JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 10623–10630 10623
DOI:10.1021/jf102233d

Cathepsin D from the Hepatopancreas of the Cuttlefish (*Sepia officinalis*): Purification and Characterization

Rafik Balti,[†] Noomen Hmidet,[†] Kemel Jellouli,[†] Naima Nedjar-Arroume,[§] Didier Guillochon,[§] and Moncef Nasri*,[†]

[†]Laboratoire de Génie Enzymatique et de Microbiologie, Ecole Nationale d'Ingénieurs de Sfax, Route Soukra Km 3,5 B.P. 1173, 3038 Sfax, Tunisia, and [§]Laboratoire de Procédés Biologiques, Génie Enzymatique et Microbien, IUT A Lille I, B.P. 179, 59653 Villeneuve d'Ascq Cedex, France

Cathepsin D from the hepatopancreas of cuttlefish (*Sepia officinalis*) was purified to homogeneity by precipitation with ammonium sulfate (30−60%, w/v), Sephadex G-100 gel filtration, Mono-S cation-exchange chromatography, Sephadex G-75 gel filtration, and Mono-S FPLC with a 54-fold increase in specific activity and 17% recovery. The molecular weight of the purified cathepsin D was estimated to be 37.5 kDa by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). On the basis of the native-PAGE and hemoglobin zymography, the purified protease appeared as a single band. The optimum pH and temperature for the cathepsin D activity were pH 3.0 and 50 °C, respectively, using hemoglobin as a substrate. The purified enzyme was completely inhibited by pepstatin A; however, no inhibition was observed with phenylmethylsulfonyl fluoride and ethylenediaminetetraacetic acid. Moreover, the activity was strongly inhibited by SDS and molybdate and enhanced by ATP. The purified cathepsin D was activated by Mg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Sr^{2+}, and Co^{2+} ions, whereas it was not affected by Na^{+}, K^{+}, and Ca^{2+} ions. The N-terminal amino acid sequence of the first 13 amino acids of the purified cathepsin D was APTPEPLS-NYMDA. *S. officinalis* cathepsin D, which showed high homology with cathepsin D from marine vertebrates and invertebrates, had a Pro residue at position 6 and a Ser residue at position 8, where Thr and Lys are common in all marine vertebrates cathepsins D. *S. officinalis* cathepsin D showed high efficiency for the hydrolysis of myofibrillar proteins extracted from cuttlefish muscle.

KEYWORDS: Biochemical characterization; purification; cuttlefish; *Sepia officinalis*; cathepsin D; hepatopancreas

INTRODUCTION

Aspartic proteinases constitute a widely distributed protein superfamily, whose members accomplish a variety of functions, including protein degradation at low pH (*1*). They share a high degree of sequence similarity and have a characteristic bilobal tertiary structure (*2*, *3*). Individual members of the superfamily differ in the topography of their active sites and in their cellular localization. These features determine physiological function (*4*,*5*).

On the basis of their different molecular characteristics and tissue/cellular localization, aspartic proteinases have been classified into several subgroups, including the cathepsins (*6*), renins (*7*), pepsins (*8*), and chymosins (*9*). In addition, other types of aspartic proteinases have been isolated from invertebrates (*10*, *11*), plants (*12*, *13*), retroviruses (*14*, *15*), and a number of microbial sources (*16*, *17*).

Among these subgroups, the intracellular aspartic proteinase cathepsin D (EC 3.4.23.5) is present in most cells in many species and is probably one of the major factors contributing to lysosomal digestive activity (*18*, *19*). Cathepsin D activity was found to be particularly elevated in a wide variety of tissues during remodeling or regression, as well as in apoptotic cells (*20*). Although important for the degradation of intracellular proteins, cathepsin D is implicated in the processing of antigens and peptide hormones (*21*, *22*), and the overexpression and secretion of cathepsin D were observed in human breast cancer (*23*). Cathepsin D has been thoroughly investigated in mammals, but a limited number of studies are available on cathepsin D from fish species (*24*−*27*).

In fish, cathepsin D, together with pepsin, has received some attention because of the potential importance of fish physiology in relation to aquaculture (*28*). Indeed, high cathepsin D activity found in fish tissues, especially during spawning, is considered to be the main factor influencing deterioration of fish products (*28*). However, most of these studies have been aimed at the identification of the enzymatic activity present in various fish tissues and rarely at elucidating the biochemical properties of invertebrate cathepsin D. Recently, the catalytic and molecular characterization of a cathepsin D from the hepatopancreas of Japanese common squid (*Todarodes pacificus*) has been reported (*29*). Mammalian cathepsin D has been purified and characterized from a variety of sources including human tissues (*30*, *31*),

*Corresponding author (phone 216 74-274-088; fax 216 74-275-595; e-mail mon_nasri@yahoo.fr or moncef.nasri@enis.rnu.tn).

Published on Web 09/15/2010

pig spleen (*32*), bovine milk (*33*), and chicken liver (*34*). The enzyme has also been purified from some fish species such as tilapia muscle (*35*), carp muscle (*24*), Antarctic icefish liver (*27*), and Atlantic cod liver (*26*).

In Tunisia, cuttlefish (*Sepia officinalis*) is relatively important in fish-catches and is utilized for human consumption. During processing, solid wastes are generated. These wastes, which may represent approximately 30% of the raw material, constitute an important source of proteolytic enzyme and proteins. In a previous work, we have purified and characterized trypsin from the hepatopancreas of cuttlefish (*S. officinalis*). The enzyme showed an optimum temperature at 70 °C and an optimum pH of 8.0. In addition, the enzyme was stable at a pH range of 6.0−10.0 and highly thermostable (*36*).

In the present study, we describe the purification of cathepsin D from the hepatopancreas of cuttlefish (*S. officinalis*) and provide basic information about its main biochemical characteristics.

MATERIALS AND METHODS

Reagents. Hemoglobin, pepstatin A, ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), benzamidine, tosyl-L-phenylalanine chloromethyl ketone (TPCK), soybean trypsin inhibitor (SBTI), L-*trans*-epoxysuccinyl-leucylamido-(4-guanido)butane (E-64), iodoacetic acid (IAA), trichloroacetic acid, glycine, ammonium sulfate, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Mexico). Sephadex G-100, Sephadex G-75, and Mono-S Sepharose were from Pharmacia Biotech (Uppsala, Sweden). PVDF membrane was purchased from Applied Biosystems (Roissy, France). Tris(hydroxymethyl)-aminomethane was procured from Panreac Quimica SA (Barcelona, Spain). All other reagents were of analytical grade.

Cuttlefish Viscera. Cuttlefish (*S. officinalis*), in the size range of 6−8 cuttlefish/kg and dorsal length 20−25 cm, were purchased from the fish market of Sfax City, Tunisia. After capture, the cuttlefish were chilled in ice and off-loaded approximately 10 h thereafter. 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.

Preparation of Crude Enzyme Extract. Defatted powder of the hepatopancreas of cuttlefish was prepared according to the method of Balti et al. (*36*). Protease was extracted from defatted powder by stirring in 10 volumes buffer A (10 mM glycine−HCl, pH 3.0) for 2 h at 4 °C. The homogenate was centrifuged at 8500*g* for 30 min at 4 °C. The pellet was discarded, and the supernatant obtained was collected and used as crude acidic protease extract.

Cathepsin D Purification. *Ammonium Sulfate Precipitation*. The crude acidic protease extract was first subjected to ammonium sulfate fractionation 0−30, 30−60, and 60−80% (w/v). The precipitate obtained in each fraction after centrifugation at 13000*g* for 30 min was suspended in buffer A and dialyzed for 24 h at 4 °C against repeated changes in the same buffer (after 8 and 16 h).

