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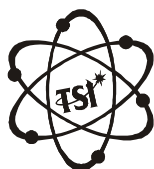

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Isolation and characterisation of visceral trypsin of Japanese threadfin bream (*Nemipterus japonicus*)

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ABSTRACT

Trypsin was purified to homogeneity and characterised from the pyloric caeca of Japanese threadfin bream (*Nemipterus japonicus*). Trypsin was optimally recovered from the tissues by homogenization at 3000 rpm for 10 minutes, followed by centrifugation at 10000 rpm for 30 minutes, precipitation at 45% ammonium sulphate saturation level, and subsequent chromatography using Sephacryl S-200, DEAE-cellulose and Sephadex G-50. Molecular mass of the enzyme was 25kDa and showed esterase activity on N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate. Trypsin showed temperature optimum of 60 °C and pH optimum of 8 for catalytic activity, and it exhibited sharp fall in its activity above 60 °C and below pH 6. The remaining activity of the trypsin pre-incubated in the presence of 10mM concentration of $CaCl_2$ was stable up to two hours when at 30 °C for pH 8, but in the presence of 10mM concentration of EDTA at 30 °C for pH 8 enzyme activity reduced sharply to negligible level within 5 hours. © 2011 Trade Science Inc. - INDIA

KEYWORDS

Trypsin;
Japanese threadfin bream;
Ranifish;
Serine protease;
Enzyme;
Temperature;
Inhibitor;
pH;
Activators.

INTRODUCTION

Export of marine products from India has set an over time record of 10048.53 crores in value and export aggregated to 678436 MT in volume during 2009-20010 with a growth rate of 12.54% in quantity and 16.74 in rupee earning, according to the Marine Product Export Development Authourity, Cochin. Seafood exports increased 23 per cent in the first four months of the current fiscal, and going by the early trends, export target of $2.5 billion set for 2010-11 seems eminently within reach, sources in seafood export industry said. The Export basket of the fisheries sector has also diversified over time[1]. Indian is in the verge of blue revolution and is turning to the hub for production of value added seafood products for international market and more than 50 items are being exported from India[2]. There are more than 560 approved seafood-processing plants in India. Japanese threadfin bream (*Nemipterus japonicus*) is exclusively used for the production of minced meat in Indian surimi production units. Seafood processing discard account for approximately three quarter of total weight of catch. Visceral wastes generally constitute about 16 % of the fish's total

weight[3]. Despite the presence of valuable components in discard, waste is not been utilised properly[4].

There is a considerable demand for enzymes with the right combination of properties for specific application in Industry. Worldwide sale of industrial enzymes are projected to exceed $ 2.9 billion by 2012[5]. Approximately 90% of all industrial enzymes are hydrolases used for depolymerization of natural substances. Some of the reasons for the under utilization of the fish proteases are that relatively few studies have been carried out on these enzymes. Marine environment contains a large pool of diversified species adapted to a variety of habitat conditions and exploitations of the unique properties of fish proteases are only at the beginning stage. Enzymes such as trypsin with unique properties for industrial usage can be recovered as by-product from fish processing visceral wastes.

Trypsin have been isolated and characterized from various fish such as Antarctic fish (*Paranotothenia magellanica foster*), trout (*Salmo gairdneri*)[6], Atlantic bonito (*Sarda sarda*)[7], mullet (*Mugil cephalus*)[8], Pacific cod (*Gadus macrocephalus*), saffron cod (*Eleginus gracilis*)[9], Monterery sardine (*Sardinops sagax caerulea*)[10], Cod (*Gadus morhua*)[11], and Tongol tuna (*Thunnus tonggol*)[12]. Majority of these fish are either not available in the Indian Ocean or not commercially exploited in Indian seafood processing units. We have made an attempt to utilise one of the common visceral waste generated in the surimi processing units in India. Hence efficient utilization of the fish waste for the production of commercially important hydrolytic enzyme will increase Indian foreign exchange in terms of value, reduce environmental burden, generate employment and increases profit margin for the already a disturbed and insecure seafood industry.

