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Biswajit Mohanty
College of Fisheries, Central Agricultural University (I), Lembucherra, Tripura, India

Corresponding Author:
Biswajit Mohanty
College of Fisheries, Central Agricultural University (I), Lembucherra, Tripura, India

Purification and characterization of trypsin from the visceral wastes of grass carp *Ctenopharyngodon idella* (Valenciennes, 1844)

Biswajit Mohanty

Abstract
The proteolytic enzyme trypsin was purified from the gastrointestinal tract waste of grass carp Ctenopharyngodon idella (Valenciennes, 1844) with ammonium sulfate fractionation (ASF) followed by DEAE-cellulose chromatography, and Benzamidine Sepharose fast flow column affinity chromatography. Trypsin was purified 26.2-fold with an 11.1% yield. The purified enzyme was active between pH 9.0 and 11.0, and maximal activity of the enzyme was observed at pH 10.0. Highest activity was found at 60°C. The activity was reduced further after reaching the maximum activity point of temperature. The trypsin enzymatic activity was decreased by 40% and 60%, when incubated at 90°C for 30 min. The Km, Kcat, and catalytic efficiency values of purified trypsin were obtained is 0.062 mM and 19.23/s, and 310.16/s/mM. Degree of hydrolysis (DH) of the proteases on muscle protein increased with increase of enzyme concentrations. The enzyme activity was also further inhibited by SBTI, PMSF, and N-α-p-tosyl-L-lysine chloromethyl ketone. The molecular weight of the purified enzyme was obtained to be 20.2 kDa by SDS-PAGE. The study showed that trypsin from grass carp visceral waste of could find use in applications where maximum activity at moderate temperature is desired.

Keywords: Grass carp; Trypsin; Enzyme purification fold; Enzyme kinetics

Introduction
Fish viscera are a potential source of digestive enzymes, especially digestive proteases. Proteases represent an important class of industrial enzymes, accounting about 50% of the total sale of the enzymes in the world [1]. Various digestive proteases such as aspartic protease pepsin and serine proteases - trypsin, chymotrypsin, and elastase are isolated from the fish viscera. Trypsin (EC 3.4.21.4) plays a pivotal role in digestive physiology. This endopeptidase hydrolyzes peptide bonds at the carboxyl end of lysine and arginine residues. Trypsin plays major roles in biological processes including digestion and activation of zymogens [2]. Proteolytic enzyme (proteases) also has the diverse industrial uses such as in detergent, food, pharmaceutical, leather industries [3]. Fish viscera have wide biotechnological potential as a source of digestive enzymes, especially digestive proteases that have high activity over a wide range of temperature and pH and exhibit high catalytic activity at a relatively low concentration [4, 5].

In most teleosts, trypsin is synthesized in the cells of pyloric caecum as an inactive precursor trypsinogen, which is secreted into the intestinal lumen and activated by enteroproteases [6]. Though trypsin and trypsin-like serine proteases are isolated from several marine species, there's scarcity of data on the digestive proteases of tropical freshwater fishes, especially of carps. Trypsin was earlier purified from the intestine of hybrid tilapia *Oreochromis niloticus* × *O. Aureus* [7] and also from the intestinal caeca of Amazonian tambaqui *Colossoma macropomum* [6].

The grass carp *Ctenopharyngodon idella* is one of the most important farmed freshwater species in rivers, lakes and ponds in India. The grass carp can utilize both plant and animal matter for food but shows a clear dietary preference for certain aquatic weeds. For a better understanding of the physiology and farming of this species (for example developing new feed protein resources and improving feeding regimes) and utilization of its processing wastes, more precise information about its digestive proteinases is needed. This paper describes the isolation, purification, inhibitory, kinetic, stability and thermodynamic properties of trypsin from the intestines of grass carp (*Ctenopharygodon idella*), and these properties are compared with those of trypsin from other fishes and aquatic organisms.

