Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2020; 8(1): 1252-1258

Received: 20-11-2019
Accepted: 24-12-2019

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Partial purification and characterization of digestive acidic and alkaline proteases from the visceral waste of grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844)

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Abstract
Acidic and alkaline proteases from visceral waste of *Ctenopharyngodon idella* (Valenciennes, 1844) were isolated, partially purified by ammonium sulphate precipisstation followed by dialysis, their kinetics and characteristics studied. The crude enzyme was partially purified and its molecular weight was studied. The enzyme showed highest activity and purification-fold when precipitated at 40–60% ammonium sulfate. The purification fold increased from 1.23 to 2.49 and 1.17 to 1.51 in acidic and alkaline protease respectively along the purification steps. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed a molecular weight of 15-35 kDa and 25-63 kDa respectively in acidic and alkaline proteases. The pH and temperature optima for acidic and alkaline proteases were 3 and 10, at 40 °C and 60 °C respectively. The acidic and alkaline protease activity was decreased by 40% and 60%, when incubated at 90 °C for 30 min. Degree of hydrolysis (DH) of the proteases on muscle protein increased with increase of enzyme concentrations. The study showed that proteases from Grass carp visceral waste of could find use in applications where maximum activity at moderate temperature is desired.

Keywords: *Ctenopharyngodon idella*, acidic proteases, alkaline proteases, enzyme purification fold

1. Introduction
Proteases constitute one of the most valuable groups of industrial enzymes used in the world today and it has multiple applications in the food industry [2]. The Protease enzymes are chiefly derived from plant, animal and microbial sources, whereas their counterparts, derived from marine and other aquatic sources, have not been extensively used. In recent years, additional applications of proteases in the seafood industry have been emerged. These include the selective removal of skin, hydrolysis of membranes and other supportive tissue that envelope roe, roe seeks and other tissues, and recovery of pigments and flavour extract [3]. There have been relatively few attempts to use fish proteases as industrial processing aids. Fish are poikilothermic and vary considerably in their feeding habits and temperature preferences, and so it is expected that their digestive enzymes will also exhibit diversity [4].
Amongst the hydrolytic enzymes, proteases represent an important class of industrial enzymes; have been employed in different applications, mostly in food, detergent, textile, leather and pharmaceutics as well as in waste management and bioremediation process [4, 5]. However, proteases require their purification and characterization before any application. Proteases contribute about 60% of the world's total enzyme production and used worldwide [6]. Presently, most of the proteolytic enzymes are extracted from bacteria, and relatively few attempts have been made on the application of fish proteases as industrial processing aids. Usually, the fishery by-products are typically used as feeds and fertilizers. Recently, interest has grown to search high-value functional bio-molecules from the fishery wastes, notably enzymes. Nevertheless, several researchers investigated proteases from the visceral wastes from marine fish [7, 8]. But characterization of fish proteases especially from the visceral wastes of freshwater fish is seldom reported.
The grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) [1] is the species of fish with the largest reported production in aquaculture globally, over five million tonnes per year. It is a large herbivorous freshwater fish species of the family Cyprinidae native to eastern Asia. A huge quantity of visceral wastes is generated in the retail fish markets due to pre-processing. Such biological wastes, if not utilized otherwise, would pose a problem of their disposal and

subsequent environmental pollution. Fish processing wastes is about 30% of the whole fish and comprised of head, scales, skins and viscera [9] and is considered as an excellent source of protein and bio-active peptides [10]. Although there is scope to recover proteins and enzymes from the visceral wastes of fish, but huge quantity of such waste is discarded without any such attempt [11]. According to Bezerra *et al.* [12], the fish visceral waste usually accounts for 5% of the total mass and includes stomach, pyloric caeca, intestines, liver, pancreas and so on and other organs like spleen and gonads. The digestive enzymes from the fish visceral waste are highly active over a wide range of pH and temperature conditions, and thereby represent an important valued by-product of fishing industry [13].

