Food Chemistry 113 (2009) 146–154

Contents lists available at ScienceDirect

Food Chemistry

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

A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterisation

Rafik Balti, Ahmed Barkia, Ali Bougatef, Naourez Ktari, 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 9 May 2008
Received in revised form 14 June 2008
Accepted 21 July 2008

Keywords:
Biochemical characterisation
Purification
Cuttlefish
Sepia officinalis
Trypsin
Hepatopancreas

ABSTRACT

Thermostable trypsin from the hepatopancreas of *Sepia officinalis* was purified by fractionation with ammonium sulphate, Sephadex G-100 gel filtration, DEAE-cellulose an ion-exchange chromatography, Sephadex G-75 gel filtration and Q-Sepharose anion-exchange chromatography, with a 26.7-fold increase in specific activity and 21.8% recovery. The molecular weight of the purified enzyme was estimated to be 24,000 Da by SDS-PAGE and size exclusion chromatography. The purified enzyme showed esterase specific activity on *N*α -benzoyl-L-arginine ethyl ester (BAEE) and amidase activity on *N*α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). The optimum pH and temperature for the enzyme activity were pH 8.0 and 70 °C, respectively, using BAPNA as a substrate. The enzyme was extremely stable in the pH range 6.0–10.0 and highly stable up to 50 °C after 1 h of incubation. The purified enzyme was inhibited by soybean trypsin inhibitor (SBTI) and phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor. The N-terminal amino acid sequence of the first 12 amino acids of the purified trypsin was **IVGGKESSPYNQ**. *S. officinalis* trypsin, which showed high homology with trypsins from marine vertebrates and invertebrates, had a charged Lys residue at position 5 and a Ser residue at position 7, where Tyr and Cys are common in all marine vertebrates and mammalian trypsins. Further, the enzyme had an Asn at position 11, not found in any other trypsins.

The trypsin kinetic constants, K_m and k_{cat} for BAPNA, were 0.064 mM and 2.32 s^{-1}, respectively, while the catalytic efficiency k_{cat}/K_m was 36.3 s^{-1} mM^{-1}.

1. Introduction

In several of the major fish-producing countries, the by-products of seafood harvesting comprise about 50% of the entire harvest. These materials, which represent an environmental problem to the fishing industry, constitute an important source of proteins and enzymes, especially digestive proteases. Proteases have diverse applications in a wide range of industries, such as the detergent, food, pharmaceutical, leather and silk industries (Gupta, Beg, & Lorenz, 2002).

In recent years, recovery and characterisation of proteolytic enzymes, from the internal organs of fish and aquatic invertebrates, have been reported and this has led to the emergence of some interesting new applications of these enzymes in food processing (Shahidi & Kamil, 2001). The digestive proteases are involved in the hydrolysis of dietary proteins and do not play a role in protein turnover within an organism. The most important digestive enzymes from fish and aquatic invertebrate 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 (EC 3.4.21.4) is a ubiquitous serine protease in animal digestive glands and is commonly synthesized as a proenzyme by pancreatic acinar cells (Kossiakoff, Chambers, Kay, & Stroud, 1977; Walsh, 1970). Trypsins 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 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) 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 caeca 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,

* Corresponding author. Tel.: +216 74 274088; fax: +216 74 275595.
E-mail addresses: mon_nasri@yahoo.fr, moncef.nasri@enis.rnu.tn (M. Nasri).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.07.059

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).

Marine vertebrate trypsins, especially those of fish, have been well characterised. However, few studies have been done on trypsins from marine invertebrates. The characteristics of the enzymes from marine invertebrates resemble those of mammalian and fish trypsins in molecular weight, cleavage specificities, pH stability and reaction with inhibitors.

