Food Chemistry 116 (2009) 644–650

Contents lists available at ScienceDirect

Food Chemistry

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

New alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*) with high activity at low temperature: Purification and characterisation

Kemel Jellouli, Ali Bougatef, Dalel Daassi, Rafik Balti, Ahmed Barkia, Moncef Nasri *

Laboratoire de Génie Enzymatique et de Microbiologie, Ecole Nationale d'Ingénieurs de Sfax, B.P. "W" 3038 Sfax, Tunisia

ARTICLE INFO

Article history:
Received 29 November 2008
Accepted 26 February 2009

Keywords:
Alkaline trypsin
Purification
Biochemical characterisation
Grey triggerfish
Balistes capriscus
Intestine

ABSTRACT

A highly alkaline trypsin from the intestine of Grey triggerfish (*Balistes capriscus*), with high activity at low temperature, was purified and characterised. The enzyme was purified to homogeneity using acetone precipitation, Sephadex G-100 gel filtration and Mono Q-Sepharose anion-exchange chromatography, with a 13.9-fold increase in specific activity and 41.3% recovery. The molecular weight of the purified alkaline trypsin was estimated to be 23.2 kDa by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and size exclusion chromatography. Purified trypsin appeared as a single band on native–PAGE. Interestingly, the enzyme was highly active over a wide range of pH, from 9.0 to 11.5, with an optimum at pH 10.5, using *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as a substrate. The relative activities at pH 9.0, 11.5 and 12.0 were 86.5%, 92.6% and 52.4%, respectively. The enzyme was extremely stable in the pH range 7.0–12.0. In addition, the enzyme had high activity at low and moderate temperatures with an optimum at around 40 °C and had more than 80% of its maximum activity at 20 °C. The purified enzyme was strongly inhibited by soybean trypsin inhibitor (SBTI) and phenylmethylsulphonyl fluoride (PMSF), a serine protease inhibitor. The enzyme showed extreme stability towards oxidising agents, retaining about 87% and 80% of its initial activity after 1 h incubation at 40 °C in the presence of 1% sodium perborate and 1% H_2O_2, respectively. In addition, the enzyme showed excellent stability and compatibility with some commercial solid detergents.

The *N*-terminal amino acid sequence of the first 12 amino acids of the purified trypsin was **IVGGYECTPNST**. *B. capriscus* trypsin, which showed high homology with trypsins from marine vertebrates, had a basic residue, Asn, at position 10, where His and Tyr are common in all marine vertebrates trypsins.

The trypsin kinetic constants, K_m and k_{cat} for BAPNA, were 0.068 mM and 2.76 s^{-1}, respectively, while the catalytic efficiency, k_{cat}/K_m, was 40.6 s^{-1} mM^{-1}.

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1. Introduction

Proteases constitute the most important group of industrial enzymes used in the world today, accounting for about 50% of the total industrial enzyme market (Rao, Tanksala, Ghatge, & Deshpande, 1998). They have diverse applications in a wide variety of industries, such as the detergent, food, agrochemical and pharmaceutical industries (Gupta, Beg, & Lorenz, 2002; Zukowski, 1992). Proteases are derived from animal, plant and microbial sources.

Today, there is an increasing demand for fish proteolytic enzymes in food processing. Viscera, one of the most important by-products of the fishing industry, have wide biotechnological potential as a source of digestive enzymes, especially proteases, that have high activity over a wide range of pH and temperature conditions (Gildberg, 1992; Shahidi & Kamil, 2001) and exhibit high catalytic activity at relatively low concentration (Haard, 1998). These characteristics have made them suitable for different applications in many food processing operations. In addition, fish enzymes could be utilised to produce bioactive peptides from fish proteins. Considering the specific characteristics of these enzymes, fish processing by-products are currently used for enzyme extraction.

The most important digestive enzymes from fish and aquatic invertebrates viscera are the aspartic protease, pepsin, and 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. Trypsin is a member of a large family of serine proteinases which specifically hydrolyse proteins and peptides at the carboxyl group of arginine and lysine residues and play major roles in biological processes, including digestion and activation of zymogens of chymotrypsin and other enzymes.

Trypsin and trypsin-like proteolytic enzymes have been isolated and characterised from the viscera of some marine invertebrates

* Corresponding author. Tel.: +216 74 274 088; fax: +216 74 275 595.
E-mail addresses: mon_nasri@yahoo.fr, moncef.nasri@enis.rnu.tn (M. Nasri).