Based on the above rationale, the present study was carried out to characterize partially purified acidic and alkaline proteases from the fish visceral wastes for determining their application in food processing operations as well as to reduce waste disposal problems. Grass carp (*Ctenopharyngodon idella*), predominantly a column feeder and feeds mainly on filamentous algae, decomposed vegetation and mud was selected for study as it is most commonly consumed freshwater fish in India amongst the carps.

2. Materials and Methods

2.1 Grass carp viscera

Viscera of Grass carp was collected from the local markets at Agartala city, packed in polyethylene bags and transported with ice. In the laboratory, viscera was washed with chilled water in order to remove the adherent blood, slimes and dirts, kept in plastic bags and stored at -20 °C until used for enzyme extraction.

2.2 Preparation of crude acidic and alkaline protease

Method suggested by Vannabun *et al.* [14] was followed for preparation of crude acidic and alkaline proteases. Initially the visceral mass was thawed and homogenization was done for 2 min with different extraction buffers, such as citrate buffer (10mM Citrate/HCl pH 3.0) for acid protease and tris buffer (10mM Tris–HCl pH 8.0, 10mM $CaCl_2$) for alkaline protease, in the ratio of 1:5(w/v). The homogenate was centrifuged at 10,000 x g for 10min at 4 °C. After homogenization, the pellet was discarded to collect the supernatant which was used as 'crude enzyme extract'.

2.3 Enzyme purification

Crude enzyme extract was subjected to two-step $(NH_4)_2SO_4$ precipitation. As per preliminary assay, $(NH_4)_2SO_4$ concentration of 40-60% gave the highest purification fold and specific activity. The crude enzyme was precipitated with 40-60% saturation of ammonium sulphate and then allowed to settle for 24h at 4 °C. The supernatant was discarded and the precipitate was dissolved in 0.02 M acetate buffer, pH 3.0 and 0.02 M Tris–HCl buffer, pH 8.0 for acidic and alkaline proteases respectively, by centrifugation at 10,000×g for 30 min at 4 °C. The enzyme thus obtained was dialyzed against the same buffer for 24 h at 4 °C with intermittent change of buffer after 12 h. After dialysis, the crude enzyme was referred as 'partially purified proteases'.

2.4 Determination of molecular weight

The molecular weight (MW) of partially purified enzyme was carried out by SDS-PAGE, following the method suggested by Laemmli [15]. A sample buffer was prepared by mixing 2.5 ml 0.5 M Tris-HCl (pH 6.8), 4 ml 10% SDS, 2 ml glycerol, 1 ml 1% b-mercaptoethanol, 0.03 ml 0.002% bromophenol blue and the final volume was made to 10 ml. Protein solutions were mixed at a 1:2 (v/v) ratio and boiled for 10 min. Samples (10 µl) were loaded on the gel made of 4% stacking and 12.5 % separating gels and fractionated for 90 min at a constant current of 400 mA. After electrophoresis, the gels were stained with 0.05g Coomassie brilliant blue R-250 in 15% methanol and 5% acetic acid, and destained with destaining solutions [solution-1 (50% methanol and 7.5% acetic acid) and solution-2 (5% methanol and 7.5% acetic acid)]. The molecular weight was estimated using protein standard (10-245kDa) (HiMedia, India).

2.5 Protein content

The protein content was estimated following Lowry's method [16] by measuring sample absorbance at 280 and 260 nm, using bovine serum albumin as standard.

2.6 Assay of protease activity

The acidic protease activity was determined as suggested by Natalia *et al.* [17] using 2% bovine haemoglobin solution containing 0.04M HCl (acid denatured) as substrate at pH 3.0 and 37 °C, whereas, method of Rawdkuen *et al.* [18] was followed to determine alkaline protease activity using casein as a substrate. The absorbance read at 280 nm and converted into µmoles of tyrosine liberated using solutions of 25-250 µg/ml concentration of tyrosine for calibration curve. Enzymatic activity was expressed as one unit equivalent to the amount of enzyme capable of hydrolyzing bovine haemoglobin to liberate 1 µmole tyrosine under standard assay conditions. Total activity and specific activity was expressed as units of enzymatic activity per ml protein (U/ml) and per mg protein (U/mg) respectively.

