Process Biochemistry 47 (2012) 1957–1964

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Calcium dependent, alkaline detergent-stable trypsin from the viscera of Goby (*Zosterisessor ophiocephalus*): Purification and characterization

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ARTICLE INFO

Article history:
Received 16 May 2012
Received in revised form 29 June 2012
Accepted 1 July 2012
Available online 7 July 2012

Keywords:
Alkaline trypsin
Biochemical characterization
Goby
Zosterissessor ophiocephalus
Calcium-dependent
Detergent-stable

ABSTRACT

An alkaline calcium dependent trypsin from the viscera of Goby (*Zosterisessor ophiocephalus*) was purified to homogeneity with a 16-fold increase in specific activity and 20% recovery. The purified trypsin appeared as a single band on sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and native-PAGE. The enzyme had an estimated molecular weight of 23.2 kDa.

The optimum pH was 9.0, and the enzyme was extremely stable in various pH buffers between pH 7.0 and 11.0. The optimum temperature for enzyme activity was 60 °C, and the activity and stability of trypsin was highly dependent on the presence of calcium ion. At 60 °C, Ca^{2+} (5 mM) stimulated the protease activity by 220%. The trypsin kinetic constants, K_m and k_{cat}, were 0.312 mM and 2.03 s^{-1}.

The enzyme showed high stability towards non-ionic surfactants and oxidizing agent. In addition, the enzyme showed excellent stability and compatibility with some commercial solid and liquid detergents.

1. Introduction

Proteases constitute the most important group of industrial enzymes and are widely used in different industrial processes such as detergent, food, leather and pharmaceutical industries [1]. Proteases with high activity and stability in high alkaline range are interesting for biotechnological applications. Their major application is in detergent industry because the pH of laundry detergents is generally in the range of 9.0–12.0, accounting for about 35% of the total enzymes sales [2]. Alkaline proteases are particularly important because they are both stable and active at high pH solutions and in the presence of detergents [3]. Most of the commercial alkaline proteases were isolated from *Bacillus* species [4]. The industrial demand of proteolytic enzymes, with appropriate specificity and stability to pH, temperature and surfactants, continues to stimulate the search for new enzymes sources. Fish viscera, one of the most important by-products of fishing industry, is a rich source of digestive enzymes [5].

The most important proteolytic enzymes from fish and aquatic invertebrate's viscera are the aspartic protease pepsin, and the serine proteases, trypsin, chymotrypsin, collagenase and elastase. Acidic proteases from fish stomachs display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0, and inactive or unstable at acidic pH. Trypsin is a member of a large family of serine proteinases which specifically cleave proteins and peptides at the carboxyl group of arginyl and lysyl residues and play major roles in biological processes, including digestion and activation of zymogens of chymotrypsin and other enzymes, including itself [6]. Trypsin (EC 3.4.21.4) is an important pancreatic serine protease synthetized as a proenzyme in the pancreatic acinar cells and secreted into the intestine of mammals.

Trypsin and trypsin-like proteolytic enzymes have been isolated and characterized from the viscera of some marine invertebrates and a wide range of cold water and warm water fish, including the digestive gland (hepatopancreas) of cuttlefish (*Sepia officinalis*) [7], the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) [8], and the entire viscera of smooth hound (*Mustelus mustelus*) [9], Grey triggerfish (*Balistes capriscus*) [10], Amazonian fish tambaqui (*Colossoma macropomum*) [11] silver mojarra (*Diapterus rhombeus*) [12], and zebra blenny (*Salaria basilisca*) [13].

The goby (*Zosterisessor ophiocephalus*) is common in Mediterranean Sea, Black Sea, and Sea of Azovthe. It reaches a maximum length of 25 cm and it is a carnivorous; juveniles feed on small crustaceans, polychaets and molluscs. It is relatively important in the fish-catches of Tunisia, and is utilised for human consumption. In Tunisia, goby (*Z. ophiocephalus*) catches were about 130 tonnes in 2004 [14].

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http://dx.doi.org/10.1016/j.procbio.2012.07.002

In a previous study, we reported the characterization of goby crude alkaline enzyme extract, which contains several proteolytic enzymes [15]. The crude enzyme extract showed an optimum activity at 50 °C and pH 8.0, using casein as a substrate.

In the present study, we describe the purification of an alkaline detergent stable trypsin from goby (*Z. ophiocephalus*), and we provide basic information about its main biochemical and kinetic characteristics.

2. Materials and methods

2.1. Reagents

Casein sodium salt from bovine milk, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulphonyl fluoride (PMSF), dithio-bis-nitrobenzoic acid (DTNB), *N*α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), benzamidine, glycine, bovine serum albumin and protein markers for molecular weights 14,000–66,000 Da were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean trypsin inhibitor (SBTI) was obtained from Fluka Biochemica (USA). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulphate, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Mexico City, Mexico). Sephadex G-100 was from Amersham Pharmacia Biotech (Uppsala, Sweden). Tris (hydroxymethyl) aminomethane was procured from Panreac Quimica SA (Barcelona, Spain). Polyvinylidene difluoride (PVDF) membrane was purchased from Applied Biosystems (Roissy, France). All other reagents were of analytical grade.

2.2. Goby viscera

Goby (*Z. ophiocephalus*) was purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with the sample per ice ratio of approximately 1:3 (weight per volume) and transported to the research laboratory within 30 min. Viscera was separated, rinsed three times with cold distilled water to remove salts and contaminants, and then used immediately for the extraction of digestive enzymes.

2.3. Enzyme purification

Purification was carried out at 4 °C. The crude enzyme extract from 160 g of viscera, prepared as previously described [15], was first subjected to ammonium sulphate fractionation. Ammonium sulphate fractions of 0–20%, 20–60% and 60–80% (w/v) were collected by centrifugation at 10,000 × *g*, and the resulting precipitates obtained in each fraction were suspended in a minimal volume of buffer A (10 mM Tris–HCl, pH 8.0). The precipitates were dialysed for 24 h at 4 °C against repeated changes of the same buffer.

The 20–60% (w/v) ammonium sulphate fraction was subjected to gel filtration on a Sephadex G-100 column (2.5 cm × 80 cm) which had been equilibrated with buffer B (25 mM glycine–NaOH pH 9.0 containing 0.5 per mille Triton X-100). Fractions of 4 ml were collected at a flow rate of 30 ml/h with the same buffer. Protein content (Absorbance at 280 nm) and protease activity (using casein) and trypsin activity (using BAPNA) were determined. The fractions with high trypsin activity were pooled.