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doi:10.1016/j.foodchem.2009.02.087

and a wide range of cold-water and warm-water fish, including the digestive gland (hepatopancreas) of the white shrimp (*Penaeus setiferus*) (Gates & Travis, 1969), cuttlefish (*Sepia officinalis*) (Balti, Barkia, Bougatef, & Nasri, 2009) and crayfish (*Astacus fluviatilis*) (Titani et al., 1983), the spleen of skipjack tuna (*Katsuwonus pelamis*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007a) and yellowfin tuna (*Thunnus albacores*) (Klomklao et al., 2006), the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic, Marshall, & Simpson, 2006), tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001), and Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yanez, Pacheco-Aguilar, Garcia-Carreno, & Toro, 2005) and the entire viscera of true sardine (*S. melanostictus*) (Kishimura, Hayashi, Miyashita, & Nonami, 2006), Japanese anchovy (*Engraulis japonica*) (Kishimura, Hayashi, Miyashita, & Nonami, 2005) and sardine (*Sardina pilchardus*) (Bougatef, Souissi, Fakhfakh, Ellouz-Triki, & Nasri, 2007).

The Triggerfish, *Balistes capriscus,* is commonly known as the Grey triggerfish. It is brownish green to grey in colour and reaches a maximum length of 40 cm. The triggerfish, *B. capriscus,* is common in the Mediterranean Sea, where spawning occurs in water temperatures above 12 °C. The grey triggerfish is relatively important in the fish-catches of Tunisia, and is utilised for human consumption. In Tunisia, Grey triggerfish (*B. capriscus*) catches were about 130 tonnes in 2004 (FAO, 2004). As in other fish species, deterioration is very rapid in muscle and especially in the digestive tract, which suggests that Grey triggerfish viscera might be a good source of enzymes. So far, no information regarding trypsins or trypsin-like enzymes from *B. capriscus* has been documented.

In the present study, we describe the purification of an alkaline trypsin from Grey triggerfish (*B. capriscus*), and 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), phenylmethylsulfonyl fluoride (PMSF), *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), benzamidine, trichloroacetic acid, 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). Sephadex G-100 and Mono Q-Sepharose were from Pharmacia Biotech (Uppsala, Sweden). Tris (hydroxymethyl) aminomethane was procured from Panreac Quimica SA (Spain). All other reagents were of analytical grade.

2.2. Grey triggerfish viscera

Grey triggerfish (*B. capriscus*) was purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. The internal organs were separated and only intestines were collected, rinsed with distilled water and then stored in sealed plastic bags at −20 °C until used for enzyme extraction.

2.3. Preparation of crude enzyme extract

Intestines from *B. capriscus* (150 g) were rinsed with distilled water, and homogenised for 60 s with 300 ml extraction buffer A (10 mM Tris–HCl, pH 8.0). The homogenate was centrifuged at 8500*g* for 30 min at 4 °C. The pellet was discarded and the supernatant was collected and used as crude protease extract.

2.4. Enzyme purification

2.4.1. Acetone precipitation

The crude enzyme extract was first subjected to acetone precipitation. Acetone fractions of 0–30%, 30–60% and 60–80% (v/v) were collected by centrifugation at 10,000*g*, and the precipitate obtained in each fraction were suspended in a minimal volume of buffer A.

2.4.2. Sephadex G-100 gel filtration

The 60–80% (v/v) acetone fraction was subjected to gel filtration on a Sephadex G-100 column (2.5 × 80 cm), equilibrated with buffer B (25 mM Tris–HCl, pH 8.0 containing 0.5‰ Triton X-100). Fractions of 5 ml were eluted at a flow rate of 30 ml/h with the same buffer. Protein content (Abs 280 nm) and protease activity were determined.

2.4.3. Mono Q-Sepharose anion-exchange chromatography

The active fractions from Sephadex G-100 were pooled and applied to a Mono Q-Sepharose column (2 × 10 cm) previously equilibrated with buffer C (25 mM Tris–HCl, pH 8.0). After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the equilibrating buffer. Fractions (5 ml each) were collected at a flow rate of 60 ml/h and analysed for protease activity and protein concentration. Active fractions were pooled and stored at −20 °C for further analyses. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.5. Trypsin activity assay

Amidase activity was measured according to the method of Erlanger, Kokowsky, and Cohen (1961), modified by Benjakul, Visessanguan, and Thummaratwasik (2000), using BAPNA as a substrate. 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 reaction buffer (0.1 M Glycine–NaOH, pH 10.5) and 200 μl of distilled water. The mixture was incubated for 10 min at 40 °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 activity was measured by the absorbance at 410 nm due to *p*-nitroaniline released. Trypsin amidase activity was then calculated using the following formula:

$$\text{Activity (U/ml)} = \frac{A - A_0 \times \text{Final volume of the mixture (ml)} \times 1000}{8800 \times \text{Time of the reaction (min)} \times \text{(ml)}}$$

where 8800 M^{-1} cm^{-1} is the extinction coefficient of *p*-nitroaniline; A and A_0 are absorbances at 410 nm of the sample and the blank, respectively.

