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Identification of a cysteine proteinase from Jumbo squid (*Dosidicus gigas*) hepatopancreas as cathepsin L

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ABSTRACT

A cysteine proteinase from Jumbo squid (*Dosidicus gigas*) hepatopancreas was partially purified by a two step procedure involving ammonium sulfate precipitation and gel filtration chromatography and further by SDS–PAGE. The molecular weight of the proteinase was 24 kDa determined by SDS–PAGE and 23.7 kDa with mass spectrometry. The activity had an optimum pH of 4.5 and optimum temperature of 55 °C under the assay for cathepsin L specific synthetic substrate Z-PAAFC. The cathepsin B and H specific synthetic substrates Z-AAAFC and H-AMC did not show any hydrolysis with the partially purified enzyme. Peptide mapping of trypsin digests of the 24 kDa band from SDS–PAGE showed the squid cysteine proteinase was homologous to cathepsin L from different animal sources. The activity of the partially purified fraction with the cathepsin L specific substrate Z-PAAFC was inhibited 75–89% by enzyme inhibitors specific for cysteine proteinases but was also significantly inhibited by serine and aspartate proteinase inhibitors.

1. Introduction

Jumbo squid (*Dosidicus* gigas d'Orbigny 1857) is an invertebrate migratory predator that is found in deep waters of the Gulf of California. It is abundant (Nevarez-Martinez et al., 2000), and is becoming a very important fishery for the northwestern region of Mexico. Jumbo squid is commercialized mainly as fresh or frozen mantle, discarding the viscera, which includes the midgut gland or hepatopancreas. The hepatopancreas is a big gland with a rich content of proteinases (Hatate, Tanaka, Suzuki, & Hama, 2000) that are partially thiol dependant as in other squid species such as *Illex* (Kolodziejska, Szyc, Karamac, & Sikorski, 1994; Raksakulthai & Haard, 2001), *Loligo* (Pignero & Rocca, 1969), *Todarodes* (Takahashi, 1960) and in *Sepia* (Boucad-Camou & Boucher-Rodhoni, 1983), it has been found that in *Illex* most of the proteinase activity could be inhibited by cysteine specific protease inhibitors (Raksakulthai & Haard, 1999).

We recently reported cysteine proteinase(s) are the main proteolytic activity in the hepatopancreas of Jumbo squid. It had important characteristics of the cysteine proteinase including high temperature optimum and stability, acid pH optimum and a wide range of pH stability (Cardenas-Lopez & Haard, 2005). In this paper, we describe the partial purification and identification of the main cysteine proteinase by peptide mapping, and molecular weight, and for the partially purified cysteine protease and the substrate Z-PAAFC we report the temperature and pH optimum and inhibition with specific enzyme inhibitors.

Along with potential uses of squid hepatopancreas proteinases in the food industry, such as cheese making and brewing industry (Gildberg, 1988; Kolodziejska et al., 1994; Raksakulthai, 2001), squid mantle tenderization (Kolodziejska, Pacana, & Sikorski, 1992), preparation of fish sauce (Raksakulthai, Lee, & Haard, 1986) and fermentation of brined squid with the improvement of flavor (Lee, Simpson, & Haard, 1982) there are other non-food uses that could be considered without the need of many purification steps, such as the decrease of viscosity of fish meal stickwaters.

2. Materials and methods

2.1. Biological materials

Jumbo squid (*Dosidicus gigas*) specimens were collected, hepatopancreas was dissected and transported cold to UCDavis IMR laboratory at Davis, California, where they were kept in a −80 °C freezer. A solution (1:25 w/v) was made with squid hepatopancreas lyophilized powder and 0.1 M sodium phosphate buffer pH 6.0 containing 1 mM EDTA, with magnetic stirring for 2 h at 4 °C, and then centrifuged at 26,000 g and 4 °C for 1 h. The supernatant was passed through Whatman filter paper # 1. The precipitate was re-dissolved in 50 mL of same buffer, extracted for another 2 h at 4 °C, and centrifuged at 26,000 g for 1 h at 4 °C. This second supernatant was passed through Whatman paper # 1 and pooled with

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the first supernatant. The combined supernatants are referred to as hepatopancreas crude extract (HCE) (Cardenas-Lopez & Haard, 2005).