2.4. Trypsin activity assay

Enzyme activity was measured according to the method of Benjakul et al. [16], using BAPNA as substrate specific for trypsin. An aliquot of the enzyme solution (200 μl), with an appropriate dilution, was added to the pre-incubated reaction mixture containing 1000 μl of 0.5 mM BAPNA in 0.1 M glycine–NaOH buffer, pH 9.0, and 200 μl of distilled water. The mixture was incubated for 10 min at 60 °C. The enzymatic reaction was terminated by adding 200 μl of 30% (v/v) acetic acid, and then centrifuged at 8000 × *g* for 3 min at room temperature. Trypsin amidase activity was measured by the absorbance at 410 nm due to *p*-nitroaniline released. One unit (U) of trypsin activity was defined as the amount that released 1 μmol of *p*-nitroaniline per minute under the established conditions using a molecular coefficient of 8800 M^{-1} cm^{-1}.

2.5. Polyacrylamide gel electrophoresis and detection of protease activity by zymography

SDS-PAGE was carried out for the control of the purity and determination of molecular weight of the purified enzyme as described by Laemmli [17], using a 5% (w/v) stacking and a 15% (w/v) separating gels. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with the SDS-PAGE sample buffer (10 mM Tris–HCl (pH 8.0), 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue). The samples were heated at 100 °C for 5 min before loading in the gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol, 10% acetic acid and destained with 5% ethanol and 7.5% acetic acid. The molecular weight of the trypsin was estimated using a low-molecular weight calibration kit as markers, consisting of: bovine serum albumin (66,000 Da); ovalbumin (45,000 Da); glyceraldehyde-3-dehydrogenase (36,000 Da); bovine trypsinogen (24,000 Da); soybean trypsin inhibitor (20,100 Da) and bovine α-lactoalbumin (14,200 Da).

Native-PAGE was performed according to the procedure of Laemmli [17], except that the sample was not heated and SDS and reducing agent were left out. Protease activity staining was performed on native-PAGE according to the method of Garcia-Carreno et al. [18] with a slight modification [15].

2.6. Determination of the N-terminal amino acid sequence of Z. ophiocephalus trypsin

The purified enzyme, from Sephadex G-100 gel filtration, was applied to SDS-PAGE. After brief staining with Coomassie Brilliant Blue R-250, the PVDF band corresponding to the trypsin was excised and the *N*-terminal amino acid sequence was determined by the Edman degradation method on an automated ABI Procise 494 protein sequencer (Applied Biosystems, Foster City, CA).

2.7. Protein determination

Protein concentration was determined by the method of Bradford [19], using bovine serum albumin as a standard. The concentration of protein during the purification studies was determined by measuring the absorbance at 280 nm.

2.8. Biochemical properties

2.8.1. Effect of pH on activity and stability

Activity of the purified trypsin was assayed at different pH values for 10 min at 60 °C using BAPNA as a substrate. To check the pH stability, enzyme was preincubated at various pHs (pH 7.0–12.0) for 60 min at 25 °C and the remaining activities were measured under the standard assay condition. The following buffer systems were used: 100 mM sodium acetate buffer, pH 6.0; 100 mM phosphate buffer, pH 7.0; 100 mM Tris–HCl buffer, pH 8.0; 100 mM glycine–NaOH buffer, pH 9.0–11.0; 100 mM Na_2HPO_4, pH 12.0.

2.8.2. Effect of temperature on activity and stability

To investigate the effect of temperature, trypsin activity was determined by incubating the reaction mixture at different temperatures ranging from 30 to 80 °C, using BAPNA as a substrate for 10 min at pH 9.0. For thermal stability, the purified enzyme was incubated at different temperatures for 60 min, and then residual activities were assayed at pH 9.0 and 60 °C for 10 min. Thermal inactivation was also examined by incubating the purified trypsin at 50 °C for 4.5 h in the absence or presence of 2 mM $CaCl_2$ or 2 mM EDTA. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions. The non-heated enzyme was considered to be the control (100% activity).

2.8.3. Effects of enzyme inhibitors, metal ions and denaturing reagents

The effects of enzyme inhibitors on trypsin activity were studied using PMSF, SBTI, benzamidine, DTNB, Pepstatin A and EDTA. The purified enzyme was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining enzyme activity was tested using BAPNA as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

The effects of various metal ions (5 mM) on trypsin activity were investigated by adding the monovalent (Na^+ or K^+) or divalent (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Mg^{2+} or Hg^{2+}) metal ions to the reaction mixture. The effect of $CaCl_2$ concentration on trypsin activity was also studied. The activity of the enzyme in the absence of metal ions was taken as control.

The effects of some surfactants (Triton X-100, Tween 80 and SDS) and oxidizing agents (sodium perborate and H_2O_2) on enzyme stability were studied by pre-incubating the purified trypsin for 1 h at 30 °C. The residual activity was measured at pH 9.0 and 60 °C. The enzyme activity of the control (without any detergent) was taken as 100%.

2.8.4. Kinetic studies

The activity of the purified trypsin was evaluated at 25 °C with different final concentrations of BAPNA, ranging from 0 to 2000 μM. The final enzyme concentration of the assay was 0.025 mg protein per ml. The determinations were repeated twice and the respective kinetic parameters, including the apparent Michaelis–Menton (K_m) and the maximum velocity (V_{max}) were calculated from Lineweaver–Burk plots [20]. The value of the turnover number (k_{cat}) was calculated from the following equation: $k_{cat} = V_{max}$ per [E], where [E] is the enzyme concentration.

2.9. Detergent compatibility

The compatibility of the purified alkaline trypsin with commercial solid laundry detergents was studied using Dixan (Henkel, Spain), Nadhif (Henkel-Alki, Tunisia), Ariel (Procter and Gamble, Suisse), New Det (Sodet, Tunisia) and Axion (Colgate-Palmolive, France). Commercial solid detergents were diluted in tap water to give a

final concentration of 7 mg per ml to simulate washing conditions. The compatibility of the purified trypsin with commercial liquid detergents was also investigated using Carrefour (Carrefour, France), Lav+ (Best LAV, Tunisia) and Tex'til (Belgique). The liquid detergents were diluted 100-fold in tap water (1%) to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65 °C prior to the addition of the enzyme. The purified alkaline trypsin was incubated with different detergents for 1 h at 30 and 40 °C, and then the remaining activities were determined under the standard assay conditions. The enzyme activity of a control, without detergent, incubated under the similar conditions, was taken as 100%.

