHODS

Silver catfish (*Pangasius sutchi*) was obtained from a local fish supplier in Kuala Terengganu, Malaysia. The viscera were carefully removed using a sharp knife without discarding any single part.

After cleaning the viscera under running water in order to remove the blood, the viscera were stored at -20 °C before proceeding to further steps. The chemicals comprising of Tris-HCl, anhydrous calcium chloride ($CaCl_2$), ammonium sulphate (NH_2SO_4), Triton X-100 %, Sephadex G-100, Bio-Gel p-100 Coomassie Brilliant Blue R-250, casein, TCA (CCl_3COOH), SDS gel, glycerol, anhydrous disodium phosphate, methanol, ethanol (95%), acetic acid, sodium acetate ($CH_3COONa.3H_2O$), glycine-NaOH, L-cysteine ($HCl.H_2O$), EDTA and phosphoric acid were purchased from Sigma Aldrich (M) Sdn Bhd.

Protein content

Protein content of the viscera was determined by Kjeldahl method according to AOAC [10].

Crude protease extraction

Crude protease was extracted according to [6]. Viscera of about 50 g was homogenized for 30 s with 50 ml extraction buffer A (10 mM Tris-HCL pH 8.0) at the ratio of 1:1. The mixture was centrifuged at 5000 rpm for 15 min. at 4 °C. The pellet was discarded and the supernatant collected is known as the crude protease extract.

Purification of Protease

The crude protease extract was mixed with ammonium sulphate up to 60% saturation whereby the precipitate was collected following centrifugation at 5000 rpm for 20 min. The precipitate was then suspended in buffer A and dialyzed for 24 h at 4 °C against changes in the same buffer [6]. The enzyme was subjected to dialysis followed by gel filtration using Bio-Gel p-100. The column was pre-equilibrated with buffer B (20 mM Tris-HCl, pH 7.5). Enzyme fractions of 4 ml each were eluted at a flow rate of 20 ml/h with the same buffer. Protein peak was identified at 660 nm [11]. The protease fraction with the highest specific activity was freeze-dried.

The crude extract, ammonium sulphate purified protease, dialysed protease and fractions from gel filtration chromatography were analysed for protein concentration, proteolytic activity and molecular weight distribution while the freeze-dried fractions from gel filtration chromatography was analysed for optimum temperature and pH.

Protein Concentration

Protein concentration was determined by the method of [12], with bovine serum albumin as the standard. Absorbance was measured at 595 nm [7].

Proteolytic Activity

Proteolytic activity was determined using Abirami *et al.*, method [13] with modification. 5 ml of 0.65% casein solution was incubated for 5 min. at 37 °C. Then 1 ml of enzyme sample was added and the mixture was further incubated for another 10 min. at the same temperature. The reaction was terminated by adding 5 ml of trichloroacetic acid. The sodium carbonate solution was also added to reduce cloudiness. Finally, 0.5 M of Folin-ciocalteau reagent was added and the absorbance was measured at 660 nm. The tyrosine standard curve was plotted. One caseinolytic activity unit is defined as the amount of enzyme needed to produce an increment of 0.01 absorbance unit per minute at the assayed condition.

Protease total activity

The protease total activity was determined using casein digestion unit analytical method (CDU) and was calculated according to [14] by the following formula:

$\frac{E_a - E_b}{E_s}$ x 50 x $\frac{11}{10}$ x DF

E_a = Absorbance of sample
E_b = Absorbance of blank
E_s = Absorbance of standard (tyrosine)
DF = dilution factor

Protease Specific Activity

The specific activity and the purification fold of the enzyme produced were calculated according to [15] by the following formula:

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Total protein (mg/ml)}}$$

$$\text{Purification fold} = \frac{\text{Specific activity of purified enzyme}}{\text{specific activity of crude enzyme}}$$

Protease % Recovery

Protease recovery was calculated according to [15] by the following formula:

$$= \frac{\text{Total activity}}{\text{Total activity of crude extract}} \text{ x } 100$$