2.2. Determination of protein concentration

Bicinchoninic acid (BCA) protein assay (Smith et al., 1985) and Bradford protein assay (Bradford, 1976) were used to determine the protein content of the enzyme preparations, and $A_{280\,nm}$ was used to monitor protein concentration in the chromatographic separations.

2.3. Electrophoresis

Electrophoresis was performed using a Hoefer SE 200 Mighty Small™ mini gel system (Hoefer Scientific Instruments, San Francisco, California). Gels were cast using an SE 245 dual gel caster, and a SE 260 mini gel (10 × 10.5 cm) system was used for electrophoresis. The thickness of gels was 0.75 mm, and a 5 well system (13 mm well width) or a 10 well system (4.8 mm well width) was used. Electrophoresis was performed under constant voltage (160 mV) with a constant power supply unit (ECPS 3000/150, Amersham Pharmacia Biotech, Piscataway, New Jersey) at 0–5 °C with a cooling unit (LAUDA RM6, Brinkmann Instruments Inc., Westbury New Jersey). One run took from 2 to 2.5 h. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed under reducing and non-reducing conditions (Laemmli, 1970). Different percentages of resolving gel (10% or 12%) with pH 8.8 and 4% stacking gel with pH 6.8 were used. After electrophoresis, densitograms of electrophoregrams were developed using a Gel Doc XR image analyzer with Quantity One 1-D Analysis Software Version 4.5.2 (Bio-Rad Laboratories, Hercules, CA).

2.4. Proteinase activity assays

Non-specific proteinase activity assay and activity on 7-amino-4-trifluoro methyl coumarin (AFC) substrates were done as described previously (Cardenas-Lopez et al., 2005).

2.5. Activity on 7-amino-4-methyl coumarin (AMC) substrates

Activity was also measured using the synthetic substrate benzyloxycarbonyl Phe-Arg-7-amino-4-methyl coumarin (Z-PAAMC). This is the most common fluorogenic substrate for cathepsin L determination (Ouali et al., 1987; Porter, Koury, & Stone, 1996; Tchoupe, Moreau, Gauthier, & Bieth, 1991). However, it was used with a spectrophotometer in the same conditions described above. One enzyme unit was defined as the absorbance at 380 nm given by 1 μM of free 7-amino-4-methyl coumarin (AMC) min^{-1}.

2.6. Activity staining

Activity staining was used to detect proteolytic activity after the polyacrylamide gel electrophoresis was performed. 12% SDS–PAGE gels containing 0.1% casein, similar to the method described by El-Shamei, Wu, and Haard (1996), run at 160 V for approximately 2:30 h. The samples were treated with sample buffer (1:1 v/v) without β-mercaptoethanol and without heat treatment. After completion of the run, gels were washed with cold water, soaked in 1% Triton X for 15 min (two times) in ice, then soaked for 30 min in iced 0.1 M sodium phosphate buffer pH 7.0 containing 1 mM EDTA and 1 mM DTT. The gels were washed in cold water, and incubated in 0.1 M sodium phosphate buffer pH 6.0 at 55 °C for 2 h. The gels were then soaked in water, dyed in 0.5% Commassie blue overnight, and destained for 2 h in a 25% ethanol 10% acetic acid solution. The presence of clearing in the blue background indicated the presence of proteolytic activity.

2.7. Isolation and purification of cysteine proteinase from Jumbo squid hepatopancreas

A purification procedure was developed for a cysteine proteinase present in Jumbo squid hepatopancreas. All the buffers for chromatography were filtered through 0.45 μm, 47 mm MAGNA nylon membrane (Osmonics Inc., Trevorse, PA), and degassed.

2.8. Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed with HCE as the initial purification step. Forty to eighty percent saturation was achieved by slowly adding finely ground ammonium sulfate to crude extract with constant magnetic stirring at 4 °C. Each mixture was centrifuged at 10,000g for 60 min and the supernatant was dialyzed against two changes of 100 mM sodium phosphate buffer, 1 mM EDTA at pH 6.0 to remove ammonium sulfate.

