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ScienceDirect
Food Chemistry 107 (2008) 777–784
Food Chemistry
www.elsevier.com/locate/foodchem

Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences

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Received 17 July 2007; received in revised form 21 July 2007; accepted 28 August 2007

Abstract

Pepsinogen from the stomach of smooth hound (*Mustelus mustelus*) was purified to homogeneity by 20–70% ammonium sulphate precipitation, Sephadex G-100 gel filtration and DEAE-cellulose anion exchange chromatography with a 9.4-fold increase in specific activity and 38.36% recovery. Upon activation at pH 2.0, *M. mustelus* pepsinogen was converted to active form in one-step pathway. Molecular weights of the purified pepsinogen and the active pepsin were estimated to be 40,000 and 35,000 Da using SDS-PAGE and gel filtration, respectively. The optimum pH and temperature for the pepsin activity were pH 2.0 and 40 °C, respectively, using haemoglobin as a substrate. Activity was completely inhibited by Pepstatin A but not by phenylmethylsulphonyl fluoride, a serine-protease inhibitor and ethylenediaminetetraacetic acid, a metalloenzyme inhibitor. The N-terminal amino acid sequences of the first 15 amino acids of the activation segment of the pepsinogen and the first 20 amino acids of the active pepsin were LLRVPLRKGKSTLDV and ATEPLSNYLDSSYFGDISIG, respectively. *M. mustelus* pepsinogen, which showed high homology to rat C pepsinogen, had Thr-Leu-Asp sequence at amino acid positions 12–14 not found in all pepsinogen sequences. A remarkable substitution was found in the activation segment of *M. mustelus* pepsinogen: the Arg-13 conserved in all gastric proteinases, whose sequences are known, is replaced by Leu-13.

Keywords: Pepsinogen; Pepsin; Biochemical characterization; Stomach; Smooth hound (*Mustelus mustelus*)

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 detergent, food, agrochemical and pharmaceutical industries (Gupta, Beg, & Lorenz, 2002; Zukowski, 1992). Proteases are mainly 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 fishing industry, are known to be a rich source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Cancre et al., 1999; Gildberg, 1992; Shahidi & Kamil, 2001). A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish. The most important proteolytic enzymes from fish viscera are the aspartic protease pepsins and serine proteases trypsin, chymotrypsin and elastase. Alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0, while acidic proteases from fish stomach, such as pepsin, display high activity between pH 2.0 and 4.0. These acidic

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doi:10.1016/j.foodchem.2007.08.077

enzymes were synthesized in the gastric mucosal glands of vertebrates, including fish, as zymogens (Haard, 1994).

Pepsinogens are synthesized as precursors containing a hydrophobic signal sequence of 15–16 amino acids at the N-terminal site serving a transport function. The signal sequence is lost during post-transcription processing. Pepsinogens are converted into the active enzymes under acidic conditions, releasing the activation segments from their NH_2-terminal regions (Bovey & Yanari, 1960; Foltmann, 1981; Fruton, 1971). The prosegment serves to stabilize the inactive form and prevent entry of substrate to active site. Pepsinogens are classified into five groups: pepsinogens A, B, C (progastricsin), F and prochymosin (Kageyama, 2002). Pepsinogens differ from each other in their primary structures and enzymatic properties of their activated forms (Kageyama, 2002).

Pepsinogens have been extracted and purified from the gastric mucosa of various vertebrates, and their primary structures have been reported in some studies; mammals such as human (Sogawa, Fujii-Kuriyama, Mizukami, Ichihara, & Takahashi, 1983), Japanese monkey (Kageyama & Takahashi, 1976), new world monkey (Kageyama, 2000), goat (Suzuki et al., 1999), bovine (Martin, Torieu-Cuot, Collin, & Ribadeau Dumas, 1982), rabbit (Kageyama & Takahashi, 1984), birds such as *Gallus gallus* (Sakamoto, Saiga, & Yasugi, 1998) and reptiles such as turtle (*Trionyx sinensis*) (Hirasawa, Athauda, & Takahashi, 1996).

