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Purification and Characterization of a Cathepsin L-Like Enzyme from the Body Wall of the Sea Cucumber *Stichopus japonicus*

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Cathepsin L-like enzyme was purified from the body wall of the sea cucumber *Stichopus japonicus* by an integral method involving ammonium sulfate precipitation and a series of column chromatographies on DEAE Sepharose CL-6B, Sephadex G-75, and TSK-GEL. The molecular mass of the purified enzyme was estimated to be 63 kDa by SDS–PAGE. The enzyme cleaved N-carbobenzoxy-phenylalanine-arginine 7-amido-4-methylcoumarin with K_m (69.92 μM) and k_{cat} (12.80/S) hardly hydrolyzed N-carbobenzoxy-arginine-arginine 7-amido-4-methylcoumarin and L-arginine 7-amido-4-methylcoumarin. The optimum pH and temperature for the purified enzyme were found to be 5.0 and 50 °C. It showed thermal stability below 40 °C. The activity was inhibited by sulfhydryl reagents and activated by reducing agents. These results suggest that the purified enzyme was a cathepsin L-like enzyme and that it existed in the form of its enzyme-inhibitor complex or precursor.

Key words: cathepsin L; characterization; protease; purification; sea cucumber (*Stichopus japonicus*)

Cathepsin L is a member of the papain superfamily of lysosomal cysteine proteases, and is one of the most powerful endopeptidases. Its usual function is regulating cellular protein turnover in lysosomes.[1–3] Previous studies have reported that cathepsin L was purified from several mammalian animal tissues, such as human and rabbit liver.[4,5] It was also purified from some fish, such as spotted mackerel[6] and anchovy.[7] Cathepsin L in the lysosomes of some fish muscle can be assumed to be involved in a gel softening effect during the gelation of surimi.[8] Compared with other cathepsins, cathepsin L catalyzed with a 10-fold higher rate of degradation a wide range of proteins, including myosin, actin, nebulin, cytosolic proteins, collagen, and elastin.[9,10] These hydrolysis exerted detrimental effects on the palatability and functional properties of muscle foods.

Sea cucumber (*Stichopus japonicus*; phylum, Echinodermata; class, Holothuroidea) farming has been rapidly expanded and intensified in China because of its abundant nutritional value and bioactive substances, but autolysis always happens in it[11] in response to a variety of environmental and mechanical factors, such as UV exposure[12] and low salinity. This autolytic character has created a major problem in product processing and the preservation of sea cucumber. Studies have shown that autolysis is a common phenomenon among many aquatic organisms. It results from high endogenous proteolytic activity in muscle and other tissues.[13,14] Cathepsin L is a representative endogenous heat-stable protease that might play an important role in autolysis. Hence, the isolation and characterization of cathepsin L-like enzyme from different tissues of sea cucumber in combination with physiology and immunity studies should provide valuable information on the cause of autolysis. Our lab has investigated the character of a cysteine protease-like enzyme in the body wall of the sea cucumber *Stichopus japonicus*.[15] As one of our series of studies on the sea cucumber, the objective of this research was to purify and characterize the

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Abbreviations: Z-Phe-Arg-NMec, N-carbobenzoxy-phenylalanine-arginine 7-amido-4-methylcoumarin; Z-Arg-Arg-NMec, N-carbobenzoxy-arginine-arginine 7-amido-4-methylcoumarin; L-Arg-NMec, L-arginine 7-amido-4-methylcoumarin; NMec, 7-amido-4-methylcoumarin; E-64, 1- (L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane; PMSF, phenylmethylsulphonyl fluoride; TI, trypsin inhibitor from soybean; EDTA, ethylenediamine tetra-acetate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBSC, sodium phosphate buffer containing L-cysteine

physicochemical and biochemical properties of cathepsin L-like enzyme from the body wall of this sea cucumber.