Materials and Methods

2.1. Maintenance of fish and collection of viscera

The Chinese major carp, *Ctenopharygodon idella* (329.17 ± 14.86 g) were collected from a local fish market and were acclimatized at outdoor conditions for 15 days. Fish were fed with artificial diet (40% protein) consisting of dried fish powder, wheat flour, cod liver oil, and vitamin and mineral premixes; pH and water temperature of water ranged from 7.85 to 8.27 and from 28.5°C to 29.0°C, respectively, during experiment. Dissolved oxygen level of water was maintained above 5 mg/l with the help of aerators. Fish were not fed for 48 h before sampling for complete evacuation of the Intestinal tract .Ten fishes were anesthetized with MS 222 (tricaine methanesulfonate) before death. Each fishes were dissected; the digestive system along with the associated visceral glands were removed from the body, cleaned, weighted perfectly, and immediately kept in a deep freezer at −20°C till further use.

2.2. Preparation of crude extract

Digestive tracts and hepatopancreas collected from ten fishes were pooled; total weight of tissue was recorded and homogenized in sample buffer (10 mM Tris–HCl and 10 mM CaCl2; pH 8.0) in the ratio of 1:3. Homogenized sample was passed through pretreated cheese cloth (kept in 1% (EDTA) for 12 h to separate excess fats. The suspension was then centrifuged at 18,000 × g at 4°C for 30 min. The floating fat was removed, and the solution was further filtered through a Buchner funnel (Borosilicate grade-2) under vacuum. The volume of the solution was measured & noted, and this sample was called as crude enzyme extract.

2.3. Purification of crude extract sample

Crude extract was the target of fractionation of ammonium sulfate [8]. Saturated ammonium sulfate (30%) was gradually applied to the raw extract by continuous stirring. The sample was then centrifuged at 18,000 × g at 4°C for 30 min, and the supernatant was brought to 50% saturation by further addition of ammonium sulfate. The sample was then centrifuged at 18,000 × g at 4°C for 30 min, and the precipitate was collected, re-suspended in the minimal volume of the sample buffer. The sample was dialyzed with dialysis bags (D 0530, 12.4 kDa; Sigma-Aldrich Corporation, St. Louis, MO, USA) overnight against the same buffer and was filtered through a 0.45-µm polyethersulfone membrane (25 mm in diameter; Whatman, Maidstone, England) syringe filter. Trypsin activity and protein concentration of filtrate were assayed. Filtrate has been introduced gradually (0.25 ml / min) to a diethylaminoethyl (DEAE)-cellulose column (0.5 × 5.5 cm column, Bio-Rad, Hercules, CA, USA). The DEAE ionic cellulose column capacity is 175 µEq / ml and the dynamic binding capacity of the matrix is > 30 mg bovine serum albumin (BSA)/ml. The column was washed with the equilibration buffer until the effluent had no detectable absorption at 280 nm. The whole process was carried out at 4°C. Trypsin-like enzymes were then eluted from the column using a step gradient of NaCl with different concentrations ranging from 100 to 500 mM NaCl in the starting buffer. The flow rate was adjusted to 24 ml/h with the help of a peristaltic pump (Amersham Biosciences, Uppsala, Sweden), and 4.5-ml fractions were collected. The sample extract were measured for protein at 280 nm. In the case of trypsin activity, the absorption spectra change was assessed at 410 nm utilizing N-5-007-benzoyl-DL-arginine-p-nitroanalide (BAPNA) as a substrate. Highest activity concentrations were grouped as PF1 and then further analyzed with affinity chromatography. The pooled fraction was adjusted to 0.2 M KCl by the addition of solid salt and applied to a Benzamidine Sepharose 4 fast flow column (1.6 × 2.5 cm, 5 ml; Amersham Biosciences, Uppsala, Sweden). Sample was applied at 0.25 ml/min, and the column was washed at a flow rate of 20 ml/h. Fraction of 2.5 ml were collected. The un-adsorbed protein was devoid of any trypsin activity. Trypsin was eluted from the column by 0.1 M acetic acid. The contents of different tubes which showed significant trypsin activity were pooled as PF2; the pooled sample was filtered through a Whatman polyethersulfone membrane (0.45 µm). The sample was then divided into aliquots and was used for various assays.