2.10. Statistical analysis

Statistical analyses were performed with statgraphics Ver. 5.1, professional edition (Manugistics Group, Rockville, MD) using ANOVA analysis. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Purification of Z. ophiocephalus trypsin

Trypsin from the viscera of *Z. ophiocephalus* was extracted and purified by the two-step procedure described in Section 2. In the first step, the crude enzyme extract was fractionated with ammonium sulphate. The precipitate formed at 20–60% (w/v) saturation showed higher specific activity (0.07 U/mg protein) than precipitates formed at 0–20% (0.007 U/mg protein) and 60–80% (0.017 U/mg protein). No activity was detected in the final supernatant. The 20–60% fraction, with the greatest specific activity for trypsin, was then subjected to Sephadex G-100 gel filtration. This procedure yielded three peaks of protease activity using casein as a substrate, and a single peak of trypsin activity using BAPNA as a substrate (results not shown).

The specific activity in the initial enzyme extract was 0.05 U/mg protein. After the final purification step, the trypsin was purified 16-fold, with a recovery of 20% and a specific activity of 0.8 U/mg protein, using BAPNA as a substrate (Table 1).

Purified trypsin from goby migrated as a single band in SDS-PAGE (Fig. 1a) and native PAGE (Fig. 1b), indicating the homogeneity of the enzyme and confirming that the purified trypsin had only one isoform. The molecular weight of trypsin was estimated to be 23.2 kDa based on SDS-PAGE (Fig. 1a), corresponding to that determined by gel filtration. Most of fish trypsins have been reported to have molecular weights in the range of 23–28 kDa [21]. The molecular weight of *Z. ophiocephalus* trypsin was similar to those from other fish species, such as walleye pollock (*Theragra chalcogramma*) [22], true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) [23], Amazonian fish tambaqui (*C. macropomum*) [11], grey triggerfish (*B. capriscus*) [10], and cuttlefish (*S. officinalis*) [7], and lower than those of trypsins from zebra blenny (*S. basilisca*) [13], and silver mojarra (*D. rhombeus*) [12].

Casein-zymogram staining, a very sensitive and rapid assay method that detects nanograms of protein, revealed a unique clear zone of proteolytic activity against the blue background of the gel, indicating the purity of the trypsin (Fig. 1b).

3.2. N-terminal amino acid sequence of Z. ophiocephalus trypsin

The N-terminal 9 amino acids of the *Z. ophiocephalus* trysin were determined to be **IVGGYECQP**. The N-terminal amino acid sequence of *Z. ophiocephalus* trypsin showed uniformity, indicating that it was isolated in a pure form and, if other isoforms are present, their amounts must be small.

The N-terminal amino acid sequence of *Z. ophiocephalus* trypsin was aligned with the sequences of other animal trypsins (Fig. 2). The alignment indicates that the first four NH_2-terminal amino acid residues (IVGG) are conserved in all trypsins. The sequence of

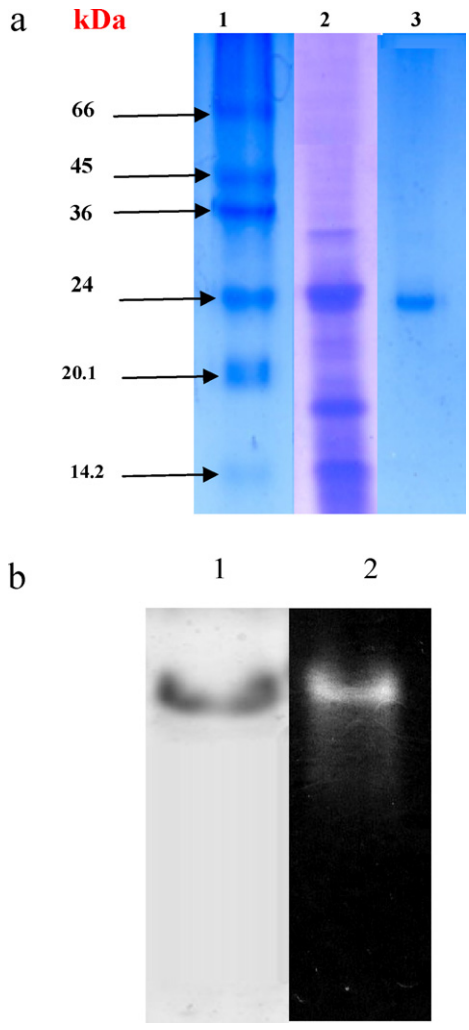

Fig. 1. (a) SDS-PAGE of the purified trypsin from *Z. ophiocephalus*. Lane 1: standard proteins marker of different molecular weights; lane 2: crude enzyme extract; lane 3: purified trypsin. (b) Native-PAGE (lane 1) and zymogram (lane 2) of the purified trypsin from *Z. ophiocephalus*.

Z. ophiocephalus trypsin was identical to that of Japanese anchovy (*Engraulis japonica*) [24]. The sequence of *Z. ophiocephalus* trypsin showed high homology with those from smooth hound [9], grey triggerfish [10] and arabesque greenling [23]. There is only one amino acid residue in the 9-terminal sequence that differs in these sequences. However, there are two amino acid residues in the 9-terminal sequence that differ with trypsins from Amazonian fish tambaqui [11], silver mojarra [12], walleye pollok [22], true sardine (TR-S) [23], antarctic fish (*Paranotothenia magellanica*) [25], mandarin fish (*Siniperca chuasti*) [26] and common Carp (*Cyprinus carpio*) (trypsin A) [27]. The sequence of *Z. ophiocephalus* trypsin showed also high homology with rat [28], dog [29], bovine [30], porcine [31] and human [32] trypsins; they differ by three amino acids at positions 6, 8 and 9.