Sephadex G-100 Gel Filtration. The 30−60% (w/v) ammonium sulfate fraction was subjected to gel filtration on a Sephadex G-100 column (2.6 cm × 165 cm) equilibrated with buffer B (25 mM sodium acetate, pH 4.0, containing 0.5‰ Triton X-100). Fractions of 5 mL were eluted at a flow rate of 28 mL/h with the same buffer. Protein content (Abs 280 nm) and proteolytic activity were determined. Fractions showing protease activity were pooled.

Mono-S Cation-Exchange Chromatography. The active fractions from Sephadex G-100 were applied to a Mono-S column (2.6 cm × 20 cm) previously equilibrated with buffer C (25 mM sodium acetate, pH 4.0). After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride (NaCl) in the range of 0−0.5 M in the equilibrating buffer. Fractions of 5 mL were collected at a flow rate of 60 mL/h and analyzed for protease activity and protein concentration. The fractions with high proteolytic activities were pooled.

Sephadex G-75 Gel Filtration. The active fractions from Mono-S were concentrated by ultrafiltration using a 10 kDa membrane and then applied to gel filtration on a Sephadex G-75 column (2.5 cm × 100 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 proteolytic activity were measured.

Mono-S FPLC Cation Exchange Chromatography. The pooled Sephadex G-75 fractions containing protease activity were concentrated by ultrafiltration using a 10 kDa membrane and further purified by cation-exchange chromatography on a Mono-S FPLC column (2.6 cm × 30 cm) equilibrated with buffer C. Nonadsorbed proteins were washed out with the same buffer containing 100 mM NaCl. The enzyme was eluted with a linear gradient of NaCl (100−250 mM). Fractions (3 mL) showing protease activities were collected at a flow rate of 120 mL/h. The enzyme-containing fractions were pooled, dialyzed, concentrated by lyophilization, and stored at −20 °C for further analyses. All of the purification steps were conducted at temperatures not exceeding 4 °C.

Assay for Protease Activity. Proteolytic activity was assayed according to the method of Anson (*37*) with slight modifications, using hemoglobin as a substrate, as described by Bougatef et al. (*38*). Enzyme (50 μL) was mixed with 100 μL of solution consisting of 2.0% acid-denatured bovine hemoglobin and 350 μL of 100 mM glycine−HCl buffer (pH 3.0). After incubation of the mixture at 37 °C for 30 min, the reaction was immediately stopped by the addition of 500 μL of 8.0% trichloroacetic acid (TCA). The mixture was centrifuged at 10000*g* for 15 min, and the absorbance of the supernatant at 280 nm was measured. One unit of enzymatic activity against hemoglobin 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. Values are the means of three independent experiments.

Polyacrylamide Gel Electrophoresis and Hemoglobin 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 (*39*), using 5% (w/v) stacking and 15% (w/v) separating gels. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Sigma), consisting of rabbit muscle phosphorylase *b* (97000 Da), bovine serum albumin (66000 Da), egg white ovalbumin (45000 Da), human carbonic anhydrase (31000 Da), soybean trypsin inhibitor (20100 Da), and α-lactalbumin (14200 Da).

Native-PAGE was performed according to the procedure of Laemmli (*39*) except that the sample was not heated and SDS and reducing agent were left out.

Hemoglobin zymography was performed on native-PAGE according to the method of Garcia-Carreno et al. (*40*).

Determination of the N-Terminal Amino Acid Sequence of *S. officinalis* Cathepsin D. The purified enzyme, from Mono-S FPLC, 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 cathepsin D was excised and the N-terminal amino acid sequence was determined by using the Edman degradation method on an Applied Biosystems Protein Sequencer Procise 492 equipped with 140 C HPLC systems (*41*).

Protein Determination. Protein concentration was determined according to the method of Bradford (*42*) using BSA as a standard and during the course of enzyme purification by measuring the absorbance at 280 nm.

Molecular Weight. To determine the molecular mass of the native enzyme, the freeze-dried proteins were resuspended in phosphate buffer, pH 7.0. Two hundred micrograms of the pure enzyme was loaded on a 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.

Biochemical Properties. *Effect of pH on Activity and Stability of Cathepsin D*. The effect of pH was determined with hemoglobin as a substrate. Cathepsin D activity was studied over the pH range of 1.0−7.0 at 37 °C for 30 min. For the measurement of pH stability, the enzyme was incubated at 25 °C for 60 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 buffer, pH 1.0−3.0; 100 mM sodium acetate buffer, pH 4.0−6.0; 100 mM Tris-HCl buffer, pH 7.0−8.0.

Effect of Temperature on Activity and Stability of Cathepsin D. The effect of temperature on cathepsin D activity was studied from 20 to 70 °C for 30 min at pH 3.0. For thermal stability, cathepsin D isolated from cuttlefish hepatopancreas was pre-incubated in 100 mM glycine−HCl, pH 3.0, at temperatures ranging from 5 to 70 °C for 30 min and immediately cooled on ice. Thereafter, the residual activity was determined at 37 °C for 30 min at pH 3.0.

Effects of Enzyme Inhibitors, SDS, and Some Chemicals. The effects of enzyme inhibitors, SDS, and some chemicals on cathepsin D activity were studied using PMSF, SBTI, benzamidine, pepstatin A, TPCK, E-64, IAA, EDTA, ATP, molybdate, and various concentrations of SDS. The purified enzyme was pre-incubated with each inhibitor or additive for 30 min at 25 °C, and then the remaining enzyme activity was tested using hemoglobin as a substrate. The activity of the enzyme assayed in the absence of any inhibitor or additive was taken as control.

Effect of Metal Ions. The effects of various metal ions (5 mM) on cathepsin D activity were investigated by adding the monovalent (Na^+ or K^+), divalent (Ca^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Ba^{2+}, Sr^{2+}, Fe^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, and Mg^{2+}), or trivalent (Fe^{3+}) metal ions to the reaction mixture. The activity of the enzyme in the absence of any metal ions was taken as control.

Effect of the Cathepsin D on Food Proteins. Proteolytic activity was examined against natural food proteins that are commonly hydrolyzed or used in protease assay. The different substrates used were hemoglobin, casein, ovalbumin, γ-globulin, BSA, and gelatin. Substrate concentration of 2% (w/v) was prepared in glycine−HCl buffer, pH 3.0. The protease activity toward hemoglobin was taken as a control.

Preparation of Myofibrillar Proteins from Cuttlefish Muscle. Myofibrillar proteins were prepared according to the method of Hashimoto et al. (*43*). All steps were performed at 4 °C to minimize proteolysis and protein denaturation. Twenty grams of cuttlefish muscle was homogenized with 10 volumes of phosphate buffer (ionic strength, I = 0.05, pH 7.5) for 1 min using a Wiset Stir HS-100 homogenizer (DAIHAN Scientific, Korea). The homogenate was centrifuged at 5000*g* for 15 min at 4 °C using a Hermele Z36HK refrigerated centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was discarded and the procedure repeated three times. The residue from the above was homogenized with 10 volumes of KCl−phosphate buffer (I = 0.5, pH 7.5) in a Waring blender (Moulinex, Paris, France) and centrifuged. The residue was similarly homogenized and centrifuged again. Both supernatants were combined and used as the myofibrillar proteins fraction. Two phosphate buffers were used for extracting myofibrillar proteins: 15.6 mM Na_2HPO_4−3.5 mM KH_2PO_4 (ionic strength, I = 0.05, pH 7.5) and 0.45 M KCl−15.6 mM Na_2HPO_4−3.5 mM KH_2PO_4 (I = 0.5, pH 7.5), respectively. All extracts were supplemented by 0.1 mM PMSF, 10 mM EDTA, and 0.01 (w/v) sodium azide to inhibit endogenous proteases and microbial growth.