2.4. Protein content determination

The total protein content was determined by using the method of (Bradford, 1976) with BSA as a standard [9]. Absorbance was assessed at 595 nm using a UV-visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

2.5. Specific trypsin activity

Trypsin activity level was evaluated with BAPNA (Sigma-Aldrich Corporation, St. Louis, MO, USA). The amount of 750 µl of BAPNA (1 mM in 50 mM Tris – HCl, pH 8.2, 20 mM CaCl2) was incubated at room temperature with 10 µl of enzyme solution, and change of absorbance was recorded under kinetic mode for 3 min at 410 nm [10]. Trypsin activity was expressed as change in absorbance per minute per milligram protein. One enzyme unit was defined as the amount of enzyme which hydrolyzed 1 mM of BAPNA per minute. Specific activity was expressed as enzyme units per milligram of protein. The molar extinction coefficient of p-nitroanalide released from BAPNA chromogenes is 8,800.

2.6. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 12% poly- acrylamide was carried out to evaluate the molecular mass and the purity of the enzyme sample [11]. Enzyme extract was filled onto each well and electrophoresis was conducted on a vertical dual mini gel electrophoresis device at 4°C controlled air temp. The gel was then washed and stained with 0.1% Coomassie brilliant blue (CBB) (Mumbai, India) in methanol/acetic acid/water (40:10:40) for two h. Destaining was through with an equivalent solution without CBB for 1 h. The gel was documented in calibrated densitometer (GS-800, Bio-Rad, CA, USA) with the assistance of Quantity One 4.5.1 Software.

2.7. Effect of pH on activity and stability of trypsin

The effect of pH was determined with casein as a substrate. Protease activity was studied in the pH range of 6.0–11.0 at the optimal temperature (60°C). In order to determine pH stability, the enzyme was incubated at 40°C for 1 h in different buffers and the residual proteolytic activity was then assessed under standard conditions of the test. The following buffer systems were used like; 100 mM sodium acetate, pH 6.0; phosphate buffer, pH 7.0; Tris–HCl buffer, pH 8.0; Glycine-NaOH buffer, pH 9.0–12.0 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).

2.8. Optimum temperature and thermal stability

The effect of temperature on trypsin activity was studied from 30 to 90°C for 15 min at pH 8.0. Thermal stability of the purified trypsin was determined by incubating the enzyme for 40 min at 40, 50, 60 and 70°C at pH 8.0. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions. The non-heated enzyme was

considered as control (100%).

2.9. Kinetic characteristics
The Michaelis-Menten constant (Km), maximum velocity (Vmax), and catalysis constant (Kcat) were evaluated [12]. The activity was assayed with varying concentrations of BAPNA ranging from 0.0156 to 2 mM. The final enzyme concentration for the assay was estimated as millimolar; Km and Vmax were evaluated by plotting the data on a Lineweaver-Burk double reciprocal graph (Prism 5 Computer Programme, Graph Pad Software, San Diego, CA, USA). Turnover number (Kcat) was calculated from the equation Kcat = Vmax/[E], where [E] is the active enzyme concentration (millimolar) and Vmax is the maximal velocity. Catalytic or the physiological efficiency of the substrate was calculated by the equation: Kcat/Km.

2.10. Effect of inhibitors
Inhibitory effect was quantified by reference to [13]. Extracted trypsin (10 µl) was incubated with various protease inhibitors (10µl), like serine-protease inhibitors phenyl methyl sulfonyl flouride (PMSF) (100 mM) and soybean trypsin inhibitor (SBTI) (250 mM); The trypsin-specific inhibitor N-5-007-p-tosyl-L-lysine chloromethyl ketone (TLCK) (10 mM) and the metalloprotease deactivator EDTA (20 mM).The residual activity was measured at 410 nm. Percentage activity in inhibition assays was reported, considering activity in the absence of an inhibitor as 100%.

