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Characterization of digestive acidic and alkaline proteolytic enzyme (proteases) from the visceral wastes of Chinese major carp, *Cyprinus carpio* (Linnaeus, 1758)

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Abstract
Acidic and alkaline proteases from visceral waste of *Cyprinus carpio* (Linnaeus, 1758) were extracted, partially purified by ammonium sulphate precipitation followed by dialysis. Their enzyme kinetics and characteristics were studied. The purification fold increased from 1.39 to 2.24 and 1.46 to 2.17 in acidic and alkaline protease respectively along the purification steps. The molecular weights were found in the range 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 4 and 11, and 40°C and 70°C respectively. The protease activity was found decreased by 40 and 60% when incubated at 90°C for 30 min. Both the proteases showed a declining activity of more than 50% at NaCl concentration of 0.5%. Degree of hydrolysis of the proteases on muscle protein increased with increase of enzyme contents. Both soybean trypsin inhibitor and EDTA exhibited high percentage of inhibition when proteases were incubated with 50 mM of both. Enzyme activators like NaCl and $CaCl_2$ (10mM) increased the activity of acidic protease by 18.56% and 29.11% respectively. The study showed that proteases from Common carp visceral waste could find use in Industrial applications.

Keywords: *Common carp*, visceral waste, acidic protease, alkaline protease, purification fold, enzyme kinetics

1. Introduction
Common carp (*Cyprinus carpio*) is considered as the most commonly consumed freshwater fish in India among the Chinese major carps. The adult of it is predominantly a bottom feeder and feeds mainly on decomposed vegetation settled down in the pond bottom. The fish's pre-processing during retailing generates huge quantity of visceral wastes, which if not utilized otherwise, would impose a threat to the environment so far biological pollution is concerned. Fish processing wastes, i.e., head, scales, skins and viscera, constitutes about 30% of the original raw material of fish [1] and an important source of protein and bio-active peptides [2]. These materials are also recognized as potential source of commercially valued products such as enzymes, which are used as processing aids in the manufacturing of food products to improve their quality, solubility and stability. Most analytical studies in enzymology have focused on the mammal or microorganism as source of the enzymes mainly, because samples from these two types of organisms are easy to obtain. 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 [3]. The fish visceral waste enzymes, especially the digestive ones are highly active over a wide range of pH and temperature conditions, and thereby constitute an important by-product of fishing industry [4].
Proteases belong to the hydrolytic group of enzymes and have been employed in different industrial applications, predominantly in food, detergent, textile, leather and pharmaceutics as well as in waste management and bioremediation process [5, 6]. Around 60% of the world's total enzyme production is contributed by the proteases and used worldwide [6]. Although, proteases are the highest sold in the enzyme market, but presently, most proteolytic enzymes are extracted from bacteria and other microorganisms. Therefore, finding of alternative sources of proteases with novel properties for wide industrial applications is a need of the day. Isolation and characterization of fish proteases have been reported but studies on the proteases from visceral wastes of freshwater fish are seldom reported.

Nevertheless, several researchers investigated proteases from the intestinal wastes from marine fish [7, 8]. The objective of this study was to characterize partially purified acidic and alkaline proteases from the visceral wastes of Common carp (*Cyprinus carpio*) for finding applications in food processing operations.

2. Materials and Methods

2.1 Raw Common carp viscera

Viscera of Common carp (*Cyprinus carpio*) was collected from the local retail fish markets at Agartala city (Tripura, India), packed in polyethylene bags, placed in ice while transportation. In the laboratory, viscera was washed properly with chilled water in order to remove the adherent blood, slimes and dirts, and used for enzyme extraction in the same day.

2.2 Preparation of crude acidic and alkaline protease

Crude acidic and alkaline proteases were prepared following the method suggested by [9]. The raw visceral mass was homogenized for 2 minutes with extraction buffers (10mM Citrate/HCl pH 3.0 for acid protease and 10mMTris–HCl pH 8.0, 10mM $CaCl_2$ for alkaline protease) in the ratio of 1:5(w/v). The obtained homogenate was then centrifuged at 10,000 xg for 10min at 4°C. The pellet was discarded and the supernatant was collected and used as 'crude enzyme extract'.

2.3 Enzyme purification

Crude enzyme extract obtained was subjected to two-step $(NH_4)_2SO_4$ precipitation method. First of all, the crude enzyme was precipitated with 40-60% saturation of ammonium sulphate $(NH_4)_2SO_4$ and then allowed to settle for 24h at 4°C. The supernatant obtained 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 (SDS-PAGE)

The molecular weight (MW) of partially purified enzyme was carried out by SDS-PAGE, following the method suggested by Laemmli (1970) [10]. 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 (enzyme) 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 Measurement of protein

The protein content of enzyme sample was estimated following Lowry's method [11] by measuring sample absorbance at 280 and 260 nm, using bovine serum albumin (BSA) as standard.