On the other hand, so far, studies on pepsinogens and pepsins from fish are quite few. Only pepsinogens from trout (*Salmo gairdneri*) (Twining, Alexander, Huibregtse, & Glick, 1983), polar cod (*Boreogadus saida*) (Arunchalam & Haard, 1984), tuna (*Thunnus thynuus orientalis*) (Tanji, Kageyama, & Takahashi, 1988), Atlantic cod (*Gadus morhua*) (Gildberg, Olsen, & Bjarnason, 1990), shark (*Centroscymnus coelolepis*) (Nguyen et al., 1998), sea bream (*Sparus latus* Houttuyn) (Zhou, Fu, Zhang, Su, & Cao, 2007) and African coelacanth (*Latimeria chalumnae*) (Tanji et al., 2007) have been reported.

In the present study, we have purified pepsinogen from smooth hound (*Mustelus mustelus*) stomach and characterized some properties of the correspondent pepsin after pepsinogen activation.

2. Materials and methods

2.1. Reagents

Haemoglobin, pepstatin A, ethylenediaminetetraacetic acid, trichloroacetic acid, glycine, ammonium sulphate and markers for molecular weights 14,000–66,000 Da were purchased from Sigma Chemical Co. (St. Louis MO, USA). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulphate, tetramethylethylenediamine, Coomassie Brilliant Blue R250 were from Bio-Rad Laboratories (Mexico). Sephadex G 100 and DEAE-cellulose were from Pharmacia (Uppsala, Sweden); PVDF membrane was purchased from Applied Biosystems (Roissy, France). All other reagents were of analytical grade.

2.2. Smooth hound viscera

Smooth hound (*M. mustelus*) was purchased from the local market at Sfax City, Tunisia. It was washed twice with water. Internal organs were then excised and separated into individual organs. Only stomach was collected. The stomach contents was removed, rinsed with cold distilled water, then immediately frozen and stored at −20 °C until used.

2.3. Preparation of crude extract

Frozen stomach was thawed using water. The samples (125 g) were cut into pieces with a thickness of 1–1.5 cm and homogenised for 1 min with 250 ml extraction buffer A (10 mM Tris–HCl pH 7.5). The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was discarded and the supernatant was collected and used as crude pepsinogen extract.

2.4. Pepsinogen purification

2.4.1. Ammonium sulphate precipitation

The crude extract was subjected to ammonium sulphate fractionation and the precipitate in the 20–70% saturation range was collected by centrifugation 15 min at 10,000*g*. The precipitate was suspended in buffer A and dialyzed 24 h at 4 °C against repeated changes in the same buffer.

2.4.2. Gel filtration

The dialyzed precipitate between 20% and 70% saturation was then subjected to gel filtration on a Sephadex G-100, from Pharmacia (Uppsala, Sweden), column (2.6 × 150 cm) pre-equilibrated with buffer B (25 mM Tris–HCl, pH 7.5 containing 0.5‰ Triton X-100). Enzyme fractions of 5 ml were eluted at a flow rate of 27 ml/h with the same buffer. Protein contents (Abs 280 nm) and protease activity were measured.

2.4.3. Anion exchange chromatography

Fractions showing protease activities were pooled from the Sephadex G-100 and applied to a DEAE-cellulose, from Pharmacia (Uppsala, Sweden), column (2 × 25 cm) previously equilibrated with buffer C (25 mM Tris–HCl, pH 7.5). After washing the column with equilibrating buffer until the absorbance at 280 nm reached baseline, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M. Fractions with protease activity were collected and stored at −20 °C for further analysis. The chromatography was carried out at a flow rate of 76 ml/h.

All the purification steps were conducted at temperatures not exceeding 4 °C.

2.4.4. Polyacrylamide gel electrophoresis and zymography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the pepsinogen and pepsin as described by Laemmli (1970), using a 5% (w/v) stacking gel and a 15% (w/v) separating gel. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris–HCl pH 8.0, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 100 °C for 5 min before electrophoresis. Gels were stained with 0.25% Coomassie Brilliant Blue R250 in 45% ethanol–10% acetic acid and destained with 5% ethanol–7.5% acetic acid. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Sigma). The molecular mass markers used are: bovine serum albumin (66,000 Da), egg white ovalbumin (45,000 Da), glyceraldehyde-3-P deshydrogenase (36,000 Da), bovine carbonic anhydrase (29,000 Da), bovine trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da) and bovine α-lactalbumin (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.

Zymography was performed on native-PAGE. Briefly, after electrophoresis, the gel was submerged in 2% acid-denatured bovine haemoglobin and incubated at 37 °C for 90 min. After washing, the gel was stained with Coomassie Brilliant Blue R250 for zymography analysis.

