Extraction, Partial Purification and Characterization of Alkaline Protease from Rainbow Trout (*Oncorhynchus Mykiss*) Viscera

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Abstract

In this study, crude alkaline proteases were recovered from rainbow trout (*Oncorhynchus mykiss*) viscera and partially purified by use of different saturation of ammonium sulfate. The enzyme exhibited highest yield, purity and activity when precipitated at a saturation of 40–60% compared to other ranges of saturation. Molecular weight for extracted protease was between 8-24 kDa. The protease had caseinolytic activity over a wide range of temperatures (30-55 °C) and pH (4-12). Soybean trypsin inhibitor and trypsin-chymotrypsin inhibitor strongly inhibited the enzyme activity but it was stable in the presence of surfactant, oxidizing reagents and organic solvents. The proteases had serine protease activity but no collagenase activity was detected. The current study showed that partially purified protease from the digestive tract of rainbow trout could be applicable in food and detergent industry because of its good activity over a wide temperature and pH range and its good thermal stability.

Keywords: Partial purification, Rainbow trout, Alkaline protease, Seafood by-product, Thermostable, Caseinolytic activity, Ammonium sulphate precipitation

1. Introduction

In addition to being a source of valuable proteins and lipids, fish processing by-products are important sources of marine bioactive compounds. Examples of bioactivities include anti-inflammatory, antioxidant, immunomodulatory, anticancer (Picot et al., 2006), blood pressure reducing properties (Slizyte et al., 2016). These properties are highly valued in food and nutraceuticals (Chandrasekaran, 2013; Shahidi and Janak Kamil, 2001). In addition, a wide range of enzymes especially proteinases has been extracted from both farmed and wild fish and their activity in different range of temperature and pH have been investigated (Debashish et al., 2005; El-Beltagy et al., 2005; Khantaphant and Benjakul, 2010; Sabtecha et al., 2014; Zamani et al., 2014). Fish enzymes have been shown to have higher activity at low temperatures compared to their homologues from warm blooded animals (Kristjansson, 1991; Shahidi and Janak Kamil, 2001; Vecchi and Coppes, 1996). This makes these enzymes interesting for various food processing industries for example caviar industry to remove supportive connective tissue where low temperatures are required (Sugihara, et al., 1971).

As a commercial fish, rainbow trout (*Oncorhynchus mykiss*) has become the most important cultured fish species in Iran (Iran Fisheries Organization, 2014). In 2015, over 140000 metric tons of rainbow trout were produced in Iran which is about 20 % of the world production (FAO, 2017). During processing, approximately 45000 metric tons of fish by-product include viscera, as a potential source of digestive enzymes, is generated that are usually discarded, used for oil extraction and fish meal or composted as fertilizer (El-Beltagy et al., 2005; Venugopal, 2008; Zamani et al., 2014). As a carnivore fish, rainbow trout viscera is considered to be rich of proteolytic enzymes, accounting for about 5% of the total body mass (Espósito et al., 2009;

Kristjansson, 1991). Alkaline proteinases extracted from fish digestive system (intestine, pyloric caeca, etc.) specifically cleaves peptide bonds mainly on the carboxyl side of lysine or arginine, except when either is followed by proline, and plays an important role in protein hydrolysis (Cao et al., 2000; Kishimura et al., 2008; Klomklao et al., 2007). At optimum enzyme conditions these enzymes have been shown to be able to recover up to 75% of total protein of fish by-product as fish protein hydrolysate (FPH) (Chakrabarti, 2002; Senphan et al., 2014). Also, proteases with high stability and activity over a broad range of temperature and pH, in addition to their stability with surfactant and oxidizing agents could be an interesting choice for detergent industry (Espósito et al., 2010)

The present study aimed to investigate the partial purification and characterization of alkaline proteases from rainbow trout viscera to provide fundamental information needed for commercial application of these enzymes in the food industry. The research aims to recover value added compound from rainbow trout by-product to help solve the problem of its disposal and create extra revenue.