2.9. Gel filtration

Aliquots of fractions obtained with 40–80% ammonium sulfate precipitation were applied to a Toyopearl® HW-55 F (TOSOHAAS Corporation, Montogomeryville, PA) column (1.6 × 98 cm). The column was equilibrated and eluted with 100 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 100 mM NaCl buffer, pH 6.0. Flow rate (1 mL/min) was controlled by using a P-3 peristaltic pump (Pharmacia Fine Chemicals, UK) and the fractions were collected using a Gilson micro fractionator (Gilson Medical Electronics, Inc., Middleton, WI). The amount of protein in each fraction was monitored by measuring the absorbance at 280 nm. The proteinase activity was measured for each alternate fraction by the aforementioned azocasein assay. The fractions with proteinase activity were pooled, then concentrated and desalted by ultrafiltration.

2.10. Concentration and desalting by ultrafiltration

The pooled fractions with proteinase activity from gel filtration chromatography were concentrated by ultrafiltration using an Amicon Ultra centrifugation filter device (Millipore Corp. Bedford, Mass.) with a 10 kDa molecular weight cut-off. The concentration was done at 4 °C and approximately 2000g in an International Clinical Centrifuge model 93913H-5 (International Equipment Co. Boston, Mass).

2.11. Peptide mapping

Peptide mapping was performed at the Molecular Structure Facility (MSF) of the University of California, Davis. For peptide mapping, the enzyme was separated by gels run on SDS–PAGE. A technique of in-gel digestion with trypsin was used to obtain peptides (Williams & Stone, 1995) from the 24 kDa band with proteolytic activity. The tryptic peptides were analyzed with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with pulsed N_2 laser (337 nm). The sequences of the trypsin-treated fragments were then compared to those proteins whose sequence is known, by using FASTAS algorithm (Mackey, Haystead, & Pearson, 2002) which compares linked peptides to a protein databank through http://fasta.bioch.virginia.edu website and BLASTP algorithm (Altschul et al., 1997) through http://www.ncbi.nlm.nih.gov molecular biology server.

2.12. Characterization of cysteine proteinase from Jumbo squid hepatopancreas

2.12.1. Temperature optimum

Temperature optimum was determined using the Z-PAAFC assay, which consisted of 480 μL of incubation buffer (75 mM sodium acetate, 8 mM DTT and 0.4 M urea at pH 4.5), and 10 μL of sample, which were pre-incubated for 2 min at the following temperatures: 4, 10, 20, 30, 35, 40, 45, 50, 55, 60, 70 and 80 °C; then 10 μL of AFC substrate was added and the reaction was followed in kinetic mode for 5 min at 380 nm. The rate is measured as Δ380 nm and converted into units by using a standard curve with free AFC.

2.12.2. pH optimum

The assay was similar to the aforementioned assays for optimum temperature, done with both Z-PAAFC and azocasein substrates, but the incubation buffer was adjusted at different pH values at 55 °C. All buffers had the same ionic strength without adding salts.

2.12.3. Molecular weight

The molecular weight was calculated by using low molecular weight standards (Sigma LMW M-3913), (Sigma Chemicals, St. Louis, Mo.) in a SDS–PAGE gel, following the procedure described previously, then performing a regression analysis of the Log of molecular weights standards vs. their relative mobility in the gel.

Molecular weight was also obtained by mass spectrometry at the molecular structure facility (MSF) at the University of California, Davis, using a Perseptive Biosystems Voyager-DE Matrix Assisted Laser Desorption Ionization (MALDI) Time of Flight (TOF) Mass Spectrometer. Sample was co-crystallized with sinapinic acid as a matrix on a disc and subjected to analysis.

2.12.4. Inhibition of activity of the cysteine proteinase

Specific Inhibitors were used to corroborate the nature of the proteinase purified by gel filtration. The inhibitors used were the following: soybean trypsin inhibitor (50 μg/mL), Pefabloc® (2 mg/mL), pepstatin (1 μg/mL), leupeptin (1 μg/mL), E-64 (1 mg/mL), chymostatin (200 μM) and TPCK (100 μg/mL). The assay was done with Z-PAAFC, and consisted of 470 μL of incubation buffer (75 mM sodium acetate, 8 mM DTT, 400 mM urea, pH 4.5), 10 μL of sample (gel filtration fractions) and 10 μL of inhibitor pre-incubated for 2 min at 55 °C and adding 10 μL of substrate, following the reaction at 380 nm for 5 min.