Hydrolysis of Myofibrillar Proteins by Purified Cathepsin D. To hydrolyze muscle proteins, purified cathepsin D (1.5 U) was added to 2 mL of reaction mixture, containing 3 mg of protein substrate in 100 mM glycine−HCl buffer, pH 3.0. The hydrolysis was conducted by incubating the reaction mixture at 50 °C for 0, 5, 10, 20, and 30 min. The control was performed by incubating the reaction mixture at 50 °C for 30 min without the addition of cuttlefish cathepsin D. The reaction was terminated by adding 100 μL of 1 N NaOH containing 1% (w/v) SDS. The mixture was further mixed with SDS-PAGE sample buffer and boiled for 1 min. The mixture was centrifuged at 8500 rpm for 10 min at room temperature (Hettich zentrifugen, Berlin, Germany) to remove debris. The protein hydrolysate was analyzed by SDS-PAGE using 12% separating and 5% staking gels.

Statistical Analysis. All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out using Duncan's multiple-range test. The SPSS statistic program (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL) was used for data analysis.

RESULTS AND DISCUSSION

Cathepsin D Purification. Cathepsin D from the hepatopancreas of *S. officinalis* was purified successively by the five-step procedure described under Materials and Methods. In the first step, the crude enzyme extract was fractionated with ammonium sulfate. Fraction F2 (30−60% w/v saturation) showed higher specific activity (20.33 U/mg of protein) than fraction F1 (0−30%; 0.577 U/mg). No activity was detected in F3 (60−80%) and in the final supernatant. The 30−60% ammonium sulfate precipitate, which gave the highest specific activity, was then successively subjected to Sephadex G-100 gel filtration, Mono-S Sepharose cation-exchange chromatography, Sephadex G-75 gel filtration, and finally Mono-S FPLC (results not shown).

Table 1. Summary of the Purification of Cathepsin D from Cuttlefish (*S. officinalis*)[a]

purification step	total activity (U)	total protein (mg)	specific activity (U/mg)	recovery (%)	purification fold
crude extract	4528	682.15	6.63	100	1
ammonium sulfate precipitation (30−60% w/v)	3758	184.8	20.33	82.99	3.06
Sephadex G-100	2856	57.0	50.10	63.07	7.55
Mono-S Sepharose	2084	12.75	163.45	46.02	24.65
Sephadex G-75	1258	5.13	245.22	27.78	36.98
Mono-S FPLC	780	2.17	359.44	17.22	54.21

[a] All operations were carried out at 4 °C. Only the precipitate formed between 30 and 60% saturation with ammonium sulfate was subjected to gel filtration on Sephadex G-100.

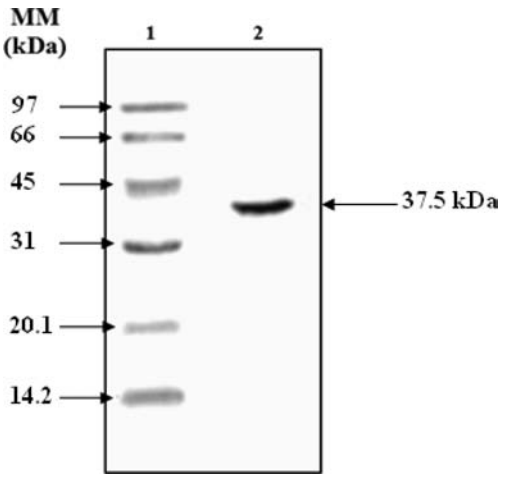

Figure 1. SDS-PAGE of the purified cathepsin D from *S. officinalis* hepatopancreas. Lanes: 1, molecular weight markers; 2, purified cathepsin D from *S. officinalis* hepatopancreas.

After the final purification step, the cathepsin D was purified 54.2-fold with a recovery of 17.2% and a specific activity of 359.4 U/mg of protein, using hemoglobin as a substrate. The results of the purification procedure are summarized in **Table 1**. The obtained total recovery of enzyme activity in the purified fractions (17.22%) is relatively high compared to other published purifications of cathepsin D from other fish species (*24*−*26*, *35*, *44*).

Purity and Molecular Weight. Analysis by SDS-PAGE of the purified enzyme showed a single band of about 37.5 kDa (**Figure 1**) corresponding to that determined by gel filtration (data not shown), indicating that the active enzyme is a single-chain monomer. Cathepsin D of common carp muscle, Atlantic cod, and Japanese common squid hepatopancreas also are single-chain enzymes (*24*, *26*, *29*).

The molecular weight of the *S. officinalis* cathepsin D was close to those from other fish species, such as white Japanese common squid (*T. pacificus*) (36.5 kDa) (*29*), carp (*Cyprinus carpio*) (36 kDa) (*24*), and herring (*Clupea harengus*) (38 kDa) (*25*).

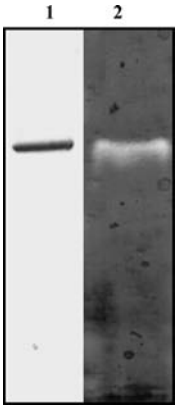

Figure 2. Native-PAGE (lane 1) and zymogram detection of proteolytic activity (lane 2) of the purified cathepsin D from *S. officinalis* hepatopancreas.

It was lower than those of cathepsins from Atlantic cod (*Gadus morhua*) (40 kDa) (*26*), Antarctic icefish (*Chionodraco hamatus*) (40 kDa) (*27*), mackerel (*Scomber australasicus*) (51 kDa), milkfish (*Chanos chanos*) (54 kDa) (*45*), banded shrimp (*Penaeus japonicus*) (61 kDa) and grass shrimp (*Penaeus monodon*) (61 kDa) (*46*).

The purity of the purified cathepsin D was also evaluated by using native-PAGE. As reported in **Figure 2**, *S. officinalis* cathepsin D migrated as a single protein band in the native-PAGE, confirming the homogeneity of the enzyme. The proteolytic activity of this protein band was confirmed by zymogram activity staining. A unique clear band of hemoglobin hydrolysis was observed in the gel, indicating the homogeneity of the purified cathepsin D (**Figure 2**).

N-Terminal Amino Acid Sequence of *S. officinalis* Cathepsin D. The N-terminal amino acid sequence of the purified cathepsin D determined by automated Edman degradation was found to be APTPEPLSNYMDA. The N-terminal amino acid sequence of *S. officinalis* cathepsin D showed uniformity, indicating that it was isolated in a pure form and, if other isoforms were present, their amounts must be small.

The N-terminal amino acid sequence of *S. officinalis* cathepsin D was aligned with the sequences of cathepsin D from other species and other known aspartic proteases (**Figure 3**). The sequence of *S. officinalis* showed high homology with cathepsins D from marine invertebrates. The cathepsin D from *S. officinalis* differs from that of Japanese flying squid (*T. pacificus*) cathepsin D by only one residue in the first 13 amino acids (*29*). The Leu_{11} in Japanese flying squid cathepsin D was replaced by Met_{11} in *S. officinalis* cathepsin D. However, there are two amino acid residues in the 13-terminal sequence that differ from cathepsins D of black tiger shrimp (*P. monodon*) (*47*) and American lobster (*Homarus americanus*) (*48*).