2. Materials and methods

2.1. Analytical reagents

Casein, bovine serum albumin (BSA), protease inhibitors Soy Bean Trypsin inhibitor (SBTI), trypsin-chymotrypsin inhibitor and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorogenic substrates were bought from Sigma Chemical Co. (St. Louis, MO, USA) and Peptide (Japan). All other chemicals were reagent grade and obtained from Merck (Darmstadt, Germany).

2.2. Sample Preparation

Fresh farmed rainbow trout (*Oncorhynchus mykiss*, average weight: 650 ± 50 g) was obtained from a local fish farm in Mazandaran, Iran. The samples were transported to the Fish Processing Laboratory in the Fishery Department at Tarbiat Modares University of Iran in less than 1 h and were immediately eviscerated. The intestines and pyloric caeca were collected and used for enzyme extraction.

2.3. Crude alkaline protease extraction

The samples were cut into pieces and ground using a Panasonic MX-GX1571 blender (Japan). This was done on ice and the samples were defatted by homogenization with cold acetone (-20 C) at a ratio of 1:3 (w/v) for 1 min using a homogenizer (Wiggen, D500, Germany) at 15,000 rpm according to the method of Bougatef et al. (2010). The homogenate was stirred continuously using IKA multi-position magnetic stirrer (Staufen, Germany) at 4 °C for 30 min and filtered on Whatman No. 4 paper under vacuum condition. This procedure was repeated two more times with cold acetone and the samples were left in vacuum to be dried at room temperature for 6 hours. The acetone dried powder was suspended in tris-buffer (10 mM Tris–HCl, pH 8.0) at a ratio of 1:30 (w/v) and stirred at 4 °C for 3 h. The homogenate was centrifuged for 30 min at 7250 g using a refrigerated centrifuge (Hettich, Universal 320R, Germany). The resultant supernatant was collected and referred to as alkaline crude protease extract (CPE).

2.4. Ammonium sulfate precipitation

The CPE was submitted to ammonium sulfate fractionation (20-40, 40-60 and 60-80%, w/v saturation) according to the method of Michail, Vasiliadou, & Zotos, (2006) with slight modification. Ammonium sulfate were slowly added to the crude protease preparations until desired saturation and stirred gradually at 4 °C for 30 min. Then, the mixture was centrifuged at 8000 g for 30 min at 4 °C. The precipitate was dissolved in the extraction buffer 1:20 (v/v) and was dialyzed for 24 h at 4 °C against three changes of the same buffer. The dialysate was kept on ice and referred to partially purified alkaline protease.

2.5. Enzyme activity assay and protein estimation

The enzyme activity was determined using casein as a substrate. The reaction mixture contained 1 ml of 0.65 % casein in 0.05 M Tris-HCl buffer, pH 8.0, and 100 µl of the enzyme solution. The mixture was stirred thoroughly and then incubated in a water bath at 55 °C for 30 min. Thereafter, the reaction was stopped and protein was precipitated with 0.5 ml of 5% (w/v) trichloroacetic acid (TCA) (Barrett and Heath, 1977). The mixture was kept on ice for 2 h. The precipitate was eliminated by centrifugation at 16500 g for 10 min. The absorbance of the soluble peptides in the supernatant were measured at 660 nm with tyrosine as reference (Ferrero et al., 1996) A blank was run in the same manner except that the protease was added after addition of TCA. One unit of protease activity was defined as the amount of enzyme required to release 1 µmol of tyrosine/min/ml. All assays were carried out in triplicate. Protein content was measured by the method of Lowry, Rosebrough, Farr, & Randall, (1951) using BSA as a standard.