2.6. Polyacrylamide gel electrophoresis

SDS–PAGE was carried out for the control of the purity and determination of molecular weight of the purified enzyme, as described by Laemmli (1970), using 5% (w/v) stacking and 15% (w/v) separating gels. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris–HCl (pH 10.5), 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 100 °C for 5 min before electrophoresis. 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 enzyme 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), carbonic anhydrase (29,000 Da), bovine trypsinogen (24,000 Da) and bovine α-lactoalbumin (14,200 Da).

Native–PAGE was performed according to the procedure of Laemmli (1970), except that the sample was not heated and SDS and reducing agent were left out.

2.7. Zymography

Zymography was performed on native–PAGE according to the method of Garcia-Carreno, Dimes, and Haard (1993). Briefly, after electrophoresis, the gel was submerged in 1% (w/v) casein in 100 mM Glycine–NaOH buffer, pH 10.0, and incubated at 50 °C for 20 min. After washing, the gel was stained with Coomassie Brilliant Blue R250 for zymography analysis. A clear zone on the blue background of the gel indicated the presence of protease activity.

2.8. Determination of the N-terminal amino acid sequence of B. capriscus trypsin

The purified enzyme, from Q-Sepharose anion-exchange chromatography, was applied to SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. 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 ABI Procise 494 protein sequencer (Applied Biosystems).

2.9. Protein determination

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard, and during the course of enzyme purification by measuring the absorbance at 280 nm.

2.10. Biochemical properties

2.10.1. Effect of pH on activity and stability

Trypsin activity was assayed over the pH range 7.0–13.0 at 40 °C for 10 min, using BAPNA as a substrate. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs for 60 min at 25 °C. The following buffer systems were used: 100 mM Glycine–HCl buffer, pH 3.0; 100 mM sodium acetate buffer, pH 4.0–6.0; 100 mM phosphate buffer, pH 7.0; 100 mM Tris–HCl buffer, pH 8.0; 100 mM glycine–NaOH buffer, pH 9.0–12.0.

2.10.2. Effect of temperature on activity and stability

To investigate the effect of temperature, trypsin activity was tested at different temperatures ranging from 0 to 70 °C, using BAPNA as a substrate for 10 min at pH 10.5. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual activity was assayed at pH 10.5 and 40 °C for 10 min. The non-heated enzyme was considered as control (100%).

2.10.3. Effects of enzyme inhibitors and denaturing reagents

The effects of enzyme inhibitors on trypsin activity were studied using phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI) and ethylene–diaminetetraacetic acid (EDTA). The purified enzyme was pre-incubated with inhibitors for 30 min at 25 °C and then the remaining enzyme activity was estimated using BAPNA as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%. The effect of some surfactants (Triton X-100, Tween 20, 80 and SDS) and oxidising agents (sodium perborate and H_2O_2) on enzyme stability was studied by pre-incubating the purified trypsin for 1 h at 40 °C. The residual activity was measured at pH 10.5 and 40 °C. The activity of the enzyme without any additive was taken as 100%.

2.10.4. Effect of metal ions

The effect of various metal ions (5 mM) on trypsin activity was 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 activity of the enzyme in the absence of any additives was taken as 100%.

2.10.5. 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 determinations were repeated twice and the respective kinetic parameters, including K_m and V_{max}, were calculated from Lineweaver–Burk plots (Lineweaver & Burk, 1934).

The value of the turnover number (k_{cat}) was calculated from the following equation:

$k_{cat} = V_{max}/[E]$, where $[E]$ is the active enzyme concentration and V_{max} is the maximal velocity.

2.11. 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 detergents were diluted in tap water to give a final concentration of 7 mg/ml 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 preparation. The purified alkaline trypsin was incubated with different detergents (7 mg/ml) for 1 h at 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%.