2.6 Assay of protease activity

The acidic protease activity was determined as suggested by Natalia *et al.* (2004) [12] using 2% bovine haemoglobin solution containing 0.04M HCl (acid denatured) as substrate at pH 3.0 and 37°C. Alkaline protease activity was determined by using casein as a substrate according to the method of Rawdkuen *et al.* (2010) [13]. The absorbance read at 280 nm was converted into µmoles of tyrosine liberated using a calibration curve. A standard curve was generated using solutions of 25-250 µg/ml concentration of tyrosine. One unit of enzymatic activity was defined as 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 Optimum pH and pH stability

The optimum pH for enzymatic activity was determined following the method of Vannabun *et al.* (2014) [9]., 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 evaluated by the method of Vannabun *et al.* (2014) [9].. 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 activity after incubation was determined and compared with the condition showed the highest value to determine relative activity (100% activity).

2.8 Optimum temperature and temperature stability

The 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.* (2014) [9].. The thermal stability of protease enzymes was determined by incubating enzyme extract for various time durations like 1, 3, 5, 10, 15, 20, 30, 40, 50, and 60 min at 90°C followed by determination of remaining activity. The blank (non-heated enzyme) was considered to be the control (100% Activity).

2.9 Effect of NaCl concentration on enzyme activity

Pure NaCl was added at desired quantity into the reaction mixture to obtain the final concentrations of 0–2.5% (w/v). The residual activity was determined for the treatment as well as for the blanks that were prepared simultaneously. The enzyme without NaCl was considered as control or blank (100% Activity) (Vannabun *et al.*, 2014) [9].

2.10 Effect of isolated enzymes on proteins hydrolysis

The 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 reaction was started by incubating the ground muscle sample (2g) completely mixed with enzyme at different concentrations (10-50 mL) for 30 minutes at 60°C. After incubation the reaction was stopped by addition of 5 mL of

20% TCA followed by centrifugation at 3300 rpm for 10 minutes to collect the 10% TCA soluble material as the supernatant. After centrifugation the protein content of the supernatant was estimated by Biuret method. Then the degree of hydrolysis was determined by the method of Hoyle and Merritt (1994) [14].

2.11 Inhibitors and activators
The effects of some inhibitors, such as soybean trypsin inhibitor (SBTI) and ethylenediaminetetraacetic acid (EDTA), as well as activators, such as $CaCl_2$ and NaCl at different concentrations (10, 20, 30, 40 and 50 mM), on the enzyme activity were determined by pre-incubation with the substrates for 10 min at the optimum temperature of enzyme. The enzyme was added and the activity was determined as described above. Inhibition and activation of enzyme activity were expressed as a percentage of the activity without modifiers.

2.12 Statistical Analysis
All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's multiple range test. 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 estimated protein content, total activity, specific activity and purification fold for acidic and alkaline proteases of visceral waste of Common carp is presented in Table 1. The average protein content was found to be 8.64 mg/ml and 9.01 mg/ml in crude acidic and alkaline proteases respectively. After the ammonium sulphate $[(NH_4)_2SO_4]$ fractionation (40-60%), the protein content was found to be decreased and the values reached to 4.83 mg/ml and 5.48 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 [15]. This may be due to the impurities of other enzymes apart from proteases, present in the crude samples which are removed after ammonium sulphate precipitation.

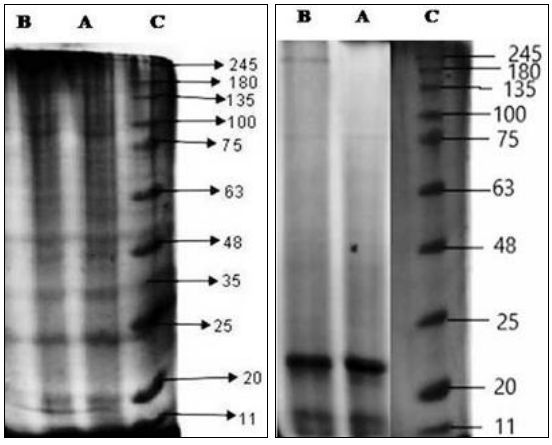
Table 1: Purification of acidic & alkaline proteases from visceral waste of Common carp

	Purification steps	Protein Content(mg/mL)	Total Activity (U/mL)	Specific Activity (U/mg)	Recovery (%)	Purification Fold
Acidic Protease	Crude	8.64±0.06	39.48±0.17	4.56±0.02	100	1
	Ammonium Sulfate Fractionation (40-60%)	4.83±0.07	30.85±0.24	6.38±0.05	78.14	1.39
	Dialysis	2.79±0.01	28.56±0.54	10.23±0.21	72.34	2.24
Alkaline Protease	Crude	9.01±0.09	59.02±0.14	5.26±0.02	100	1
	Ammonium Sulfate Fractionation (40-60%)	5.48±0.05	42.01±0.90	7.66±0.16	71.17	1.46
	Dialysis	3.11±0.07	35.60±0.38	11.44±0.51	60.31	2.17

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

In dialysis step, the purification of proteases showed a further decline of protein content in the $(NH4)_2SO_4$ precipitated proteases. In partially purified acidic and alkaline proteases the average protein content decreased to 2.79 mg/ml and 3.11 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
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 protease in the range of 17-90 kDa. El-Beltagy *et al.* (2004) [16] reported that molecular weight of acidic protease from *Tilapia nilotica* was found to be 31.0 kDa after gel filtration on Sephadex G-100. Lopez-Liorca (1990) [17] reported that the acidic protease from viscera of fish had a molecular weight around 32 kDa. The molecular weight of acidic proteases was also reported as 28.5 kDa [18].