2.6. Determination of specific proteolytic activities

The activities of trypsin, chymotrypsin, collagenase, cathepsin B, and myofibril-bound serine proteinase (MBSP) were tested according to the method of Barrett & Kirschke (1981). Briefly, a proper dilution of the rainbow trout digestive proteases was equally mixed to appropriate assay buffer (Buffer A for trypsin, chymotrypsin and MBSP: 150 mM bis-Tris, pH 8.0; Buffer B for cathepsin B: 150 mM bis-Tris, 30 mM EDTA, 6 mM DTT, pH 6.0; Buffer C for collagenases 150 mM bis-Tris, 7.5 mM CaCl2, pH 6.0). After incubating at 30 °C for 10 min, the reaction was initiated by adding 100 μL 0.09375 mM substrate solution to all samples and blank (adding distilled water instead of enzyme to the reaction mixture). After 15 min of incubation at the desired temperature, the reaction was terminated by adding 3.0 ml 1% SDS in 50 mM bis-Tris buffer, pH 7.0. The test tubes were instantly put on ice. Proteolytic activity was measured as amount of AMC liberated from the fluorogenic substrates (for cathepsin Z-Arg-Arg-AMC). Amount of liberated AMC was determined with Fluorescence Spectrometer 3000, Perkin Elmer, UK after 15 min at 360 nm (10 nm slits) excitation wavelength and 460 nm (10 nm slits) emission wavelength. Activities were calculated based on increasing in fluorescence intensity during incubation and expressed as an arbitrary units (U). The analyses were run in triplicate.

2.7. Molecular weight distribution

The molecular weight distribution of partial purified enzyme was analyzed using gel filtration on a FPLC system. The sample was separated on a SuperdexTM 10/300 GL column, and its molecular weight was estimated by plotting the partition coefficient (K_{av}) against the logarithm of the molecular weight of the standards. The elution volume (V_e) was determined for each standard and enzyme. The standards used were Aprotinin (6.51 kDa), Cytochrome C (12.33 kDa), trypsin (23.30 kDa) and pepsin (35.00 kDa).

2.8. Biochemical Characterization

2.8.1. Optimum pH and pH stability

The optimum pH of the partially purified protease was assayed over a pH range of 3.0–12.0 (50 mM acetate buffer for pHs 3.0-6.0; 50 mM Tris-HCl buffer for pHs 7.0-8.0 and 50 mM glycine-NaOH for pHs (9.0-12.0) using casein as a substrate at 37 °C for 20 min. The effect of pH on enzyme stability was assayed by determination of the residual proteolytic activity after incubation in the different buffer solutions (1:1 v/v) for 60 min under standard assay conditions.

2.8.2. Optimum temperature and thermostability

The protease activity was investigated at different temperatures (30 to 70 °C) using casein as a substrate. The assay was conducted at pH 8.0 for 20 min. For thermal stability, the sample was incubated at different temperatures for 30 min in water bath (model W350, Memmert, Germany). The residual activity was measured using casein as a substrate under standard assay conditions. The sample without prior thermal treatment was considered as a 100%.

2.8.3. Effect of monovalent and divalent metal ions on the protease activity

The influence of various metal ions (5 mM) on protease activity was studied by adding the monovalent (Na^+ and K^+) or divalent (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Fe^{2+}, Mg^{2+} and Hg^{2+}) metal ions to the reaction mixture. The activity of the sample without any metallic ions was used as control (100%).

2.8.4. Effect of protease inhibitors, oxidizing agents and surfactants on the enzyme activity

The effect of enzyme inhibitors (pepstatin A, soybean trypsin inhibitor (SBTI), trypsin-chymotrypsin inhibitor) was determined at 1 mg/ml concentration and effect of ethylenediamine tetraacetic acid (EDTA) at 5 mM.

The effects of some surfactants (Triton X-100, Tween 80, and SDS) and oxidizing agent (H_2O_2) at a final concentration of 1% (v/v) was evaluated by pre-incubating the reaction mixtures at room temperature for 1 h. A control assay incubated under similar conditions without any additive was taken as 100%.

2.8.5. Effect of organic solvents on protease activity

The effect of organic solvents on enzyme activity was assayed as described by Annamalai et al. (2014) with a slight modification. The partially purified protease was incubated with different organic solvents (methanol, ethanol, acetone and hexane) in the ratio of 1:4 (v/v) (solvent/enzyme) at room temperature (24 °C) for 1 h and the residual activity was measured.