Determination of Molecular Weight

The molecular weight of protease was determined by SDS-PAGE with 4% stacking gel and 12% resolving gel. About 10 µL (0.05 g/ml) of samples was prepared and mixed with 2.5 µL NuPage sample buffer and 7.5 µL distilled water. The mixture was heated at 70 °C for 10 min. Low molecular weight marker (Seeblue plus2 Prestained Standard) ranging from 10 to 220 kDa was used. About 10 µL of the prepared sample was loaded into each well of the gel. Electrophoresis was performed for 1.0 h at constant current of 100 mA/gel. After electrophoresis, the gel was stained in Coomassie Brilliant Blue solution (NuPAGE Tris-Acetate Gel protocol).

Determination of Protease Optimum Temperature

The effect of temperature on protease activity was studied ranging from 30 to 80 °C with interval of 10 °C for 15 min at pH 8.0. Aliquot was withdrawn at desired time intervals to test the remaining activity at standard conditions using proteolytic activity steps [6].

Determination of Protease Optimum pH

The effect of pH was determined with casein as a substrate. Protease activity was studied in the pH range of 6.0–11.0 at room temperature for 1 h in different buffers and then the proteolytic activity was determined. The following buffer systems used in this method were: 100 mM sodium acetate, pH 6.0; phosphate buffer, pH 7.0; Tris–HCl buffer, pH 8.0; glycine-NaOH buffer, pH 9.0–11.0 [6].

Statistical Analysis

All measurements were carried out in triplicate. Statistical analysis was carried out using statistical analysis software SAS 9.3.2 with the minimum level of significant difference ($p < 0.05$).

RESULTS AND DISCUSSION

Protein Content of The Viscera

Pangasius sutchi fish viscera contains 13.79% protein which falls within the similar range in other fish such as pink salmon, pollock and cod ranging from 13 to 15.3% as stated in the previous findings [16]. High protein content of the viscera is essential for obtaining high yield of protease.

Proteolytic Activity

The pH was fixed to 8.0 to prevent sudden changes in pH that could irreversibly affect folding, solubility, and the function of the enzyme [17]. According to [6], crude enzyme treated with 30-60% of ammonium sulphate showed a highest specific activity compared with those that have been treated with 0-30% ammonium sulphate. Thus, the maximum percentage of ammonium sulphate which is 60% was selected in order to obtain the maximum activity. It has been suggested that each purification step reduced the impurity of protein [18]. Table 1 shows the proteolytic activity of protease from *Pangasius sutchi* viscera at each purification stage.

Total activity in each stage decreased from 536.1 U in crude extract to 344.08 U after the enzyme was subjected to various stages of purification with gel filtration chromatography showed the lowest activity. A total of 35.8% decreased was recorded at this final stage of purification. The total activity of crude extract from a hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) viscera showed almost similar value which is 526.5 U however, decreased to 66.8 U [19]. While the total activity for Amazonian fish pirarucu viscera crude extract was 179 U and decreased to 31.3 when crude extract was subjected to ammonium sulphate precipitation (30–90%), size exclusion chromatography with Sephadex G-75 followed by affinity chromatography with benzamidine-agarose where the crude extract was heated prior to ammonium sulphate precipitation [2].

Table 1: The proteolytic activity of protease from viscera of *Pangasius sutchi* silver catfish at each purification stage

Purification stages	Volume (mL) per 50 g	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	80.83	71.48	536.1	7.77	1	100
60% ammonium sulphate precipitation	38.5	26.82	496.13	18.67	2.48	92.59
Dialysis	28.67	16.83	410.11	25.18	3.37	76.56
Gel filtration chromatography	24	9.888	344.08	31.92	3.5	63.16

Specific activity increased from 7.77 U/mg to 31.92 U/mg through each stage of purification with gel filtration chromatography exhibiting the highest value. Similarly, in [11] studies on visceral protease from a small carp-like fish, Tunisian barbell (*Barbus callensis*), a 27 fold increase of specific activity was observed.