(Common carp Alkaline**)** **(**Common carp Acidic**)**

Fig 1: (Electrophoretic pattern of crude and partially purified alkaline and acidic protease isolated from the viscera of Common carp**)** *Where C- Protein marker, B- Purified enzyme extract, A- Crude enzyme extract

The molecular weight of alkaline proteases from fish viscera was reported as 23.5 kDa [3], 23-28 kDa [19], 23 kDa [16], 24-30 kDa [20]. It is revealed from this study, 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 [21], 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 [22, 23].

3.3 Assay of Proteolytic Activity

The average total activity of crude acidic and alkaline Common carp viscera waste was determined to be 39.48 U/ml and 59.02 U/ml respectively. Total proteolytic activity was 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 71.17% and 60.31% 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 78.14 and 72.34 respectively (Table 1). Purification might remove other catheptic enzymes those were probably present in the visceral waste, and resulted decrease of the total activity. Such decrease of protease activity after purification was found similar to that reported by several authors before [24, 25].

The average specific activity after dialysis was found to be 11.44 U/mg and 10.23 U/mg in case of alkaline and acidic proteases respectively. Such increase of specific activity along with 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 several researchers [18, 3, 16]. This study also revealed that the specific activity of acidic proteases post-dialysis was more than the alkaline proteases, although, the later showed more total activity and also pre-dialysis specific activity compared to the acidic one. Since the Common 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 [26].

The specific activity of the enzyme also further determines the purification fold. In this two-step purification system, the purification fold exhibited 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 1.46 to 2.17, whereas, it was 1.39 to 2.24 in case of acidic proteases. Increase of purification fold following dialysis has also been reported by several researchers [18, 16]. A 2.7 fold increase in purity of trypsin-like enzyme from anchovy digestive tract after ammonium sulphate precipitation (20-70%) was also reported by Martinez *et al.* (1988) [27].

3.4 Optimum pH and pH stability

Partially purified acid and alkaline proteases was found to be active over a pH range of 1.0–12.0 using casein and acid-denatured bovine haemoglobin as substrates for alkaline and acidic proteases respectively. The acidic protease showed high activity in the pH range from 2-4 with an estimated maximum at pH 4.0 and then decreased significantly ($p<0.05$) with increasing of pH (Fig. 2). More than 50% of the relative activity was lost over pH 5.0. According to reports the optimum pH for the hydrolysis of acid denatured bovine haemoglobin by partially purified acidic protease from *Tilapia nilotica* was found to be 2.5 [16]. Our results corroborate well with the findings of Bougatef *et al.* (2009) [1], who reported pH optima for acidic proteases in the range of 2-4. The alkaline protease exhibited maximum activity at pH 11.0, 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]. The optimum pH of an enzyme is considered as an important indicator for its potential application in different purposes.

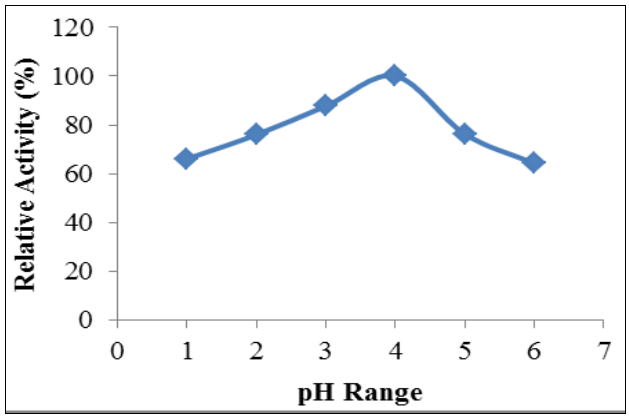


Fig 2: (Acidic Protease)

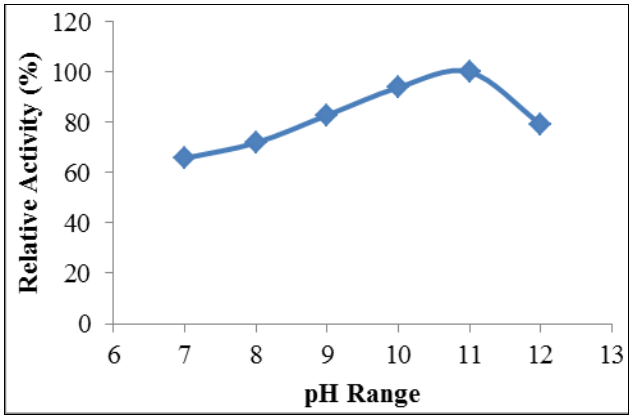


Fig 3: (Alkaline Protease)