2-9. Substrate specificity

The substrate specificity of the rainbow trout viscera proteases was determined with different protein substrates such as casein, azocasein, gelatin, haemoglobin, egg albumin, whey and BSA. The reaction mixture containing 100 µl of partially purified protease (30 µg/ml) and 400 µl of substrate (1 mg/ml) were incubated at 37 ◦C for 20 min and the relative activity was measured by a standard assay (Annamalai et al., 2014)

2.10. Statistical Analysis

The data was subjected to analysis of variance (ANOVA) to determine significant differences. If a significant difference was observed, a comparison of means was carried out by Duncan's tests. Experiments were run in triplicate. Statistical analysis was performed using the SPSS® program, version 22.0 (SPSS Statistical Software, Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Partial purification of the crude alkaline protease extraction

Partial purification of alkaline proteases from rainbow trout viscera by ammonium sulfate precipitation (Table 1) indicate that the precipitation using 40-60% saturation of ammonium sulfate gave significantly higher activity than precipitation with 20-40 or 60-80 %. The recovery of enzyme activity by use of 20-40, 40-60 and 60-80 percent of ammonium sulfate was 0.91, 66.6, 16.79 %, respectively, compared to crude protein extract (CPE). The purity of CPE increased 3.29-fold for the 40 to 60% ammonium sulfate precipitate. These results show that 40-60% is optimal saturation of ammonium sulfate for the first step of CPE fractionation. There was no precipitation with the saturation of 0-20% (Data not shown). Ammonium sulfate precipitation have been reported as an effective initial step for purification of proteolytic enzymes to remove other protein from CPE (El-Beltagy et al., 2005; Kurtovic et al., 2006; Marcuschi et al., 2010). Surface tension of water increases with the addition of ammonium sulfate, which leads to an increase in hydrophobic interaction between water and protein. To reduce contact with water, protein decrease its surface area and this resulting in precipitation (Wingfield, 2001). Senphan et al. (2015) reported that ammonium sulfate at 40–60% saturation showed the highest yield and purity of protease from hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*) compared to other concentrations

of ammonium sulfate. Kurtovic et al. (2006) achieved a recovery of 24.3 % of original activity and a specific activity of 0.29 U mg-1 when they used $(NH_4)_2SO_4$ as an initial step to purify a trypsin-like protease from pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*).

3.2. Determination of specific proteolytic activities

A series of various specific fluorogenic substrates were used to identify the substrate specificity of the alkaline protease from the viscera of rainbow trout (Table 2). The enzyme exhibited highest activity against t-butoxy-Gln-Ala-Arg-AMC (80856 U/mg) and Boc-Phe-Ser-Arg-AMC (40096 U/mg). The activity against substrates with Arg in P1 position imply presence of trypsin-like activity and myofibril bound serine protease (MBSP) like activity that both belong to serine proteases (Yongsawatdigul et al., 2014). In addition, partially purified enzyme from rainbow trout exhibited slight activity against Succinyl- Leu- Leu-Val-Tyr-AMC (1152 U/mg) and Z-Arg-Arg-AMC (120 U/mg) that represent chymotrypsin and cathepsin B activity, respectively. On the other hand, the enzyme was unable to cleave collagenase substrates (succinyl-Gly-Pro-Leu-Gly-Pro-AMC). These results indicate that the rainbow trout proteases contain trypsin and MBSP-like proteases as major enzymes whereas does not contain collagenase. Also, there are some chymotrypsin and cathepsin B activity.

3.3. Molecular weight distribution

The molecular weight distribution of the partially purified enzyme was determined to be in range of 8.2-23.6 kDa by gel filtration using SuperdexTM 10/300 GL column (Table 5). The results indicated that the enzyme from rainbow trout digestive system contain several protein peaks, representing sarcoplasmic proteins including proteases. Enzymes with estimated molecular weight

in range of 20 to 30 kDa were previously isolated from chinook salmon (Kurtovic et al., 2006), pacific white shrimp (Senphan et al., 2015), skipjack tuna (*Katsuwonus pelamis*) (Klomklao et al., 2005) and tongol tuna (*Thunnus tonggol*) (Klomklao et al., 2006) .

3.4. Optimum temperature and thermal stability

Temperature activity profile as well as the stability of rainbow trout alkaline proteases are summarized in Fig. 1 (b and d). The enzyme was highly active against casein at temperatures from 30 to 65 °C with a maximum activity at 55 °C. The proteases retained 85% and 66% of maximum activities (55 °C) at 30 and 70 °C, respectively.