3.3. Enzyme characterization

3.3.1. Effects of enzyme inhibitors on trypsin activity

Proteases can be classified by their sensitivity to various inhibitors. To confirm the nature of the purified protease, activities were measured in the presence of different enzyme inhibitors (Table 2). Trypsin from *Z. ophiocephalus* was strongly inhibited by the well-known trypsin inhibitor investigated, namely, SBTI (1 mg/ml) and by PMSF and benzamidine, serine protease

Table 1
Summary of the purification of trypsin from *Z. ophiocephalus* viscera.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purity (fold)
Crude extract	45	850	0.05	100	1
Ammonium sulphate precipitation (20–60%)	30	430	0.07	0.66	–
Sephadex G-100	8.9	11.36	0.8	20	16

All operations were carried out at 4 °C.

inhibitors. On the other hand, the metalloprotease inhibitor (EDTA, 5 mM) inhibited the enzyme by 59%. The relatively high activity of the trypsin in the presence of EDTA is very useful for application as detergent additive because chelating agents are components of most detergents. Chelating agents function as water softeners and also assist in the removal of stain. These agents specifically chelate metal ions making them unavailable in the detergent solution.

In addition, Pepstatin A, an aspartic protease inhibitor and DTNB, specific for thiol proteases, did not show any inhibitory effects against the purified trypsin. The inhibition results indicate that the purified enzyme is a serine-protease based on inhibition by PMSF and authentic trypsin based on its catalytic specificity for BAPNA and inhibition by SBTI.

3.3.2. Effect of pH on activity and stability of Z. ophiocephalus trypsin

The pH activity profile of the purified *Z. ophiocephalus* trypsin was investigated at 25 °C between pH 6.0 and pH 12.0. As can be seen in Fig. 3a, the optimum pH value of the purified enzyme was 9.0. The relative activities at pH 10.0 and 11.0 were about 91% and 50%, respectively, of that at pH 9.0. Therefore, *Z. ophiocephalus* trypsin could be a potential candidate for addition in commercial laundry detergents, because the pH in laundry detergent is generally in the range of 9.0–11.0. The optimum pH activity of *Z. ophiocephalus* trypsin was higher than that of trypsin from *S. officinalis*, which showed maximum activity at pH 8.0 [7] and that of trypsin from *D. rhombeus* which exhibited higher enzyme activity at pH 8.5 [12]. However, the optimum pH was lower than that of *B. capriscus* trypsin which exhibited maximum activity at pH 10.5 and showed about 91% activity at pH 11.0 [10].

The pH stability profile of trypsin from *Z. ophiocephalus* is shown in Fig. 3b. The enzyme is highly stable in a broad pH range, and more than 95% activity was retained even after incubation for 1 h at 25 °C at pH between 7.0 and 11.0. However, it was unstable at pH 12.0. The pH stability of *Z. ophiocephalus* trypsin is higher than monterey sardine trypsin, which was stable in the pH range from 7.0 to 8.0 [33] and *D. rhombeus* trypsin, which maintained over 85% of its optimum activity between pH 8.5 and 11.0 [12]. These results suggest that the viscera of goby would be a potential source of trypsin for certain food processing operations that require high alkaline conditions.

3.3.3. Effect of temperature on the activity and stability of Z. ophiocephalus trypsin

Temperature profile of trypsin from *Z. ophiocephalus* is depicted in Fig. 3c. The enzyme was active at temperatures from 30 to 80 °C with an optimum around 60 °C. The relative activity at 50 °C was about 85% of that at 60 °C. However, enzyme activity decreased rapidly at higher temperatures. The sharp decrease in hydrolysis of BAPNA might be attributed to irreversible protein denaturation

Table 2
Effect of various enzyme inhibitors, metal ions and additives on the activity of the purified trypsin from *Z. ophiocephalus* viscera.

Ions, inhibitors, surfactant, oxidizing agent, detergent	Concentration	Target proteases	Relative activity (%)
None	–	–	100
EDTA	5 mM	Metallo-proteases	41
DTNB	5 mM	Cysteine proteases	99
Pepstatin A	0.1 mM	Some aspartic proteases	100
PMSF	1 Mm	Serine proteases	58
	5 mM	–	0
Benzamidine	5 mM	Serine proteases	1.15
SBTI	1 mg/ml	Trypsine-like serine proteases	0
Na^+	5 mM		102
K^+	5 mM		98
Mg^{2+}	5 mM		112
Mn^{2+}	5 mM		110
Zn^{2+}	5 mM		10
Cu^{2+}	5 mM		0
Hg^{2+}	5 mM		0
Ca^{2+}	5 mM		220
Ba^{2+}	5 mM		95
SDS	0.1% (w/v)		52
	0.5%		40
	1%		20
Tween 80	1% (v/v)		100
	5%		100
Triton X-100	1% (v/v)		100
	5%		96
Sodium perborate	0.2% (w/v)		89.5
	1%		51.7
H_2O_2	1% (v/v)		66.8
	5%		40.1

Purified enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25 °C and the remaining activity was determined at pH 9.0 and 60 °C. Enzyme activity measured in the absence of any inhibitor was taken as 100%.
The effect of metal ions on the activity of the purified trypsin was determined by incubating the enzyme in the presence of various metal ions for 10 min at 60 °C and pH 9.0. The enzymes were pre-incubated with surfactants, oxidizing agents for 1 h at 30 °C and the remaining protease activities were measured under standard conditions. The activity is expressed as a percentage of the activity level in the absence of additives.

Gobie	I	V	G	G	Y	E	C	Q	P
Japanese anchovy	I	V	G	G	Y	E	C	Q	P
Grey Trigger fish	I	V	G	G	Y	E	C	T	P
Smooth hound	I	V	G	G	Y	E	C	K	P
Arabesque greenling	I	V	G	G	Y	E	C	T	P
Antarctic fish	I	V	G	G	K	E	C	S	P
Mandarin fish	I	V	G	G	Y	E	C	E	A
Walleye pollok	I	V	G	G	Y	E	C	T	K
Silver mojarra	I	V	G	G	Y	E	C	T	M
True sardine	I	V	G	G	Y	E	C	K	A
Tambaqui	I	V	G	G	Y	E	C	K	A
Cuttlefish	I	V	G	G	K	E	S	S	P
Zebra blenny	I	V	G	G	R	E	C	T	E
Common carp (trypsin A)	I	V	G	G	Y	E	X	E	P
Rat	I	V	G	G	Y	T	C	P	E
Dog	I	V	G	G	Y	T	C	S	A
Bovine	I	V	G	G	Y	T	C	G	A
Porcine	I	V	G	G	Y	T	C	A	E
Human	I	V	G	G	Y	N	C	E	E