2.12.5. Activity with other methylcoumaride substrates

Activity of the gel filtration fractions was measured with Z-Arg-Arg AFC (Z-AAAFC) and H-Arg-AMC (HAAMC) in a similar assay as described above, only with 480 μL of incubation buffer at pH 5.0 and 55 °C, 10 μL of sample and then 10 μL of substrate.

3. Results and discussion

3.1. Purification of cysteine proteinase from Hepatopancreas

The crude extract was fractionated by precipitation with ammonium sulfate as described above into 3 fractions: 0–40%, 40–80% saturation, and the supernatant of this last fraction (>80%). Most of the activity towards azocasein was found in the fraction that precipitated at 40–80% saturation with $(NH_4)_2SO_4$. When an aliquot of this fraction was applied to a gel filtration chromatography column, a major peak of proteinase activity was obtained (Fig. 1). The fractions with proteinase activity (fractions 31–37, with a total of 7 mL) were pooled and concentrated approximately twofold using an ultrafiltration membrane. Protein concentration and proteolytic activity were determined with the BCA method and the azocasein assay, respectively. A purification table (Table 1) shows that in only these two steps of purification a 15-fold of the cysteine proteinase is achieved with a 45% recovery.

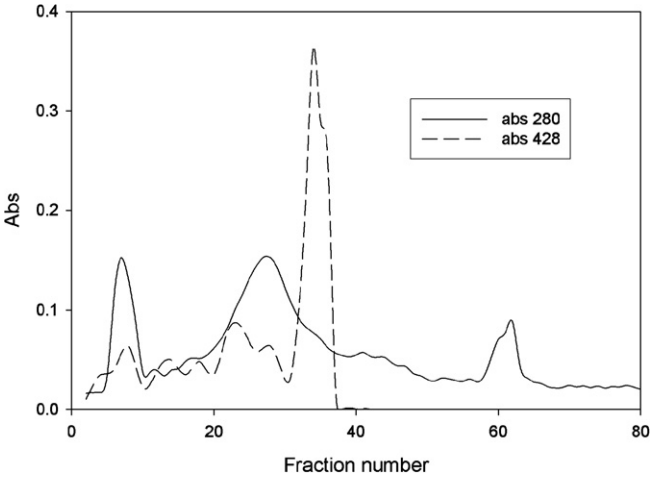

Fig. 1. Gel filtration chromatogram on Toyopearl® HW-55 F column (1.6 × 98 cm) showing total proteinase activity assay with azocasein substrate. Continuous line represents protein concentration and discontinuous line represents total proteolytic activity.

3.2. Peptide mapping

After trypsin digestion from the 24 kDa SDS–PAGE band, peptide fragments were obtained and sequenced as described previously. The peptide fragments sequences (peptide tags) were: TFLTLGNLK, GSDSR, PDMPDWR, VATVTGFVDV, using the one letter amino acid convention. These peptide tags were submitted to NBRF PIR1 protein databases library using FASTS for comparison with known proteins, obtaining the best scores with the longest peptide tag VATVTGFVDV (10 amino acid residues) for a human cathepsin L precursor, with a 70% identity; mouse cathepsin L precursor, with a 70% identity; rat cathepsin L precursor, with a 70% identity; chicken cathepsin L, with a 60% identity; threonine dehydratase (EC 4.2.1.16) from *Escherichia coli*, with 60% identity; and a toxin from sea anemone (*Radianthus paumotensis*), with 50% identity. The comparison is shown in Table 2. The identity of the fragment is consistent with Cathepsin L (EC 3.4.22.15) from different animal sources. There is an obvious difference in position of the fragment with other non-cathepsin L proteins, such as threonine dehydratase and sea anemone toxin, as well as lower identity values. It is important to point out that cathepsin L sequences from different sources have a high degree of homology despite the source. The approach of the use of proteomic tools such as trypsin digestion of an isolated protein from a PAGE gel and then sequence with mass spectrometry, although not in great use in the study of marine bio-

Table 1
Purification table of cysteine proteinase from Jumbo squid hepatopancreas

Sample	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold	Recovery (%)
Crude extract	150.4	11045	110	1.0	100
40–80% AS[a]	48.2	5905	197	1.8	53
Gel filtration	0.6	4931	1644	15.0	45

Activity was measured with Z-PAAFC synthetic substrate.
[a] ammonium sulfate saturation.