2.4.5. Protein concentration

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.

2.5. Assay for protease activity

Potential pepsin activity of pepsinogen as well as pepsin activity against haemoglobin was determined by the method of Anson (1938) with slight modification. Fifty microlitres of appropriately diluted enzyme sample were mixed with 100 μl of solution consisting of 2.0% acid-denatured bovine haemoglobin and 350 μl of 100 mM glycine–HCl buffer (pH 2.0). After incubation of the mixture at 37 °C for 15 min, the reaction was immediately stopped by addition of 500 μl of 8.0% trichloroacetic acid (TCA). The mixture was centrifuged at 10,000*g* for 15 min and the absorbance of the supernatant at 280 nm was measured. One unit of pepsin activity against haemoglobin was defined as the amount of enzyme that catalyzed an increase of 1.0 in the absorbance at 280 nm per minute under the assay conditions.

2.6. N-terminal amino acid sequence of pepsinogen and pepsin

The purified enzyme was subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF). After the membrane was briefly stained by Coomassie brilliant blue, the region containing the protease band on the PVDF membrane was excised and the protein N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

2.7. Molecular weight

To determine the molecular mass of the native enzyme, the freeze-dried proteins were resuspended in phosphate buffer, pH 7. Two hundred micrograms of the pure enzyme was loaded on size exclusion HPLC column Protein Bio. Sil SEC. 125 (300 × 7.8 cm) equilibrated in phosphate buffer. Elution was performed with phosphate buffer at 0.5 ml/min.

2.8. Biochemical properties

2.8.1. Pepsinogen activation

The conversion reaction was carried out in different tubes with a volume of 100 μl. Pepsinogen dissolved in buffer C was acidified to pH 2.0 by the addition of 0.1 M HCl and incubated at 25 °C. At each reaction interval, 10 μl of 16% trichloroacetic acid were immediately added to terminate the conversion reaction. Supernatant in each tube was collected then analyzed by SDS-PAGE.

2.8.2. Effect of pH on activity and stability of pepsin

The effect of pH was determined with haemoglobin as a substrate. Pepsin activity was studied in the pH range of 1.0–6.0 at 37 °C. For the measurement of pH stability, the enzyme was incubated at 30 °C for 30 min in different buffers and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM glycine–HCl, pH 1.0–4.0; sodium acetate, pH 5.0–6.0.

2.8.3. Optimum temperature and thermal stability

The effect of temperature on pepsin activity was studied from 20 to 70 °C for 15 min at pH 2.0. Thermal stability of the active pepsin was determined by incubating the enzyme 60 min at 20, 30, 40, 50, 60 and 70 °C at pH 2.0. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions. The non-heated enzyme was considered as control (100%).

2.8.4. Effects of enzyme inhibitors

The effects of inhibitors on pepsin activity were studied using pepstatin A, phenylmethylsulphonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA). The pepsin was preincubated with inhibitors for 60 min at 30 °C and then the remaining enzyme activity was estimated using haemoglobin as a substrate. The inhibitory effect of pepstatin A was also studied at different concentrations.

3. Results and discussion

3.1. Purification of pepsinogen

Pepsinogen from the stomach of *M. mustelus* was purified by the three-step procedure described in Section 2. In the first step, the crude extract was fractionated with ammonium sulphate. The fraction F2 (20–70% saturation), which gave the highest specific activity (3.02 U/mg of protein) (Table 1), was subjected to gel filtration on a Sephadex G-100 column. This procedure yielded a single peak of protease activity (data not shown). Fractions containing protease activity were pooled and then loaded on DEAE-cellulose column pre-equilibrated with buffer C. Protease activity appeared in a single peak together with adsorbed fractions (data not shown). The results of the purification procedure are summarised in Table 1. After the final purification step, the pepsinogen was purified 9.48-fold with a recovery of 38.36% and a specific activity of 7.68 U/mg of protein. The homogeneity of purified pepsinogen was checked by SDS-PAGE under reducing conditions. As shown in Fig. 1a, the purified pepsinogen had a molecular weight of ≈40,000 Da when analysed using SDS-PAGE, corresponding with that determined by gel filtration. Purity of pepsinogen was also evaluated by using native gel electrophoresis. As shown in Fig. 1b, pepsinogen migrated as a single protein band indicating the homogeneity of the zymogen.