2.7 Total activity

The total enzymatic activity was estimated using the following equation.

$$\text{Total Activity } \left(\frac{\text{U}}{\text{ML}}\right) \frac{\text{µmole of tyrosine equivalent released} \times \text{Total Volume of reaction assay (mL)}}{\text{Volume of enzyme used (mL)} \times \text{Time of assay(min)} \times \text{Volume used in Cuvette (mL)}}$$

2.8 Specific activity of enzyme

The specific activity of both the enzymes was determined using the equation as suggested by El-beltagy *et al.* [19].

$$\text{Specific activity } \left(\frac{\text{U}}{\text{mg}}\right) = \frac{\text{Total activity } \left(\frac{\text{U}}{\text{mL}}\right)}{\text{Protein content } \left(\frac{\text{mg}}{\text{mL}}\right)}$$

2.9 Enzyme purification fold

The level of purification was evaluated by determining the purification fold following the equation given by El-beltagy *et al.* [19].

$$\text{Purification fold} = \frac{\text{Specific Activity}}{\text{Specific Activity of crude extract}}$$

2.10 Optimum pH and pH stability
The optimum pH for enzymatic activity was determined following the method of Vannabun *et al.* [14], by assaying protease activity at different pH conditions using 100mM buffer solutions ranging from pH 1.0 to 12.0 (Glycine- HCl buffer for pH (1.0-3.0); sodium acetate buffer for pH (4.0-6.0); Tris-HCl buffer for pH (7.0–9.0); and Glycine–NaOH buffer for (9.0-12.0), at the optimum temperature for activity previously determined. The effect of pH on enzyme stability was determined by the method of Vannabun *et al.* [14]. The enzyme was incubated at various pH (1.0–12.0) using different buffers of 100mM Glycine–HCl (1.0–3.0), Na-acetate (4.0–6.0), Tris–HCl (7.0–9.0) and Glycine–NaOH (10.0–12.0) for 30 min along with the blanks prepared simultaneously. The residual enzymatic activity after incubation was evaluated and compared with the condition that showed the highest value (100% activity).

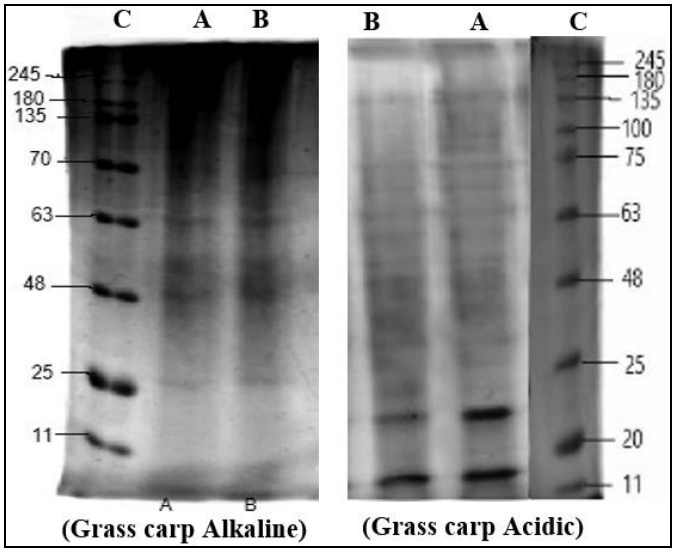
2.11 Optimum temperature and temperature stability
Protease activity at different temperatures (30–90°C) was performed by using different buffers like Glycine–HCl (pH 3.0) and Tris–HCl (pH 8.0) for acidic and alkaline protease activity respectively according to the method given by Vannabun *et al.* [14]. To determine thermal stability of proteases, enzyme extract was incubated for various time durations like 1,3,5,10,15,20,30,40,50, and 60 min at 90°C and the remaining enzymatic activity was determined. The control was not pre-incubated and considered as 100% activity.

