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Extraction, Purification and Characterization of Fish Chymotrypsin from *Catla catla*

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Abstract

Indian major carps, Catla (*Catla catla*) viscera chymotrypsin was purified by fractionation with ammonium sulfate (30–70 % saturation), gel filtration, affinity, and ion exchange chromatography. Chymotrypsin molecular weight was 29 kDa according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, shown a single band in zymogram. Electrofocusing study suggested being an anionic enzyme, exhibiting maximal activity at pH 9 and 50 °C, using Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. Enzyme was effectively inhibited by phenyl methyl sulfonyl fluoride with 99 percent. Enzyme activity was affected by the following ions in decreasing order: Hg^{2+}, Fe^{2+}, Cu^{2+}, Li^{1+}, Mg^{2+}, K^{1+}, Mn^{2+}, while Ca^{2+} had no effect. Chymotrypsin activity decreased continuously as NaCl concentration increased from 0 to 30 percent. The K_m and V_{max} values were 0.83 ± 1.1 mM and 1.10 ± 0.09 µmol/min/mg of protein respectively. Results suggest the enzyme has a potential application where low processing temperatures are needed, such as in fish sauce production.

Introduction

The waste parts of fish processing (heads, viscera, leather and frames) are extracted in different processes, each stream can be collected separately and used for the production of additional products. Fish processing waste contains high value proteins that can be reused to produce valuable products such as fertilizers, fish oils and residues (IFC, 2007). Fish waste is also known to contain essential acids including omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), enzymes (pepsin, trypsin, chymotrypsin), collagen and fat (Byun *et al.*, 2003); Swatschek *et al.*, 2002; Kim *et al.*, 2006). These essential compounds can be used in the medical and food industries while oils can be converted into biosel

and used in the transportation industry. Therefore, the proper use of fish waste can lead to a large commercial value (Chong *et al.,* 2002).

Currently, chymotrypsin is widely used in skin applications, in the chemical and clinical industries. In the industry, chymotrypsin is often produced in fresh beef or pork chops and is usually made in pill form for oral or injectable fluid. The price of chymotrypsin is related to the purity of the products. Using fish waste, instead of fresh cattle or pig milk, can significantly reduce the cost of chymotrypsin production. There have been many studies on chymotrypsin from higher invertebrates such as cattle and pigs but little information has been found on fish chymotrypsin (Einarsson *et al.,* 1996). Currently, ammonium sulphate is used to reduce raw chymotrypsin from raw material and purified by chromatography. The purpose of this study was to test the feasibility of using reversible micelles to purify chymotrypsin fish.

Material and Method

Extraction and purification of chymotrypsin: Generally, the initial recovery steps of chymotrypsinogen are extraction which includes preparation of crude material, homogenization using a buffer to extract crude chymotrypsin (chymotrypsingen) from the prepared material and centrifugation to separate the crude chymotrypsin and precipitation or fractionation to collect chymotrypsingen. The processes that can be used extract chymotrypsin from fish waste are shown in Fig. 1.

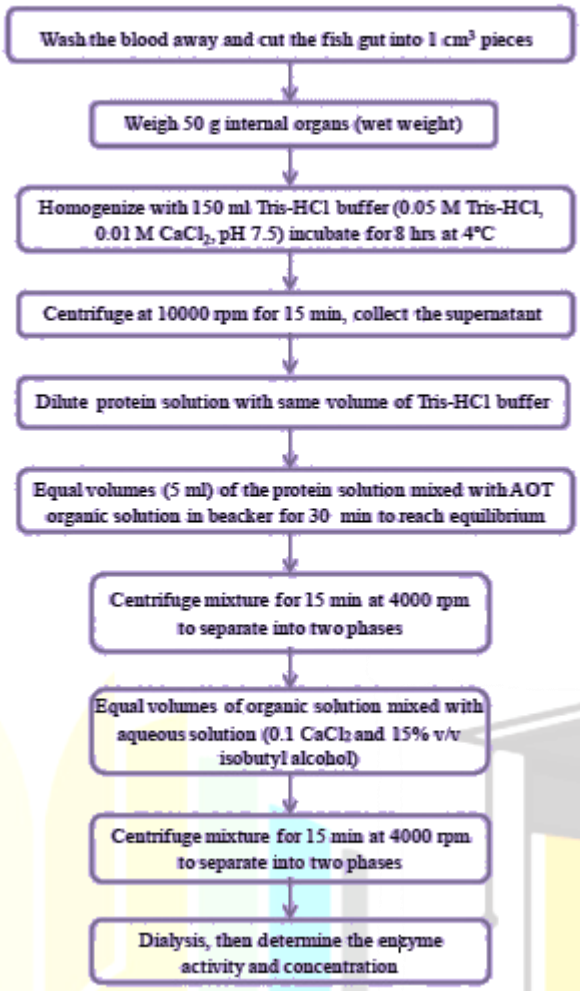

Figure 1. Extraction of chymotrypsin from fish waste by using reverse micelles method.