2.11. 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 [14].

2.12. 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).

Results and Discussion
3.1. Purification of trypsin
In the initiative of purification, crude enzyme extract (0.7 U/mg protein) was concentrated by ammonium sulfate precipitation. The precise enzyme activity of the sample after ammonium sulfate precipitation (30% to 50%) and followed by dialysis was 3.9 U/mg protein (Table 1). Ammonium sulfate precipitation served as an initial step to get rid of other proteins within the crude enzyme extract. Dialysate reveals 5.8-fold purification compared to the crude enzyme extract with a recovery of 41.7 percent. The elution profiles of the trypsin activity and proteins after filtration on a DEAE-cellulose column were shown in Figure 1. Step gradient of NaCl within the range of 100 to 500 mM NaCl was applied to the DEAE- cellulose matrix. Fractions from 1 to 43 correspond to the unbound when washed with the equilibration buffer. During a 100-mM NaCl elution, more specific trypsin activity with BAPNA was found between the fractions 53 and 66. The precise activity of the pooled fraction PF1 (50 to 69) of 100-mM NaCl elution was 11.0 U/mg protein; 16.4-fold purification was found compared with the crude extract. Pooled PF1 fraction has been further accustomed to affinity chromatography.

Table 1: Purification summary of trypsin from the visceral waste of grass carp

Fraction	Total volume	Total protein	Protein (mg/ml)	Total activity	Specific activity	Yield (%)	Purification fold
	(ml)	(mg)		(U)	(U/mg)		
Crude sample extract 30%-50% $(NH_4)_2$ SO_4 precipitation	195.0	679.9	3.5	454.7	0.7	100.0	1.0
Dialysis	14.0	49.0	3.5	189.6	3.9	41.7	5.8
DEAE-cellulose column	100.0	7.3	0.1	79.8	11.0	17.6	16.4
Benzamidine column	8.0	2.9	0.4	50.4	17.6	11.1	26.2

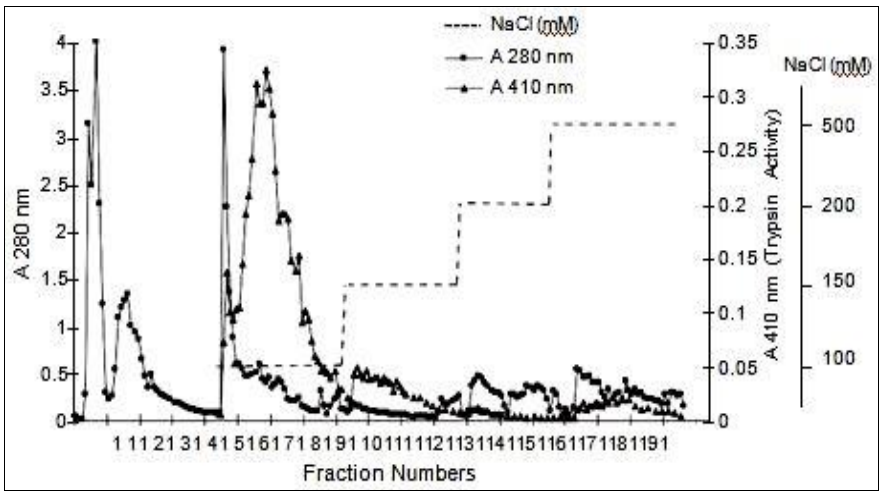

Fig 1: Chromatography pattern of grass carp trypsin using DEAE-cellulose column. Column was immobilized with 10 mM Tris-HCl and 10 mM $CaCl_2$ (pH 8.0) comprising 100, 150, 200 and 500 mM NaCl. The primary activity quantities were elucidated by 10 mM Tris – HCl and 10 mM $CaCl^2$ (pH 8.0) containing 100 mM NaCl. Elution flow was 24 ml/h. Content of protein is expressed as absorbance at 280 nm. Enzyme activity is expressed as absorbance at 410 nm.