S. officinalis cathepsin D, which showed high homology with cathepsin D from marine vertebrates, had a Pro residue at position 6 and a Ser residue at position 8, whereas Thr and Lys are common in all marine vertebrate cathepsins D (*25*, *49*). Compared with the N-terminal amino acids of herring (*C. harengus*) cathepsin D, cuttlefish cathepsin differed only at residues 6 and 8.

The N-terminal amino acid sequence was also compared with those of other cathepsins and aspartic proteinases. The sequence showed high homology with cathepsin from lizard. Both sequences differed by only two amino acids. Compared with those from chicken and human, there are three and four amino acid residues in the 13-terminal sequence that differed from cuttlefish cathepsin D.

Effects of Enzyme Inhibitors, SDS, and Some Chemicals on Cathepsin D Activity. Proteases can be classified by their sensitivity to various inhibitors (*50*). To determine the nature of the purified protease, the effects of a variety of enzyme inhibitors, such as chelating agent and specific group reagents, on the cathepsin D activity were investigated (**Table 2**).

Purified cathepsin D from *S. officinalis* was strongly inhibited by pepstatin A, which inhibits only aspartyl proteases (*51*), indicating that the enzyme belonged to the aspartic acid proteases. Furthermore, E-64 and iodoacetic acid (IAA), which are

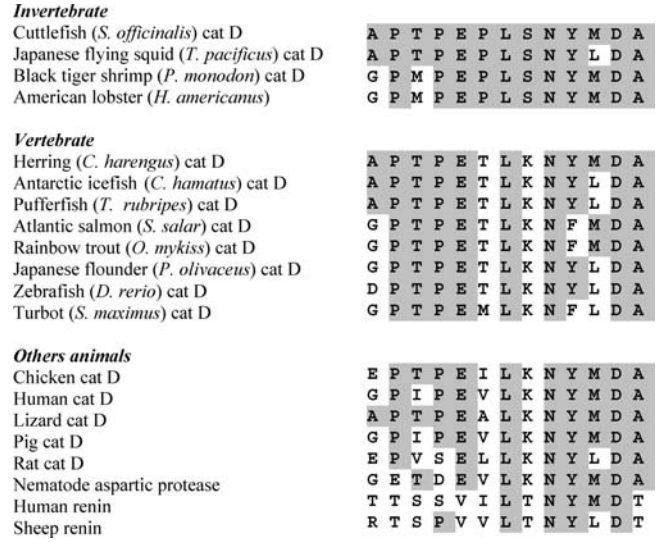

Figure 3. Alignment of the N-terminal amino acid sequence of the purified cathepsin D from cuttlefish (*S. officinalis*) with the sequences of other aspartic proteinases. Residues identical with *S. officinalis* cathepsin D are shaded. Cuttlefish, *S. officinalis* cathepsin D (cat D) (this work); Japanese flying squid (*T. pacificus*) cat D (*29*); black tiger shrimp (*Penaeus monodon*) cat D (*47*); American lobster (*Homarus americanus*) cat D (*48*); herring (*Clupea harengus*) cat D (*25*); Antarctic icefish (*Chionodraco hamatus*) cat D (*27*); pufferfish (*Takifugu rubripes*) cat D (*71*); Atlantic salmon (*Salmo salar*) cat D (*49*); rainbow trout (*Oncorhynchus mykiss*) cat D (*72*); Japanese flounder (*Paralichthys olivaceus*) cat D (*73*); zebrafish (*Danio rerio*) cat D (*74*); turbot (*Scophthalmus maximus*) cat D (*75*); chicken cat D (*76*); human cat D (*77*); lizard (*Podarcis siculus*) cat D (*78*); pig cat D (*79*); rat cat D (*80*); nematode aspartic proteinase (*81*); human renin (*82*); sheep renin (*83*).

Table 2. Effects of Various Enzyme Inhibitors, SDS, and Some Chemicals on the Activity of the Purified Cathepsin D from Hepatopancreas of Cuttlefish (*S. officinalis*)[a]

inhibitor	concentration	remaining activity[b] (%)
control		100
pepstatin A	1.5 μM	0*
PMSF	5 mM	100
benzamidine	5 mM	100
SBTI	1 mM	100
TPCK	1 mM	100
E-64	1 mM	83.0 $\pm$ 1.1*
iodoacetic acid (IAA)	1 mM	76.0 $\pm$ 0.5*
EDTA	5 mM	97.0 $\pm$ 1.8
ATP	1 mM	385.5 $\pm$ 1.4*
molybdate	1 mM	2.3 $\pm$ 0.4*
	0.05 (w/v)	52.9 $\pm$ 2.1*
SDS	0.1 (w/v)	43.4 $\pm$ 2.5*
	0.2 (w/v)	28.0 $\pm$ 0.3*

[a] Purified enzyme was pre-incubated with various enzyme inhibitors or additives for 30 min at 25 °C, and the remaining activity was determined at pH 3.0 and 37 °C, using haemoglobin as a substrate. Enzyme activity measured in the absence of any inhibitor or additive was taken as 100%. [b] Mean $\pm$ SD from triplicate determinations. *, Significantly different from control at $p < 0.05$.

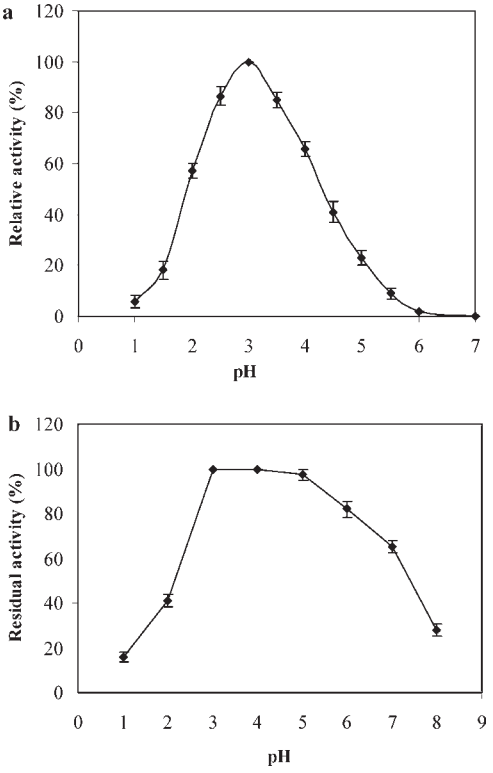

Figure 4. pH profile (**a**) and pH stability (**b**) of the purified cathepsin D from cuttlefish (*S. officinalis*) hepatopancreas. Results are mean ± SD of triplicates.

specific for cysteine proteases (*52*), partially inhibited the activity of the enzyme. The enzyme retained 83 and 76% of its initial activity, after 30 min of incubation at 25 °C, in the presence of E-64 and iodoacetamide, respectively. However, PMSF, a serine protease inhibitor, SBTI, a trypsin specific inhibitor, and EDTA, a metalloprotease inhibitor, did not affect the enzyme activity.

The hemoglobin activity of the purified cathepsin D was strongly inhibited by the strong anionic surfactant (SDS) (**Table 2**). The enzyme retained 43.2 and 28% of its activity after 30 min of incubation at 25 °C in the presence of 0.1 and 0.2% SDS, respectively. Watabe et al. (*53*) and Jiang et al. (*45*) showed that 0.057 and 0.1% SDS completely inactivated the cathepsin D from porcine adrenal cortex and milkfish (*C. chanos*) muscle, respectively.

Because SDS would bear a net negative charge at pH 3.0, this compound would distort the conformation of cathepsin D and consequently inhibit activity of this proteinase.