Other studies showed a decreased when using three-phase partitioning method of purification [20]. Purification fold increased from 1 to 3.5 indicating that *Pangasius sutchi* viscera protease increased in purity at each stage of purification achieving 63.16% recovery. In studies on farmed giant catfish visceral protease, purification fold of 5.0 was obtained with 163.1% recovery [20]. They attributed high recovery exceeding 100% to the three phase partitioning (TPP) in the presence of t-butanol and ammonium sulphate whereby TPP led to the simultaneous activation of the enzyme.

Optimum Temperature and pH

Figure 1a shows the effect of temperature on *Pangasius sutchi* viscera protease activity. Proteolytic activity ranges from 43 -52 U when subjected to exposure at 30 to 80 °C. Slight increase in activity occurs between 30-50°C followed by sharp increased at 60 °C. Subsequently, the activity reduced at higher temperature which was probably due to thermal denaturation. The optimum activity of visceral proteases as reported in other studies was between 45 to 55 °C [11, 5].

Pangasius sutchi viscera protease is slightly acidic in which the highest activity was recorded at pH 6 (Figure 1b). The activity increases from pH 4 until pH 6 and decreases thereafter. For acidic protease, the highest proteolytic activity was at pH between 2 to 4 while alkaline protease activity was highest at pH 8 to 10 [5, 21]. The differences in pH stability are influenced by the different molecular properties and enzyme conformation amongst different species and location [17].

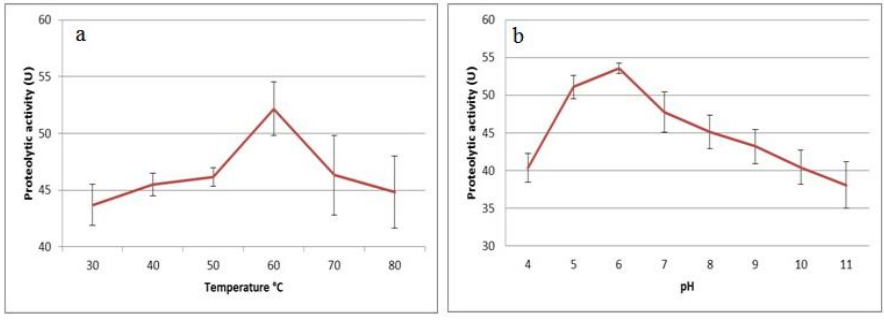

Figure 1: Effect of temperature (a) and pH (b) on Pangasius sutchi viscera protease activity at the final purification stage; gel filtration chromatography freeze-dried fraction.

Molecular Weight Distribution

Pangasius sutchi viscera showed bands between 10 to 220 kDa with three clear bands appearing at 35, 75 and 220 kDa (Figure 2). Crude protease extract showed bands between 10 to 70 kDa. Most of the bands were faded with purification stages leaving only one band observed at 35 kDa following gel filtration stage.

However, when the protease was freeze-dried some of the bands reappeared. This could be due to purification of the protein in which water evaporated during freeze drying. The presence of clear band at 35 kDa has also been reported previously [22]. Viscera from sardine showed clear band at 25 kDa, hybrid catfish at 24 kDa and silver mojarra at 36.6 kDa [6, 22, 19, 23].