From this study it was revealed that both the acidic and alkaline proteases were highly stable over a wide range of pH, maintaining more than 90% of its original activity between pH 1.0-5.0 and pH 8.0-12.0 for acid and alkaline proteases respectively after 30 minutes of incubation at 37°C (Fig. 4, 5). The pH stability of proteases depends on the differences in molecular properties, i.e., bonding and stability of the structure as well as enzyme conformation amongst various species and anatomical location [28]. Similar findings regarding pH stability of acidic protease from fish has also been reported by Castillo-Yanez *et al.* (2004) [4] for Monterey sardine. The pH stability of alkaline proteases in the range of 6-12, has been reported by several authors [23, 22, 29]. Decrease in protease activity for about 15-20% at pH over 6.0 for acid protease and below of pH 7.0 for alkaline protease were found.

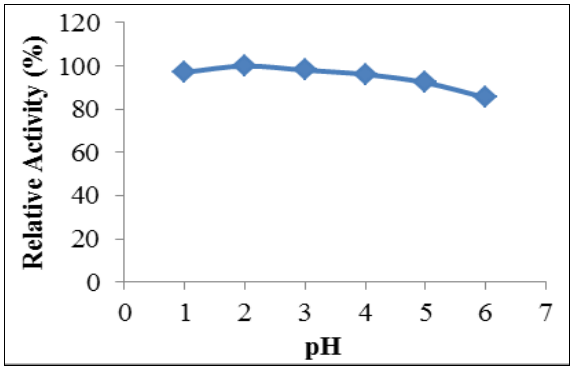


Fig 4: (Acidic Protease)

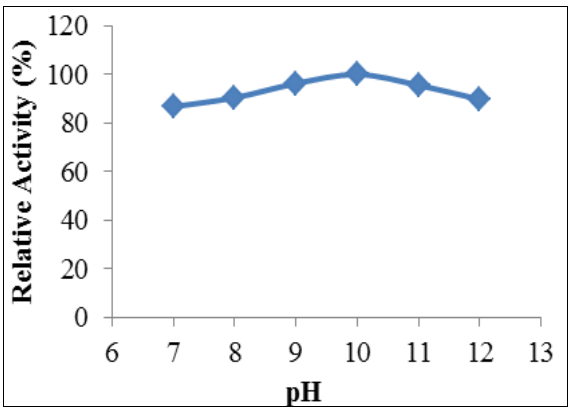


Fig 5: (Alkaline Protease)

3.5 Optimum temperature and thermo stability

The partially purified acidic protease exhibited optimum enzymatic activity at 40°C (Fig. 6), similar to those reported from other fish species such as pepsins of Sardinelle [30] and smooth hound [1]. The optimal temperature of alkaline protease was 70°C (Fig. 7) and the result was similar to that of trypsin from pyloric caeca of Chinook salmon [29], and Japanese seabass [31]. 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 (3D) structure of the enzyme active site [29]. The native conformations of enzyme might be governed by environmental and genetic factors among the different species [32].

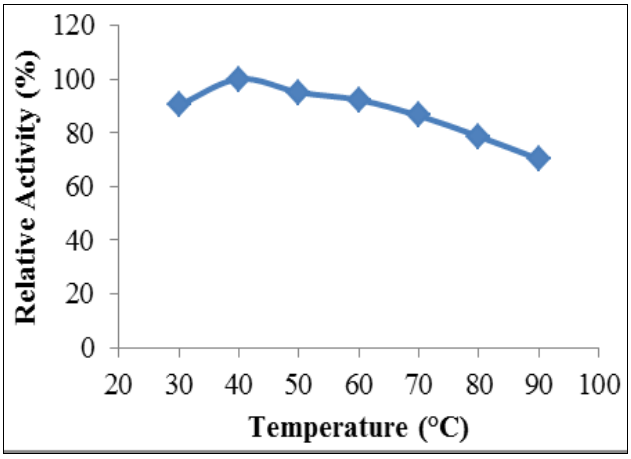


Fig 6: Acidic Protease

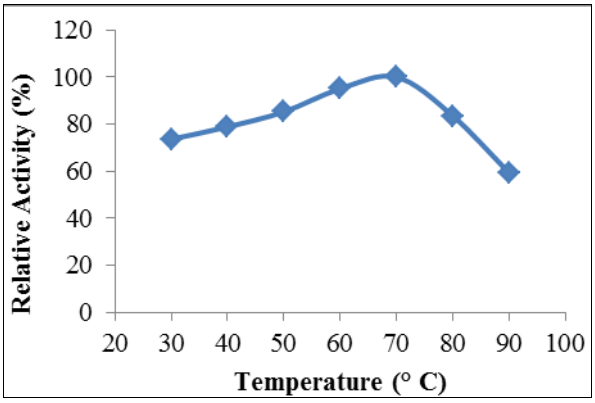


Fig 7: Alkaline Protease

Decreasing activity of acidic and alkaline proteases by 40 and 60% was observed when incubated at 90°C for 30 min (Fig. 8 & 9). Unfolding of the enzyme molecule during thermal treatment resulted inactivation of enzymatic activity [33]. Vannabun *et al.* (2014) [9] also reported similar findings while characterizing acidic and alkaline proteases from the viscera of farmed giant cat fish. Thermal stability of an enzyme is a parameter that is governed or dependent by the fish habitat, environment and genetic features [34].