2.12 Effect of isolated protease enzymes on proteins hydrolysis
Extracted acidic and alkaline proteases were used to hydrolyze the ground fish muscle protein to determine the degree of hydrolysis of enzyme on the fish muscle. The ground muscle (2g) was incubated with enzyme at different concentrations (10-50 mL) for 30 minutes at 60°C. The reaction was stopped by adding 5 mL of 20% TCA followed by centrifugation at 3300 rpm for 10 minutes to collect the 10% TCA soluble material as the supernatant. The protein content of the supernatant was estimated by Biuret method. The degree of hydrolysis was determined by the method of Hoyle and Merritt [20].

$$\% \text{ DH} = \frac{\text{10\% TCA soluble protein in the sample}}{\text{Total protein content of sample}} \times 100$$

2.13 Statistical Analysis
Analysis of variance (ANOVA) followed by Duncan's multiple range test was carried out to determine differences between means. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows version 16.0, SPSS, Inc., Chicago, IL).

3. Results and Discussion
3.1 Partial purification of proteases
The protein content, total activity, specific activity and purification fold for acidic and alkaline proteases of visceral waste of Grass carp is presented in Table 1. The average protein content was found to be 6.31 mg/ml and 7.79 mg/ml in acidic and alkaline crude proteases respectively. After ammonium sulphate fractionation (40-60%), the protein content decreased in the crude proteases and the values reached to 3.72 mg/ml and 4.15 mg/ml in acidic and alkaline crude proteases respectively. The saturated ammonium sulphate solution selectively precipitates proteins from the crude enzyme extract by the salting-in and salting-out mechanism to form a partially purified enzyme extract [9]. This may be due to the impurities present in the crude sample which are removed after ammonium sulphate precipitation. Dialysis, a step in the purification of proteases, exhibited a further decline of protein content in the $(NH4)_2SO_4$ precipitated proteases. In acidic and alkaline proteases the average protein content decreased to 1.68 mg/ml and 2.96 mg/ml respectively. Such decrease of protein content after dialysis may be due to further removal of other proteins, not removed by ammonium sulphate fractionation.

3.2 Molecular weight of proteases


*Where C- Protein marker, B- Purified enzyme extract, A- Crude enzyme extract

Fig 1: Bands Showing Different Molecular Weight of Protease with Reference to Protein Marker

The electrophoretic pattern showed several clear bands indicating the presence of different proteases of varying molecular mass in case of both alkaline and acidic protease samples (Fig. 1). In case of crude and partially purified acidic and alkaline proteases 3-4 bands were observed ranging from 15-35 kDa and 25-63 kDa respectively. Several authors reported the molecular weights of visceral alkaline and acidic proteases in the range of 17-90 kDa. Molecular weights of fish visceral alkaline proteases have been reported as 23.5 kDa [12], 23-28 kDa [21], 23 kDa [22], and 24-30 kDa [23]. The present study revealed that the molecular weights of alkaline proteases are higher compared to acidic proteases. Presence of several bands in the electrophoretic separation of digestive proteases was explained as due to constituent enzymes like trypsin, chymotrypsin, collagenase, gastricin, pepsin, elastase, carboxypeptidase and carboxyl esterase [24], and also due mostly to the different molecular weights of individual enzyme. The present study justified the observations reported by earlier researchers in the context of molecular weight distribution of digestive proteases [25, 26].

3.3 Assay of proteolytic activity
The average total activity of crude acidic and alkaline Grass carp viscera waste was determined to be 18.33 U/ml and

34.11 U/ml respectively. Total proteolytic activity reduced after $(NH_4)_2SO_4$ fractionation (ASF) and further reduction took place after dialysis. In case of alkaline proteases the recovery percentage was found to be 63.67 and 58.95 after ASF and dialysis respectively (Table 1). Similar was observed in case of acidic proteases, wherein, recovery of total activity after ASF and dialysis was found to be 72.76 and 66.28 respectively (Table 1). Purification might have removed other catheptic enzymes which were probably present in the visceral waste, and resulted decrease of the total activity. Such decrease of protease activity after purification was also reported [27, 28].