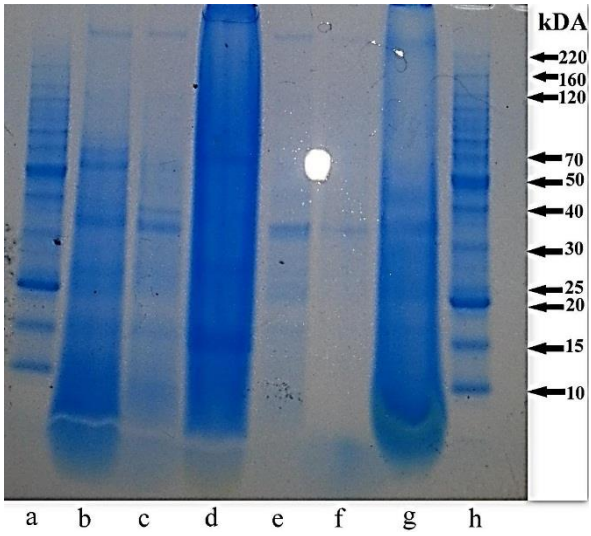

Figure 2: The electrophoresis pattern of (a and h) benchmark protein ladder, (b) raw viscera of *Pangasius sutchi*, (c) crude protease extract, (d) protease purified at 60% ammonium sulphate precipitation, (e) dialyzed protease, (f) protease purified using gel filtration chromatography, and (g) freeze-dried protease

CONCLUSION

Pangasius sutchi viscera protease could be extracted and further purified by ammonium sulphate precipitation, gel filtration chromatography and freeze-drying methods producing protease with a relatively high proteolytic activity.

REFERENCES

[1] N. Ktari, H.B. Khaled, R. Nasri, K. Jellouli, S. Ghorbel, and M. Nasri, 2012. Trypsin from Zebra Blenny (*Salaria basilisca*) Viscera: Purification, Characterization and Potential Application as a Detergent Additive. *Fd. Chem*., vol. 130, pp. 467-474.

[2] A.C. Freitas-junior, H.M. Costa, M.Y. Icimoto, I.Y. Hirata, M. Marcondes, and L.B. Carvalho Jr., 2012. Giant Amazonian Fish Pirarucu (*Arapaima gigas*): Its Viscera as a Source of Thermostable Trypsin. *Fd. Chem*., vol. 133, pp. 1596-1602.

[3] B. Hayet, N. Rym, B. Ali, G. Sofiane, and N. Moncef, 2011. Low Molecular Weight Serine Protease from the Viscera of Sardinelle (*Sardinella aurita*) With Collagenolytic Activity: Purification and Characterization. *Fd. Chem*., vol. 124, pp. 788-794.

[4] B. Sabtecha, J. Jayapriya, and A. Tamilselvi. 2014. Synthesis, Spectral and Antimicrobial Investigation of Some Ternary Schiff Base Transition Metal Complexes. *Int. J. of Chemtech. Res*., vol. 6, pp. 4504-4509.

[5] F.J. Yanez, R.P. Aguilar, F.L. Carreno, and M.L. Toro, 2004. Characterization of Acidic Proteolytic Enzymes from Monterey Sardine (*Sardinops sagax caerulea*) viscera. *Fd. Chem*., vol. 85, pp. 343-350.

[6] A. Bougatef, N. Souissi, N. Fakhfakh, Y.Ellouz-Triki, and M.Nasri, 2007. Purification And Characterization of Trypsin from the Viscera of Sardine (*Sardina pilchardus*). *Fd. Chem*., vol. 102, pp. 343-350.

[7] S. Prakash, R. Ramasubburayan, P. Iyapparaj, and S. Sankaralingam, A. Palavesam, 2014. Optimization and Partial Purification of a Protease Produced By Selected Bacterial Strains Grown on Trash Fish Meal Substrate and Its Antagonistic Property against Bacterial Pathogens. G.Immanuel, *Biocatalysis and Agric. Biotech*., vol. 3, pp. 288-295.

[8] S.M. Safa, Y. Faridah, and S.J. Mohammed, 2014. Extraction And Screening of Various Hydrolases from Malaysian Channel Catfish (*Ictalurus punctatus*) Viscera. *Int. J. of Chem. and Env. Eng*., vol. 5, pp. 79-82.

[9] F. Shahidi, and J. Kamil, 2001. Enzymes from Fish and Aquatic Invertebrates and Their Application in the Food Industry. *Trends in Fd. Sci. & Tech*., vol. 12, pp. 435-464.

