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Isolation of Crude Proteases from Freshwater Fishes *Catla catla* and *Labeo rohita*: Optimizing the Hydrolysis Conditions of Crude Proteases

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ABSTRACT

Proteases from gastrointestinal (GI) tract of fresh water fish Catla catla and Labeo rohita have been studied. This study is prelude to use proteases form GI tract of fresh water fish for the production of gelatin protein hydrolysates. The optimum conditions for the crude proteases obtained from GI tract have been standardized. The optimum temperature for the activity of enzymes from two species of fish found to be 55 ^{o}C and hydrolysis time of 15 min. The pH optima for the proteases from catla were found to be 10.5 and that from rohu was 8.5 and can be considered as alkaline proteases. The substrate used to assay the proteolytic activity was casein. The enzymes from two species had number of subunits in molecular weight range of 6.5–97 kDa as revealed by electrophoretic mobility under reduced conditions.

Key words: *Freshwater fish, digestive proteases, proteolytic activity, SDS-PAGE*

INTRODUCTION

The world fish production from fresh water sources (cultured and captured) is estimated to be around 47.1 million tons during 2014 [1]. The fish production in India from fresh water sources was 6.58 million tons during the year 2014, out of which culture practices contribute nearly 80% of total production[2]. The species used for aquaculture practice include Indian major carps IMC (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*) and cat fish (*Ictalurida sps*). The Indian major carps contribute more than 85% for total production of aquaculture in India. It is projected that, by 2030 the fish production from aquaculture in India will be 10 million metric tons. Such a huge production of fish for human consumption bound to generate large amount of processing waste. The fish processing waste includes viscera, skin, bones and air bladder which generally discarded [3]. Many attempts have been made to utilize the fish processing waste including visceral mass for product utility.

Proteases are a class of enzymes and are associated naturally in GI tracts of vertebrates and invertebrates. Proteases are used extensively in several bioremediation processes, pharmaceutical, nutraceutical, food and detergent industries.

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The fish viscera is one of the major by products of the fish processing industry, are known to be a rich source of digestive enzymes, especially proteases that have a high activity over a wide range of pH and temperature conditions[4]. 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) [5]. Acidic proteases from fish viscera display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin and chymotrypsin , are most active between pH 8.0 - 11.0. Proteases with high activity and stability in high alkaline range are interesting for bioengineering and biotechnological applications[6].

Several studies have been reported on isolation and characterization of digestive enzymes; proteases from fish viscera and processesing waste, digestive proteases of *Catla catla, Labeo rohita* and *Hypophthalmichthys molitrix*[7]; Pepsins from pectoral rattail (*Coryphaenoides pectoralis*)[8]; trypsin from *Catla catla* [9]; digestive protease from *Labeo rohita*[10]; alkaline proteases from thornback ray (*Raja clavata*)[4]; alkaline trypsin from *Liza aurata*[11]. The protein hydrolysates comprising of bioactive peptides has been found to be one of the important functional foods. The bioactive peptides are known to possess anti-hypertensive activity, immuno-modulatory activity and antioxidant properties[4]. Protein hydrolysates can be obtained from various food proteins by application of proteolysis. Fish skin, bones and fins are rich source of collagen and can be converted to gelatin by appropriate process. The protein hydrolysates from gelatin can be prepared using proteases which will add value and thus helps in utilizing fish processing waste. With this rationale, the objectives of present investigation was to isolate crude proteases from two important fresh water aquacultured fishes and optimizing the hydrolysis conditions with reference to temperature, time of hydrolysis and pH of reaction mixture.

MATERIALS AND METHODS

Fish

Two species of Indian major carps (IMC) *Catla catla* and *Labeo rohita* were used in the present study. The fishes were purchased from local fish market in iced condition and brought to the laboratory. The fishes were washed in water, beheaded, gutted and digestive tract was collected stored at -20 $^{\circ}$C and used for enzyme extraction within two days. All the chemicals used were either analytical grade reagent (AR) or guaranteed grade reagent (GR).

Preparation of digestive proteases

Crude extract was carried out according to the method as described by Phanturat *et al.*[12], with slight modification given in flow chart. The stored digestive tracts of *C. catla* and *L. Rohita*, were partially thawed using running water, cut into small pieces and homogenized in 10mM Tris-HCl buffer (pH 8.0 containing 10mM $CaCl_2$) at a ratio of 1:10 (w/v) for 3 min at 11,000 rpm using homogenizer (ULTRA-TURRAX T25, IKA Labortechnik, Germany). The homogenate was centrifuged at 11,500 x g for 30 min at 4°C, using a refrigerated centrifuge (Sorvall Legand XTR centrifuge, Thermo Fisher Scientific, New Hampshere, USA) to remove the debris. The supernatant was collected and filtered through a Whatman filter paper No 1. (Whatman Plc, Maidstone, Kent, UK). The filtrate obtained referred as 'crude proteases - catla proteases (CP) and rohu proteases (RP)'. The protein content of crude proteases was measured according to Lowry *et al.*[13] using BSA as standard.