These results are similar to results from earlier studies on rainbow trout (Kristjansson, 1991; Michail et al., 2006) and carp (*Cyprinus carpio*) (Cao et al., 2000). Jellouli et al. (2009) found 40 °C as the optimum for grey triggerfish (*Balistes capriscus*) trypsin, while Pacific white shrimp and cuttlefish (*Sepia officinalis*) trypsin had an optimum temperature at 80 and 70 °C, respectively.

The extracted proteases from rainbow trout viscera were highly stable at temperatures below 55 °C, retaining more than 87% of its activity after 60 min of heating at 40 °C (Fig. 1d). However, there was rapid enzyme inactivation above 55 °C and it was totally inactivated at 70 °C. While it lost about 68% of its initial activity after 60 min of incubation at 60 °C ($p < 0.05$).

The results showed that partially purified protease from rainbow trout have good thermal properties, which make it a good choice to be used by the industry in mild and low temperatures processes.

3.5. Optimum pH and pH stability

The activity of partially purified protease was determined over a wide range of pH (3.0-12.0). The enzyme was highly active between pH 6.0 and 11.0 retaining more than 80% of activity in this range (Fig. 1a). At 55 °C, the optimum pH for the enzyme was 7.0. However, a significant decrease was observed in enzyme activity above pH 11.0 and below 6. The optimum pH for rainbow trout proteases was similar to that reported by Kristjansson (1991) and lower than proteases from *Serranus scriba*, *Sardinops sagax*, *Pangasianodon gigas* *and* tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001; Castillo-Yáñez et al., 2005; Nasri et al., 2015; Vannabun et al., 2014). The rainbow trout alkaline protease was highly stable at pH 8, and retained more than 60 % of its original activity at pH 4 and 12 after 60 min of incubation at 25 °C (Fig. 1c). Generally, stability of proteins including proteases, is related to their net charge at a particular pH (Nasri et al., 2015). The stability of proteases over a wide range of pH make them suitable for industrial applications.

3.6. Effect of monovalent and divalent metal ions on the protease activity

Relative activity of the rainbow trout alkaline proteases in the presence of various metal ions, is summarized in table 3. The enzyme was found to lose its activity in the presence of most metallic ions at a concentration of 5 mM, except for Na^{+} and Cu^{2+}. Ca^{2+}, Ba^{2+} and Mn^{2+} moderately reduced residual activity of the protease whereas $FeSO_4$ reduced the enzyme activity to 41%. The inhibitory effect of K^{+}, Mg^{2+} and Ba^{2+} on the rainbow trout proteases was similar to that measured for Nile tilapia (*Oreochromis niloticus*) alkaline proteases (Bezerra et al., 2005).

3.7. Effect of protease inhibitors, oxidizing agents and surfactants on the enzyme activity

The influence of several inhibitors on the activity of the partial purified proteases from rainbow trout digestive tract was investigated as shown in Table 2. The enzyme was strongly inhibited by

SBTI (80.38%) and Trypsin-Chymotrypsin Inhibitor from soybean (85.16%) ($p < 0.05$) whereas pepstatin A as an aspartic proteinases inhibitor slightly reduced enzyme activity. SBTI form an inactive complex by binding strongly to the active site of enzyme (Klomklao et al., 2006; Senphan et al., 2015).

Regarding the effect of metalloenzyme inhibitor on the enzyme activity, significant inhibition was found with EDTA (56.91%). This may indicate the presence of metalloprotease in the partially purified protease from rainbow trout viscera (Annamalai et al., 2014; Michail et al., 2006). Generally, these results are in agree with those reported by Kristjansson (1991) and Michail et al. (2006). Klomklao et al. (2006) reported that enzyme from the spleen of tongol tuna was highly sensitive to SBTI and TLCK, but was not affected by pepstatin A.

Regarding the stability towards surfactant, the rainbow trout alkaline proteases is stable or stimulated in the presence of Tween 80 and Triton X (non-ionic surfactant). However, the enzyme activity was markedly inhabited after incubation for 1 h at room temperature in the presence of 1% SDS (anionic surfactant). Further, the proteases from rainbow trout were found to be affected by H_2O_2 (1%) and retained 62% of its initial activity.