After the ultimate purification in affinity chromatography, enzyme sample showed one peak like the fraction number 44 (Figure 2); 26.2-fold purified trypsin (PF2) was obtained with 11.1% recovery. The precise activity of the purified sample was 17.6 U/mg proteins. (Cohen *et al*. 1981) also obtained that 17% yield after purification of the common carp trypsin. An identical study suggests, 60-fold purification of alkaline protease was obtained from the intestine of *Clarias batrachus* by natural process chromatography on DEAE-cellulose column [15].

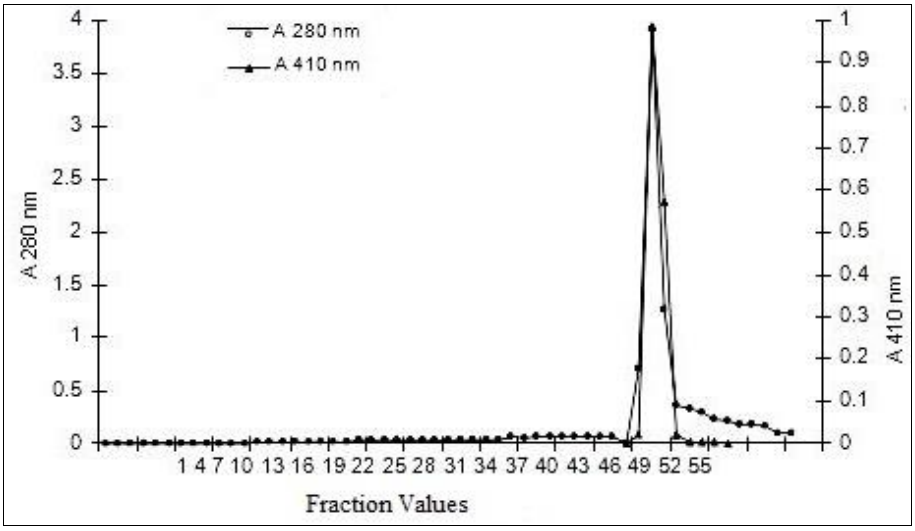

Fig 2: Purification profiles of trypsin of grass carp on Benzamidine Sepharose 4 fast flow column. Trypsin was eluted using 0.1 M ethanoic acid. The fractions eluted from the column were immediately adjusted to pH 8.0 by adding 700 µl of 1 M Tris–HCl buffer (pH 9.0). Elution flow was 20 ml/h. Content of protein is expressed as absorbance at 280 nm. Enzyme activity is expressed as absorbance at 410 nm.

3.2. Molecular mass determination (SDS-PAGE)

The purified enzyme showed one band on SDS-PAGE (Figure 3). The molecular mass of the band was 20.2 kDa. The presence of one band in SDS-PAGE confirmed the purity of the sample. Within the common carp, the molecular mass of trypsin was approximately 25.0 kDa [16]. The molecular mass of trypsin of hybrid tilapia was 22.0 kDa [7]. In seafood, tambaqui (*Colossoma macropomum*), the purified enzyme showed one band of 27.5 kDa [6].

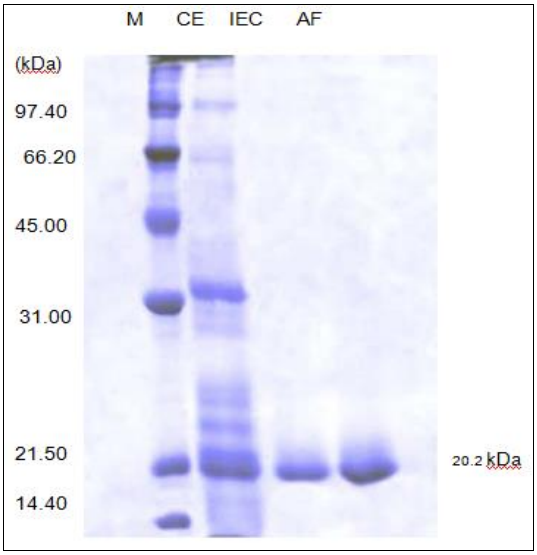

Fig 3: SDS-PAGE of enzyme sample of grass carp at various stages of purification. The sample was mixed in the ratio of (1:1) with the sample buffer. The used molecular molecular marker comprises phosphorylase b (97,400), bovine albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). After electrophoresis, the gel was stained with CBB for two h and was destained. CE-crude extract; IEC-purified fraction obtained by natural process chromatography; AF-purified fraction obtained by affinity chromatography.