Table 2
Identity of trypsin digested peptide fraction VATVTGFVDV from the 24 kDa SDS PAGE band with known proteins in database

Accesion number	Protein	Sequence VATVTGFVDV	Identity (%)
KHMSL	Mouse cathepsin L precursor	GSCKYRAEFAVANDTGFVDIPQQEKALMKA 210	70
KHRTL	Rat cathepsin L precursor	GSCKYRAEYAVANDTGFVDIPQQEKALMKA 210	70
KHHUL	Human cathepsin L precursor	ESCKYNPKYSVANDTGFVDIPKQEKALMKA 210	70
KHCHL	Chicken cathepsin L	EDCRYKAEYNAANDTGFVDIPQGHERALMKA 100	60
DWECTD	Threonine dehydratase	SIISGGNIDLSRVSQITGFVDA[a] 310	60
TZAZR3	Sea anemone RpIII Toxin	KCDDEGPNVRTAPLTGYVDLGYCNEGWEK 4	50

Residue number in the sequence corresponds to first residue shown.
[a] End of sequence.

technology (Pineiro, Barros-Velazquez, Vazquez, Figueras, & Gallardo, 2003) has a great potential and it will increase as long as the bioinformatics databases include more marine organisms sequences.

3.3. Temperature optimum

Temperature optimum was 55 °C determined with the substrate Z-PAAFC (Fig. 2). Temperature optimum is an operational parameter, depending on the conditions of the assay (Whitaker, 1994). However, the determined temperature optimum with Z-PAAFC is comparable to the results from cathepsin L from other animal sources and tissues, including hepatopancreas from carp (Aranishi, Ogata, Hara, Osatomi, & Ishihara, 1997), an invertebrate *Paramecium* (Volkel et al., 1996), anchovy muscle (Heu, Kim, Cho, Godber, & Pyeun, 1997), Pacific whiting (An, Weerasinghe, Seymour, & Morrissey, 1994) and mackarel (Lee, Chen, & Jiang, 1993). The implications of a relatively high temperature optimum of cathepsin L on the production of surimi has been discussed (Yamashita & Konagaya, 1990). Although squid has not been an ideal choice for the production of surimi due to poor gelation capacity (Gomezguillen, Solas, Borderias, & Montero, 1996), the elaboration of other squid products could benefit from the properties of this proteinase. An Arrhenius plot was constructed with the Z-PAAFC substrate activity, with a calculated energy of activation of 11.32 ± 0.14 kcal mol^{-1} (47.52 ± 0.84 kJ mol^{-1}), showing a moderate temperature dependence. However, when assayed with azocasein there was a calculated Q_{10} using temperatures 30 °C and 40 °C of 2.7 ± 0.4, which is an unusually high value, indicating a heat activation at this temperature range (Whitaker, 1994). This characteristic has been studied in Pacific whiting cathepsin L and it has an important impact on surimi product manufacturing (An et al., 1994).

3.4. Optimum pH

The optimum pH was 4.5 with Z-PAAFC substrate as shown in Fig. 3. The results are on the lower end of optimum pH for cathepsin L from several sources. A similar pH optimum was determined for human cathepsin L on elastin as substrate (Mason, Green, & Barrett, 1985), from rat kidney on hemoglobin (Reddy & Dhar, 1992), from chicken liver on azocasein and Z-PAAMC (Dufour, Dive, & Toma, 1988; Dufour et al., 1987), and from rat mammary gland on azocasein (Recklies & Mort, 1985). The other important implication is that the pH optimum of this enzyme is close to the range found in jumbo squid mantle after several days of refrigeration storage (Ramirez-Olivas, 2000). The hepatopancreas is removed from the squid after arrival at the processing plants on shore, but seepage of enzymes from hepatopancreas to mantle could occur after several hours of ice chilling in the boat, depending on the success of the jigging capture. It is important to study if this represents any implication on the quality of mantle due to proteinase activity from the hepatopancreas.