3. Results and discussion

3.1. Purification of the B. capriscus trypsin

Trypsin from the intestine of *B. capriscus* was extracted and purified successively by the three-step procedure described in the "Materials and methods" section. In the first step, the crude enzyme extract was precipitated with acetone. Acetone fraction 60–80% (v/v) showed higher specific activity (0.83 U/mg of protein) than did 0–30% (0.08 U/mg of protein) or 30–60% (0.09 U/mg of protein). No activity was detected in the final supernatant. The 60–80% acetone fraction, which gave the highest specific activity, was successively subjected to Sephadex G-100 gel filtration and Mono Q-Sepharose anion-exchange chromatography (data not shown).

The results of the purification procedure are summarised in Table 1. After the final purification step, the trypsin was purified 13.9-fold, with a recovery of 41.3% and a specific activity of 4 U/mg of protein, using BAPNA as a substrate.

3.2. Purity and molecular weight

The molecular weight of trypsin was estimated by using SDS–PAGE. As shown in Fig. 1a, the purified enzyme has a molecular weight of approximately 23.2 kDa corresponding with that deter-

Table 1
A summary of the purification of trypsin from *B. capriscus*.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	163	560	0.29	100	1
60–80% acetone fraction	104	123	0.83	63.7	2.86
Sephadex G-100	75.2	36.5	2.06	46.3	7.10
Mono-Q	67.1	16.6	4.03	41.3	13.9

All operations were carried out at 4 °C.

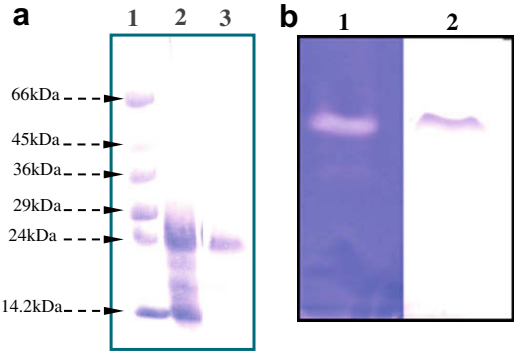

Fig. 1. (a) SDS–PAGE of the purified trypsin from *B. capriscus*. Lane 1: standard proteins marker of different molecular weights; Lane 2, crude enzyme extract; Lane 3, purified trypsin. (b) Zymogram (Lane 1) and Native–PAGE (Lane 2) of the purified trypsin from *B. capriscus*.

Grey Trigger fish (*B. capriscus*)	I V G G Y E C T P N S T
Arabesque greenling (*P. azonus*)	I V G G Y E C T P H T Q
Common carp (*C. carpio*) (trypsin B)	I V G G Y E X T P H S Q
Walleye pollok (*T. chalcogramma*)	I V G G Y E C T K H S Q
Japanese anchovy (TR-II) (*E. japonica*)	I V G G Y E C Q P Y S Q
Jacopever (TR-J) (*S. schlegellii*)	I V G G Y E C K P Y S Q
Antarctic fish (*P. magellanica*)	I V G G K E C S P Y S Q
Sardine (*S. pilchardus*)	I V G G Y E C Q K Y S Q
True sardine (TR-S) (*S. melanostictus*)	I V G G Y E C K A Y S Q
Common Carp (*C. carpio*)	I V G G Y E X E P Y S T
Mandarin fish (*S. chuasti*)	I V G G Y E C E A H - -
Bluefish (*P. saltatrix*)	I V G G Y E C K P K S A
Cuttlefish (*S. officinalis*)	I V G G K E S S P Y N Q
Brown hakeling (*P. japonicus*)	I V G G Y E C P K H S Q
Rat	I V G G Y T C P E H S V
Dog	I V G G Y T C S A N S V
Bovine	I V G G Y T C G A N T V
Porcine	I V G G Y T C A E N S V
Human	I V G G Y N C E E N S V

Fig. 2. Alignment of the *N*-terminal amino acid sequence of the purified trypsin from Grey triggerfish (*B. capriscus*) with the sequences of other trypsins. Amino acid residues identical to *B. capriscus* trypsin are shaded. Grey triggerfish, *B. capriscus* trypsin (present study); arabesque greenling (*P. azonus*) (Kishimura et al., 2006); Common carp (*C. carpio*) (trypsin B) (Cao et al., 2000); walleye pollok (*T. chalcogramma*) (Kishimura et al., 2008); Japanese anchovy (TR-II) (*E. japonica*) (TR-II) (Kishimura et al., 2005); jacopever (TR-J) (*Sebastes schlegellii*) (Kishimura et al., 2007); antarctic fish (*Paranotothenia magellanica*) (Genicot, Rentier-Delrue, Edwards, Van Beeumen, & Gerday 1996); sardine (*S. pilchardus*) (Bougatef et al., 2007); true sardine (TR-S) (*S. melanostictus*) (Kishimura et al., 2006); common Carp (*C. carpio*) (trypsin A) (Cao et al., 2000); mandarin fish (*Siniperca chuasti*) (Lu et al., 2008); bluefish (*Pomatomus saltatrix*) (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2007b); cuttlefish (*S. officinalis*) (Balti et al., 2009); rat (MacDonald, Stary, & Swift, 1982); dog (Pinsky, LaForge, & Scheele, 1985); bovine (LeHuerou, Wicker, Guilloteau, Toullec, & Puigserver, 1990); porcine (Hermodson, Ericsson, Neurath, & Walsh, 1973); human (Emi et al., 1986).