[10] AOAC (Association of Official Analytical Chemist). Official methods of analysis (15th ed.). 1990; Washington, VA.

[11] A. Sila, R. Nasri, M. Jridi, M. Nasri, R. Balti, and A. Bougatef, 2012. Characterisation of Trypsin Purified from the Viscera of Tunisian Barbel (*Barbus Callensis*) and Its Application for Recovery of Carotenoproteins From Shrimp Wastes. *Fd. Chem*., vol. 132, pp. 1287-1295.

[12] M.M. Bradford, 1976. A Rapid And Sensitive Method for The Quantitation of Microgram Quantities of Protein Utilizing The Principle of Protein-Dye Binding. *Anal. Biochem*. vol. 72, pp. 248-254.

[13] V. Abirami, S.A. Meenakshi, K. Kanthymathy, R. Bharathidasan, R. Mahalingam, and A. Panneerselvam, 2011. Pelagia Research Library. *Eur. J. of Exp. Biol*., vol. 1, pp. 114-123.

[14] G.R. Dapeau. Met. in Enzymology 1976; (Vol. 45). (L. Lorand, Ed.) New York: Academic press.

[15] A. El-Beltagy, T. El-Adawy, E. Rahma, and A. El-Bedawey, 2004. Purification and Characterization of an Acidic Protease from the Viscera of Bolti Fish (*Tilapia nilotica*). *Fd. Chem.*, vol. 86, pp. 33-39,

[16] P.J. Bechtel, 2003. Properties of Different Fish Processing By-Products from Pollock, Cod and Salmon. *J. of Fd. Proc. and Pres*., 27(2), 101-116.

[17] A.Vannabun, S. Ketnawa, S. Phongtha, 2014. Characterization of Acid and Alkaline Proteases from Viscera of Farmed Giant Catfish. *Fd. Biosci*., vol. 6, pp. 9-16.

[18] T. Nurhayati, S. Rusyadi, R. Suwandi, and R. Nugraha, 2013. Purification and Characterization of a Cathepsin Inhibitor from Catfish (*Pangasius sp.)* of Indonesian Water. *Int. Fd. Res. J.*, vol. 20, pp. 941-946.

[19] S. Klomklao, S. Benjakul, H. Kishimura, and M. Chaijan, 2011. 24 kDa Trypsin: A Predominant Protease Purified from the Viscera of Hybrid Catfish (Clarias macrocephalus × Clarias gariepinus). *Fd. Chem*., vol. 129, pp. 739-746.

[20] S. Rawdkuen, A.Vanabun, and S.Benjakul, 2012. Recovery of Proteases from the Viscera of Farmed Giant Catfish (*Pangasianodon gigas*) By Three-Phase Partitioning. *Proc. Biochem.*, vol. 47, pp. 2566-2569.

[21] H.B. Khaled, O.G. Bellaaj, N. Hmidet, K. Jellouli, N. EL- Hadj Ali, and S. Ghorbel, 2011. A Novel Aspartic Protease from the Viscera of Sardinelle (*Sardinella aurita*): Purification and Characterization. *Fd. Chem*., vol. 128, pp. 847-853.

[22] A. Bougatef, R. Balti, S.B. Zaied, N. Sousii, and M. Nasri, 2008. Pepsinogen and Pepsin from the Stomach of Smooth Hound (*Mustelus mustelus*): Purification, Characterization and Amino Acid Terminal Sequences. *Fd. Chem*., vol. 107, pp. 777-784.

[23] J.F. Silva, T.S. Esposito, M. Marcuschi, K. Ribeiro, R.O. Cavalli, and V. Oliveira, 2011. Purification And Partial Characterisation of a Trypsin from the Processing Waste of the Silver Mojarra (*Diapterus rhombeus*). *Fd. Chem.*, vol. 129, pp. 777-782.