Proteolytic activity of crude proteases

Proteolytic activities of crude proteases were determined using casein as substrate according to the method described by Kunitz[14] and Walter[15] with some modifications. The reaction mixture consisted of 2.5 ml of 2% casein and 0.5 ml of crude proteases, incubated in water bath for 30 min at 37°C. A total of 2.5 ml of 10% Trichloro aceticacid (TCA) was added to reaction mixture to terminate the hydrolytic reaction. This mixture was then filtered through a Whatman filter paper No.4. Absorbance of supernatant was recorded at 280 nm and the amount of tyrosine

liberated was calculated using a tyrosine standard curve. The proteolytic activity was expressed in terms of specific activity. The blank was prepared by adding the TCA prior to incubation. One unit of specific activity was defined as the amount of enzyme required to catalyze the formation of 1µM of tyrosine per min per mg protein of protease (µmol mg^{-1} min^{-1}).

The optimum conditions for enzymatic hydrolysis such as temperature, pH and time of hydrolysis were optimized using the casein as a substrate.

Effect of temperature on proteolytic activity of crude proteases

A 2% casein homogenous solution was prepared. The optimization of temperatures studied for two proteases varied from 30˚C to 70 ˚C was carried out by keeping the pH and time of hydrolysis as constant.

Effect of pH on proteolytic activity of crude proteases

A 2% casein homogenous solution was prepared. The range of pH used for different enzymes were 7.5 to 12.5. The pH of the homogenate was adjusted using either 1M HCl or 1M NaOH. The hydrolysis reaction was carried out at 55 ˚C temperature by adding the enzyme to the pre-incubated homogenate. Hydrolysis time for two proteases was constant.

Effect of time of hydrolysis on proteolytic activity of crude proteases

The time of hydrolysis chosen for two proteases in the study varied from 0 to 60 min. The proteolytic activities of visceral enzyme were studied at 55 ˚C temperatures using 2% casein as substrate at the pH of 10.5 for catla and 8.5 for rohu proteases.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic mobility under reduced condition of crude proteases was carried out by the method as described by Laemmli[16]. Electrophoresis was carried out using polyacrylamide gel slabs of 10 × 8 cm (length × width) in a vertical slab electrophoresis apparatus (Model mighty small II, SE 250 / SE 260, Hoefer Pharmacia Biotech Inc., Halliston, USA). Crude proteases (1mg/ml) were dissolved in 5% SDS solution and incubated at 85 ˚C for 60 min, followed by centrifugation at 8000 × g for 10 min at room temperature. The supernatants were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 0.03 mM of coomassive brilliant blue-G) and boiled for 3 min. Samples (10µl) were loaded onto a polyacrylamide gel made of (12% running gel and 5% stacking gel). The molecular weight of the proteins was estimated using standard wide range molecular-weight protein markers (Sigma, St. Louis, MO, USA).

RESULTS AND DISCUSSION

Effect of temperature on proteolytic activity of crude proteases

The effect of temperature of hydrolysis on the proteolytic activities of crude proteases is given in Fig.1A. The proteolytic activities of visceral enzyme were studied at different temperatures ranging from 27 to 80˚C, using 2% casein as substrate. The optimum temperature of hydrolysis for catla and rohu was 55˚C, respectively. It was found that high proteolytic activity for Liza aurata and thornback ray at temperature range of 40 - 60˚C [4, 11]. The optimal temperature of catla and rohu proteases was similar to that of Nile tilapia and red scorpion fish[17, 18]. The enzyme activity of proteases increased in reaction rates to a certain point and decreases with increase in temperature. This is due to changes in the structural conformation of protein resulting from the breakdown of the weak ionic bonding that stabilizes the three dimensional structure of the enzyme active site[19]. Klomklao *et al.,*[8] stated that enzymes were inactivated at high temperatures, possibly due to the partial unfolding of the enzyme module and thermal denaturation.