Fig. 2. Alignment of the N-terminal amino acid sequence of the purified trypsin from *Z. ophiocephalus* with the sequences of other trypsins. Amino acid residues identical to *Z. ophiocephalus* trypsin are shaded: walleye pollok (*T. chalcogramma*) [22]; arabesque greenling (*P. azonus*) and true sardine (TR-S) (*S. melanostictus*) [23]; antarctic fish (*Paranotothenia magellanica*) [25]; Japanese anchovy (*E. japonica*) [24]; grey triggerfish (*B. capriscus*) [10]; cuttlefish (*S. officinalis*) [7]; smooth hound (*S. aurita*) [9]; zebra blenny (*S. basilisca*) [13]; mandarin fish (*Siniperca chuasti*) [26]; common Carp (*C. carpio*) (trypsin A) [27]; silver mojarra (*D. rhombeus*) [12]; Tambaqui (*C. macropomum*) [11], rat [28], dog [29], bovine [30], porcine [31] and human [32].

due to the partial unfolding of the enzyme molecule. The optimal temperature for *Z. ophiocephalus* protease was similar to trypsin from *S. basilisca* [13], higher than that of trypsin from *B. capriscus* [10], which had an optimum temperature at 40 °C and lower than that of cuttlefish trypsin which had an optimum temperature at 70 °C [7].

The thermal stability profile showed that the purified trypsin was highly stable at 30 and 40 °C for 1 h incubation, retaining 100% and 96% of its activity, respectively, but was inactivated at temperatures over 40 °C (Fig. 3d). The enzyme retained 52% and 13% of its initial activity after 60 min incubation at 50 and 60 °C, respectively. The thermal stability profile is similar to trypsins from grey triggerfish [10] and zebra blenny [13].

3.3.4. *Effect of calcium on trypsin thermostability*

The increased rate of proteolysis of proteases at elevated temperatures is one of the factors responsible for the rapid thermal inactivation of enzymes. Several works reported that calcium ion plays a major role in enzyme stabilization at high temperatures. To determine whether Ca^{2+} enhanced thermal stability of trypsin during thermal inactivation, the stability was examined by incubating the purified trypsin for 4 h 30 min at 50 °C in the absence and presence of 2 mM $CaCl_2$ or 2 mM EDTA. The thermal stability profiles reported in Fig. 4a showed a continuous decrease in trypsin activity in the absence of $CaCl_2$ or in the presence of EDTA with increasing incubation time. The enzyme retained 50% and 34% of its activity after 60 min incubation in the absence of $CaCl_2$ or in the presence of EDTA, respectively. However, the stability of the enzyme was

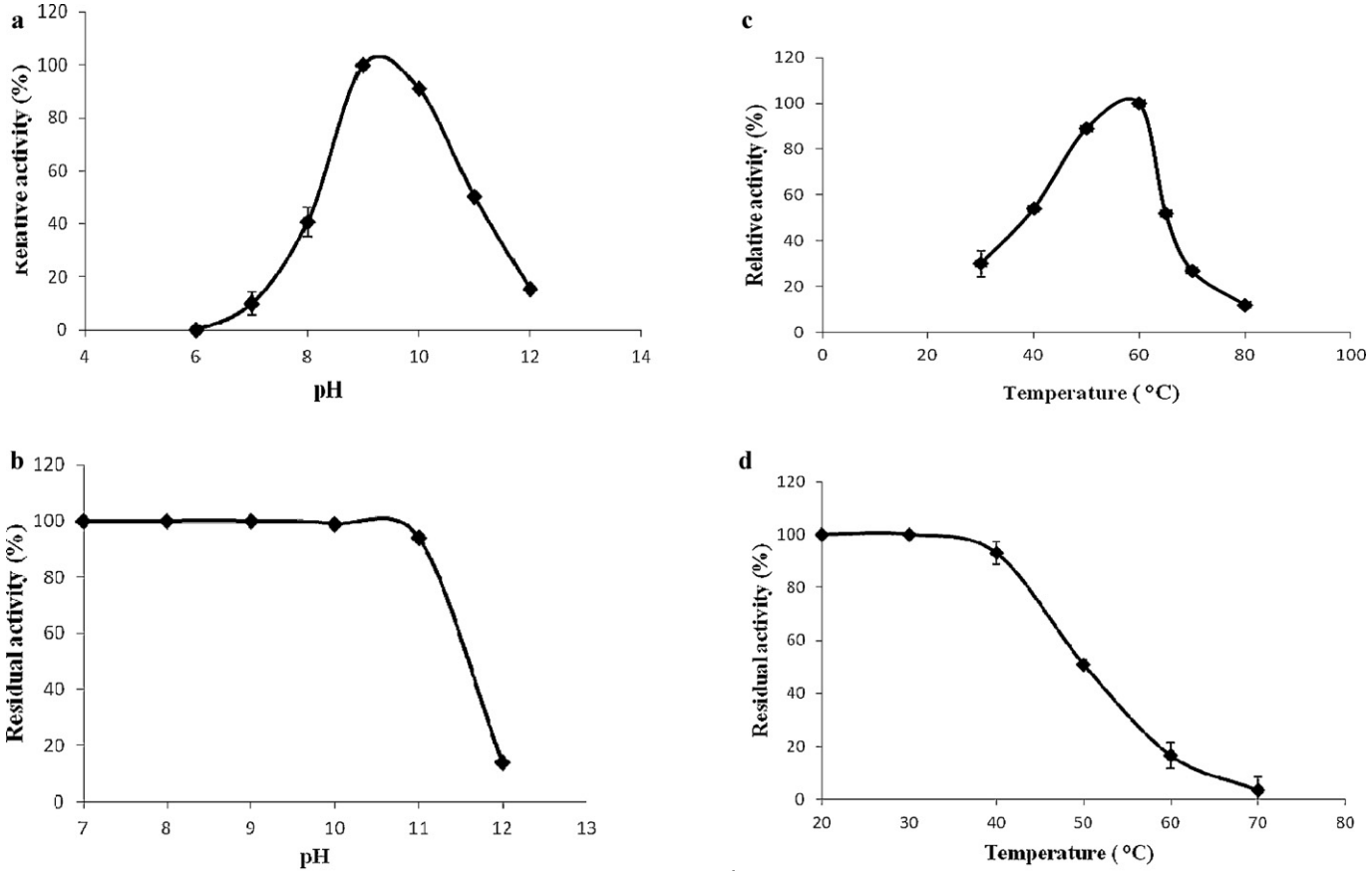

Fig. 3. pH (a) and temperature profiles (c), and pH (b) and thermal (d) stability of the purified trypsin. The pH profile was studied at 60 °C at various pHs. For temperature profile, the activity of trypsin was determined at pH 9.0 using BAPNA as a substrate. For pH and thermal stability, the remaining activities were assayed at pH 9.0 and 60 °C using BAPNA as a substrate. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2.