3.8. Effect of organic solvents on protease activity

Investigating the effect of organic solvents on enzyme activity showed that the protease was activated by the addition of ethanol (107.36%), hexane (111.14%) and methanol (113.03%), whereas acetone (98.87%) had no significant effect on the enzyme activity (Table 4). Wang et al. (2009) reported that purified enzyme have better stability compared to alkaline protease in the presence of organic solvent. Normally, addition of organic solvent to the reaction solution inactive

316 enzymes (Annamalai et al., 2014). But, stability of rainbow trout proteases against organic solvent
317 reveals that it could be useable and functional for different types of systems.
318
319 *3.9. Substrate specificity*
320 Partially purified enzyme from rainbow trout digestive system effectively hydrolyzed several
321 tested protein substrates except gelatin. It showed highest activity when casein (100%) and
322 hemoglobin (97%) were used as a substrate followed by BSA (76%), azocasein (66%) and whey
323 (49%) respectively, but no activity was found with gelatin. Similarly, Dadshahi et al. (2016) have
324 reported an alkaline protease from pacific white shrimp that has higher activity towards casein
325 relative to other proteins. These results indicated that proteases from rainbow trout have the
326 potential to digest various sources of protein and convert them to peptides and amino acids
327 (Annamalai et al., 2014; Senphan et al., 2014).
328
329 **4. Conclusions**
330 Biochemical characterization and evaluation of how the alkaline protease activity is affected by
331 various chemicals and substrates indicated that extraction and partial purification of enzyme from
332 the digestive tract of rainbow trout could produce value added compounds from by-product and
333 also help to solve environmental pollution caused by fish processing discards. The proteases,
334 mainly consisting of serine proteases, are highly active at low temperature (30 °C) and stable over
335 a wide range of pH (4-12) and temperature (30-55 °C) which makes it a good choice for the food
336 industry such as caviar production, meat tenderization and fish descaling. Considering the stability
337 of the enzyme with metal ions, oxidizing reagents and ionic and non-ionic surfactants, rainbow
338 trout alkaline proteases could potentially be applicable in detergent industries.

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Table 1

Partial purification of rainbow trout proteases with ammonium sulfate ($(NH_4)_2SO_4$).

Fraction	Total protease activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Crude protease extract	152.24	433	0.35	100	1.00
$(NH_4)_2SO_4$ (20–40%)	1.39	4.1	0.34	0.91	0.96
$(NH_4)_2SO_4$ (40–60%)	101.36	88	1.16	66.6	3.29
$(NH_4)_2SO_4$ (60–80%)	25.56	43	0.59	16.79	1.67

Activity was determined at pH 8.0 with casein as substrate. Values represent Mean ; n = 3.

Table 2

Specific proteolytic activities of alkaline proteases from rainbow trout viscera, given as increase in fluorescence intensity/mg protein/min. Values represent Mean ± Standard deviation of Mean; n = 3.

Fluorogenic substrate	ΔFI/mg protein
Trypsin-like (*t*-butoxy-Gln-Ala-Arg-AMC)	80856±4670
MBSP-like (Boc-Phe-Ser-Arg-AMC)	40096±2516
Chymotrypsin (Succinyl- Leu- Leu-Val-Tyr-AMC)	1152±61
Catepsin B (Z-Arg-Arg-AMC)	120±24
Collagenase (succinyl-Gly-Pro-Leu-Gly-Pro-AMC)	0±0

Table 3

Effect of various metal ions on the alkaline protease of rainbow trout viscera. The non-treated enzyme was considered as control (100%). Values represent Mean ± Standard deviation of Mean; n = 3.

Metal ions	Concentrations (mM)	% Relative activity
Control	No added	100
NaCl	5	93.52±3.27
KCl	5	80.04±4.40
$CaCl_2$	5	70.65±4.26
$CuCl_2$	5	96.97±5.40
$ZnCl_2$	5	67.70±4.43
$MnCl_2$	5	75.12±6.21
$BaCl_2$	5	74.78±0.64
$CoCl_2$	5	78.26±1.74
$FeSO_4$	5	41.60±5.91
$Al_2(so_4)_2$	5	79.25±3.26

Activity was determined at pH 8.0 with casein as substrate. Values represent Mean ± Standard deviation of Mean; n = 3.