mined by gel filtration. Fish trypsins have been reported to have molecular weights in the range 23–28 kDa. The molecular weight of *B. capriscus* trypsin was similar to those of trypsins from other fish species, such as walleye pollock (*Theragra chalcogramma*) (Kishimura, Klomklao, Benjakul, & Chun, 2008), true sardine (*S. melanostictus*) and Arabesque greenling (*Pleuroprammus azonus*) (Kishimura et al., 2006), jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*) (Kishimura et al., 2007), yellowfin tuna (*Thunnus albacores*) (Klomklao et al., 2006), skipjack tuna (*K. pelamis*) (Klomklao et al., 2007a), sardine (*S. pilchardus*) (Bougatef et al., 2007) and *Sepia officinalis* (Balti et al., 2009).

Purity of the purified trypsin was also evaluated by using native gel electrophoresis. As shown in Fig. 1b, *B. capriscus* trypsin migrated as a single protein band in the native–PAGE, indicating the homogeneity of the enzyme. The proteolytic activity of this protein band was confirmed by zymogram activity staining. As shown in Fig. 1b, a unique clear band of casein hydrolysis was observed in the gel, indicating the homogeneity of the purified trypsin and its proteolytic activity.

3.3. N-terminal amino acid sequence of B. capriscus trypsin

The *N*-terminal 12 amino acids of the *B. capriscus* trypsin were determined to be **IVGGYECTPNST**. The *N*-terminal amino acid sequence of *B. capriscus* trypsin showed uniformity, indicating that it was isolated in a pure form.

The *N*-terminal amino acid sequence of *B. capriscus* trypsin was aligned with the sequences of other animal trypsins (Fig. 2). The *N*-terminal four amino acid sequence of *B. capriscus* trypsin (**IVGG**) was conserved with all other animal trypsins. The sequence of *B. capriscus* showed high homology with those from Arabesque greenling (*P. azonus*) (Kishimura et al., 2006), Common carp (*Cyprinus carpio*) trypsin B (Cao et al., 2000), Walley pollok (*T. chalcogramma*) (Kishimura et al., 2008), and Japanese anchovy (*E. japonica*) (Kishimura et al., 2005). There are only three amino acid residues, in the 12-terminal sequence, that differ in the sequences.

B. capriscus trypsin had a basic amino acid residue (Asn) at position 10, where His and Tyr are common in marine vertebrates and invertebrates. Asn at position 10 was found only in mammalian trypsins. In addition, *B. capriscus* trypsin had Tyr at position 12, where Val or Gln are common in marine and mammalian trypsins.

3.4. Effect of enzyme inhibitors on trypsin activity

Proteases can be classified by their sensitivity to various inhibitors (North 1982). In order to determine the nature of the purified protease, the effects of different enzyme inhibitors, such as chelating agent and a specific group reagent on the protease activity were investigated (Table 2).

Protease from *B. capriscus* was strongly inhibited by the well-known trypsin inhibitor investigated, namely, SBTI, and also by PMSF. On the other hand, metalloprotease inhibitor (EDTA, 5 mM) was practically without influence on the activity of the purified trypsin. These results indicate that the *B. capriscus* enzyme is a serine protease and belongs to the trypsin family.

3.5. Effect of pH on activity and stability of B. capriscus trypsin

The pH activity profile of the purified Grey triggerfish trypsin is shown in Fig. 3a. The purified enzyme was active between pH 9.0 and 12.0, with an optimum around pH 10.5. The relative activities at pH 9.0, 11.0 and 12.0 were about 86.5%, 96% and 52.4%, respectively, of that at pH 10.5. Therefore, *B. capriscus* trypsin could be a potential candidate for addition to commercial laundry detergents, because the pH in laundry detergent is generally in the range of 9.0–11.0. The pH activity of *B. capriscus* trypsin was higher than

Table 2
Effect of various enzyme inhibitors and metal ions on the activity of the purified trypsin from *B. capriscus*. Purified enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25 °C and the remaining activity was determined at pH 10.5 and 40 °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 40 °C and pH 10.5.