Effect of pH on proteolytic activity crude of proteases

The effect of pH on the proteolytic activities of crude proteases is given in Fig.1B. The proteolytic activities of crude proteases were assayed over the pH range of 7.5 to 12.5 at 55˚C for the incubation period of 30 min, using

2% casein as substrate. The optimum pH of hydrolysis for catla and rohu was found to be 10.5 and 8.5 respectively. It was stated that the digestive protease activity of catla, rohu, red scorpion fish and *Liza aurata* found to be high in the pH range 8.0-11.0 [7, 18, 11]. Trypsins are generally reported to be more active at alkaline pH ranging from 7.5 to 10.5 [5]. Kanno *et al.* 2010 [20] reported that the optimum pH of trypsin from masu salmon fish was found to be 8.5. Hidalgo *et al.*[21] found higher enzymatic activity at pH 8.5 for rainbow trout (*Oncorhynchus mykiss)*, gilthead seabream (*Sparus aurata*), European eel (*Anguilla Anguilla*), common carp (*Cyprinus carpio*), goldfish (*Carassius auratus)*, and tench *Tinca tinca.*

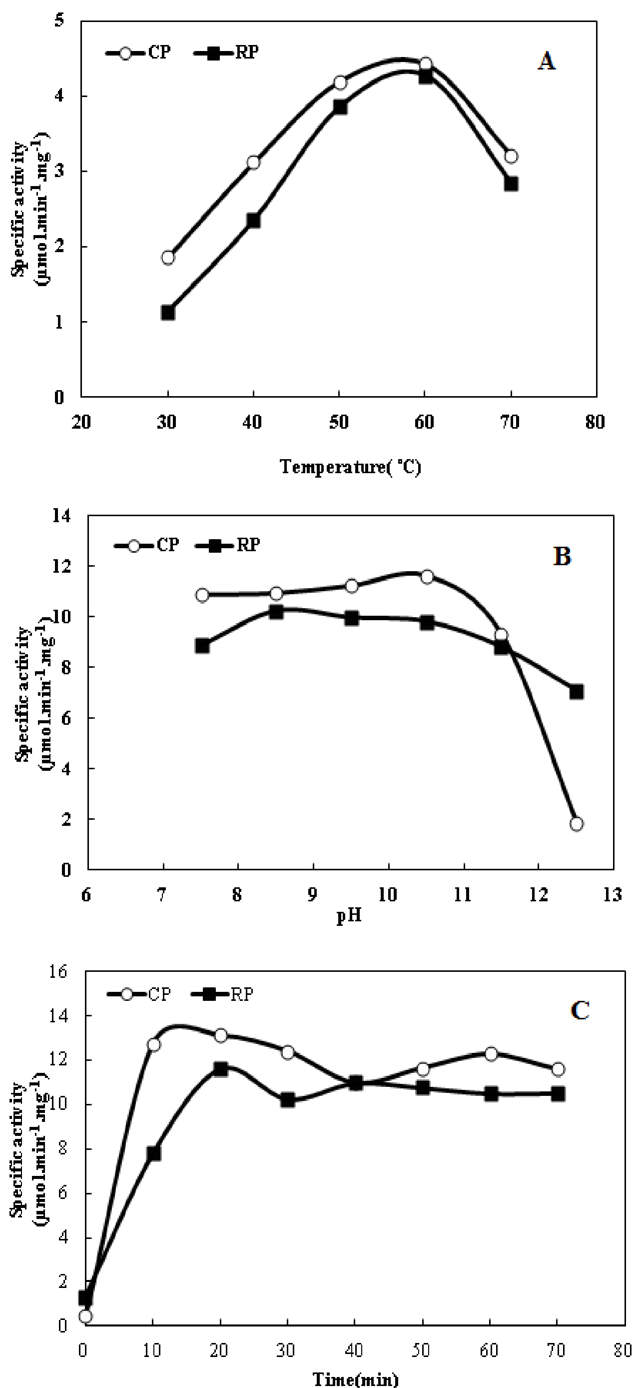
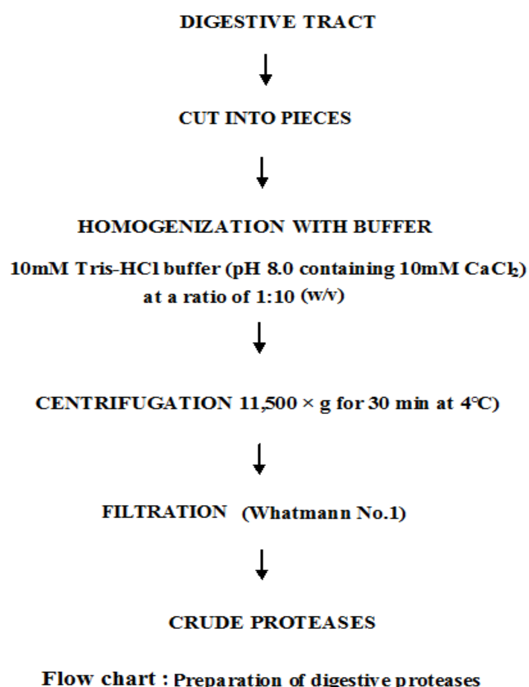