Materials and Methods

Chemicals. DEAE Sepharose CL-6B and Sephadex G-75 were purchased from Waterman (Maidstone, Kent, UK). TSK-GEL was purchased from Tosoh (Japan, type, G4000pWXL; column size, 7.8 mm ID × 30.0 cm L). N-carbobenzoxy-phenylalanine-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), N-carbobenzoxy-arginine-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NMec), L-arginine 7-amido-4-methylcoumarin (L-Arg-NMec), 7-amido-4-methylcoumarin (NMec), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), antipain, leupeptin, phenylmethylsulphonyl fluoride (PMSF), trypsin inhibitor (TI) from soybean, and ethylenediamine tetra-acetate (EDTA) were purchased from Sigma Chemical (St. Louis, MO). Iodoacetic acid, L-cysteine, dithiothreitol (DTT), and low molecular weight protein markers were purchased from Takara Biochemical. (Dalian, China). Bovine serum albumin (BSA) was purchased from Bo Aoxing (Beijing, China). All of the other chemicals used were of analytical grade.

Sea cucumber samples. The sea cucumber *Stichopus japonicus* used in this study was obtained from a local company located in Dalian China. Fresh sea cucumbers were maintained at 0 °C in sea water and transported to the laboratory. Autolysis was carefully avoided until use.

Protease purification. The viscera and heads were removed from the fresh sea cucumber. Body wall (100 g) was homogenized 3 times with 50 mM sodium acetate buffer (containing 1 mM EDTA and 0.2% Triton X-100, pH 5.0) at 4 °C. The resulting homogenate was kept overnight at 4 °C. Debris was removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. A 20%–80% saturation ammonium sulfate fraction of the supernatant was taken and the precipitated protein was dissolved in PBSC (20 mM sodium phosphate buffer containing 5 mM L-cysteine, pH 6.0) and then dialyzed against the same buffer at 4 °C for 24 h. The crude protease solution was stored at −80 °C. The crude protease was first applied to a column of DEAE Sepharose CL-6B (2.0 cm by 15 cm) equilibrated with PBSC. After washing with two column volumes of the same buffer, the bound protease was eluted with 0.1 M NaCl in PBSC until the absorbance at 280 nm (A_{280}) was less than 0.05. Then the column was eluted with a linear gradient from 0.1 M to 1.5 M NaCl in PBSC at 4 °C at a flow rate of 0.5 ml/min. Each elution was monitored at 280 nm. The fractions with peak absorbance at 280 nm were pooled, dialyzed, and concentrated for further purification. The sample was then applied to a Sephadex G-75 column (1.5 cm by 90 cm) equilibrated with PBSC containing 0.1 M NaCl. The column was eluted with the same buffer at 4 °C at a flow rate of 0.5 ml/min. Fractions containing the protease were pooled and dialyzed for further purification. Size exclusion chromatography was finally carried out on a TSK-GEL column connected with a HPLC pump and a UV detector. The sample was injected into the column. Proteins were eluted with ultrapure water at 4 °C at a flow rate of 0.3 ml/min with monitoring of absorbance at 280 nm, and collected according to the various peaks.

Protein determination. The protein concentration was determined by the method of Lowry *et al.*[16] using bovine serum albumin (BSA) as a standard.

Assay of protease activity. Hydrolytic activity on substrate Z-Phe-Arg-NMec was determined according to the method of Barrett and Kirschke,[17] with a slight modification. Briefly, 150 µl of sample was added to 75 µl of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, and 8 mM DTT, pH 5.5, prepared just before use). The mixture was preincubated at 37 °C for 2 min. Freshly prepared substrate (75 µl) was added to a final concentration of 5 µM and the mixture was incubated under the same conditions for another 10 min. The reaction was terminated by adding 300 µl of chloroacetate buffer (100 mM sodium acetate, 100 mM acetic acid, and 100 mM chloroacetate, pH 4.3). The fluorescence of aminomethylcoumain (NMec) liberated by hydrolysis was measured with a spectrofluorometer (LS55, Perkin-Elmer, Shelton, CT) at an excitation wavelength of 346 nm and an emission wavelength of 440 nm. NMec was used as a standard substance. A blank was run by adding the sample after chloroacetate buffer was added. One unit of enzyme activity was defined as 1 µM of NMec released per min.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli.[18] Sample was mixed 1:1 (v/v) with SDS–PAGE sample buffer and loaded on a gel made of 5% stacking and 12% separating gels. Electrophoresis was run at a constant voltage of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 25% ethanol and 10% acetic acid, and further destained with 25% ethanol and 10% acetic acid. Low molecular weight protein markers were used, including lysozyme (14.3 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29.0 kDa), ovalbumin (44.3 kDa), albumin (66.4 kDa), and phosphorylase (97.2 kDa).