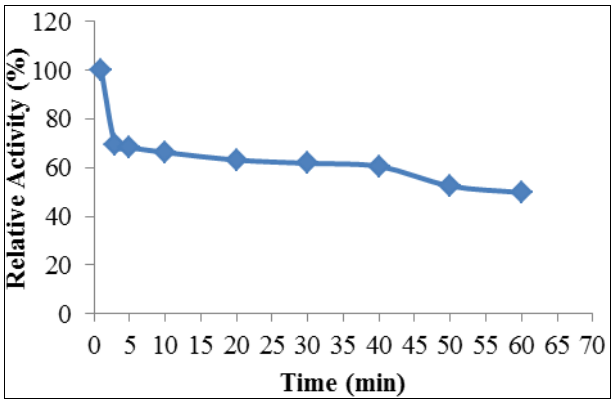


Fig 8: Acidic Protease

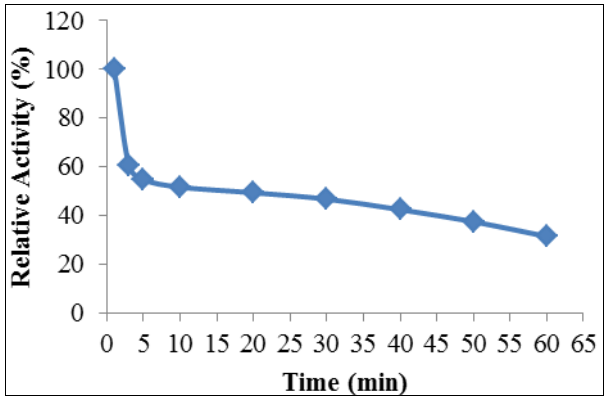


Fig 9: Alkaline Protease

3.6 Effect of isolated enzymes on proteins hydrolysis

Degree of hydrolysis (DH), which indicates the percentage of peptide bonds broken down [35], is essential because several properties of protein hydrolysates are dependent on DH. Using ground muscle meat of fish as substrate, hydrolysis was conducted at optimum temperature and pH with certain time period for both the enzymes. The degree of hydrolysis (DH) as a function of the enzyme concentration is given in Fig. 10 & 11. The result indicated that the higher amount of proteases

in enzyme fraction cleaved more peptide bonds and similar observation was also reported by some researcher [36].