Different chemicals were also used to elucidate their impact on the activity of cathepsin D (**Table 2**). ATP, an activator of cathepsin D (*54*), enhances the activity by 385%, whereas molybdate inhibitor of cathepsin D affects considerably the activity of the enzyme. Pillai and Zull (*54*) had also reported that cathepsin D was generally activated by ATP when BSA, hemoglobin, and parathyroid hormone were used as substrates. However, the enzyme was fully inhibited by molybdate, a cathepsin D inhibitor.

Effect of pH on the Activity and Stability of Cuttlefish Cathepsin D. The pH activity profile of the purified cuttlefish cathepsin D is shown in **Figure 4a**. The purified enzyme was active between pH 1.5 and 5.0, with an optimum around pH 3.0. The relative activities at pH 2.0, 4.0, and 5.0 were about 57, 65.7, and 23%, respectively, of that at pH 3.0. However, cathepsin D activity decreased significantly above pH 5.0 and was only 2% at pH 6.0. The optimum pH of *S. officinalis* cathepsin D was similar to those

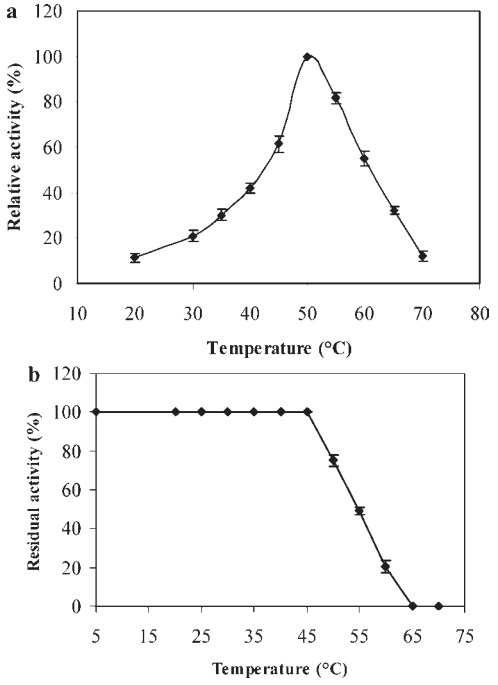

Figure 5. Temperature profile (**a**) and thermal stability (**b**) of the purified cathepsin D from the hepatopancreas of the cuttlefish (*S. officinalis*). Results are mean ± SD of triplicates.

from Japanese flying squid (*T. pacificus*) (*29*), Atlantic cod (*G. morhua* L.) (*26*), and Antarctic icefish (*C. hamatus*) (*27*). However, the pH optimum may vary in the acidic range depending on the protein substrate (*55*, *56*).

It is recognized that most cathepsins D from different sources have an optimum between pH 3.0 and 5.0 (*57*−*60*). However, some cathepsins D had two optimum pH values (*61*−*63*). The pH curves for cathepsin D obtained from different sources were slightly different. This suggested that the pK_a values for the carboxyl groups were slightly different. Accordingly, the catalytic groups in the active site of the corresponding proteinases from different sources might be aspartate residues, but the environment around this residue was not similar.

The pH stability of cuttlefish cathepsin D is shown in **Figure 4b**. Cathepsin D was highly stable in a broad pH range, maintaining 100% of its original activity at pH 3.0 and 4.0 after 60 min of incubation at 25 °C. At pH 2.0 and 5.0, the enzyme retained 41.0 and 97.3% of its initial activity, respectively.

The pH stability results showed that *S. officinalis* cathepsin D has lower pH stability than those from banded shrimp (*P. japonicus*) and grass shrimp (*P. monodon*) cathepsin D, which were stable in the pH ranges of 5.0−7.0 and 4.0−8.0, respectively (*46*).

Effect of Temperature on the Activity and Stability of Cuttlefish Cathepsin D. The cathepsin D from cuttlefish was active at temperatures from 20 to 70 °C with an optimum around 50 °C. At temperatures above the optimum, activity quickly decreases (**Figure 5a**). The relative activities at 55, 60, and 65 °C were about 81.7, 55.1, and 32%, respectively, of that at 50 °C. The optimum temperature for *S. officinalis* cathepsin D was similar to those from porcine spleen (*64*), rat spleen (*65*), carp muscle (*62*), and banded shrimp (*P. japonicus*) muscle (*46*) and higher than those of cathepsins D from grass shrimp (*P. mondon*) and tilapia (*Tilapia nilotica* × *Tilapia aurea*) muscle, which had optimal temperatures at 45 and 37 °C, respectively (*35*, *46*).

The thermal stability profile of the purified cathepsin D showed that the enzyme is highly stable at temperatures below 45 °C but declined rapidly at temperatures exceeding 50 °C (**Figure 5b**).

Table 3. Effects of Metal Ions (5 mM) on Cathepsin D Activity[a]

ion	relative activity[b] (%)
control	100
Na^{+}	100
K^{+}	100
Mg^{2+}	137.6 ± 1.2*
Mn^{2+}	32.5 ± 0.4*
Ni^{2+}	176.0 ± 2.5*
Zn^{2+}	123.7 ± 1.2*
Cu^{2+}	157.0 ± 1.1*
Cd^{2+}	191.3 ± 1.4*
Hg^{2+}	26.2 ± 1.2*
Fe^{2+}	68.0 ± 0.3*
Ca^{2+}	103.1 ± 0.1
Sr^{2+}	212.5 ± 1.5*
Ba^{2+}	54.0 ± 1.1*
Co^{2+}	239.4 ± 2.8*
Fe^{3+}	12.4 ± 0.5

[a] The activity of the cathepsin D was determined by incubating the enzyme in the presence of various metal ions for 30 min at 37 °C and pH 3.0. [b] Mean ± SD from triplicate determinations. *, significantly different from control at $p < 0.05$.

The enzyme retained more than 75.2 and 49% of its initial activity after 30 min of incubation at 50 and 55 °C, respectively. Although the optimal temperature for the cathepsin D from cuttlefish was 50 °C, 25% of the activity of this protease was lost after 30 min of incubation at 50 °C (**Figure 5b**). These results indicated that cathepsin D from *S. officinalis* was most likely stabilized against thermal inactivation by its substrate during incubation. The enzyme is labile at relatively high temperatures (>55 °C) and retained only 20.5% after incubation for 30 min at 60 °C, whereas no activity was detected after incubation at 65 °C. Thermostability of the isolated cathepsin D was similar to those from porcine spleen, rat spleen, and bovine spleen, which retained about 60−70% of the hemoglobin hydrolytic activity after incubation for 30 min at 50 °C (*64*−*67*).

Interestingly, the thermal stability of *S. officinalis* cathepsin D was higher than those from other marine vertebrate and invertebrate cathepsins D. Cathepsin D from the liver of Atlantic cod (*G. morhua* L.) (*26*) was completely inactivated after 15 min at 40 °C. Mackerel (*Scomber australasicus*) cathepsin D retained < 50% of its original activity after 20 min of incubation at 50 °C (*45*).

Effects of Metal Ions. The effects of some metal ions, at a concentration of 5 mM on the activity of *S. officinalis* cathepsin D were studied at pH 3.0 and 37 °C (**Table 3**) by the addition of metal ions to the reaction mixture. The hemoglobin hydrolytic activity of the purified cathepsin D was activated by Mg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Sr^{2+}, and Co^{2+} ions, whereas it was not affected by Na^{+}, K^{+}, and Ca^{2+} ions. However, the addition of Ba^{2+}, Mn^{2+}, Hg^{2+}, and Fe^{3+} decreased the activity by 46, 67.5, 73.8, and 87.6%, respectively. Cathepsins D from tilapia muscle, rat spleen, and human erythrocyte membrane acid proteinase (EMAP) were also inhibited by Hg^{2+} and Fe^{3+} (*35*, *65*, *68*). Hg^{2+} has been reported to bind to SH groups of the target enzyme and subsequently inhibit the enzymatic activity (*69*). Inhibition of cuttlefish cathepsin D by IAA and Hg^{2+} suggests that the active site of these proteases might also contain cysteine.