Table 1: Purification of acidic & alkaline proteases from visceral waste of Grass carp

	Purification steps	Protein Content (mg/mL)	Total Activity (U/mL)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
Acidic Protease	Crude	6.31±0.08	18.33±0.06	2.90±0.05	100	1
	Ammonium Sulfate Fractionation (40-60%)	3.72±0.04	13.37±0.02	3.59±0.01	72.76	1.23
	Dialysis	1.68±0.05	12.15±0.28	7.23±0.15	66.28	2.49
Alkaline Protease	Crude	7.79±0.06	34.11±0.11	4.38±0.01	100	1
	Ammonium Sulfate Fractionation (40-60%)	4.15±0.03	21.72±0.76	5.23±0.04	63.67	1.17
	Dialysis	2.96±0.03	20.11±0.61	6.79±0.07	58.95	1.51

*Values given in the table are means ± SD, n=3.

The average specific activity after dialysis was found to be 6.79 and 7.23 in case of alkaline and acidic proteases respectively. Such increase of specific activity along the purification steps may be explained as the removal of interfering proteins during $(NH_4)_2SO_4$ fractionation and further during dialysis, resulting enhanced activity. Increase of specific activity with the progress of the purification was also reported by Liu *et al.* [29], Bezerra *et al.* [12] and El-Beltagy *et al.* [22]. This study also revealed that the specific activity of post dialysis acidic proteases was more than the alkaline proteases. Since the Grass carp being lacking of true stomach, might be the reason for low amount of acidic proteases secretion in the gut content of viscera, as stomach constitutes an important source of digestive proteolytic enzymes [30].

The specific activity of the enzyme determines the purification fold. In the two-step purification system, the purification fold experienced an increase from step two to step three in case of both acidic and alkaline proteases. In case of alkaline proteases the result showed that increase in purification fold was from 1.17 to 1.51, whereas, it was 1.23 to 2.49 in case of acidic proteases. Increase of purification fold following dialysis has also been reported by El-Beltagy *et al.* [22] and Liu *et al.* [29]

3.4 Optimum pH and pH stability

Partially purified acid and alkaline proteases was found to be active over a range of pH 1.0–12.0 using casein and acid-denatured bovine haemoglobin as substrates for alkaline and acidic proteases respectively. The acidic protease exhibited high activity in the pH range from 2-4 with an estimated maximum at 3.0 and then decreased significantly ($p<0.05$) with increasing pH (Fig. 2). Relative activity of about more than 50% was lost over pH 4. In other study, the optimum pH for hydrolysis of acid denatured bovine haemoglobin by partially purified acidic protease from *Tilapia nilotica* was found to be 2.5 [22]. Our results corroborate well with the observation of Bougatef *et al.* [31], who reported pH optima for acidic proteases in the range of 2-4.

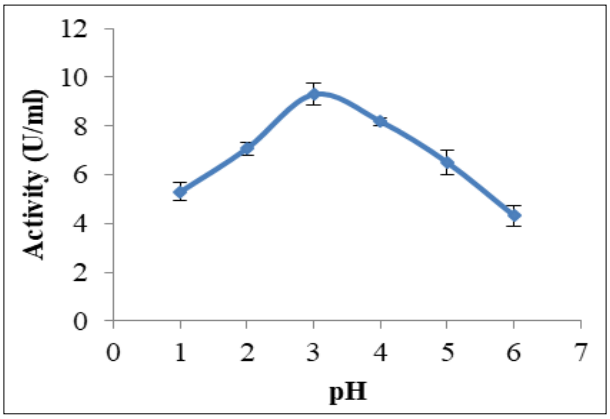


Fig 2: Optimum pH of Acidic Protease

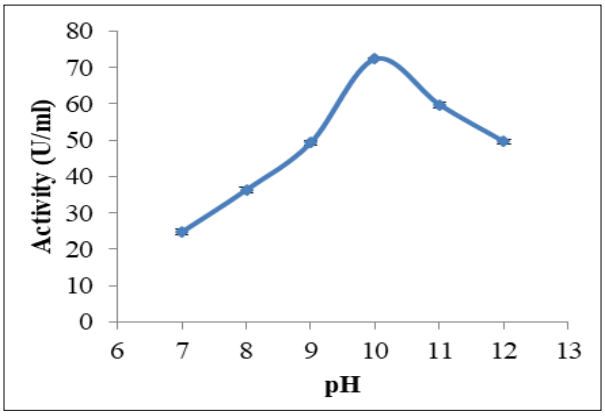


Fig 3: Optimum pH of Alkaline Protease

The alkaline protease exhibited maximum activity at pH 10, and then decreased significantly at higher pH levels (Fig. 3). Optimum pH for maximum activity of alkaline protease was reported in the range of 8-10 [8]. Determination of pH optima of an enzyme is very essential as this is considered to be an important indicator for its potential application in different purposes.