MATERIALS AND METHODS

Enzyme preparation

Japanese threadfin bream (*Nemipterus japonicus*) caught using trawl nets from the Arabian Sea were obtained from the fishing boats landed in 'Bunder area', Mangalore between August and September month. The time lapsed between catching and landing may not exceed over four to five hours. A 10 kg portion of the freshly caught Japanese threadfin bream belongs to size group of 15 to 18 cm long; each weighing around 100 to 120 grams was selected for the present study. The material was brought in an insulated container after adequately icing them in the proportion of 1:1 fish to ice, to the laboratory within two hours. The samples were gutted by split opening the belly using sharp knife and visceral portions were separated. Visceral portions were packed in plastic bags, labeled, frozen at – 40 °C, and stored at – 20 °C in a deep freezer until further use. The samples were thawed at room temperature of 28 °C. The pyloric caeca were selected, weighed and homogenized using the potter-elvehjem homogenizer (Rotek Instruments, Kerala,) using 10 mM Tris-HCl buffer at pH 8 containing 1 mM $CaCl_2$ at homogenization speed of 600, 1200, 1800, 2400, or 3000 revolutions per minutes for 10 minutes at 4 °C. Crude homogenate obtained were assayed for Total protein content[13] and trypsin[14]. The crude homogenate with highest protein content were centrifuged at 2000, 4000, 6000, 8,000, or 10,000 revolutions per minute for 30 minutes at 4 °C in a cooling centrifuge (REMI Instruments Ltd. Mumbai). Each supernatant and sediment collected was assayed for protein content using Lowry's method and trypsin activity using N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) as a substrate. Extract with highest specific activity for trypsin were fractionated with ammonium sulfate at 25, 35, 45, 55 or 65 % saturation levels as described by Deutscher[15], followed by dialysis against 10 mM Tris-HCl buffer of pH 8.0 at 4 °C using cellulose dialysis tubing of 12,000-14,000 (Hi Media Laboratories Ltd, Mumbai) for up to 24 hours with the change of buffer on every 2 hours. Further purification was carried out in Sephacryl S-200 (3.9X63 cm) equilibrated with the approximately two bed volumes of 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM $CaCl_2$. The column was latter eluted using the same buffer at a flow rate of 0.5 mL/min. Fractions of 5 mL is collected, and the fractions with TAME activity are pooled together. Pooled fractions were dialyzed using 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM $CaCl_2$ for 12 hours. Further purification is done using DEAE-cellulose chromatography (2.2X18 cm) at 28 °C equilibrated with 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM $CaCl_2$. The samples were loaded in

to the column at the rate of 0.5 mL/min and column was washed using 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM $CaCl_2$ with linear sodium chloride concentrations gradient of 0.25- 0.26M at the flow rate of 0.5 mL/min. Trypsin fractions collected in this process were further purified using same columns. Fractions with TAME activity were pooled and dialyzed using 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM $CaCl_2$ for 12 hours. The resulting samples were loaded onto a Sephadex G-50 (3.9x64) column at flow rate of 0.5 mL/min. Fractions of 3 mL were collected and fraction with TAME activity were used for subsequent study.

Electrophoresis

SDS-PAGE was performed by Laemmli gel method[16] utilizing vertical gel electrophoresis (Bhat-Biotech, Bangalore) apparatus using ready to mix SDS-PAGE gel kit (Bhat-Biotech, Bangalore) as per the manufacturers instructions. The SDS gel was run at 100 volts for 1 hour. Purified trypsin was identified in gel lanes through visualization Coomassie blue staining procedure (Bhat-Biotech, Bangalore).

Assay of trypsin

Trypsin activity is determined using the substrate TAME as described by Hummel[14]. 20 μL of sample with appropriate dilutions were mixed with 3.0 mL of 1 mM TAME in 10 mM Tris-HCl buffer at pH 8.0 and incubated at 30 °C for 20 minutes. Increase in absorbance at 247 nm due to production of *p*-tosyl-L-arginine is recorded. One unit of trypsin activity is defined as the quantity of the enzyme hydrolyzing one micromole of TAME in a minute.