Other pepsinogens were reported to have molecular weight of about 40,000–43,000 Da (Arunchalam & Haard, 1984; Tanji et al., 1988).

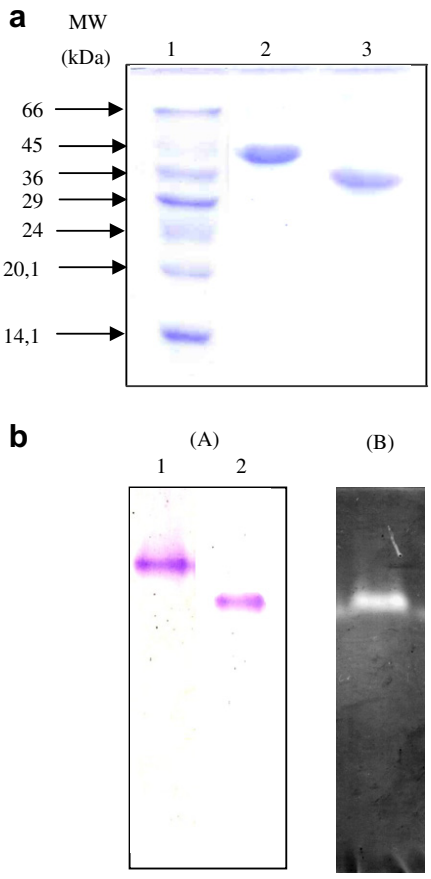

Fig. 1. (a) SDS-polyacrylamide gel electrophoresis of the purified pepsinogen from the stomach of smooth hound (*M. mustelus*). Lane 1: standard protein markers of different molecular weights; lane 2: purified pepsinogen; lane 3: active form of pepsinogen. (b) (A) Native-PAGE of smooth hound pepsinogen (Lane 1) and pepsin (Lane 2). (B) Zymography of smooth hound pepsin. Pepsin on native-PAGE was incubated with 2.0% acid-denatured haemoglobin at 37 °C for 90 min.

3.2. Activation of pepsinogen

Conversion of pepsinogens to pepsins proceeds autocatalytically at acidic pH by two different pathways, a one-step pathway to release the intact activation segment directly, and a stepwise pathway through a pseudopepsin(s) (Dykes & Kay, 1976). Fig. 2 shows the time course of the activation of the pepsinogen at 25 °C and pH 2.0. The activation resulted in direct conversion of propepsin into pepsin without the formation of an intermediate forms. Complete activation of pepsinogen was carried out in 30 min. Furthermore, only one band was detected on native-PAGE and on the zymogram indicating the homogeneity of the pepsin (Fig. 1b).

The release of pepsin in one step by proteolytic cleavage of the peptide bound between the C-terminal of the prosegment and the N-terminal of the active enzyme was also reported for bullfrog progastricsin (Yakabe et al., 1991)

Table 1
A summary of the purification of pepsinogen from smooth hound (*M. mustelus*)

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	183.51	224.73	0.81	100	1
Step 1: Ammonium sulphate precipitation					
$(NH_4)_2SO_4$ (20–70%)	128.73	42.65	3.02	70.14	3.71
Step 2: Gel filtration					
Sephadex G-100	97.16	22.69	4.28	52.94	5.28
Step 3: Anion-exchange					
DEAE-cellulose	70.4	9.16	7.68	38.36	9.48

All operations were carried out at 4 °C. Only precipitate formed between 20% and 70% saturation with ammonium sulphate was subjected to gel-filtration on Sephadex G-100.

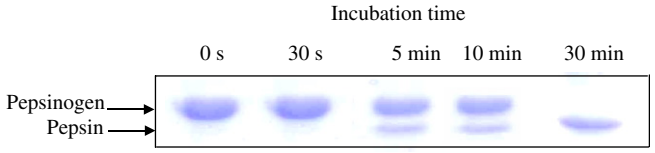

Fig. 2. Time course of activation of pepsinogen analyzed by SDS-PAGE. Pepsinogen was incubated at 25 °C and pH 2.0 as described in Section 2. Conversion reactions were stopped at appropriate intervals and subjected to SDS-PAGE.

and pepsinogen B from dog stomach (Narita, Oda, Moriyama, & Kageyama, 2002). However, many works reported the conversion of pepsinogens to mature pepsins through intermediates forms (Tanji et al., 1988, 2007).