Both the acidic and alkaline proteases were highly stable over a wide pH range, maintaining more than 90% of its original activity between pH 1.0-5.0 and pH 8.0-12.0 in respect of acid and alkaline proteases respectively after 30 minutes incubation at 37°C (Fig. 4, 5). The pH stability of proteases depends on the differences in molecular properties, which includes bonding and stability of the structure; conformation of enzyme in different anatomical locations amongst various species [32]. Similar findings regarding pH stability of acidic protease from fish has also been reported by Castillo-Yanez *et al.* [13] for Monterey sardine. Acidic protease activity showed a decrease of about 15-20% at pH over 6.0 whereas; a similar decrease was shown by alkaline protease at pH below 7.0.

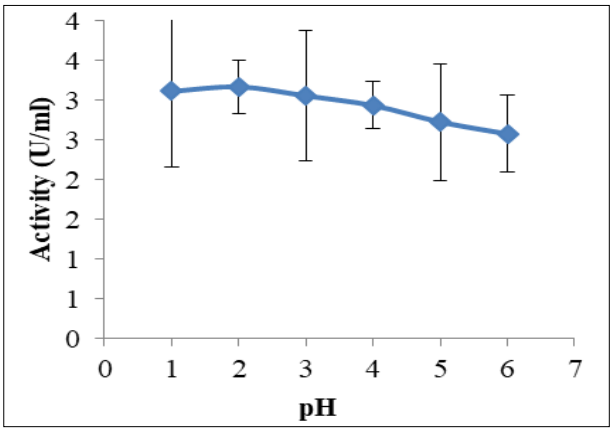


Fig 4: pH stability (Acidic Protease)

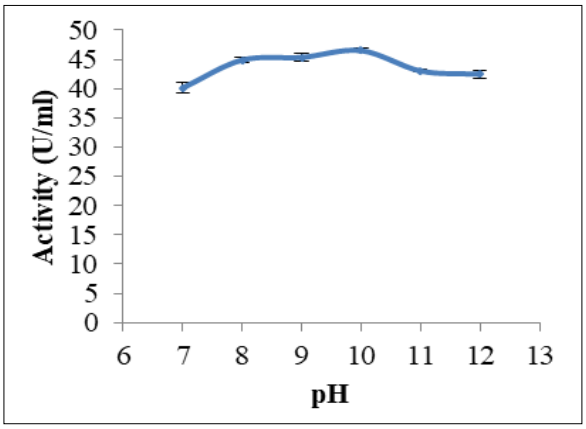


Fig 5: pH stability (Alkaline Protease)

3.5 Optimum temperature and thermostability

In this study, the optimum activity of acidic protease was found at 40°C (Fig. 6) that is similar to the earlier reports from other fish, viz., pepsins from *Sardinelle* spp. by Ben Kahled *et al.* [33] and smooth hound by Bougatef *et al.* [31]. The optimal temperature of alkaline protease activity was found as 60°C (Fig. 7) and the similar result was reported by Klomklao *et al.* [34] and Cao *et al.* [35] for trypsin from the pyloric caeca of Chinook salmon (*Oncorhynchus tshawytscha*) and Japanese seabass (*Lateolabrax japonicas*) respectively. The result showed that the enzyme activity of proteases increased to a certain point followed by a decrease with increase in temperature forming a bell shaped curve. At temperature above optimum the native conformation of protein is changed due to breakdown of weak intramolecular bonds responsible of stabilization of three dimensional structure of the enzyme active site [34]. As opined by Klomklao *et al.* [9], environmental and genetic factors among the different species might be responsible for the native conformations of enzymes.