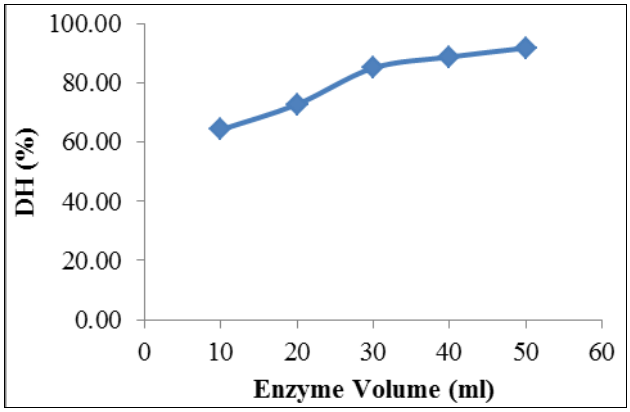


Fig 10: Acidic Protease

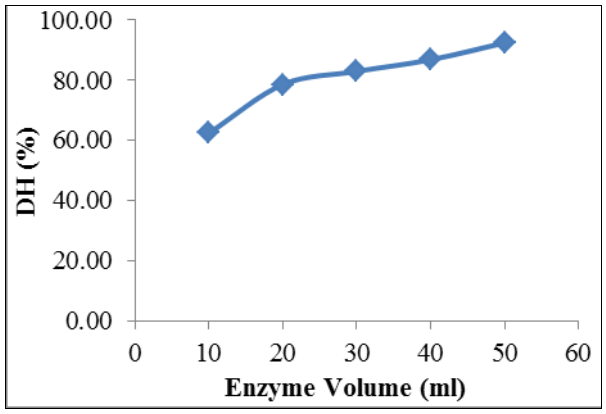


Fig 11: Alkaline Protease

3.7 Effect of inhibitors and activators on the enzyme activity

The enzyme was pre-incubated with inhibitor or activator solution at the temperature of the assay for 10 min. The reaction in each case was initiated by the addition of the substrate. Inhibition and activation of enzyme activity was expressed as a percentage of the activity without modifiers. Soybean trypsin inhibitor (SBTI) and ethylenediaminetetraacetic acid (EDTA) was used as inhibitor of metalloproteases (EC 3.4.24). A high inhibition percentages of the total activity of acidic protease was obtained when the enzyme was incubated with 50 mM of both SBTI and EDTA (90.9% and 68.8%, respectively), and 23.9% and 10.5% respectively, when the concentration was 10 mM (Fig. 12). Almost similar percent inhibition was obtained in case of alkaline protease (Fig. 13). Our result is in agreement with the findings of some researchers for the acidic protease [37, 38, 16]. In a study with tilapia digestive proteases, a high inhibition of approx. 40% was reported using very low concentration of SBTI [39, 40]. Inhibition of Rohu alkaline proteases at 250 µM concentration of SBTI was reported to be 78.1% [41].

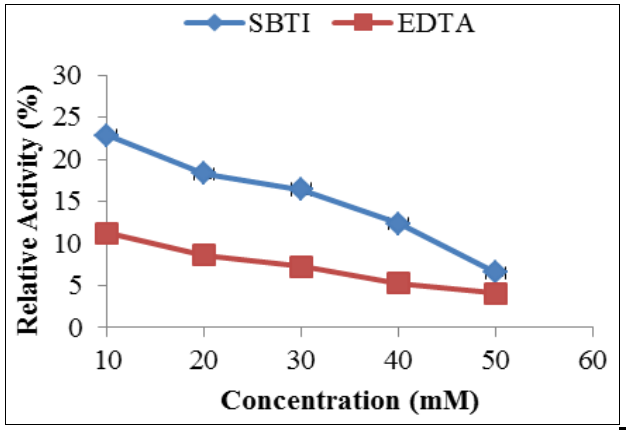


Fig 12: Acidic Protease

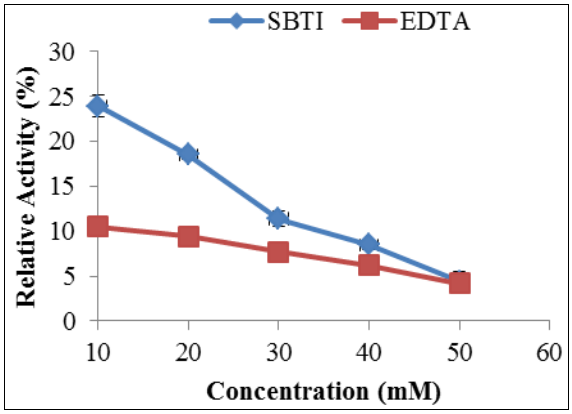


Fig 13: Alkaline Protease

Fig. 14 & 15 depicted the effects of various concentrations (10-50 mM) of $CaCl_2$ and NaCl on the activity of partially purified acidic and alkaline proteases of Common carp visceral waste. Pre-incubation of substrates with both NaCl and $CaCl_2$ (10mM) increased the activity of acidic protease by 18.56% and 29.11% respectively, while in the same reaction condition, the activity of alkaline protease was increased by 6.23% and 11.4% respectively. The activation percentage was increased with increase of activator and reached 46.87% and 53.47% respectively when 50 mM of both NaCl and $CaCl_2$ were used. In the same reaction condition, the activity of alkaline protease increased by 27.48% and 31.29% respectively. Almost parallel data was reported by some findings of some researchers [42] for squid aminopeptidase. Our results are not quite in line to that of Squires, Haard and Felthame (1982) [43] who reported that porcine pepsin showed slight inhibition at higher levels of NaCl, while cod protease was unaffected by the presence of NaCl. However, the protease activity of Green land Cod was dramatically increased when NaCl was used. In this study, activation of Common carp alkaline protease was found to be less compared to acidic in presence of activators like NaCl and $CaCl_2$. Our result was also supported the report of El-Beltagy *et al.* (2004, 2005) [38, 16].