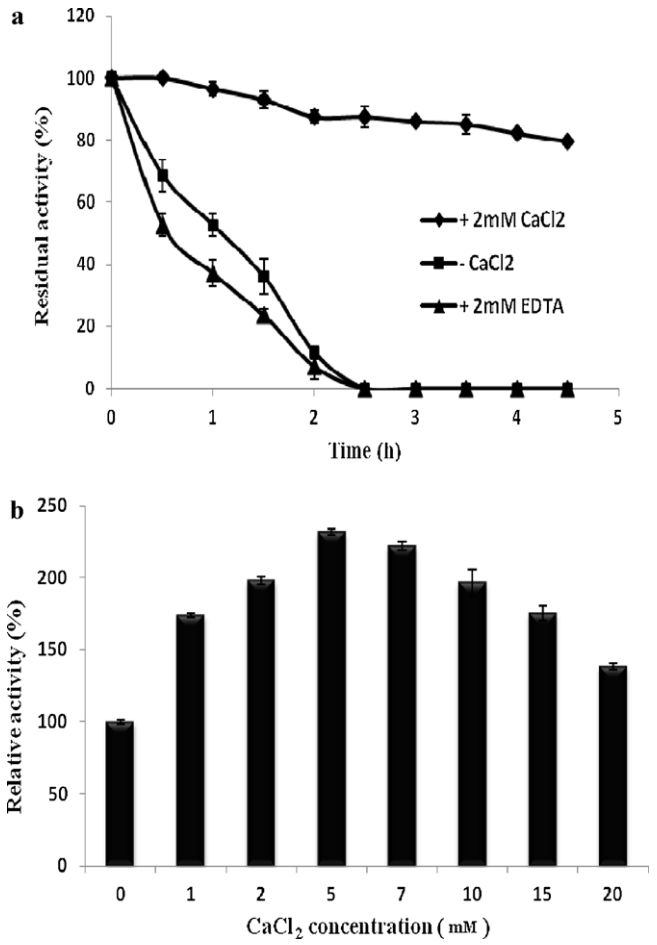

Fig. 4. (a) Effect of calcium ion (2 mM) and EDTA (2 mM) on the stability of the purified tryspsin from *Z. ophiocephalus*. The stability was tested by incubating the purified enzyme at 50 °C in the absence or presence of $CaCl_2$ or EDTA and residual enzyme activity was determined from 0 to 4 h 30 min at 30-min intervals. The non-heated enzyme was considered as control (100% activity). (b) Effect of $CaCl_2$ concentrations on the trypsin activity. The activity of the enzyme without $CaCl_2$ was considered as 100%.

considerably enhanced by Ca^{2+}. After incubation for 150 min the enzyme retained more than 85% of its activity, while no activity was detected in the absence of $CaCl_2$. The half-lives of the purified trypsin at 50 °C were determined to be of the order of 1 h and >10 h, in the absence and presence of 2 mM $CaCl_2$, respectively.

The improvement in protease stability against thermal inactivation in the presence of Ca^{2+} may be explained by the strengthening of interactions inside protein molecules and by the binding of calcium ion to autolysis sites. In fact, many studies showed that serine-proteases contain binding site with a higher affinity for calcium ions, which play an important role in stabilizing the enzyme against thermal denaturation and autodegradation [34]. These results suggest that the purified trypsin possesses a primary calcium-binding site like mammalian and other fish species trypsins.

3.3.5. Effects of metal ions

The effects of some metal ions on the trypsin activity were studied (Table 2). Addition of $CaCl_2$ to the assay medium enhanced the protease activity to 220%. However calcium at 5 mM increased the activity of the crude protease extract from goby to only 110% [15]. Further, Mg^{2+} and Mn^{2+} exhibited a slight stimulatory effect on the enzyme. On the contrary, Mn^{2+} decreased the protease activity of the crude extract by 52.5% [15]. It is known that calcium ion promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis. Similarly, trypsins from other fish species were activated by calcium ion [13,27]. On the contrary, trypsin from pyloric ceca of the starfish *Asterina pectinifera* was neither activated nor stabilized by calcium ion [34].

Protease activity was not affected by Ba^{2+}, NaCl and KCl at a concentration of 5 mM. The trypsin activity was reduced by Zn^{2+} to 10%. Cu^{2+} and Hg^{2+} completely inhibited trypsin activity.

Since $CaCl_2$ enhances trypsin activity, the effect of its concentration on enzyme activity was also studied. As shown in Fig. 4b, maximum activity was obtained with 5 mM $CaCl_2$ and there was 220% increase in the activity compared to that realized without $CaCl_2$. Beyond 5 mM Ca^{2+}, trypsin activity decreased. These results indicated that the enzyme require Ca^{2+} for its optimal activity. The calcium requirement for trypsin activity is highly specific since other metal ions (such as Mn^{2+}, Mg^{2+}, and Ba^{2+}) are not able to enhance, or slightly stimulated the enzyme activity.

3.3.6. Effects of oxidizing agents and surfactants on protease stability

The suitability of *Z. ophiocephalus* trypsin as a detergent additive was determined by testing its stability against oxidants and surfactants. As shown in Table 2, the alkaline trypsin is stable in the presence of the non-ionic surfactants, like Tween 80 and Triton X-100. Interestingly *Z. ophiocephalus* trypsin was little influenced by oxidizing agent, and retained about 89% and 52% of its activity after incubation for 1 h at 40 °C in the presence of 0.2% and 1% sodium perborate, respectively. In the presence of 1% and 5% hydrogen peroxide the enzyme retained about 67% and 40% of its initial activity, respectively. The relative stability of the enzyme in the presence of oxidizing agents is a very important characteristic for its eventual use in detergent formulations. The stability of *Z. ophiocephalus* trypsin against sodium perborate was higher than *S. basilisca* trypsin which retained about 40% of its initial activity after incubation for 1 h at 30 °C in the presence of 1% sodium perborate [13]. The *Z. ophiocephalus* trypsin was, however, unstable against the strong anionic surfactant (SDS) and retained 40% and 20% activity in the presence of 0.5% and 1% SDS, respectively.