Effect of Cathepsin D on Food Proteins. To study the possible food applications of the cathepsin D of *S. officinalis*, the activity of the protease using different protein substrates was studied. Among all proteins, hemoglobin was the most susceptible to hydrolysis, followed by casein, BSA, and then gelatin (**Table 4**), whereas ovalbumin and γ-globulin were resistant to hydrolysis by cathepsin D.

Table 4. Hydrolysis of Food Protein Substrates by Purified Cathepsins D

substrate	relative activity[a] (%)
hemoglobin	100
casein	46.3 ± 1.15*
BSA	21.4 ± 2.83*
ovalbumin	0[a]
gelatin	12.9 ± 1.66*
γ-globulin	2.6 ± 0.04*

[a] Mean ± SD from triplicate determinations. *, significantly different from hemoglobin at $p < 0.05$.

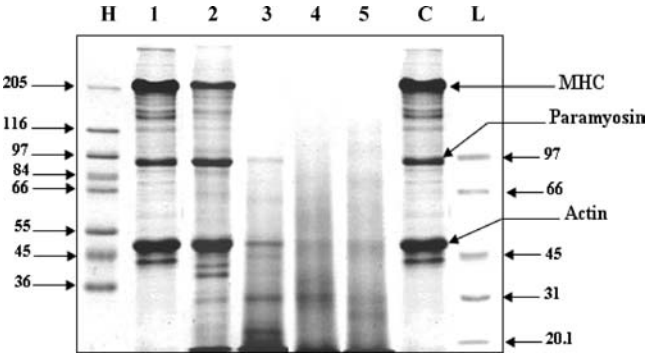

Figure 6. Hydrolysis of myofibrillar protein by purified cathepsin D from the hepatopancreas of cuttlefish at 50 °C. Lanes 1, 2, 3, 4, and 5 were 0, 5, 10, 20, and 30 min, respectively. H, high molecular weight standard; L, low molecular weight standard; C, control (incubated without enzyme addition for 30 min at 50 °C); MHC, myosin heavy chain.

Hydrolysis of Myofibrillar Proteins from Cuttlefish Muscle by the Purified Cathepsin D from Hepatopancreas of Cuttlefish. The myofibrillar proteins fraction, extracted from cuttlefish muscle, contained three major protein bands, corresponding to myosin heavy chain (MHC), actin, and paramyosin (**Figure 6**). The molecular mass of paramyosin from different species ranges from 95 to 125 kDa (*70*).

The digestion of myofibrillar protein from cuttlefish muscle by the purified cathepsin D at 50 °C was investigated by SDS-PAGE. **Figure 6** shows the degradation of the MHC, paramyosin, and actin. Among all proteins, MHC was the most susceptible to hydrolysis, followed by paramyosin. MHC was degraded rapidly within 5 min by the purified cathepsin D (**Figure 6**). Total disappearance of MHC was observed after 10 min of incubation at 50 °C. For actin and paramyosin, the degradation increased during the incubation time. However, the degradation rate was lower than that of MHC. On the other hand, paramyosin was completely hydrolyzed by cathepsin D after 20 min of incubation, whereas actin was more resistant to hydrolysis by cathepsin D. Actin was retained to some extent after 30 min of incubation at 50 °C. From the result, cathepsin D from cuttlefish hepatopancreas hydrolyzed myofibrillar proteins effectively, particularly MHC, which is the dominant protein in fish muscle. Therefore, cathepsin D can be used for further application, especially for the production of protein hydrolysates.

Conclusion. In the present study, a new cathepsin D from *S. officinalis* was purified and characterized. The purified cathepsin D was homogeneous on SDS-PAGE, and its molecular mass was estimated to be 37.5 kDa. The enzyme showed an optimum temperature at 50 °C and an optimum pH of 3.0. The N-terminal sequence of cathepsin D showed high homology with vertebrate and invertebrate cathepsins D.

Further work is needed to isolate and sequence analyze the cDNA encoding cuttlefish cathepsin D and to determine the

properties of this protease as a possible biotechnological tool in the fish-processing and food industries.

LITERATURE CITED

(1) Davies, D. R. The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189−215.

(2) Foltmann, B. Gastric proteinases: structure, function, evolution and mechanism of action. *Essays Biochem.* **1981**, *17*, 52−84.

(3) Cooper, J. B.; Khan, G.; Taylor, G.; Tickle, I. J.; Blundell, T. L. X-ray analyses of aspartic proteinases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. *J. Mol. Biol.* **1990**, *214*, 199−222.

(4) Takeda-Ezaki, M.; Yamamoto, K. Isolation and biochemical characterization of procathepsin E from human erythrocyte membranes. *Arch. Biochem. Biophys.* **1993**, *304*, 352−358.

(5) Sato, K.; Nagai, Y.; Suzuki, M. Purification and characterization of an acid proteinase from *Dirofilaria immitis* worms. *Adv. Exp. Med. Biol.* **1995**, *362*, 299−304.

(6) Tang, J.; Wang, R. N. S. Evolution in the structure and function of aspartic protease. *J. Cell Biochem.* **1987**, *33*, 53−63.

(7) Inagami, T.; Murakami, K.; Misono, K.; Workman, R. J.; Cohen, S.; Suketa, Y. Renin and precursor: purification, characterization, and studies on active site. *Adv. Exp. Med. Biol.* **1977**, *95*, 225−247.

(8) Roberts, N. B.; Taylor, W. H. The preparation and purification of individual human pepsins by using diethylaminoethyl-cellulose. *Biochem. J.* **1987**, *169*, 607−615.

(9) Foltman, B.; Andersen, B. The primary structure of prochymosin (prorennin) EC 3.4.4.3. Some tryptic fragments of a maleylated preparation. *FEBS Lett.* **1971**, *17*, 87−89.

(10) Kay, J.; Tyas, L.; Humphreys, M. J.; Hill, J.; Dunn, B. M.; Berry, C. Aspartic proteinases from parasites. *Adv. Exp. Med. Biol.* **1996**, *389*, 247−250.

(11) Francis, S. E.; Sullivan, D. J., Jr.; Goldberg, D. E. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* **1997**, *51*, 97−123.

(12) Guruprasad, K.; Tormakangas, K.; Kervinen, J.; Blundell, T. L. Comparative modelling of barley-grain aspartic proteinase: a structural rationale for observed hydrolytic specificity. *FEBS Lett.* **1994**, *352*, 131−136.

(13) Brodelius, P. E.; Cordeiro, M. C.; Pais, M. S. Aspartic proteinases (cyprosins) from *Cynara cardunculus* spp. *flavescens* cv. Cardoon; purification, characterisation, and tissue-specific expression. *Adv. Exp. Med. Biol.* **1995**, *362*, 255−266.

(14) Phylip, L. H.; Mills, J. S.; Parten, B. F.; Dunn, B. M.; Kay, J. Intrinsic activity of precursor forms of HIV-1 proteinase. *FEBS Lett.* **1992**, *314*, 449−454.

(15) von der Helm, K. Retroviral proteases: structure, function and inhibition from a non-anticipated viral enzyme to the target of a most promising HIV therapy. *Biol. Chem.* **1996**, *377*, 765−774.