3.3. Optimum pH and temperature

The effect of pH on enzyme activity decided over a pH range of seven. 0 to 12.0. The purified enzyme was active between pH 9.0 and 11.0. Most enzymes suffer irreversible denaturation at very low or very high alkaline conditions. The very best activity was observed at pH 10.0 (Figure 4). The optimum pH of two isoforms of trypsin obtained from grass carp were 8.0 (GT-A) and eight. 5 (GT-B); optimum pH for hybrid tilapia trypsin was 9.0 [17, 7]. Two particular features were observed in carp enzymes, instability in low pH and trypsin showing as an anionic protein [16, 18]. Proteases purified from the intestine of *C. batrachus* also have showed optimum activity at pH 8.0 [15].

The purified trypsin was incubated at various temperatures starting from 30°C to 90°C to review the effect of temperature on enzyme activity (Figure 5). The activity increased with the

increasing temperature. The very best activity was found at 60°C. Activity gradually decreased with the rise of temperature. The activity was significantly reduced at a temperature of 90°C. This might flow from to thermal denaturation. The result showed that the enzyme activity of proteases increased to a particular point followed by a decrease with increase in temperature forming a bell shaped curve. At temperature above optimum the native conformation of protein is modified thanks to breakdown of weak intramolecular bonds responsible of stabilization of three dimensional structure of the enzyme site [19]. As opined by Klomklao *et al.* 2007 [20], environmental and genetic factors among the various species could be liable for the native conformations of enzymes. This study showed that the grass carp trypsin may have a possible application value where optimum processing temperature and better enzyme activity is required.

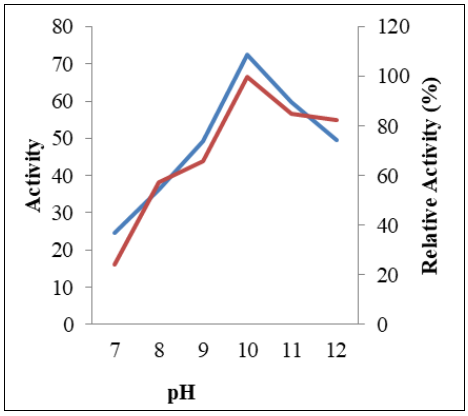

Fig 4: Optimum pH for trypsin activity: Activity was measured at various pH values ranging from 7.0 to 12.0 using 1 mM BAPNA as substrate at 25°C. Percentage of enzyme activity was estimated considering 100% - the highest activity detected in the assay.

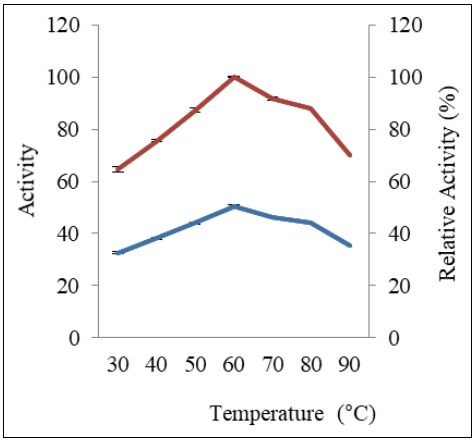

Fig 5: Optimum temperature for trypsin activity: Activity was assayed at various temperatures ranging from 30°C to 90°C using 1 mM BAPNA as substrate (pH 8.2). Percentage of enzyme activity was estimated considering 100% - the highest activity detected in the assay