Buffer extraction: The fish stomach and intestines are removed from fish and separated as soon as the fish has been killed and washed with cold water or isotonical saline solution to get rid of blood in the tissue. The inhibitors in blood can reduce chymotrypsin activity (Chong *et al.*, 2002 Boeris *et al.*, 2009).

Purification: The chymotrypsinogen purification processes account for up to 80% of the total cost of chymotrypsin production. Precipitation is the technique most widely used in the chymotrypsin production industry because it can effectively purify and concentrate chymotrypsin at a very low cost (Matsudo *et al.*, 2003; Boeris *et al.*, 2009).

***Ammonium sulfate method*:** Ammonium sulfate is the most reported polyelectrolyte in chymotrypsinogen production (Chatterjee *et al.*, 2004). Folk (1970) used ammonium sulfate fractionation as second step inchymotrypsin purification process after extracted crude chymotrypsin with acetone powder. Same method had been used by Castillo-Yañez *et al*. (2006), Lam *et al*. (1999) and Möckel and Barnard (1969a). In the precipitation process, poly-charged molecules which contain opposite electrical charges to the

chymotrypsinogen are added into protein solutions to form a chymotrypsinoge n-polyelectrolyte complex and generate insoluble aggregates.

Activation of chymotrypsinogen*:* Chymotrypsinogen is an inactive form of chymotrypsin existing in the pancreas and the extraction and purification processes result in pure chymotrypsinogen. Although chymotrypsinogen has enzymatic activity, the low level of activity makes it hard to detect in normal conditions. In order to evaluate the properties of chymotrypsin, chymotrypsinogen activation is a necessary process (Blow, 1976). However, the optimal pHs of activators are different. The optimum pH for trypsin is 7.5, the optimal pH of Aspergillus oryzae is 3.0-5.0 and the optimal pH of Kaufman and Erlanger ranges from 3.2-3.4 (Prokuryakov, 1970). Since chymotrypsinogen is a single polypeptide chain made up of 245 amino acids, different forms of chymotrypsin can be activated by controlling the reaction condition (Boeris *et al.*, 2009).

Trypsin concentration: Trypsin is an activator of chymotrypsinogen which can dramatically accelerate the speed of zymogen activation even at a very low concentration (0.01 mg/g of homogenate). The relative activity of chymotrypsin was during the zymogen activation process in the presence or absence of trypsin. (Berg *et al.*, 2007. Glazer and Steer (1976) found that an increase in trypsin concentations at low concentations (0.5-1.0%) did significantly increase the rate of activation but trypsin concentrations above 10% showed little increase in activation.

Incubation time: The incubation time required for chymotrypsinogen activation process depends on the activation method applied. There are two types of activation: classical activation (pH 7.5, 5°C, trypsin free, 48 incubation time) and rapid activation (activated by trypsin with incubation time varying from 1-48 h) (Bettelheim and Neurath, 1954; Miller *et al.*, 1971). With the addition of trypsin, the enzymogen activation significantly increases in 1 h with the decrease of thehomogenate solution viscosity.

High performance liquid chromatography (HPLC)*:* HPLC separations were carried out by a Knauer Smartline 1000 pump equipped with a Smartline UV detector 2500 (Berlin, Ger- many), and a Rheodyne 7725 injection valve (Cotati, CA, USA). The method was opti- mized at the 0.8 *ml/min* flow rate and 210 *nm* wavelength UV detection. Separation was carried out over C_{18} column with 4.6 *mm* diameter and 250 *mm* length from Grace Company (Munich, Germany) with a mixture of water and acetonitrile (95:5) as mobile phase. Extra pure *E.coli* LPS (Sigma, Saint Louis, USA) was used as standard.

Assaying of chymotrypsin

Enzyme concentration: Three main methods are commonly used to evaluate chymotrypsin concentration: (a) absorbance at 280 nm (b) Bradford method and (c) Lowery method. Protein in solution has a maximum absorbance of ultraviolet light at 280nm. When measuring enzyme concentration, the wavelength must be adjusted to 280 nm and the system calibrated to zero with buffer solution. The absorbance of protein solution is then measured and the concentration (mg/mL) is calculated by the following equations (Layne, 1957; *et al*.; 2009; Stoscheck, 1990). This method had been used by Yang, Castillo-Yañez *et al*. (2006, 2009) and Möcke *et al*. (1969a). For protein mixture:

$$Concentration = \frac{\Delta AU_{280}}{path\ length\ (cm)} \qquad (1)Equation$$