3.3. N-terminal amino acid sequence of *M. mustelus* pepsinogen and pepsin

The 15 N-terminal amino acid sequence of the purified pepsinogen from *M. mustelus* was determined to be LLRVPLRKGKSTLDV. The N-terminal amino acid sequence of the pepsinogen showed uniformity, indicating that it was isolated in a pure form, and if other isoforms are present, their amounts must be small. On the other hand, many works reported that fish species secrete at least two different pepsinogens with different pH optima (Gildberg & Raa, 1983; Zhou et al., 2007).

The sequence of *M. mustelus* pepsinogen was aligned with the sequences of other pepsinogens: human A (Sogawa et al., 1983), porcine A (Ong & Perlmann, 1968), porcine B (Nielsen & Foltmann, 1995), chicken A (Baudys & Kostka, 1983), bovine A (Harboe, Anderson, Foltmann, Kay, & Kassel, 1974), human C (Hayano, Sogawa, Ichihara, Fujii-Kuriyama, & Takahashi, 1988), dog B (Narita et al., 2002), bullfrog C (Yakabe et al., 1991), rat C (Ishihara et al., 1989), Tuna 1, 2, 3 (Tanji et al., 1988) and African coelacanth 1, 2, 3 (Tanji et al., 2007) (Fig. 3a). Pepsinogen from *M. mustelus* showed high homology with rat C pepsinogen. The *M. mustelus* pepsinogen differs from that of rat C by 5 amino acids in the first 15 amino acids. The N-terminal eight amino acid sequence of *M. mustelus* pepsinogen (LLRVPLRK) was identical with that of rat pepsinogen C (Ishihara et al., 1989). Interestingly, in the amino acids positions 12–14, *M. mustelus* pepsinogen had Thr-Leu-Asp sequence not found in all described pepsinogens. In particular, *M. mustelus* pepsinogen had a Leu residue at amino acid position 13; however, all pepsinogens had a charged Arg residue at position 13.

Basic amino acid residues at positions 3, 8, 10, 13 and 36 in the activation segment have been shown to be important in the stabilization of the pepsin moiety at neutral pH (James & Sielecki, 1986; Richter, Tanaka, & Yada, 1998). Basic residues in the prosegment are engaged in electrostatic interactions with acidic residues in the active enzyme moiety. The change of Arg-13 to Leu-13 may cause a conformational change in the activation segment, resulting in a change in the mechanism of activation of the enzyme.

a

Smooth hound	-	L	L	R	V	P	L	R	K	G	K	S	T	L	D	V	-
Rat C	-	L	L	R	V	P	L	R	K	M	K	S	I	R	E	T	-
Tuna 1	-	L	L	Q	V	P	L	E	K	G	Q	S	A	R	E	Y	-
Bullfrog C	-	I	I	K	V	P	L	K	K	F	K	S	M	R	E	V	-
Porcine B	-	M	E	R	I	I	L	R	K	G	K	S	I	R	E	A	
Dog B	-	V	E	R	I	I	L	K	K	G	K	S	I	R	Q	V	-
Human C	A	V	V	K	V	P	L	K	K	F	K	S	I	R	E	-	-
Chicken A	S	I	H	R	V	P	L	K	K	G	K	S	L	R	K	-	-
Human A	I	M	Y	K	V	P	L	I	R	K	K	S	L	R	R	-	-
Porcine A	-	L	V	K	V	P	L	V	R	K	K	S	L	R	Q	N	-
Tuna 2	-	F	H	K	L	P	L	I	K	G	K	T	A	R	E	E	-
Tuna 3	-	-	I	N	V	P	L	T	R	H	K	S	M	R	E	S	L
Coelacanth 3	-	L	I	S	V	P	L	K	R	F	X	S	I	R	E	S	-
Bovine A	S	V	V	K	I	P	L	V	K	K	K	S	L	R	Q	-	-
Coelacanth 1	-	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I	-
Coelacanth 2	-	I	A	K	V	P	L	I	K	E	X	P	L	R	A	I	-