3.4. Stability of the alkaline trypsin with commercial solid and liquid detergents

In order to confirm the potential of the purified *Z. ophiocephalus* trypsin as a detergent additive, we examined its compatibility and stability towards some commercial liquid and solid detergents after incubation for 1 h at 30 and 40 °C.

The data presented in Fig. 5a shows that the alkaline trypsin showed excellent stability and compatibility in all liquid detergents tested. The enzyme retained 100% activity in the presence of Carrefour and Lav^{+}, and about 91, 87 and 94% in Tex'til, Ariel and Persil, respectively, after 1 h incubation at 30 °C. The enzyme showed also relative stability and compatibility at 40 °C.

The data presented in Fig. 5b shows that the enzyme is also highly stable in the presence of solid detergents, retaining 90, 100, 60, 93 and 100% of its initial activity after 1 h incubation at 30 °C in the presence of Nadhif, Ariel, Axion, New-Det and Dixan, respectively. The enzyme was, however, less stable at 40 °C than 30 °C. The enzyme was found to be affected by axion, retaining only 43% of its initial activity after 1 h incubation at 40 °C.

Since the proteolytic activity varied with each laundry detergent tested, the obtained results clearly indicated that the performance of enzymes in detergents depends on number of factors, including the detergents compounds. Esposito et al. [35] reported also the stability of tambaqui proteases in the presence of several commercial detergents.

Table 3
Kinetic constants of *Z. opheocephalus* trypsin and other trypsins.

Trypsins	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	References
Goby (*Z. ophiocephalus*)	0.312	2.03	6.51	This study
Cuttlefish (*S. officinalis*)	0.064	2.32	36.25	[7]
Grey triggerfish (*B. capriscus*)	0.068	2.76	40.6	[10]
Bigeye snapper (*P. macracanthus*)	0.312	1.06	3.4	[36]
Montery sardine (*S. sagax cearula*)	0.051	2.12	41.0	[33]
Silver mojarra (*D. rhombeus*)	0.266	0.93	3.48	[12]
Zebra blenny (*S. basilisca*)	0.6	1.38	2.32	[13]

3.5. Kinetic properties

Kinetic constants K_m and k_{cat} of the purified *Z. ophiocephalus* trypsin were determined using Lineweaver–Burk plots. The K_m and k_{cat} of the purified enzyme, using BAPNA, were 0.312 mM and 2.03 s^{-1}, respectively, and were close to those reported for trypsins from bigeye snapper (*Pricanthus macracanthus*) [36] and silver mojarra [12]. The catalytic efficiency (k_{cat}/K_m) of *Z. ophiocephalus* trypsin, 6.51 s^{-1} mM^{-1}, was close to trypsins from silver mojarra [12] and bigeye snapper [36] (Table 3).

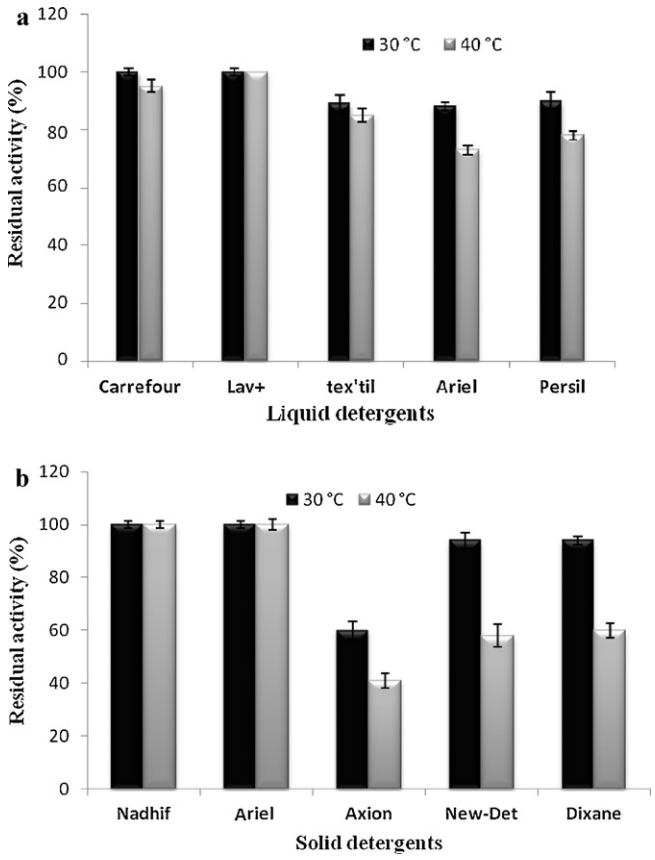

Fig. 5. Stability of trypsin from *Z. ophiocephalus* in the presence of various commercial laundry detergents. The enzyme at 200 U/ml was incubated 1 h at 30 and 40 °C and pH 9.0 in the presence of liquid detergents (a) diluted 100-fold in tap water (1/100) and solid detergents (b) at a final concentration of 7 mg/ml, and the remaining activities were determined at pH 9.0 and 60 °C using casein as a substrate. Enzyme activity of control sample without any detergent, incubated under the similar conditions, was taken as 100%.

4. Conclusion

In this report, an alkaline calcium dependent trypsin from *Z. ophiocephalus* was purified and identified based on molecular weight, inhibitor sensibility, substrate specificity and N-terminal amino acids sequencing. The enzyme showed an optimum temperature at 60 °C and optimum pH of 9.0. The enzyme was highly stable at a pH range of 7.0–11.0 and at temperatures below 40 °C. Further, the activity of the enzyme was highly enhanced by the addition of calcium ion in the reaction mixture. On the other hand, the enzyme possesses some other special characteristics, stability against the non-ionic surfactants and oxidizing agents, and compatibility with some commercial liquid and solid detergents.

Considering these properties, *Z. ophiocephalus* trypsin may find application in certain industrial applications that require alkaline conditions, such detergent industry. Further research is needed to determine properties of *Z. ophiocephalus* trypsin as a possible biotechnological tool in the fish processing and food industries.