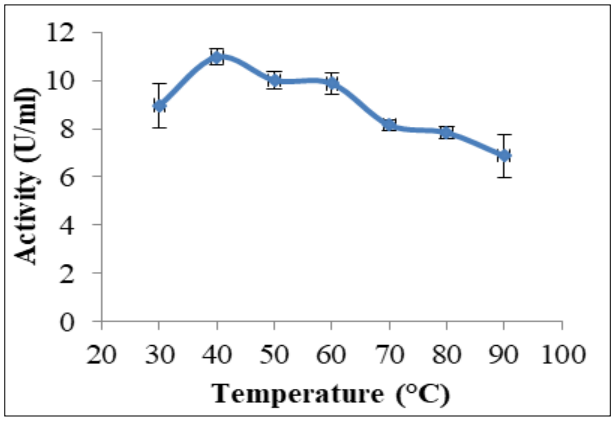


Fig 6: Optimum Temperature (Acidic Protease)

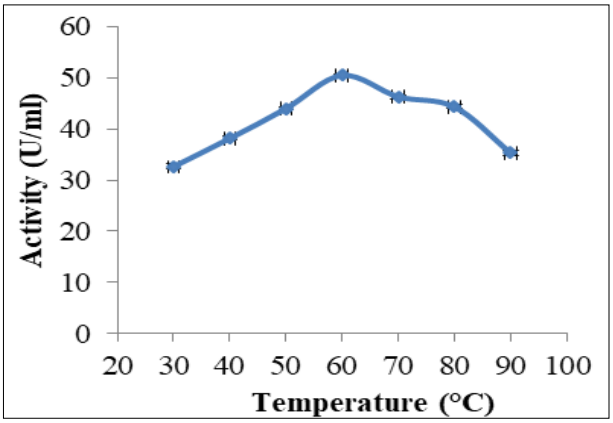


Fig 7: Optimum Temperature (Acidic Protease)

The study also revealed that acidic and alkaline proteases' activity decreased by 40 and 60% respectively when the incubation condition was 90 °C for 30 min (Fig. 8 & 9). This may be explained as the inactivation of enzymatic activity following stretching out of the enzyme's native conformation during thermal treatment [34]. Vannabun *et al.* [14] also reported similar findings while characterizing visceral acidic and alkaline proteases of farmed giant cat fish. As proposed by Sabtecha *et al.* [36], stability of a fish enzyme in different temperature is influenced by their habitat, environment and genetic characters.

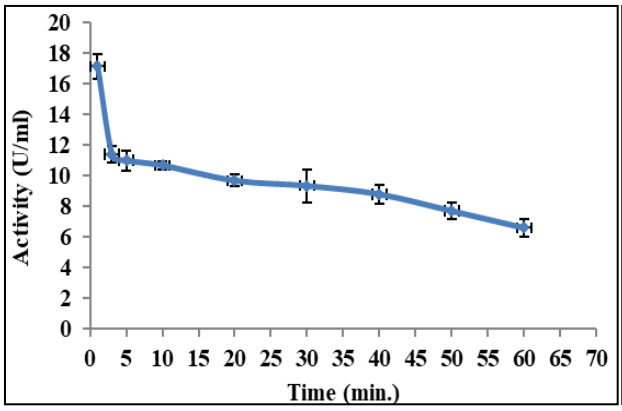


Fig 8: Thermostability (Acidic Protease)

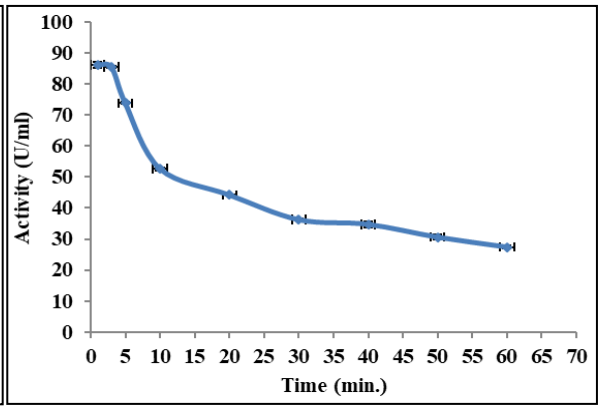


Fig 9: Thermostability (Alkaline Protease)