Effect of pH on enzyme activity

Analysis of pH effect upon hydrolysis of artificial substrate by TAME was determined using 50mM buffer solutions of acetic acid sodium acetate buffer (pH 4-7), Tris-buffer (pH 7-9) and glycerine-NaOH (pH 9-11) at 30 °C. Measurements of pH were made on a pH-meter (Systronics, Mumbai). All other aspects of the trypsin assay were as described in standard assay conditions. The specific activity of the trypsin was determined at pH 4-11 and 30 °C, and stability of the trypsin was determined by estimating remaining specific activity of trypsin at 30 °C and pH 8 of the samples pre-incubated at 30 °C for 30 minutes at varying pH of 4, 5, 6, 7, 8, 9, 10 or 11.

Effect of temperature on enzyme activity

Temperature effect upon trypsin activity was examined by establishing the temperature of the enzyme assay solution, within each, individual test tubes at 10, 20, 30, 40, 50, 60, or 70 ºC at pH 8. The specific activity of the trypsin was determined at different temperature for pH 8, and thermal stability of the trypsin was determined by estimating remaining specific activity of trypsin at 30 °C for pH 8 of the samples that has been pre-incubated at different temperature and pH 8 for 30 minutes. As temperature was the only parameter being changed in this line of analysis, all other aspects of the assay were identical to those already described for the standard enzyme assay. Reaction temperature was obtained through use of either a water bath or in cooling BOD incubator (Rotex, Kerala).

Effect of effectors on enzyme activity

The remaining specific activity of the trypsin that has been pre-incubated in the presence of 10mM concentration of either $CaCl_2$ or EDTA at pH 8 for various intervals of the time is performed, at pH 8 for various intervals of time was performed. This is the only parameter being changed in this line of analysis, all other aspects of the assay were identical to those already described for the standard enzyme assay

Chemicals

All the chemicals used were of analytical grade and were obtained from Merck Limited (Mumbai, India).

Statistical analysis

One- and two-way ANOVA was performed using Statographics 2.1 (STSC Inc., Rock vile, MD). The difference in means was analyzed using a Turkey HSD test ($p<0.05$).

RESULTS AND DISCUSSIONS

Trypsin was isolated, concentrated, and purified from the pyloric caeca of the Japanese threadfin bream is illustrated in the figure 1. Pyloric ceca were homogenized at the speed of 600, 1200, 1800, 2400, or 3000 rpm at 4 °C for 10 minutes. The homogenization speed of 3000 rpm for 10 minutes resulted in

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2556 ml of homogenate releasing 2170mg of protein and total enzyme activity of 1067 units. We have selected a homogenization speed of 3000 rpm for 10 minutes for further purification, because at this level maximum ($p<0.01$) quantity of protein was released compared to other parameters. Usually during cell disruption, yield is measured in terms of the total protein released, rather than the specific activity of the enzyme[17]. At a centrifugal speed of 10,000 rpm for 30 minutes at 4 ºC, quantity of insoluble sediment was more. It is interesting to note here that specific activity of trypsin in the supernatant increased significantly ($p<0.01$) with the increase in the speed of centrifugation from 2000 to 10000 rpm, and in the sediment specific activity decreased proportionately.