b

Smooth hound	-	-	-	-	A	T	E	P	L	S	N	Y	L	D	S	S	Y	F	G	D	I	S	I	G
Dog B	N	D	A	V	A	Y	E	P	F	T	N	Y	L	D	S	Y	Y	F	G	E	I	S	I	G
Porcine B	-	-	A	V	A	Y	E	P	F	T	N	Y	L	D	S	F	Y	F	G	E	I	S	I	G
Bullfrog C	-	-	A	T	A	F	E	P	L	A	N	Y	M	D	M	S	Y	Y	G	E	I	S	I	G
Camel A	-	-	-	-	D	E	Q	P	L	E	N	Y	L	D	T	E	Y	F	G	T	I	S	I	G
Tuna 2	-	Y	Q	D	G	T	E	P	M	T	N	D	A	D	L	S	Y	Y	G	V	V	S	I	G
Cod	-	-	-	R	T	V	E	Q	M	K	N	E	A	D	T	E	Y	Y	G	V	I	S	I	G
Tuna 3	-	A	T	A	N	Y	M	Y	I	N	Q	Y	A	D	T	I	Y	Y	G	A	I	S	I	G

Fig. 3. (a) Comparison of N-terminal amino acid sequence of the purified pepsinogen from smooth hound (*M. mustelus*) with other pepsinogens: rat C (Ishihara et al., 1989); human A (Sogawa et al., 1983); porcine A (Ong & Perlmann, 1968); porcine B (Nielsen & Foltmann, 1995); chicken A (Baudys & Kostka, 1983); bovine A (Harboe et al., 1974), human C (Hayano et al., 1988); bullfrog C (Yakabe et al., 1991); dog B (Narita et al., 2002); Pgs 1, 2, 3 tuna (Tanji et al., 1988); Pgs 1, 2, 3 coelacanth (Tanji et al., 2007). Amino acid residues different from *M. mustelus* pepsinogen are booxed. (b) Comparison of N-terminal amino acid sequence of the pepsin from smooth hound (*M. mustelus*) with other pepsins: cod pepsin (Karlsen et al., 1998); tuna pepsins 2 and 3 (Tanji et al., 1988); porcine pepsin B (Hartsuck et al., 1992); arabian camel pepsin A (Kappeler et al., 2006); dog pepsin B (Narita et al., 2002) and bullfrog pepsin C (Yakabe et al., 1991). Amino acid residues different from *M. mustelus* pepsin are boxed.

We also determined 20 amino acids sequences from the NH_2-termini of the activated forms of pepsinogen, pepsin. The 20 N-terminal amino acid sequence was ATEPLSNYLDSSYFGDISIG. As shown in Fig. 3b, the N-terminal amino acid sequence alignment analysis of pepsin from *M. mustelus* showed 75% identity with dog pepsin B (Narita et al., 2002) and porcine pepsin B (Hartsuck, Koelsch, & Remington, 1992), 70% identity with bullfrog pepsin C (Yakabe et al., 1991), 65% identity with camel pepsin A (Kappeler et al., 2006), 55% identity with tuna pepsin 2 (Tanji et al., 1988), 45% identity with cod pepsin (Karlsen, Hough, & Olsen, 1998), and 40% identity with tuna pepsin 3 (Tanji et al., 1988).

3.4. Biochemical characterization of pepsin

3.4.1. Molecular weight

The molecular weight of the active pepsin was estimated to be 35,000 Da, by SDS-PAGE using molecular weight markers and by gel filtration (Fig. 1a). The molecular weight of *M. mustelus* pepsin was similar to the alkali labile aspartic proteases from sardine (Noda & Murakami, 1981) and pepsins A and C from *Xenopus* (Ikuzawa, Inokuci, Kobayachi, & Yasumasu, 2001).

3.4.2. Effect of pH on pepsin activity and stability

The effect of pH on enzyme activity was determined over a pH range of 1.0–6.0. The pepsin was active between pH 1.0 and 6.0, with an optimum around pH 2.0 (Fig. 4a). The relative activities at pH 1.0 and 4.0 were about 60.3% and 53.2%, respectively, of that at pH 2.0. As seen in Fig. 4a, protease activity decreased significantly above pH 4.0. Optimum pH between 2.0 and 4.0 has been reported for pepsin activities of fish species such as Sea bream (*S. latus* Houttuyn) (Zhou et al., 2007), African coelacanth, (*L. chalumnae*) (Tanji et al., 2007) and tuna (*T. thynuus orientalis*) (Tanji et al., 1988).