511 Table 4

512 Effect of protease inhibitors, detergents (ionic, non-ionic) and organic solvents on the alkaline

513 protease of rainbow trout viscera. The non-treated enzyme was considered as control (100%).

Inhibitor	Concentration	% Relative activity
Control		100
Trypsin + Chymotrypsin Inhibitor	1 g/L	14.84±0.20
SBTI	1 g/L	19.62±0.17
EDTA	5Mm	43.09±0.62
Pepstatin A	1 g/L	93.45±0.41
Detergent		
SDS	1 % (v/v)	16.91±0.85
Tween 80	1 % (v/v)	120.43±1.18
Triton X	1 % (v/v)	119.53±2.00
H_2O_2	1 % (v/v)	62.00±7.56
Organic solvents		
Ethanol		107.36±2.95
Aceton		98.87±4.55
Metanol		113.03±4.33
Hexan		111.14±3.75

514 Activity was determined at pH 8.0 with casein as substrate. Values represent Mean ± Standard

515 deviation of Mean; n = 3.

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Table 5

Retention volume peaks and estimated molecular weight for the partial purified enzyme from rainbow trout viscera.

Retention time (min)	V_e (mL)	K_{av}	Logarithm of molecular weight	Molecular weight (kDa)
26.14	13.07	0.32	4.36	22.936
30.77	15.385	0.46	4.15	14.22
36.06	18.03	0.63	3.92	8.235