Chemicals	Concentration (mM)	Activity (%)
None	–	100
PMSF	1	20
	5	0
β-Mercaptoethanol	5	100
EDTA	1	100
	5	100
SBTI	1 mg/ml	0
Pepstatin A	1	100
Ca^{2+}	5	100
Ba^{2+}	5	92.6
Zn^{2+}	5	97
Cu^{2+}	5	100
Mg^{2+}	5	105
Mn^{2+}	5	100
Hg^{2+}	5	42.7
K^{+}	5	100
Na^{+}	5	100

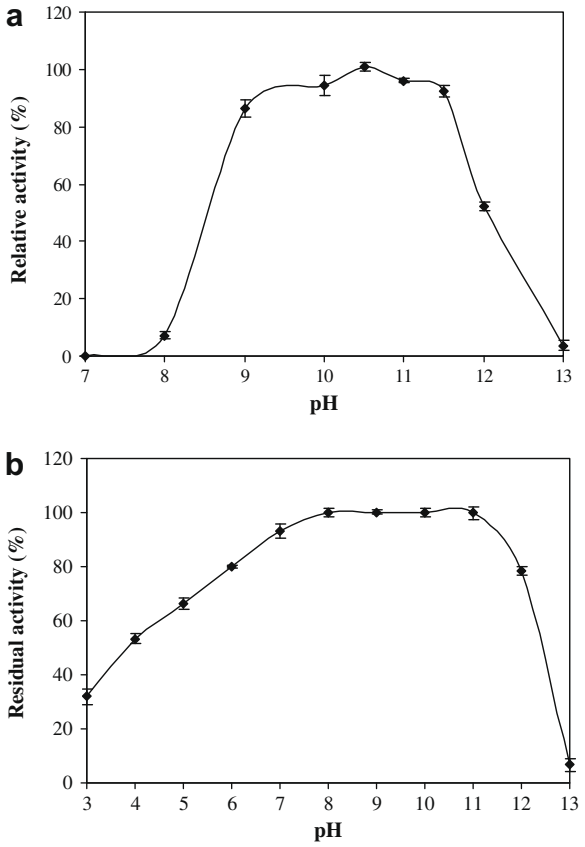

Fig. 3. pH profile (a) and pH stability (b) of the purified trypsin from the intestine of the Grey triggerfish (*B. capriscus*). Trypsin activity was assayed in the pH range 7.0–13.0 at 40 °C. The maximum activity obtained at pH 10.5 was considered as 100% activity. The pH stability was determined by incubating the enzyme in different buffers for 60 min at 25 °C and the residual enzyme activity was determined at pH 10.5 and 40 °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.

those of most described trypsins, which showed maximum activity at pH 8.0.

The pH stability test showed that the Grey triggerfish trypsin was highly stable over a broad pH range, maintaining more than 90% of its original activity between pH 7.0 and 11.0, and more than 75% of its activity at pH 12.0 (Fig. 3b). The pH stability of Grey triggerfish protease is higher than monterey sardine trypsin, which was stable in the pH range 7.0–8.0 (Castillo-Yanez et al., 2005).

3.6. Effect of temperature on the activity and stability of *B. capriscus* trypsin

The effect of temperature on activity was determined by assaying enzyme activity at different temperatures (Fig. 4a). The trypsin from Grey triggerfish was active at temperatures from 0 to 65 °C with an optimum around 40 °C. The relative activities at 20, 30 and 50 °C were about 80.3%, 97% and 71.7%, respectively, of that at 40 °C. The optimum temperature for *B. capriscus* protease was similar to those of trypsins from cold-water fish, which had optimal temperatures in the range 40–45 °C (Lu et al., 2008; Simpson, 2000). High activity of trypsin at low temperatures may be interesting for many biotechnological and food processing applications (Haard, 1992).

The thermal stability profile of the purified trypsin showed that the enzyme was highly stable at temperatures below 40 °C but was inactivated at higher temperatures (Fig. 4b). The enzyme retained more than 78.7% and 8.5% of its initial activity after 60 min of incubation at 50 and 60 °C, respectively.