For investigation of the pH stability, the enzyme was incubated in different buffers of varying pH values for 30 min at 30 °C and the residual activities were determined at 37 °C and pH 2.0. Pepsin was very stable in a broad pH range (Fig. 4b). At pH 2.0, the enzyme retained 100% of its activity. At pH 1.0 and 4.0, the enzyme retained 85.3 and 61.5 of its initial activity, respectively.

3.4.3. Effect of temperature on pepsin activity and stability

The effect of temperature on pepsin activity was determined by assaying enzyme activity at different temperatures (Fig. 5a). The optimum temperature for the pepsin activity was 40 °C. The relative activities at 30 and 50 °C were about 62.8% and 49.6%, respectively, of that at 40 °C. The optimum temperature for *M. mustelus* pepsin was similar to those from other fish species such as palometa (*Parona signata*) (37 °C) (Pavlisko, Rial, Vecchi, & Coppes, 1997), capelin (*Mallotus villosus*) (pepsin I) (38 °C) (Gildberg & Raa, 1983) and lower than those from sea bream (*S. latus* Houttuyn) which have optimum temperature between 45 and 50 °C (Zhou et al., 2007).

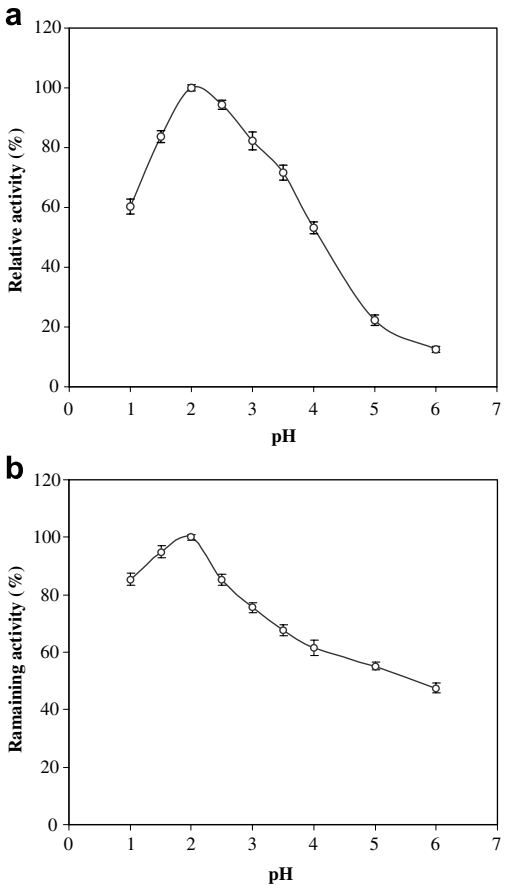

Fig. 4. Effect of pH on activity (a) and stability (b) of the pepsin from stomach of smooth hound (*M. mustelus*). Activity was evaluated using bovine haemoglobin as a substrate. The pepsin was assayed in the pH range of 1.0–6.0 using buffers of different pH values at 37 °C. The maximum activity obtained at pH 2.0 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 30 min at 30 °C and the residual activity was measured at pH 2.0 and 37 °C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2.

The thermal stability profile of the pepsin showed that the enzyme is highly stable at temperatures below 50 °C but was inactivated at higher temperatures (Fig. 5b). The enzyme at 30 °C remains fully active even after 60 min of incubation, indicating that this protease might be used in mild heating process. The enzyme retained more than 95% and 80.66% of its initial activity after 1 h incubation at 40 °C and 50 °C, respectively, while 70.5% and 10% of the maximal activity remained within 15 min of incubation at 60 and 70 °C, respectively.

3.4.4. Effect of enzyme inhibitors on pepsin activity

The effect of a variety of enzyme inhibitors, such as chelating agent and a group specific agent on the pepsin activity was investigated. The enzyme was completely inhibited by the aspartic protease inhibitor pepstatin A (20 μM) indicating that the protease belonged to the aspartic proteases. The