For protein mixture with possible nucleic acid

$$Concentration = \ 1.55 \times \Delta AU_{280} - 0.76 \times \Delta AU_{260} \qquad (2)Equation$$

In the Bradford method, Coomassie Blue reagent is mixed with the enzyme solution and the absorbance is read at 595 nm after incubation for 15 min. Concentrations are determined relative to standard curve based on Bovine Serum Albumin (BSA). The method had been used by Tsai (1986). The method developed by Lowery *et al* (1951) is a relatively sensitive method but more complicated and time consuming compared to the Bradford Method. The enzyme solution is treated with Folin-ciocaltea reagents to create a blue compound and allowed to incubate for 10 min. Absorbance is read at 660 nm and standard using BSA is required.

Enzyme activity: A number of substrates are used to assay chymotrypsin activity including N-acetyl-LTyrosine Ethyl Ester (ATEE), Benzoyl-Tyrosine Ethyl Ester (BTEE) and N-Suc-Ala-Ala-Pro-Phe-*p*-Nitroanilide (SAAPPNA) (Hummel, 1959; Erlanger *et al.,* 1961; Ramakrishna *et al.,* 1987; Sabapathy and Teo, 1994; Heu *et al.,* 1995; Chong *et al.,* 2002; Chakrabarti *et al.,* 2005; Li *et al.,* 2005; Sveinsdóttir *et al.,* 2006). The activity of chymotrypsin is determined as change in absorbance of chymotrypsin used in the assay per mg protein per min (Chakrabarti *et al.,* 2006). When ATEE is used as substrate, one unit of enzyme activity is defined as the decrease in measured absorbance of 0.0075 min-1 at 237 nm and 25°C. The enzyme is mixed with ATEE solution in potassium phosphate buffer and the absorbance is measured every half min for 5 min. The activity is calculated using the following equation-3. When BTEE is used as substrate, one unit of enzyme activity is defined as one unit of enzyme

$$Activity = \frac{\Delta AU_{237}/min \times dilution}{0.0075\ 0.2\ \text{mg enzyme/ml original solution}} \qquad (3) Equation$$

Results

Crude extraction: Crude protein was extracted from the intestine (50 g) of Indian major carp, *Catla catla*. The total volume (TV) was measured after homogenization, centrifugation and dilution. The activity of enzyme (AE), total activity (TA), specific activity (SA), protein concentration (Cp), purification fold (PF) and recovery yield (RY) were determined (Table 1). After centrifugation, the total volume decreased from 177 to 138 ml (20.11%). The total activity decreased from 13.91 to 12.94 U (6.81%). The concentration decreased from 4513.8 to 2018.4 µg/ml (53.16%). The enzyme activity increased from 0.08 to 0.87 U/ml (18.02%). The specific activity increased from 0.188 to 0.489 U/mg (172.4%). The purification fold of the centrifugation step was 2.44 and the recovery yield was 94.1%.

Table- 1. The results of crude extraction

Different extraction steps	Total volume (ml)	Activity of enzyme (Unit/ml)	Total activity (Unit)	Protein concentration (µg/ml)	Specific activity (Unit/mg)	Purification folds (-)	Recovery Yield (%)
After Homogenizing	177	0.81	139.1	4513.8	0.188	0	0
After Centrifuging	138	0.87	129.4	2018.4	0.489	2.44	94.1
After Dilution and pH adjustment	261	0.40	128.6	967.6	0.491	0	100

*Sample size: 50 gm

Conclusion

Chymotrypsin is an important digestive enzyme which widely exists in mammal pancreatic tissues and fish guts (intestine). It is a endopeptide compound with 245 amino acid and molecular weights ranging for 22.000-30,000 Da. Chymotrypsin specifically hydrolyzes peptide bounds with α-amino acid carbonyl groups, nonpolar aromatic group and nonpolar groups. The optimal pH range for fish chymotrypsin is in between 7.5-11 in which is slightly higher than mammalian chymotrypsin. Fish chymotrypsin is more stable in basic environment than the acidic one. The optimal temperature for fish chymotrypsin is lower than mammal chymotrypsin. The factors affecting activity and the amount of chymotrypsin in fish are water temperature, fish species, fish age, fish weight, fish weight, starvation and nutrition. Buffer extraction, ammonium sulphate precipitation are usually used to produce chymotrypsin from fish. Each method has its own merit and demerit. Buffer extraction and ammonium sulphate precipitation can be used in large scale applications but the enzyme purity and activity is very low which can only be used in the leather or

chemical industry. Further study should focus on the optimization of purifying chymotrypsin from fish processing waste.

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