(16) Toogood, H. S.; Prescott, M.; Daniel, R. M. A pepstatin-insensitive aspartic proteinase from a thermophilic *Bacillus* sp. *Biochem. J.* **1995**, *307*, 783−789.

(17) Hill, J.; Phylip, L. H. Bacterial aspartic proteinases. *FEBS Lett.* **1997**, *409*, 357−360.

(18) Metcalf, P.; Fusek, M. Two crystal structures for cathepsin D: the lysosomal targeting signal and active site. *EMOBJ.* **1993**, *12*, 1293−1302.

(19) Tsuji, H.; Akasaki, K. Identification and characterization of lysosomal enzymes involved in the proteolysis of phenobarbital-inducible cytochrome P450. *Biol. Pharm. Bull.* **1994**, *17*, 568−571.

(20) Yang, X.; Schnellmann, R. G. Proteinases in renal cell death. *J. Toxicol. Environ. Health* **1996**, *48*, 319−332.

(21) Diment, S.; Martin, K. J.; Stahl, P. D. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. *J. Biol. Chem.* **1989**, *264*, 13403−13406.

(22) van Noort, J. M.; van der Drift, A. C. The selectivity of cathepsin D suggests an involvement of the enzyme in the generation of T-cell epitopes. *J. Biol. Chem.* **1989**, *264*, 14159−14164.

(23) Garcia, M.; Platet, N.; Liaudet, E.; Laurent, V.; Derocq, D.; Brouillet, J. P.; Rochefort, H. Biological and clinical significance of cathepsin D in breast cancer metastasis. *Stem Cells* **1996**, *14*, 642−650.

(24) Goldman-Levkovitz, S.; Rimon, A.; Rimon, S. Purification properties and specificity of cathepsin D from *Cyprinus carpio*. *Comp. Biochem. Physiol.* **1995**, *112*, 147−151.

(25) Nielsen, L. B.; Nielsen, H. H. Purification and characterization of cathepsin D from herring muscle (*Clupea harengus*). *Comp. Biochem. Physiol.* **2001**, *128*, 351−363.

(26) Wang, P. A.; Stenvik, J.; Larsen, R.; Mæhre, H.; Olsen, R. L. Cathepsin D from Atlantic cod (*Gadus morhua* L.) liver. Isolation and comparative studies. *Comp. Biochem. Physiol.* **2007**, *147*, 504−511.

(27) Capasso, C.; Lees, W. E.; Capasso, A.; Scudiero, R.; Carginale, V.; Kille, P.; Kay, J.; Parisi, E. Cathepsin D from the liver of the antarctic icefish *Chionodraco hamatus* exhibits unusual activity and stability at high temperatures. *Biochim. Biophys. Acta* **1999**, *1431*, 64−73.

(28) Gildberg, A. Aspartic proteinases in fishes and aquatic invertebrates. *Comp. Biochem. Physiol.* **1988**, *91*, 425−435.

(29) Komai, T.; Kawabata, C.; Amano, M.; Lee, B. R.; Ichishima, E. Todarepsin, a new cathepsin D from hepatopancreas of Japanese common squid. *Comp. Biochem. Physiol.* **2004**, *82*, 373−382.

(30) Wright, L. M.; Levy, E. S.; Patel, N. P.; Alhadeff, J. A. Purification and characterization of cathepsin D from normal human breast tissue. *J. Protein Chem.* **1997**, *16*, 171−181.

(31) Wittlin, S.; Rösel, J.; Stover, D. R. One-step purification of cathepsin D by affinity chromatography using immobilized propeptide sequences. *Eur. J. Biochem.* **1998**, *252*, 530−536.

(32) Huang, J. S.; Huang, S. S.; Tang, J. Cathepsin D isozymes from porcine spleens. Large scale purification and polypeptide chain arrangements. *J. Biol. Chem.* **1979**, *254*, 11405−11417.

(33) Larsen, L. B.; Benfeldt, C.; Rasmussen, L. K.; Petersen, T. E. Bovine milk procathepsin D and cathepsin D: coagulation and milk protein degradation. *J. Dairy Res.* **1996**, *63*, 119−130.

(34) Barrett, A. J. Cathepsin D. Purification of isoenzymes from human and chicken liver. *Biochem. J.* **1970**, *117*, 601−607.

(35) Jiang, S. T.; Wang, Y. T.; Chen, C. S. Purification and characterization of a proteinase identified as cathepsin D from tilapia muscle (*Tilapia nilotica* × *Tilapia aurea*). *J. Agric. Food Chem.* **1991**, *39*, 1597−1601.

(36) Balti, R.; Barkia, A.; Bougatef, A.; Ktari, N.; Nasri, M. Heat-stable trypsin from cuttlefish (*Sepia officinalis*) hepatopancreas: purification and characterization. *Food Chem.* **2009**, *113*, 146−154.

(37) Anson, M. L. The estimation of pepsin, trypsin, papain, and the cathepsin with hemoglobin. *J. Gen. Physiol* **1938**, *22*, 79−89.

(38) Bougatef, A.; Balti, R.; Ben Zaied, S.; Souissi, N.; Nasri, M. Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences. *Food Chem.* **2008**, *107*, 774−784.

(39) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(40) Garcia-Carreno, F. L.; Dimes, L. E.; Haard, N. F. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.* **1993**, *214*, 65−69.

(41) Hewick, R. M.; Hunkapiller, M. W.; Hood, L. E.; Dreyer, W. J. A gas−liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **1981**, *256*, 7990−7997.

(42) 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*, 248−254.

(43) Hashimoto, K.; Watabe, S.; Kono, M.; Kazuki, S. Musle protein composition of sardine and mackerel. *Bull. Jpn. Soc. Sci. Fish.* **1979**, *45*, 1435−1441.

(44) Gildberg, A. Purification and characterization of cathepsin D from the digestive gland of the pelagic squid *Todarodes sagittatus*. *J. Sci. Food Agric.* **1987**, *39*, 85−94.

(45) Jiang, S. T.; Heir, Y. H.; Lee, J. J.; Wang, J. H. Comparaison of the cathepsin D from mackerel (*Scomber australasicus*) and milkfish (*Chanos chanos*). *J. Agric. Food Chem.* **1993**, *57*, 571−577.

(46) Jiang, S. T.; Nei, F. P.; Chen, H. C.; Wang, J. H. Comparative Study on the Cathepsin D from banded shrimp (*Penaeus japonicus*) and