Acknowledgement

This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.

References

[1] Gupta R, Beg Q, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59:15–32.
[2] Kalisz HM. Microbial proteinases. In: Fietcher A, editor. Advances in biochemical engineering/biotechnology (enzymes studies), vol. 36. Berlin, Heidelberg, NY: Springer; 1988. p. 3–61.
[3] Gupta R, Gupta K, Saxena RK, Khan S. Bleach-stable, alkaline protease from *Bacillus* sp. Biotechnol Lett 1999;21:135–8.
[4] Priest FG. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev 1977;41:711–53.
[5] Shahidi F, Kamil JYVA. Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Technol 2001;12:435–64.
[6] Rypniewski WR, Perrakis A, Vorgias CE, Wilson KS. Evolutionary divergence and conservation of trypsin. Protein Eng 1994;7:57–64.
[7] Balti R, Barkia A, Bougatef A, Nasri M. Heat-stable trypsin from cuttlefish (*Sepia officinalis*) hepatopancreas: purification and characterization. Food Chem 2009;113:146–54.
[8] Kurtovic I, Marshall SN, Simpson BK. Isolation and characterization of a trypsin fraction from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*). Comp Biochem Physiol 2006;143B:432–40.
[9] Bougatef A, Balti R, Nasri R, Jellouli K, Souissi N, Nasri M. Biochemical properties of anionic trypsin acting at high concentration of NaCl purified from the intestine of a carnivorous fish: Smooth hound (*Mustelus mustelus*). J Agric Food Chem 2010;58:5763–9.
[10] Jellouli K, Daassi D, Bougatef A, Barkia A, Nasri M. New alkaline trypsin from intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: Isolation and characterization. Food Chem 2009;116:644–50.
[11] Marcuschi M, Esposito TS, Machado MFM, Hirata IY, Machado MFM, Silva MV, et al. Purification, characterization and substrate specificity of a trypsin from the Amazonian fish tambaqui (*Colossoma macropomum*). Biochem Biophys Res Commun 2010;396:667–73.

[12] Silva JF, Esposito TS, Marcuschi M, Ribeiro K, Cavalli RO, Oliveira V, et al. Purification and partial characterization of a trypsin from the processing waste of the silver mojarra (*Diapterus rhombeus*). Food Chem 2011;129:777–82.
[13] Ktari N, Ben Khaled H, Nasri R, Jellouli K, Ghorbel S, Nasri M. Trypsin from zebra blenny (*Salaria basilisca*) viscera: purification, characterization and potential application as a detergent additive. Food Chem 2012;130:467–74.
[14] FAO-Sofia. The state of world fisheries and aquaculture (SOFIA) 2004. Rome: FAO Fisheries Department; 2004.
[15] 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. J Amino Acids 2011;2011:9.
[16] Benjakul S, Visessanguan W, Thummaratwasik P. Isolation and characterization of trypsin inhibitors from some Thai legume seeds. J Food Biochem 2000;24:107–27.
[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[18] Garcia-Carreno FL, Dimes LE, Haard NF. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinases inhibitors. Anal Biochem 1993;214:65–9.
[19] Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–54.
[20] Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:665–6.
[21] Whitaker JR. Principles of enzymology for the food sciences. 2nd ed. New York, NY: Marcel Dekker; 1994. p. 63–115.
[22] Kishimura H, Klomklao S, Benjakul S, Chun BS. Characteristics of trypsin from the pyloric ceca of walleye pollock (*Theragra chalcogramma*). Food Chem 2008;106:194–9.
[23] Kishimura H, Hayashi K, Miyashita Y, Nonami Y. Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). Food Chem 2006;97:65–70.
[24] Kishimura H, Hayashi K, Miyashita Y, Nonami Y. Characteristics of two trypsin isozymes from the viscera of Japanese anchovy (*Engraulis japonica*). J Food Biochem 2005;29:459–69.
[25] Genicot S, Rentier-Delrue F, Edwards D, VanBeeumen J, Gerday C. Trypsin and trypsinogen from an Antarctic fish: molecular basis of cold adaptation. Biochim Biophys Acta 1996;1298:45–7.
[26] Lu BJ, Zhou LG, Cai QF, Hara K, Maeda A, Su WJ, et al. Purification and characterisation of trypsins from the pyloric caeca of mandarin fish (*Siniperca chuatsi*). Food Chem 2008;110:352–60.
[27] 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 Sci 2000;66:1172–9.
[28] MacDonald RJ, Stary SJ, Swift GH. Two similar but nonallelic rat pancreatic trypsinogens. J Biol Chem 1982;14:222–4.
[29] Pinsky SD, LaForge S, Scheele G. Differential regulation of trypsinogen mRNA sequences encoding two oppositely charged trypsinogen isozymes in the dog pancreas. Mol Cell Biol 1985;5:2669–76.
[30] LeHuerou I, Wicker C, Guilloteau P, Toullec R, Puigserver A. Isolation and nucleotide sequence of cDNA clone for bovine pancreatic anionic trypsinogen: structural identity within the trypsin family. Eur J Biochem 1990;193:767–73.
[31] Hermodson MA, Ericsson LH, Neurath H, Walsh KA. Determination of the amino acid sequence of porcine trypsin by sequenator analysis. Biochemistry 1973;12:3146–53.
[32] Emi M, Nakamura Y, Ogawa M, Yamamoto T, Nishide T, Mori T, et al. Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. Gene 1986;41:305–10.
[33] Castillo-Yanez FJ, Pacheco-Aguilar R, Garcia-Carreno FL, Toro MAN. Isolation and characterization of trypsin from pyloric caeca of Monterey sardine *Sardinops sagax caerulea*. Comp Biochem Physiol 2005;140B:91–8.
[34] Kishimura H, Hayashi K. Isolation and characteristics of trypsin from pyloric ceca of the starfish *Asterina pectinifera*. Comp Biochem Physiol 2002;B132:485–90.
[35] Esposito TS, Amaral IPG, Buarque DS, Oliveira GB, Carvalho Jr LB, Bezerra RS. Fish processing waste as a source of alkaline proteases for laundry detergent. Food Chem 2009;112:125–30.
[36] Hau PV, Benjakul S. Purification and characterization of trypsin from pyloric caeca of bigeye snapper (*Pricanthus macracanthus*). J Food Biochem 2006;30:478–95.