3.5. Molecular weight determination

The molecular weight was estimated by SDS–PAGE. The molecular weight of the proteinase was estimated to be in the order of 24 kDa by SDS–PAGE.

There is a wide range of molecular weights of cathepsin L from different sources, with 23 kDa for cathepsin L from rabbit spleen determined by gel filtration (Maciewicz & Etherington, 1988) at the lower end, 24 kDa from rabbit muscle (Okitani, Matsumura, Kato, & Fujimaki, 1980), 27 kDa from *Paramecium* (Volkel et al., 1996), 29 kDa from rat kidney (Reddy & Dhar, 1992), 30 kDa from carp hepatopancreas (Aranishi et al., 1997) and salmon muscle (Yamashita & Konagaya, 1990), 34.9 kDa from rat brain (Marks & Berg, 1987), 35 kDa from rat mammary gland (Recklies & Mort, 1985), and 40 kDa from human macrophages (Reilly et al., 1989).

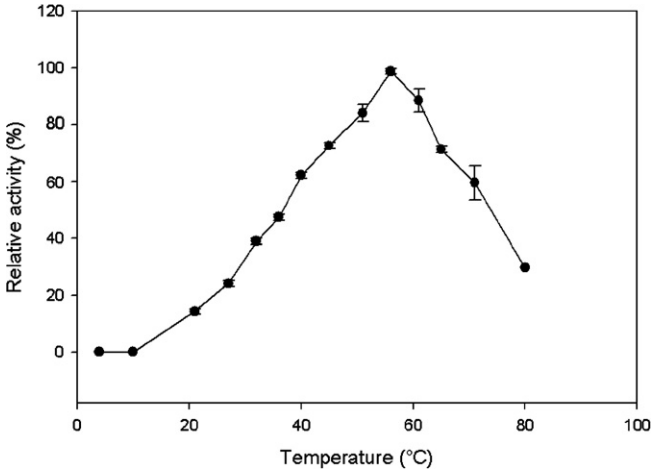

Fig. 2. Temperature optimum of cysteine proteinase from jumbo squid hepatopancreas measured with Z-PAAFC substrate at pH 4.5.

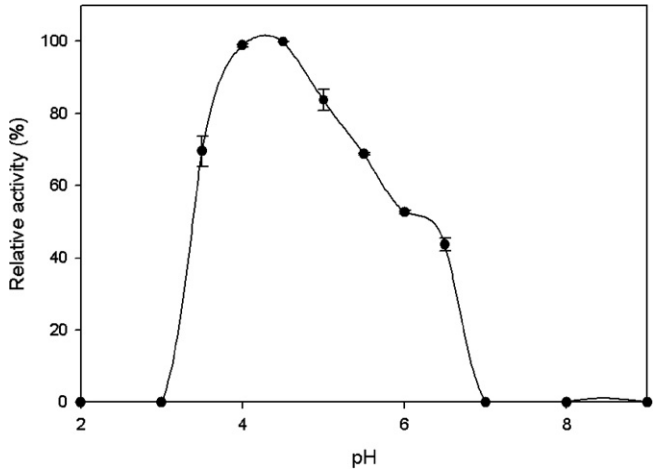

Fig. 3. Optimum pH of cysteine proteinase from Jumbo squid hepatopancreas on Z-PAAFC substrate at 55 °C.

Cathepsin L has been considered a monomeric enzyme (Kirschke, Barrett, & Rawlings, 1998), although, recently there was a report of a novel heterodimeric cathepsin L from the larvae of brine shrimp (*Artemia franciscana*) found to be composed of two subunits of 28.5 kDa and 31.5 kDa (Butler, Alton, & Warner, 2001) In Fig. 4, a zymogram shows activity of the cysteine proteinase purified by gel filtration enhanced by the presence of β-mercaptoethanol (BME) in the sample buffer and no activity when samples were heat treated. The presence of 2 bands very close in their migration in the zymogram could indicate isoforms with close molecular weights that show as one broad band in the BME treated sample because of the enhancement of the activity. This enhancement of cysteine cathepsin activity by BME has been documented in the past (Barrett, Rawlings, & Woessner, 1998). From the MALDI-TOF mass spectrogram a molecular mass $[M + H]^{1+}$ of 23.79 kDa, and its parent $[M + H]^{2+}$ were obtained as the main peaks, there were some other peaks at 16.793 and 17.613 kDa. The main peak mass corresponds to that of the main 24 kDa band found using SDS–PAGE.