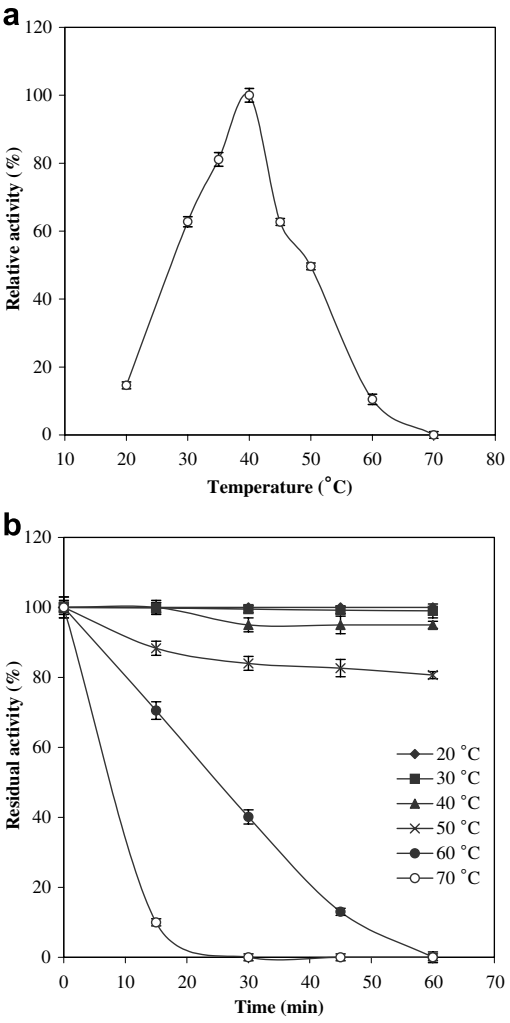

Fig. 5. Effect of temperature on activity (a) and stability (b) of the pepsin from stomach of smooth hound (*M. mustelus*). Activity was evaluated using bovine haemoglobin as a substrate. The temperature profile was determined by assaying pepsin activity at temperatures between 20 and 70 °C. The activity of the enzyme at 37 °C was taken as 100%. The temperature stability was determined by incubating purified enzyme at 20, 30, 40, 50, 60 and 70 °C from 0 to 60 min at pH 2.0 and residual enzyme activities were estimated at regular intervals under standard conditions. The non-heated enzyme was considered as control (100%).

enzyme was not affected by serine-protease inhibitor (PMSF, 2 mM) and metalloprotease inhibitor (EDTA, 2 mM).

Further study on the inhibitory effect of pepstatin at different concentrations showed that a pepstatin/pepsin ratio of 16 mol/mol gave total inhibition. In this respect, smooth hound pepsin resembles mammalian pepsin C. This result is in agreement with the result found by Tanji et al. (1988) in which pepsin 3 from tuna (*T. thynuus orientalis*) was inhibited by about 17-fold molar excess of pepstatin.

4. Conclusion

Pepsinogen was isolated from the stomach of *M. mustelus* by ammonium sulphate precipitation (20–70% saturation), Sephadex G-100 gel filtration and DEAE-cellulose anion exchange chromatography. After the final purification step, the pepsinogen was purified 9.4-fold with a specific activity of 7.68 U/mg of protein and 38% recovery. The purified pepsinogen was homogenous on SDS-PAGE and on native-PAGE and its molecular weight was estimated to be 40,000 Da using SDS-PAGE and gel filtration. *M. mustelus* pepsinogen was converted directly to pepsin after 30 min incubation at pH 2.0 and 25 °C. The molecular weight of *M. mustelus* pepsin was estimated by SDS-PAGE and gel filtration as 35,000 Da. The enzyme showed an optimum temperature at 40 °C and optimum pH of 2.0. *M. mustelus* pepsin was stable at a pH range of 1.0–4.0 and was highly stable at temperatures below 50 °C but was inactivated at higher temperatures.

The N-terminal amino acid sequences of the activation segment of the pepsinogen and pepsin were determined. It' is interesting to note that no similarity of amino acid sequence at positions 12–14 was found between *M. mustelus* pepsinogen and all other pepsinogens. In particular, *M. mustelus* pepsinogen has a residue Leu at the position 13 whereas the corresponding residue in the sequences of all pepsinogens is Arg. The sequence of *M. mustelus* pepsinogen was much more similar to that of rat C. There are only 5 amino acid residues, in the 15-terminal amino acid sequence, that differ in the two sequences. Smooth hound pepsin is suggested to be pepsin C, from its sensitivity to pepstatin and analysis of NH_2-terminal sequence.

Further characterization of this pepsinogen, including the determination of the full length primary structure and properties of its active form, pepsin, as a possible biotechnological tool in the fish processing and food industries is necessary.

Acknowledgement

This work was funded by the "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie, Tunisia".

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