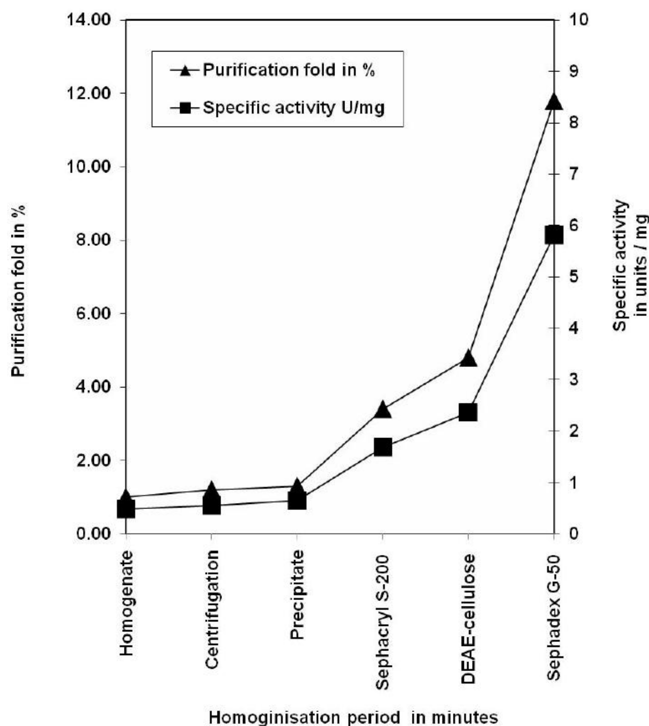

Figure 1 : Purification details of trypsin from Japanese threadfin bream (*Nemipterus japonicus*) at various stages

Centrifugation speed of 10000 rpm for 30 minutes at 4ºC was optimum in removing the impurities and proceeded for further purification. Ammonium sulphate at 45% saturation level and subsequent dialysis extracted maximum ($p<0.01$) enzymes in to precipitate with the specific activity of the precipitate for trypsin was 0.65 units/mg of protein. During ammonium sulfate precipitation substantial concentration occurred, as the total volume reduced from the initial value of 2556 mL to the final volume of 322mL. Effective purification at this stage of unit process was 1.3-folds and yield at the end of this process was 74% compared to the initial tissue homogenate. Kristjansson[18] reported that ammonium sulfate precipitation at 30-70% saturation level of trypsin isolated from the pyloric caeca of rainbow trout resulted in 4.9-fold increase in the specific activity. Subsequent chromatography on Sephacryl S-200, DEAE-cellulose and Sephadex G-50, total volume reduce from the initial volume of 322mL to the final volume of 12mL, and during this process total protein content reduced from 1210 mg to 11mg and specific activity increased from 0.65 to 5.82 units/mg. A purification of 11.8-folds with the yield of 6% was achieved during chromatographic purification. Chromatographic fractions constituting the sharp peak were pooled to provide the source of enzyme used in the characterization. Purified trypsin appeared as a single band with a molecular weight of 24 kDa was observed. Molecular weight of Japanese threadfin bream trypsin was similar to Japanese anchovies[19], yellowfin tuna[12], true sardine and arabesque greenling[10].

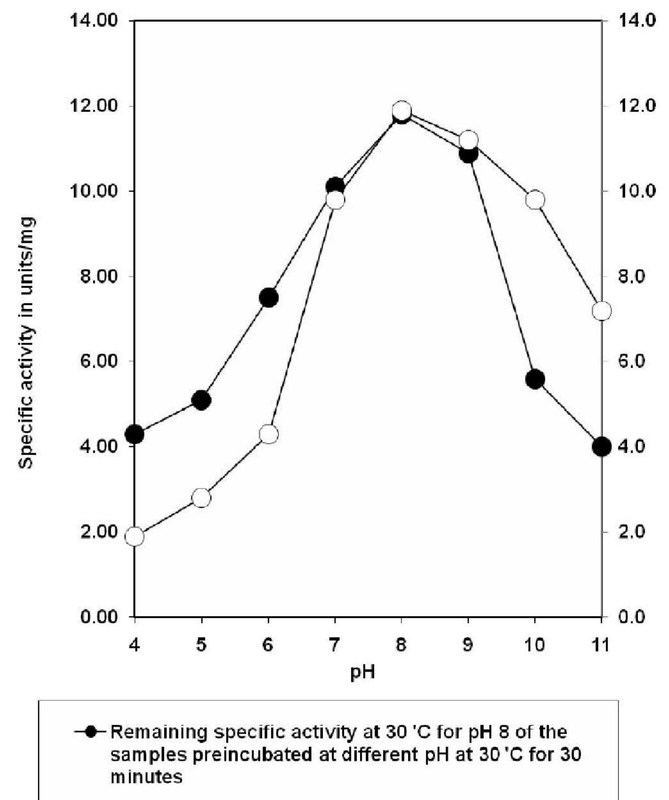

Figure 2 : Activity and stability of trypsin from Japanese threadfin bream (*Nemipterus japonicus*) at various pH levels