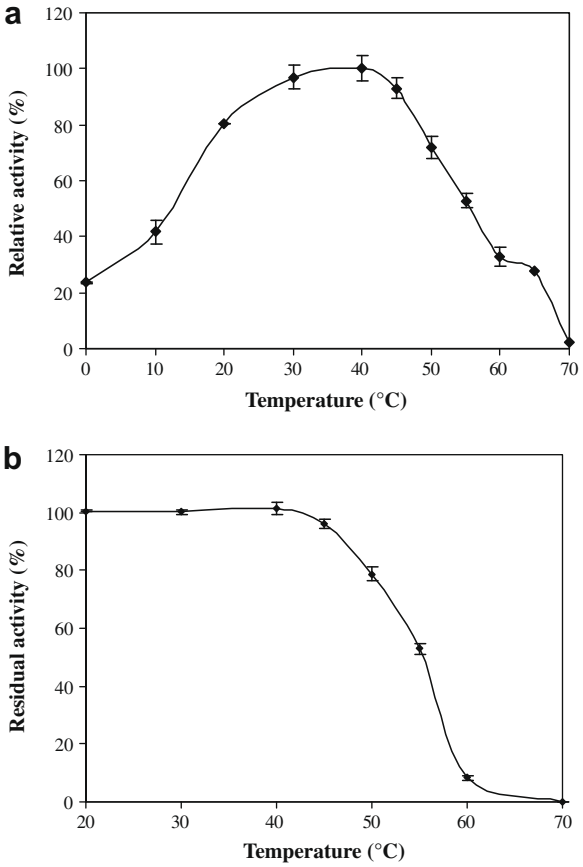

Fig. 4. Temperature profile (a) and thermal stability (b) of the purified trypsin from the intestine of the Grey triggerfish (*B. capriscus*). Enzyme activity was assayed at different temperatures ranging from 0 to 70 °C at pH 10.5, using BAPNA as a substrate. The activity of the enzyme at 40 °C was taken as 100%. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual enzyme activity was assayed at pH 10.5 and 40 °C. The non-heated enzyme was considered as control (100%).

Table 3
Stability of *B. capriscus* trypsin in the presence of various surfactants and oxiclising agents. The enzyme was incubated with different surfactants and oxidising agents for 1 h at 40 °C and the remaining activity was measured under standard conditions. The activity is expressed as a percentage of the activity level in the absence of additives.

Tensioactifs/oxidising agents	Concentration (mM)	Residual activity (%)
None	–	100
SDS	0.1 (w/v)	28.8
	0.5	23.8
Triton X-100	5 (v/v)	125
Tween 20	5 (v/v)	115
Tween 80	5 (v/v)	115
H_2O_2	1 (v/v)	79.8
	5	39
Sodium perborate	0.1 (w/v)	96.4
	1	86.8

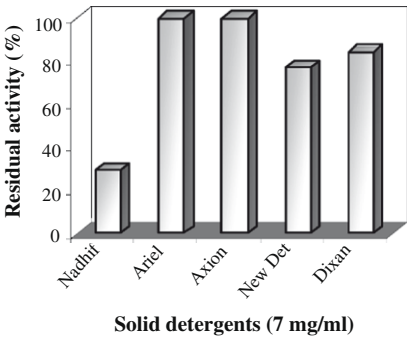

Fig. 5. Stability of trypsin from *B. capriscus* in the presence of various commercial solid detergents. The enzyme, at 200 U/ml, was incubated 1 h at 40 °C and pH 10.0 in the presence of solid detergents at a final concentration of 7 mg/ml and the remaining activities were determined at pH 10.0 and 55 °C, using casein as a substrate. Enzyme activity of control sample without any detergent, incubated under the similar conditions, was taken as 100%

3.7. Effect of metal ions

The effects of some metal ions, at a concentration of 5 mM, on the activity of *B. Capriscus* trypsin were studied at pH 10.5 and 40 °C by the addition of metal ions to the reaction mixture (Table 2). The protease activity was slightly affected by Zn^{2+}. Hg^{2+} greatly affected the enzyme activity, with more than 57% inhibition. The enzyme activity was not affected by any other metallic ions tested.

3.8. Effect of oxidising agents and surfactants on protease stability

In order to be effective during washing, a good detergent protease must be compatible and stable with all commonly used detergent compounds, such as surfactants, oxidising agents and other additives, which might be present in the formulation (Gupta, Gupta, Saxena, & Khan, 1999; Kumar & Takagi, 1999).

As shown in Table 3, the activity was increased in the presence of the non-ionic surfactants, e.g. Tween 80 and Triton X-100. In addition, *B. Capriscus* trypsin was little influenced by oxidising agents, and retained about 80% and 87% of its activity after incubation for 1 h at 40 °C in the presence of 1% hydrogen peroxide and sodium perborate, respectively. The relative stability of the enzyme in the presence of oxidising agents is a very important characteristic for its eventual use in detergent formulations.