3.6. Inhibition of activity of the partially purified cysteine proteinase

The activity measured on the synthetic substrate Z-PAAFC was reduced drastically when leupeptin, chymostatin and E-64 inhibitors were present in the assay. These are specific inhibitors for cysteine proteases. However, the inhibitors specific for other types of proteases did have reduction in the activity, although not in the same magnitude, as presented in Table 3. This could be due to working with a partially purified fraction that could have some other proteases present. Recently, a publication described two Kunitz type serine proteinase inhibitors from ticks *Boophilus microplus* and *Rhipicephalus sanguineus* that inhibited cathepsin L (Sasaki, Cotrin, Carmona, & Tanaka, 2006), although the substrate Z-PAAFC has been recognized as specific for cathepsin L, so further experimentation needs to be done.

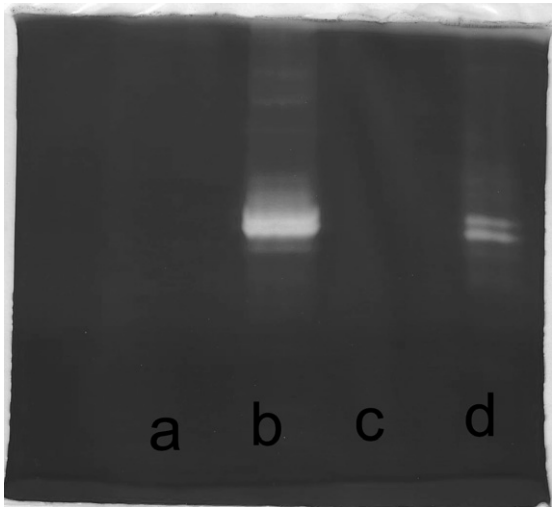

Fig. 4. Zymogram of hepatopancreas cysteine proteinase with: (a) heat treatment and BME, (b) no-heat treatment and BME, (c) heat treatment and no BME, and (d) no heat treatment, no BME.

Table 3
Residual proteinase activity on Z-PAAFC substrate of Jumbo squid hepatopancreas cysteine proteinase with different specific inhibitors

Inhibitor	Mean residual activity (%)	Standard deviation (%)
E-64[a]	24.76	20.42
Chymostatin	32.24	4.77
Leupeptin	11.06	0.55
TPCK[b]	66.63	1.79
Pefabloc	78.43	15.24
Pepstatin	58.06	0.34
SBTI[c]	63.12	8.09

[a] L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane.
[b] Tosyl phenylalanyl chloromethyl ketone.
[c] Soybean trypsin inhibitor.

3.7. Activity with other synthetic substrates

No activity was found when gel filtration fractions were assayed with Z-AAAFC and H-Arg-AMC substrates, these substrates have been used for specific activity of cathepsin B and Cathepsin H proteinases respectively, from different animal sources.

4. Conclusions

A cysteine proteinase was partially purified from Jumbo squid hepatopancreas in a two step purification procedure involving ammonium sulfate precipitation and gel filtration chromatography. There was a 45% recovery and a 15-fold purification. The cysteine proteinase had a molecular weight of 24 kDa, determined by SDS–PAGE. The enzyme further purified by SDS–PAGE had a high degree of homology with cathepsin L from several different animal sources. SDS–PAGE indicated that squid cathepsin L exists in multimeric form but this was not indicated by the results of MALDI-TOF mass spectrogram. The catalytic characteristics of this cysteine proteinase are also consistent with cathepsin L purified from other marine animals and terrestrial mammals. These characteristics include a pH optimum of 4.5–5.0, a temperature optimum of 55 °C with the assay under study. The Z-PAAFC synthetic substrate proved to be a useful tool for measuring the activity of this proteinase.

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