The specific activity of the trypsin was determined at pH 4-11 and 30 °C, and stability of the trypsin was determined by estimating remaining specific activity of trypsin at 30 °C at pH 8 of the samples pre-incubated for 30 minutes at pH of 4, 5, 6, 7, 8, 9, 10 or 11 and at temperature 30 °C (Figure 2). Trypsin from Japanese threadfin bream hydrolysed the TAME effectively at alkaline pH, similar to that of other trypsin[20-25]. Trypsin exhibited the maximum specific activity of 11.8 units/mg for pH 8, and reduced by around three fold in both acidic and alkaline pH values. Deviation of pH values of the buffer away from the optimum pH value changes the charge distribution and conformation of the enzymes, as most of the enzymes gets irreversibly denatured in very acidic or alkaline solution resulting in loss of stability molecules[26]. Trypsin showed high stability in the pH range of 7-9, but lost substantial activity below pH 6, same as those of trypsin isolated from Japanese anchovies[19], yellowfin tuna[12] true sardine and rainbow trout[27] and arabesque greenling[20].

The specific activity of the trypsin was determined at different temperature for pH 8, and thermal stability of the trypsin at was determined by estimating remaining specific activity of trypsin at 30 °C for pH 8 of the samples that has been pre-incubated for 30 minutes at different temperature for pH 8 (Figure 3). Trypsin isolated from Japanese threadfin bream showed highest trypsin activity at 60 °C for pH 8, which is higher than the trypsin isolated from the frigid zone fish such as Atlantic code[25], Elkhorn Sculpin[28], with the optimum temperature ranging from 40-45 °C[28]. Difference in the optimum temperature between trypsin of temperate and tropical fish is due to the temperature adoptability to the surrounding environment. Trypsin isolated from Japanese threadfin bream that have been pre-incubated at temperatures below 60 °C for 30 minutes and pH 8 were thermal stability, but exhibited sharp fall in its activity above 60 °C under same conditions and negligible activity above 60 °C.

Specific activity of the trypsin that have been pre-incubated in the presence of 10mM concentration of