Fig.1 Effect of Temperature, pH and time of hydrolysis on proteolytic activity of Catla proteases (CP) and Rohu proteases (RP) against Casein as substrate



Flow chart : Preparation of digestive proteases

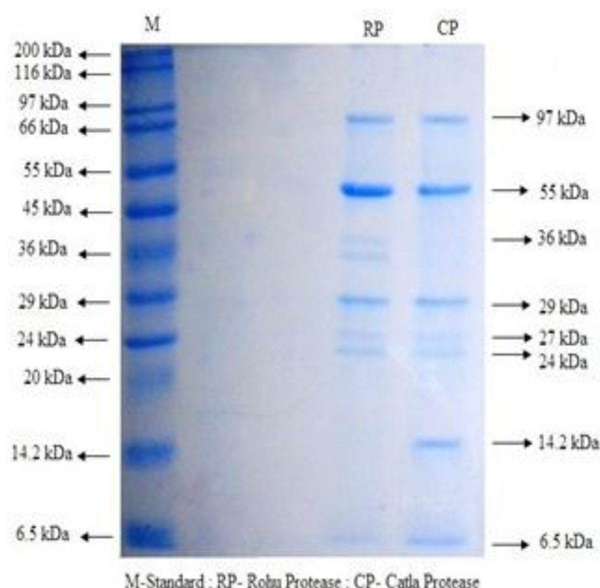


Fig. 2: SDS-PAGE of crude proteases

The optimum pH reflects the pH at which maximum number of enzyme molecules combines with the substrate to form ES (Enzyme-Substrate) complex, leading to maximal product formation and results in a bell-shaped curve [22]. However, the decreased activity at other pH may be due to the irreversible denaturation of most enzymes in very acidic or alkaline solutions since a change in pH affects both the substrate and enzyme by changing the charge distribution and confirmation of the molecules[23].

Effect of time of hydrolysis on proteolytic activity of crude proteases

The effect of time of hydrolysis on the proteolytic activities of crude proteases from two fish species are given in Fig.1C. The proteolytic activities of visceral enzyme were studied at 55˚C temperatures using 2% casein as substrate at the pH of 10.5 for catla and 8.5 for rohu. The hydrolysis of casein by crude proteases of catla and rohu increased up to 20 min. Thereafter, the hydrolysis curve remained almost constant. Hence the optimum time of hydrolysis was taken as 20 min. The different optimum time for enzymes from different sources could be due to the rate of cleavage of peptide bonds at different incubation periods[24]. The units of proteolytic activity at optimum condition for catla was 13.1 µmol mg^{-1} min^{-1} while that of rohu 11.6 µmol mg^{-1} min^{-1}. The proteases obtained from bacterial sources especially, alkaline proteases, the activity have been found to be in the range of 7.8 to µmol mg^{-1} min^{-1} from different bacterial sources [25]. The activity of crude extract in the present study appears to be promising for commercial applications.

SDS PAGE of proteases

The SDS-PAGE patterns of crude proteases are presented in Fig.2. SDS–PAGE of catla and rohu proteases showed multiple bands with different molecular masses in the range 6.5 – 97 kDa. There are certain differences in subunits of crude proteases from catla and rohu. Low molecular weight molecules in the range of 6.5 – 14.2 kDa found in protease from rohu. The molecular weight of crude proteases from GI tract of rohu has been reported in the range of 14.4 – 90 kDa [10]. The molecular weight of trypsin and chymotrypsin from catla were found to be in the range 20.3 - 30 kDa [5, 9]. The results of SDS–PAGE of present investigation may not reveal the nature of activity on active site for which further studies have to carry out.

CONCLUSION

These crude proteases from GI tract of Catla *catla* and *Labeo rohita* have been isolated and optimum conditions for hydrolysis with casein as substrate has been standardized. The optimum temperature and time of hydrolysis for catla and rohu found to be 55°C and 20 min, respectively. The pH optima for the proteases from catla were found to be 10.5 and that from rohu was 8.5 and can be considered as alkaline proteases. These alkaline proteases from fish waste (visceral organs) have potential application in food and fish processing industries. Furthermore, it is needed to purify alkaline proteases to produce protein or gelatin hydrolysates and as a possible biotechnological tool promising for commercial applications.

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