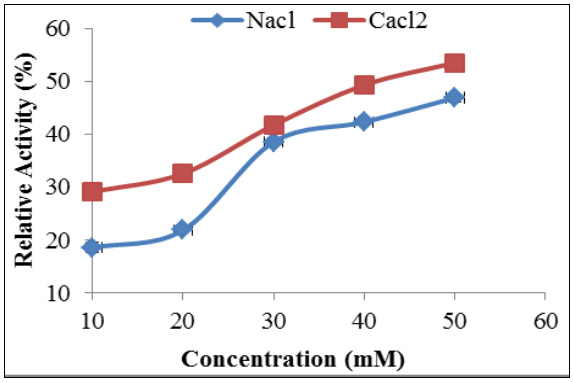


Fig 14: Acidic Protease

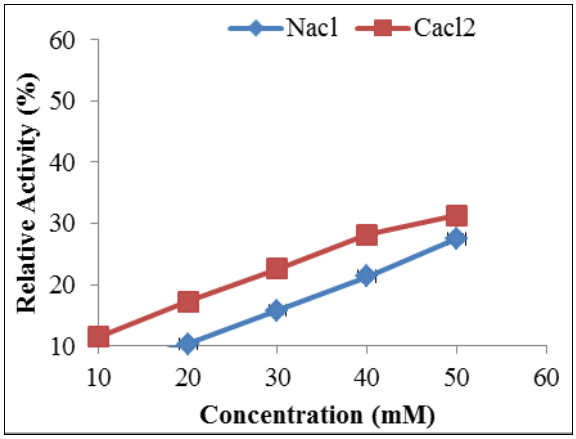


Fig 15: Alkaline Protease

4. Conclusion

The viscera of Common carp fish contained considerable amounts of acidic and alkaline proteases that can be used as different food processing applications, thereby contributing to reducing the waste disposal problem leading to environmental pollution. The enzymes extracted have high activity in the acid and alkaline condition. In addition, they exhibited the maximal activity at 40°C for acid protease and 70°C for alkaline protease. The isolated enzymes could find use in applications where maximum activity at moderate temperature is desired. The obtained crude acidic and alkaline protease characteristics are close to those reported previously of some marine fishes.

5. Acknowledgements

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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.

7. References

1. Bougatef A, Hajji M, Balti R, Lassoued I, Triki-Ellouz Y, Nasri M. Antioxidant and free radical-scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. Food chemistry. 2009; 114(4):1198-1205.
2. Arnesen JA, Gildberg A. Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. Bioresource Technology. 2007; 98(1):53-57.
3. Bezerra RS, Lins EJ, Alencar RB, Paiva PM, Chaves ME, Coelho LC, Carvalho Jr LB. Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). Process Biochemistry. 2005; 40(5):1829-1834.
4. Castillo-Yañez FJ, Pacheco-Aguilar R, Garcia-Carreño FL, de los Angeles Navarrete-Del M. Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax* caerulea) viscera. Food Chemistry. 2004; 85(3):343-350.
5. Anwar A, Saleemuddin M. Alkaline proteases: a review. Bioresource technology. 1998; 64(3):175-183.
6. Gupta R, Beg Q, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology. 2002; 59(1):15-32.
7. Klomklao S, Kishimura H, Nonami Y, Benjakul S. Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*). Food Chemistry. 2009; 115(1):155-162.
8. Nasri R, Younes I, Lassoued I, Ghorbel S, Ghorbel-Bellaaj O, Nasri M. Digestive alkaline proteases from *Zosterisessor ophiocephalus*, Raja clavata, and Scorpaena scrofa: characteristics and application in chitin extraction. Journal of Amino Acids, 2011.
9. Vannabun A, Ketnawa S, Phongthai S, Benjakul S, Rawdkuen S. Characterization of acid and alkaline proteases from viscera of farmed giant catfish. Food Bioscience. 2014; 6:9-16.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680-685.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of biological chemistry. 1951; 193:265-275.
12. Natalia Y, Hashim R, Ali A, Chong A. Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue Scleropages formosus (Osteoglossidae). Aquaculture. 2004; 233(1-4):305-320.
13. Rawdkuen S, Chaiwut P, Pintathong P, Benjakul S. Three-phase partitioning of protease from Calotropis procera latex. Biochemical Engineering Journal. 2010; 50(3):145-149.
14. Hoyle NT, Merrltt JH. Quality of fish protein hydrolysates from herring (*Clupea harengus*). Journal of food Science. 1994; 59(1):76-79.
15. Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK. Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol*). Journal of Agricultural and Food Chemistry. 2006; 54(15):5617-5622.
16. El-Beltagy AE, El-Adawy TA, Rahma EH, El-Bedawey AA. Purification and characterization of an acidic