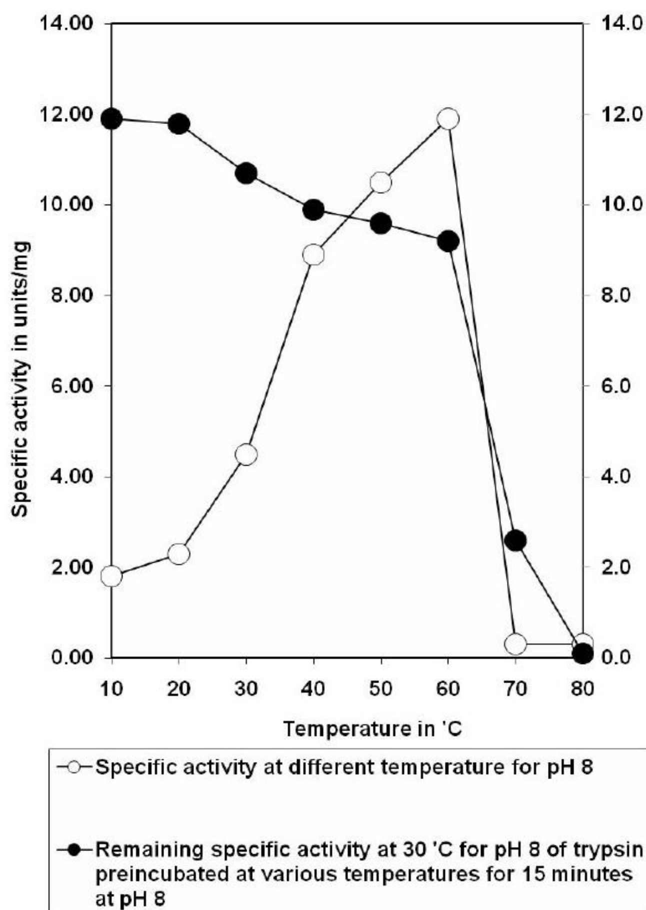

Figure 3 : Activity and stability of trypsin from Japanese threadfin bream (*Nemipterus japonicus*) at various temperature.

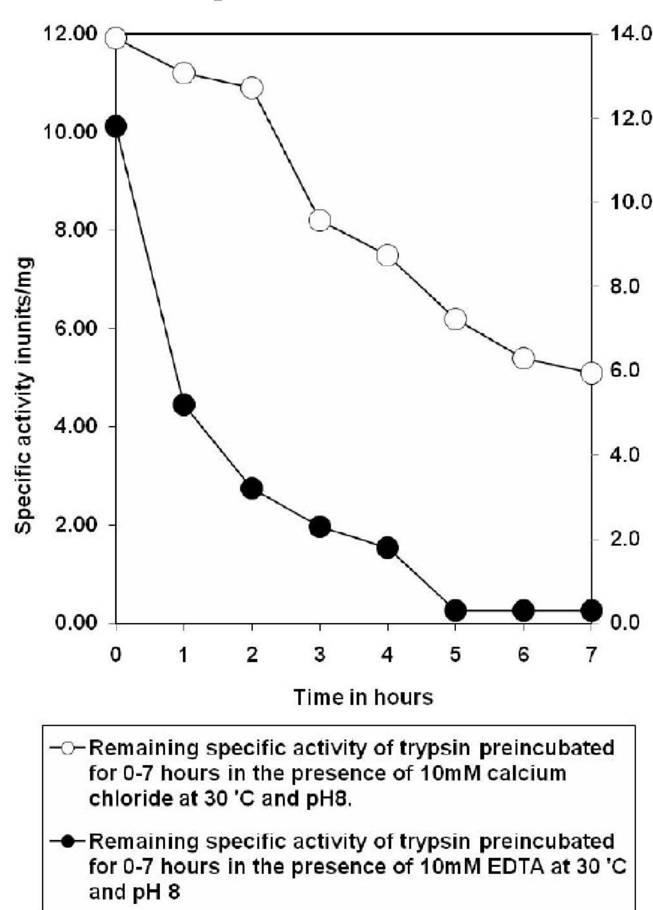

Figure 4 : Stability of trypsin from Japanese threadfin bream (*Nemipterus japonicus*) in the presence of effectors.

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either $CaCl_2$ or EDTA for pH 8 for various intervals of the time is represented in the figure 4. The remaining activity of the trypsin pre-incubated in the presence of 10mM concentration of $CaCl_2$ was stable ($p<0.05$) for about 3 hours at 30 °C, as binding of the calcium to a single binding site stabilises the enzymes against denaturation[27]. Stabilisation by calcium ions against thermal denaturation is also reported in trypsin of fish such as yellowfin tuna[30], true sardine and arabesque[31]. On the other hand remaining specific activity of the trypsin been pre-incubated in the presence of 10mM concentration of EDTA for pH 8 decreased with increase in the incubation period. EDTA chelates the metal ions required for the enzyme activity, lowering the trypsin activity to almost negligible by 5 hours.

CONCLUSION

Based on the SDS-PAGE analysis and activity for specific substrate, TAME and susceptibility to effectors, EDTA and $CaCl_2$ the enzyme isolated from the pyloric caeca of Japanese threadfin bream was trypsin. The characteristics of enzyme isolated were similar to trypsin isolated from tropical regions, but differs from the temperate zone. Interesting feature of the trypsin is its optimum activity at pH 8 and at temperature 60 °C, as its potential use in food processing industry.

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