3.4. pH and Temperature stability

Trypsin was highly stable over a good pH range, maintaining quite 90% of its original activity between pH 8.0-12.0 after half-hour incubation at 37°C (Fig.6). The pH stability of proteases depends on the differences in molecular properties, which incorporates bonding and stability of the structure; conformation of enzyme in several anatomical locations amongst various species [21]. Similar findings regarding pH stability of acidic protease from fish has also been reported by Castillo-Yanez *et al.*, 2005 [22] for Monterey sardine. Acidic protease activity showed a decrease of about 15-20% at pH over 6.0 whereas; an identical decrease was shown by alkaline protease at pH below 7.0.

The study also revealed that trypsin activity was decreased by 60%, when it had been incubated at the condition of 90°C for 30 min (Fig. 7). This might be explained because the inactivation of enzymatic activity following stretching out of the enzyme's native conformation during thermal treatment [19]. An immediate correlation was found between the temperature of the fish habitat and therefore the thermal stability of trypsin [23]. Trypsins from tropical fish showed higher thermal stability compared with those in fish that adapted to cold environment. This might flow from to lesser hydrophilicity and stronger hydrophobic interactions within the protein centre [20, 24]. Vannabun *et al.*, 2014 [25] also reported similar findings while characterizing visceral acidic and alkaline proteases of farmed giant cat fish. As proposed by Sabtecha *et al.*, [26], stability of a fish enzyme in several temperatures is influenced by their habitat, environment and genetic characters. An immediate correlation was found between the temperature of the fish habitat and therefore the thermal stability of trypsin [23]. Trypsins from tropical fish showed higher thermal stability compared with those in fish that adapted to cold environment. This might flow from to lesser hydrophilicity and stronger hydrophobic interactions within the protein centre [20, 24].

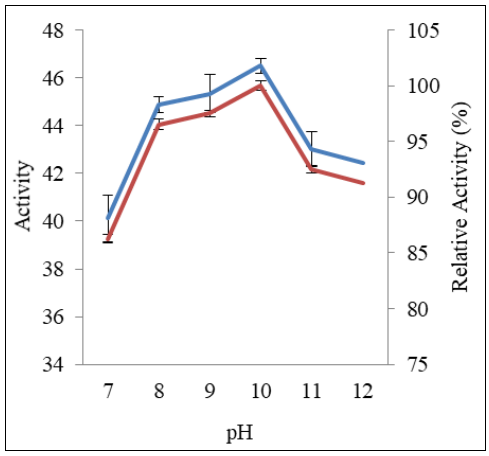

Fig 6: Effect of pH on enzyme stability: Activity was measured at various pH values ranging from 7.0 to 12.0 using 1 mM BAPNA as substrate at 25°C. Percentage of enzyme activity was estimated considering 100% - the highest activity detected in the assay.

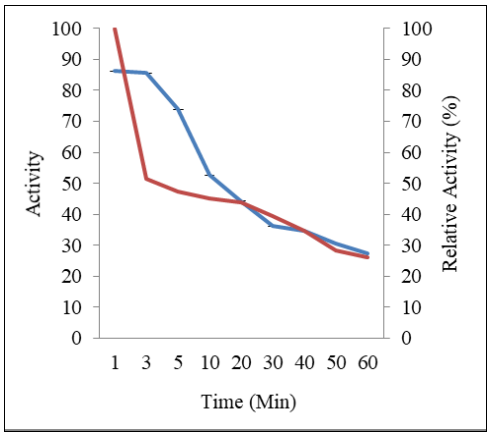

Fig 7: Effect of Temperature on enzyme stability: Activity was measured at various Time values ranging from 1 min to 60 min using 1 mM BAPNA as substrate at 90°C. Percentage of enzyme activity was estimated considering 100% - the highest activity detected in the assay.