Cuttlefish (*Sepia officinalis*) is relatively important in the fish-catches in Tunisia, and is utilised for human consumption. During processing, solid wastes are generated. These wastes, which may represent 30% of the raw material, constitute an important source of proteolytic enzymes and proteins. Traditionally, fish processing by-products have been converted to powdered fish flour for animal feed, fertilizer and fish silage (Gildberg & Almas, 1986). However, most of these products have low economic value. Novel processing methods are needed to convert the cuttlefish processing by-products into more profitable and marketable products. One way is to produce powders used as a substrate of fermentation for microbial growth and bioproduct production. In a previous paper, Souissi, Ellouz-Triki, Bougatef, Blibech, and Nasri (2008) showed that protease synthesis is strongly induced when bacterial strains are grown in media containing cuttlefish (*Sepia officinalis*) by-product powder as a complex carbon source. Balti et al. (2008) showed that selective enzymatic hydrolysis of cuttlefish by-product proteins improved their functional and biological activities.

In the present study, we describe the purification of trypsin from the hepatopancreas of cuttlefish (*S. officinalis*), 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-L-arginine ethyl ester hydrochloride (BAEE), benzamidine, trichloroacetic acid, glycine, ammonium sulphate, 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) and *N*α -benzoyl-DL-arginine-p-nitroanilide (BAPNA) were 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 G-75, Sepharose mono Q and diethylaminoethyl (DEAE)-cellulose were from Pharmacia Biotech (Uppsala, Sweden). PVDF membrane was purchased from Applied Biosystems (Roissy, France). Tris (hydroxymethyl) aminomethane was procured from Panreac Quimica SA (Spain). All other reagents were of analytical grade.

2.2. Cuttlefish viscera

Cuttlefish (*S. officinalis*) were obtained 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 the hepatopancreas was collected and then stored in sealed plastic bags at −20 °C until used for enzyme extraction.

2.3. Preparation of crude enzyme extract

Hepatopancreas samples from *S. officinalis* were washed with water then with buffer A (10 mM Tris-HCl, pH 8.0). The cleaned hepatopancreas (64 g) was defatted by homogenisation with cold acetone for 30 s in a tissue homogenizer. The homogenate was filtered. The acetone-insoluble material was washed several times with cold acetone, and then dried at room temperature overnight. The acetone dried powder was homogenised for 2 h with buffer A at 4 °C (ratio of 1:10). The precipitate was removed by centrifugation at 8,500 × *g* for 30 min at 4 °C, and the supernatant obtained was collected and used as the crude protease extract.

2.4. Trypsin purification

2.4.1. Ammonium sulphate precipitation

The crude protease extract was subjected to ammonium sulphate fractionation (0–20%, 20–60% and 60–80%). The precipitates obtained after centrifugation at 13,000 × *g* for 30 min were suspended in buffer A and dialysed for 24 h at 4 °C against repeated changes in the same buffer (after 8 and 16 h).

2.4.2. Sephadex G-100 gel filtration

The dialysed precipitate between 20% and 60% saturation was subjected to gel filtration on a Sephadex G-100 column (2.6 cm × 150 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 trypsin activity were determined. Fractions showing protease activities were pooled.

2.4.3. DEAE-cellulose anion-exchange chromatography

The active fractions from the Sephadex G-100 were applied to a DEAE-cellulose column (2 cm × 25 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 NaCl, in the range 0–0.5 M, in the equilibrating buffer. Fractions of 5 ml were collected at a flow rate of 70 ml/h. The fractions with high trypsin activities were pooled.

2.4.4. Sephadex G-75 gel filtration

The active fractions from DEAE-cellulose were concentrated by ultrafiltration using 10 kDa membrane, then applied to gel filtration on a Sephadex G-75 column (2.5 cm × 90 cm) pre-equilibrated with buffer B and eluted at a flow rate of 30 ml/h with the same buffer. Protein content (Abs 280 nm) and trypsin activity were measured.

2.4.5. Q-Sepharose anion-exchange chromatography

Fractions from the Sephadex G-75 showing protease activities were pooled and applied to a Q-Sepharose column (2 cm × 10 cm) previously equilibrated with buffer C (25 mM Tris-HCl, pH 8.0). Cationic proteins were washed out in the unadsorbed fractions. After being washed with the same buffer, adsorbed proteins were eluted with a linear gradient of NaCl (0-0.5 M) in the equilibrating buffer. Fractions (5 ml) showing trypsin activities were collected at a flow rate of 60 ml/h. The fractions showing protease activities were pooled and stored at −20 °C for further analyses. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.5. Assay for trypsin activity

2.5.1. Amidase activity

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