protease from the viscera of bolti fish (*Tilapia nilotica*). Food Chemistry. 2004; 86(1):33-39.
17. Lopez-Llorca LV. Purification and properties of extracellular proteases produced by the nematophagous fungus Verticillium suchlasporium. Canadian Journal of Microbiology. 1990; 36(8):530-537.
18. Liu ZY, Wang Z, Xu SY, Xu LN. Two trypsin isoforms from the intestine of the grass carp (Ctenopharyngodon idellus). Journal of Comparative Physiology B. 2007; 177(6):655-666.
19. Balti R, Barkia A, Bougatef A, Ktari N, Nasri M. A heat-stable trypsin from the hepatopancreas of the cuttlefish (Sepia officinalis): Purification and characterisation. Food Chemistry. 2009; 113(1):146-154.
20. Sekizaki H, Itoh K, Murakami M, Toyota E, Tanizawa K. Anionic trypsin from chum salmon: activity with p-amidinophenyl ester and comparison with bovine and Streptomyces griseus trypsins. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2000; 127(3):337-346.
21. Barkia A, Bougatef A, Nasri R, Fetoui E, Balti R, Nasri M. Trypsin from the viscera of Bogue (Boops boops): isolation and characterisation. Fish physiology and biochemistry. 2010; 36(4):893-902.
22. Younes I, Nasri R, Bkhairia I, Jellouli K, Nasri M. New proteases extracted from red scorpionfish (Scorpaena scrofa) viscera: Characterization and application as a detergent additive and for shrimp waste deproteinization. Food and Bioproducts Processing. 2015; 94:453-462.
23. Sila A, Nasri R, Jrid M, Balti R, Nasri M, Bougatef A. Characterisation of trypsin purified from the viscera of Tunisian barbel (Barbus callensis) and its application for recovery of carotenoproteins from shrimp wastes. Food chemistry. 2012; 132(3):1287-1295.
24. Subash A, Shobana A, Shanmugakani G, Swetha VP, Josephine JL. Utilization of visceral waste of ribbon fish (trachipterus trachypterus) for the extraction, purification and characterization of protease. Journal of Cell and Tissue Research. 2011; 11(1):2589.
25. Kim SK, Ngo DH, Vo TS. Marine fish-derived bioactive peptides as potential antihypertensive agents. In Advances in food and nutrition research. Academic Press. 2012; 65:249-260.
26. Simpson BK. Digestive proteinases from marine animals. Food Science and Technology-New York-Marcel Dekker, 2000, 191-214.
27. Martínez A, Olsen RL, Serra JL. Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy Engraulis encrasicholus. Comparative biochemistry and physiology. B, Comparative biochemistry. 1988; 91(4):677.
28. Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK. Trypsin from the pyloric caeca of bluefish (Pomatomus saltatrix). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2007; 148(4):382-389.
29. Klomklao S, Benjakul S, Kishimura H, Chaijan M. 24 kDa Trypsin: A predominant protease purified from the viscera of hybrid catfish (Clarias macrocephalus× Clarias gariepinus). Food chemistry. 2011; 129(3):739-746.
30. Ben Khaled H, Bougatef A, Balti R, Triki-Ellouz Y, Souissi N, Nasri M. Isolation and characterisation of trypsin from sardinelle (Sardinella aurita) viscera. Journal of the Science of Food and Agriculture. 2008; 88(15):2654-2662.
31. Cao MJ, Osatomi K, Suzuki M, Hara K, Tachibana K, Ishihara T. Purification and characterization of two anionic trypsins from the hepatopancreas of carp. Fisheries science. 2000; 66(6):1172-1179.
32. Klomklao S, Benjakul S, Visessanguan W, Kishimura H, Simpson BK, Saeki H. Trypsins from yellowfin tuna (Thunnus albacores) spleen: purification and characterization. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2006; 144(1):47-56.
33. Klomklao S, Benjakul S, Kishimura H, Chaijan M. 24 kDa Trypsin: A predominant protease purified from the viscera of hybrid catfish (Clarias macrocephalus× Clarias gariepinus). Food chemistry. 2011; 129(3):739-746.
34. Sabtecha B, Jayapriya J, Tamilselvi A. Extraction and characterization of proteolytic enzymes from fish visceral waste: potential applications as destainer and dehairing agent. International Journal of Chem Tech Research. 2014; 6(10):4504-4510.
35. Adler-Nissen J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. Journal of agricultural and food chemistry. 1979; 27(6):1256-1262.
36. Klompong V, Benjakul S, Kantachote D, Hayes KD, Shahidi F. Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. International journal of food science & technology. 2008; 43(6):1019-1026.
37. Dıaz-López M, Moyano-López FJ, Alarcón-López FJ, Garcıa-Carreno FL, del Toro MAN. Characterization of fish acid proteases by substrate-gel electrophoresis. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1998; 121(4):369-377.
38. El-Beltagy AE, El-Adawy TA, Rahma EH, El-Bedawey AA. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). Journal of food biochemistry. 2005; 29(5):445-458.
39. Moyano FJ, Diaz M, Alarcón FJ, Sarasquete MC. Characterization of digestive enzyme activity during larval development of gilthead seabream (Sparus aurata). Fish Physiology and Biochemistry. 1996; 15(2):121-130.
40. López FM, Dıaz IM, López MD, López FA. Inhibition of digestive proteases by vegetable meals in three fish species; seabream (Sparus aurata), tilapia (*Oreochromis niloticus*) and African sole (Solea senegalensis). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1999; 122(3):327-332.
41. Kumar S, Garcia-Carreno FL, Chakrabarti R, Toro, MAN, Cordova-Murueta JH. Digestive proteases of three carps Catla catla, Labeo rohita and Hypophthalmichthys molitrix: partial characterization and protein hydrolysis efficiency. Aquaculture Nutrition. 2007; 13(5):381-388.
42. Raksakulthai R, Haard NF. Purification and characterization of aminopeptidase fractions from squid (Illex illecebrosus) hepatopancreas. Journal of food biochemistry. 1999; 23(2): 123-144. Squires EJ, Haard NF, Feltham LAW. Gastric proteases of the Greenland cod Gadus ogac. II. Structural properties. Biochemistry and Cell Biology. 1986; 64(3):215-222.