3.5. Kinetic characteristics

The Km value of trypsin was 0.062 mM (Figure 8). Kcat value was calculated as 19.23/s. The catalytic efficiency of the purified trypsin was found to be 310.16/s/mM. The Km and Kcat values and therefore the catalytic efficiency of grass carp within the present study were higher compared thereupon of the common carp. Within the common carp, the Km and Kcat values and therefore the catalytic efficiency were 0.039 mM, 3.10/s, and 79.5/s/mM, respectively [18].

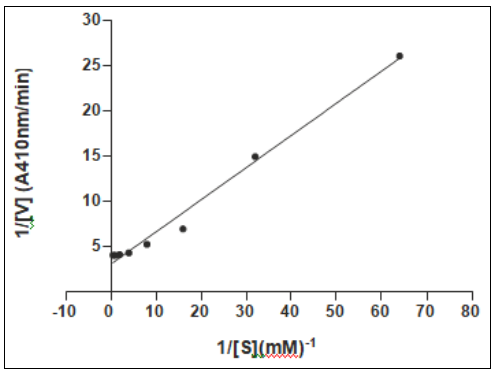

Fig 8: Line weaver – Burk plot for trypsin kinetics. 1/[V] and 1/[S] are the reciprocals of velocity and substrate.

3.6. Effect of inhibitors

Proteases are often classified by their sensibility to varied inhibitors [27]. Trypsin activity was completely inhibited by the serine protease inhibitors, SBTI and PMSF, and therefore the specific inhibitor of trypsin, TLCK. These results suggested that the purified enzyme may be a serine protease and classified as trypsin-like enzyme. The metalloprotease inhibitor EDTA inhibited 59.53% of the enzyme activity. This shows the importance of ions in enzyme activity. An identical result was found in zebra blenny *Salaria basilisca* [27]. (Jany 1976) [28] Reported the presence of serylprotease trypsin in stomach less goldfish gibelio (Bloch).

3.7. Effect of isolated enzymes on proteins hydrolysis

As degree of hydrolysis (DH) is that the indicative of the extent of peptide bonds weakened [19] 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 the trypsin enzyme. The degree of hydrolysis (DH) as a function of the enzyme concentration is given in Fig 9. The result of this study signifies that higher amount of trypsin in enzyme fraction cleaved more peptide bonds and similar observation was also reported by Klompong *et al.*, 2008 [29].

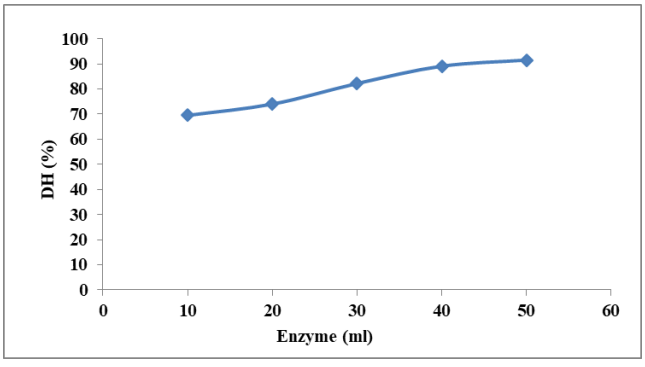

Fig 9: Effect of isolated enzymes on proteins hydrolysis

Conclusions

The present investigation has established some important biochemical properties of trypsin purified from the gastrointestinal system of grass carp. The protease had a similarity with trypsin from other fishes. Stability at high pH and coldness indicates the potential application of this protease in detergent and within the food industry. Enzymes extracted from the fish viscera (the waste a part of the fish body) could also be utilized in the food processing industry and thus making beneficial and productive use of the fish processing wastes.

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Practical applications

Recently proteases are gaining its successful application in several industrial fields, mostly in food, detergent, textile, leather and pharmaceutics also 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. during this way, utilization of fish visceral wastes as a source of proteases would minimize the main bio-pollutants generating during retailing of fish, and on the opposite hand, would be the judicious economic use of the wastes. During this aspect, characterization of the trypsin is utmost essential for his or her selection for specific application. This study would offer the essential characteristics of the trypsin from the fish visceral wastes which might be helpful for his or her application-specific uses.

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