grass shrimp (*Penaeus monodon*). *J. Agric. Food Chem.* **1992**, *40*, 961−966.
(47) Tassanakajon, A.; Wongpanya, R. Cathepsin D of *Penaeus monodon*. Accession no. ABQ10738, GenBank database, 2007.
(48) Rojo, L.; Muhlia-Almazan, A.; Saborowski, R.; Garcia-Carreno, F. L. Aspartic cathepsin D endopeptidase contributes to extracellular digestion in clawed lobsters *Homarus americanus* and *Homarus gammarus*. *Mar. Biotechnol.* **2010**, in press.
(49) Andreassen, R.; Lunner, S.; Hoyheim, B. Characterization of full-length sequenced cDNA inserts (FLIcs) from Atlantic salmon (*Salmo salar*). *BMC Genomics* **2009**, *10*, 502.
(50) North, M. J. Comparative biochemistry of the proteinases of eukaryotic microorganisms. *Microbiol Rev.* **1982**, *46*, 308−340.
(51) Yoshida, H.; Okamoto, K.; Iwamoto, T.; Sakai, E.; Kanaoka, K.; Hu, J.-P.; Shibata, M.; Hotokezaka, H.; Nishishita, K.; Mizuno, A.; Kato, Y. Pepstatin A, an aspartic proteinase inhibitor, suppresses RANKL-induced osteoclast differentiation. *J. Biochem.* **2006**, *139*, 583−590.
(52) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. L-*trans*-Epoxysuccinyl-leucylamido (4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **1982**, *201*, 189−198.
(53) Watabe, S.; Taguchi, S.; Ikeda, T.; Takada, M.; Yogo, N. Characterization of cathepsin D in porcine adrenocortical lysosomes. *J. Biochem.* **1982**, *92*, 45−55.
(54) Pillai, S.; Zull, J. E. Effects of ATP, vanadate, and molybdate on cathepsin D-catalyzed proteolysis. *J. Biol. Chem.* **1985**, *260*, 8384−8389.
(55) Doke, S. N.; Ninjoor, V.; Nadkarni, G. B. Characterization of cathepsin D from the skeletal muscle of fresh water fish, *Tilapia mossambica*. *Agric. Biol. Chem. Tokyo* **1980**, *44*, 1521−1528.
(56) Stoknes, I.; Rustad, T. Purification and characterization of a multicatalytic proteinase from Atlantic salmon (*Salmo salar*) muscle. *Comp. Biochem. Physiol.* **1995**, *111*, 587−596.
(57) Asghar, A.; Henrickson, R. L. Postmortem stimulation of carcasses: effects on biochemistry, biophysics, microbiology and qualityof meat. *Crit. Rev. Food Sci. Nutr.* **1982**, *18*, 51−58.
(58) Asghar, A.; Bhatti, A. R. Endogenous proteolytic enzymes in skeletal muscle: their significance in muscle physiology and during postmortem aging events in carcasses. *Adv. Food Res.* **1987**, *31*, 343−451.
(59) Bond, J. S.; Butler, P. E. Intracellular proteases. *Annu. Rev. Biochem.* **1987**, *56*, 333−364.
(60) Zeece, M. G.; Katoh, K. Cathepsin D and its effects on myofibrillar proteins: a review. *J. Food Biochem.* **1989**, *13*, 157−178.
(61) Iodice, A. A.; Leong, V.; Weinstock, L. M. Separation of cathepsin A and D of skeletal muscle. *Arch. Biochim. Biophys.* **1966**, *117*, 477−486.
(62) Makinodan, Y.; Kyaw, N. N.; Ikeda, S. Intracellular distribution of carp muscle cathepsin D and electron micrograph of muscle lysosomal fraction. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 1501−1509.
(63) Takeda, M.; Ueno, E.; Kato, Y.; Yamamoto, K. Isolation and catalytic and immunochemical properties of cathepsin D-like acid proteinase from rat erythrocytes. *J. Biochem.* **1986**, *100*, 1269−1277.
(64) Cunningham, M.; Tang, J. Purification and properties of cathepsin D from porcine spleen. *J. Biol. Chem.* **1976**, *251*, 4528−4536.
(65) Yamamoto, K.; Katsuda, N.; Himeno, M.; Kato, K. Cathepsin D of rat spleen, affinity purification and properties of two types of cathepsin D. *Eur. J. Biochem.* **1979**, *95*, 459−467.
(66) Turk, V.; Kregar, I.; Gubensek, F.; Lapanje, S.; Urh, I.; Kovacic, M. Studies on bovine spleen cathepsin D. *Acta. Biol. Med. Ger.* **1977**, *36*, 1531−1535.
(67) Draper, A. M.; Zeece, M. G. Thermal stability of cathepsin D. *J. Food Sci.* **1989**, *54*, 1651−1652.
(68) Yamamoto, K.; Takeda, M.; Yamamoto, H.; Tatsumi, M.; Kato, Y. Human erythrocyte membrane acid proteinase (EMAP): sidedness and relation to cathepsin D. *J. Biochem.* **1985**, *97*, 821−830.
(69) Klee, C. B. Interaction of calmodulin with Ca^{2+} and target proteins. In *Molecular Aspects of Cellular Regulations, Vol. 5, Calmodulin*; Cohen, P., Ed.; Elsevier: Amsterdam, The Netherlands, 1988; Chapter 3, pp 35−56.
(70) Sikorski, Z. E. The myofibrillar proteins in seafoods. In *Seafood Proteins*; Sikorski, Z. E., Pan, B. S., Shahidi, F., Eds.; Chapman and Hall: New York, 1994; pp 40−57.
(71) Kurokawa, T.; Uji, S.; Suzuki, T. Identification of pepsinogen gene in the genome of stomachless fish, *Takifugu rubripes*. *Comp. Biochem. Physiol.* **2005**, *140*, 133−140.
(72) Brooks, S.; Tyler, C. R.; Carnevali, O.; Coward, K.; Sumpter, J. P. Molecular characterization of ovarian cathepsin D in the rainbow trout, *Oncorhynchus mykiss*. *Gene* **1997**, *201*, 45−54.
(73) Park, E. M.; Kim, Y. O.; Nam, B. H.; Kong, H. J.; Kim, W. J.; Lee, S. J.; Kim, K. K. Cloning and expression analysis of cathepsin D in the olive flounder *Paralichthys olivaceus*. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1856−1859.
(74) Riggio, M.; Scudiero, R.; Filosa, S.; Parisi, E. Sex- and tissue-specific expression of aspartic proteinases in *Danio rerio* (zebrafish). *Gene* **2000**, *260*, 67−75.
(75) Jia, A.; Zhang, X. H. Molecular cloning, characterization and expression analysis of cathepsin D gene from turbot *Scophthalmus maximus*. *Fish Shellfish Immunol.* **2009**, *26*, 606−613.
(76) Retzek, H.; Steyrer, E.; Sanders, E. J.; Nimpf, J.; Schneider, W. J. Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. *DNA Cell Biol.* **1992**, *11*, 661−672.
(77) Faust, P. L.; Kornfeld, S.; Chirgwin, J. M. Cloning and sequence analysis of cDNA for human cathepsin D. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4910−4914.
(78) De Stasio, R.; Borrelli, L.; Kille, P.; Parisi, E.; Filosa, S. Isolation, characterization and molecular cloning of cathepsin D from lizard ovary: changes in enzyme activity and mRNA expression throughout ovarian cycle. *Mol. Reprod. Dev.* **1999**, *52*, 126−134.
(79) Shewale, J. G.; Tang, J. Amino acid sequence of porcine spleen cathepsin D. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3703−3707.
(80) Birch, N. P.; Loh, Y. P. Cloning, sequence and expression of rat cathepsin D. *Nucleic Acids Res.* **1990**, *18*, 6445−6446.
(81) Jolodar, A.; Miller, D. J. Preliminary characterization of an *Onchocerca volvulus* aspartic protease. *Int. J. Parasitol.* **1997**, *27*, 1087−1090.
(82) Imai, T.; Miyazaki, H.; Hirose, S.; Hori, H.; Hayashi, T.; Kageyama, R.; Ohkubo, H.; Nakanishi, S.; Murakami, K. Cloning and sequence analysis of cDNA for human rennin precursor. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 7405−7409.
(83) Aldred, G. P.; Fu, P.; Crawford, R. J.; Fernley, R. T. The sequence and tissue expression of ovine renin. *J. Mol. Endocrinol.* **1992**, *8*, 3−11.

Received for review February 26, 2010. Revised manuscript received August 27, 2010. Accepted August 30, 2010. This work was funded by the Ministry of Higher Education and Scientific Research, Tunisia.