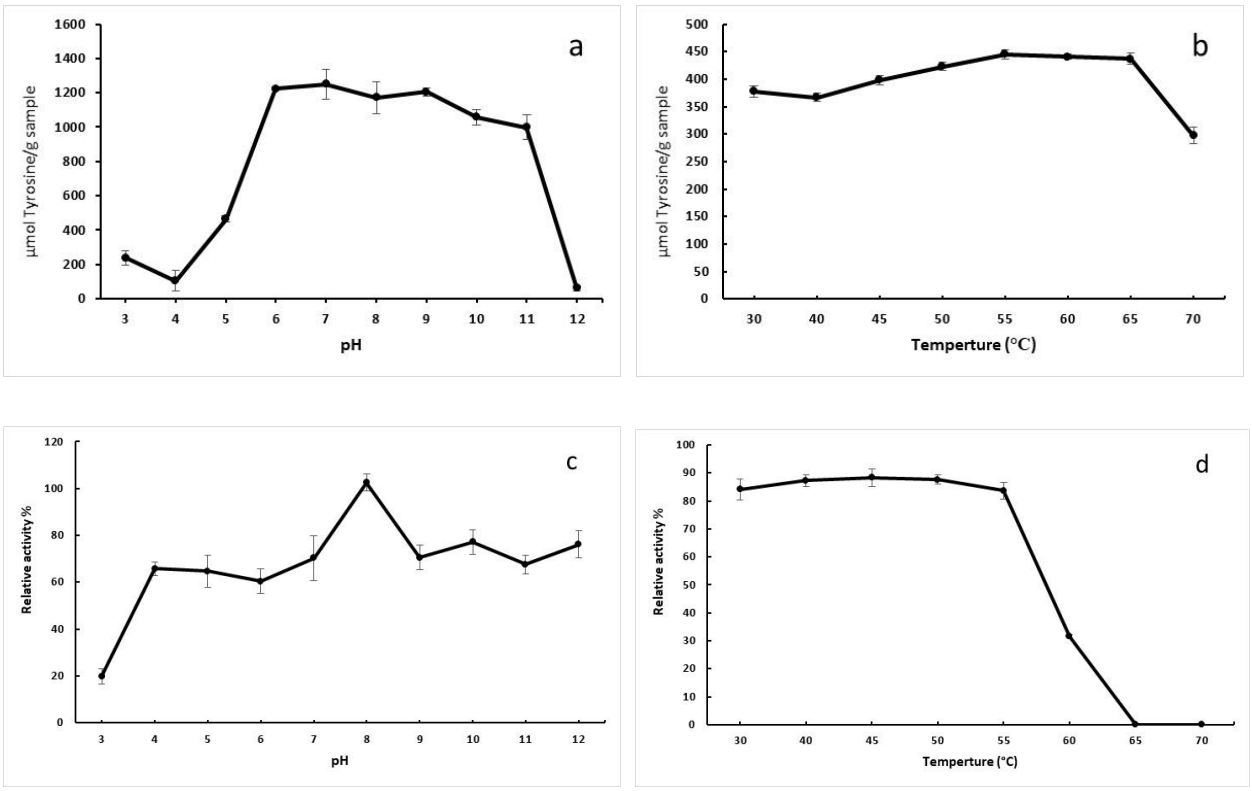
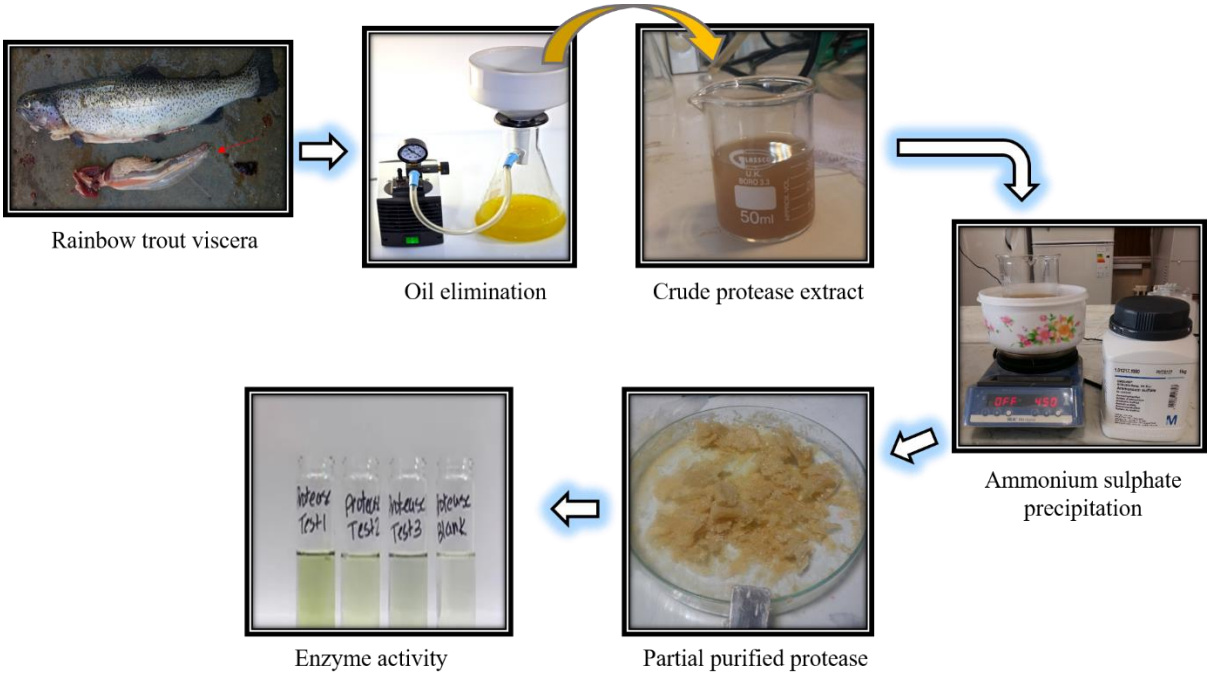


Fig. 1. pH optimum in the pH range of 3.0–12.0 at 55 °C (a) temperature optimum at different temperatures ranging from 30 to 70 °C at pH 8.0 using casein as a substrate (b) pH stability by incubating the enzyme in different buffers for 60 min at 25 °C (c) and thermostability by incubating at different temperatures for 60 min (d) for the alkaline proteases from the viscera of *Oncorhynchus mykiss*. The activity for temperature and pH profile expressed as µmol Tyrosine/g sample and for thermal and pH stability the activity of the enzyme was taken as 100%.


Rainbow trout viscera
Oil elimination
Crude protease extract
Ammonium sulphate precipitation
Partial purified protease
Enzyme activity

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