3.6 Effect of isolated enzymes on proteins hydrolysis

As degree of hydrolysis (DH) is the indicative of the extent of peptide bonds broken down [37], its determination is crucial since several characteristics of protein hydrolysates is DH dependent. Using ground muscle meat of fish as substrate, hydrolysis was conducted at temperature 37°C and optimum pH for both the enzymes. The degree of hydrolysis (DH) as a function of the enzyme concentration is given in Fig. 10 & 11. The result of this study signifies that higher amount of proteases in enzyme fraction cleaved more peptide bonds and similar observation was also reported by Klompong *et al*. [38].

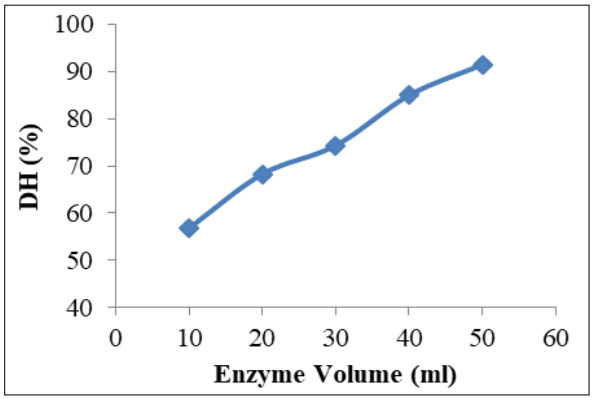


Fig 10: Degree of hydrolysis (Acidic Protease)

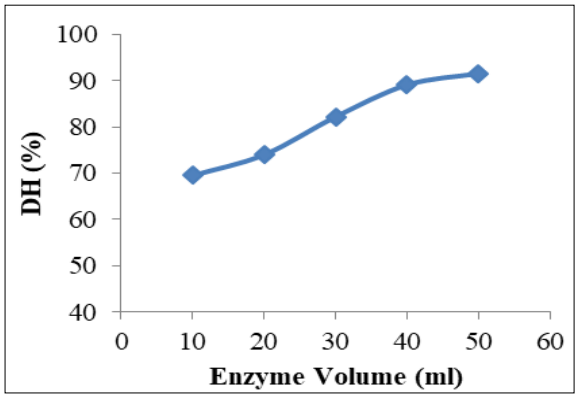


Fig 11: Degree of hydrolysis (Alkaline Protease)

4. Conclusion

This study has revealed that considerable amounts of acidic and alkaline proteases are present in the visceral waste of Grass carp fish, and those in purified form have potential for application as different food processing aids, and on the other hand, would contribute to solve bio-waste disposal problem to a great extent. Both the proteases exhibited substantial activity in both acidic and alkaline conditions. As a requirement for their application purpose, the maximum activity of acidic and alkaline protease was found to be at 40°C and 60°C respectively. Nevertheless, the stability of these enzymes at elevated temperature was not found to be satisfactory. Based on the present study, the enzymes from Grass carp visceral waste could find use in applications where maximum activity at moderate temperature is desired.

5. Acknowledgements

The authors would like to thank Dean, All the staff of the Dept. of Fish Processing Technology, College of Fisheries (CAU), Lembucherra, Tripura (W), India for their technical support to conduct this research.

6. Practical applications

Recently proteases are gaining its successful application in different industrial fields, mostly in food, detergent, textile, leather and pharmaceutics as well as in waste management and bioremediation processes. Extraction and utilization of proteases from fish visceral wastes bear a promising potential in substituting the proteases of bacterial origin. In this way, utilization of fish visceral wastes as a source of proteases would minimize the major bio-pollutants generating during retailing of fish, and on the other hand, would be the judicious economic use of the wastes. In this aspect, characterization of the proteases is utmost essential for their selection for specific application. This study would provide the basic characteristics of the proteases from the fish visceral wastes which would be helpful for their application-specific uses.

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