3.9. Stability of the alkaline trypsin with commercial solid detergents

The high activity and stability of the purified alkaline trypsin in the pH range 9.0–12.0, and its relative stability towards surfactants and oxidising agents are very useful for its eventual application as a detergent additive. To check the compatibility of the alkaline trypsin with solid detergents, the purified enzyme was pre-incubated in the presence of various commercial laundry detergents for 1 h at 40 °C.

The data presented in Fig. 5 show that the alkaline protease was extremely stable in the presence of Ariel and Axion, retaining 100% of its activity even after 1 h of incubation at 40 °C. In the presence of Dixan and New Det the enzyme retained, respectively, about 84.5% and 76.9% of its initial activity. However, the alkaline trypsin was found to be less stable in the presence of Nadhif, retaining only 29.7% of its activity. These results clearly indicated that the performance of enzymes in detergents depends on number of factors, including the detergent compounds, since the proteolytic activity varied with each laundry detergent.

3.10. Kinetic properties

Kinetic constants K_m and k_{cat} of the purified *B. Capriscus* trypsin were determined using Lineweaver–Burk plots (Table 4). The K_m and k_{cat} of the purified enzyme, using BAPNA, were 0.068 mM and 2.76 s^{-1}, respectively, and were close to those reported for trypsins from carp (*C. carpio*) (Cohen, Gertler, & Birk, 1981), Monterey sardine (*S. sagax cearula*) (Castillo-Yanez et al., 2005), anchovy (*E. japonica*) (Heu, Kim, & Pyeun, 1995) and salmon (*O. keta*) (Sekizaki, Itoh, Murakami, Toyota, & Tanizawa, 2000). The catalytic efficiency (k_{cat}/K_m) of *B. Capriscus* trypsin, 40.6 s^{-1} mM^{-1}, was close to those of trypsins from Monterey sardine (*S. sagax cearula*) (41.0 s^{-1} mM^{-1}) (Castillo-Yanez et al., 2005) and *S. officinalis* (36.3 s^{-1} mM^{-1}) (Balti et al., 2009).

4. Conclusions

In the present study, an alkaline protease from *B. Capriscus* was purified and characterised. The purification, to homogeneity, of the protease was achieved by acetone precipitation (60–80%), gel

Table 4
Kinetic constants of *B. capriscus* trypsin and other trypsins.

Trypsins	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	References
Grey triggerfish (*B. capriscus*)[a]	0.068	2.76	40.6	This study
Cuttlefish (*S. officinalis*)[a]	0.064	2.32	36.3	Balti et al. (2009)
Sardinelle (*S. aurita*)[a]	1.67	3.87	2.31	Ben Khaled et al. (2008)
Anchovy (*E. japonica*)[a]	0.049	1.55	31.0	Heu et al. (1995)
Salmon (*O. keta*)[a]	0.029	2.29	79.0	Sekizaki, Itoh, Murakami, Toyota, and Tanizawa (2000)
Carp (*C. carpio*)[a]	0.039	3.10	79.5	Cohen et al. (1981)
Bigeye snapper (*Priacanthus macracanthus*)	0.312	1.06	3.4	Hau and Benjakul (2006)
Anchovy (*E. encrasicholus*) A[a]	0.830	1.55	1.86	Martinez, Olson and Serra (1988)
Anchovy (*E. encrasicholus*) B[a]	0.660	3.2	4.84	Martinez, Olsen, and Serra (1988)
Montery sardine (*S. sagax cearula*)[a]	0.051	2.12	41.0	Castillo-Yanez et al. (2005)

[a] Substrate: *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA).

filtration through Sephadex G-100 and a Mono Q-Sepharose column. After the final purification step, the enzyme was purified 13.9-fold with a specific activity of 4 U/mg and 41.3% recovery. The purified protease was homogeneous on SDS–PAGE and its molecular weight was estimated to be 23.2 kDa. The enzyme showed good activity over a wide temperature range, with an optimum temperature at 40 °C, which may be beneficial for potential application. The trypsin was highly active and stable at high pH (10.0–11.0). In addition, the activity of the enzyme was not affected by the metalloenzyme inhibitor (EDTA). The high activity of the trypsin in the presence of EDTA is very useful for application as a 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.

Considering the high activity and stability in high alkaline pH, activity at low temperature, stability in the presence of surfactants and oxidising agents, *B. capriscus* trypsin may find application in laundry detergents.

Acknowledgement

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

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