Profiles of pH and temperature. Optimum pH on the purified protease was assayed in a pH range of 3.0 to 10.0 using different buffers (0.2 mM Gly-HCl buffer, pH 3.0; 0.2 mM sodium acetate buffer, pH 4.0–5.5; 0.2

mM Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0–8.0; 0.2 mM Gly-NaOH, pH 9.0–10.0), containing 8 mM DTT at 37 °C for 10 min. Determination of the optimal temperature of the purified protease was carried out ranging from 20 °C to 80 °C using sodium acetate buffer (pH 5.0). To determine thermal stability, the purified protease was incubated at various temperatures for 30 min. Then the activity was determined using Z-Phe-Arg-NMec as a substrate under standard assay conditions.

Inhibitor and activator studies. The purified protease was incubated for 30 min at room temperature with equal volumes of 0.2 mM E-64, 2 mM indoacetic acid, 2 mM PMSF, 0.2 g/l TI, 2 mM 1, 10-phenanthroline, 0.2 mM antipain, 0.2 mM leupeptin, 2 mM EDTA, and 2 mM DTT to give final concentrations as listed in Table 2. Then the activity was determined using Z-Phe-Arg-NMec as a substrate under standard assay conditions.

Metal ion study. The effect on purified protease activity of specific metal ions (K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} as chloride salts) was measured. The corresponding divalent cations were adjusted to a final concentration of 1 mM. Protease activity was detected after incubation with these cations for 30 min.

Substrate specificity and kinetic studies. Substrate specificity was determined by measuring hydrolytic activities on synthetic substrates, including Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec. The kinetic constants of cathepsin L-like enzyme for the hydrolysis of Z-Phe-Arg-NMec and Z-Arg-Arg-NMec at various concentrations ranging from 2 to 25 μM were determined. Protease activity was assayed under standard conditions. All of the determinations were repeated 3 times, and the respective kinetic parameters were evaluated by plotting the data on a Lineweaver Burk double-reciprocal graph.[19] The values of k_{cat} were calculated by the following equation: $Vmax/[E] = k_{cat}$, where [E] is the active enzyme concentration.

Results and Discussion

Purification of protease

The crude protease was first purified by ion-exchange chromatography on DEAE Sepharose CL-6B. A large peak of inactive protein was eluted by chromatography with 0.1 M NaCl (Fig. 1A). Three other protein peaks were eluted by the salt gradient. The first two peaks contained proteolytic activity against Z-Phe-Arg-NMec, with the strongest value measured in the first peak. The active fractions were then applied to a Sephadex G-75 column (Fig. 1B). Chromatography gave a broad peak of activity with low absorption at 280 nm. The protease solution was collected in fractions 29 to 38, leading to a 6.17-fold increase in purity (Table 1). Pooled protease was subsequently separated on TSK-GEL based on molecular sieving, and monitored at 280 nm. Four different molecular weight proteins were obtained, but the proteolytic activity against Z-Phe-Arg-NMec remained mostly in the first peak at a retention time of 17.336 min (Fig. 1C). TSK-GEL effectively separated the cathepsin L-like protease from other molecular weight proteins. In the entire purification process, an increase in purity of 42.81-fold was observed as compared with the crude extract.

The activity of the second peak on DEAE Sepharose CL-6B was decreased compared with the first peak, so it might have been an isoenzyme, or contaminated by some other protease. We concluded that the first peak with higher activity was purer than the second, so we took a sample of the first peak to complete the other experiments.

Molecular mass of the purified protease

SDS–PAGE of the purified protease indicated a single protein band with the estimated molecular mass of 63 kDa (Fig. 2), larger than earlier reports. The molecular masses of cathepsin L from arrowtooth flounder muscle[20] and rabbit liver[5] were reported to be 27 kDa and 29 kDa. The cathepsin L purified from other fishes and some mammalians were previously reported to be in the range between 28 kD and 30 kDa.[9,17,21] All of them existed as active free forms.

It has been reported that multiple isoenzymic forms of cathepsin L exist in the muscle of fish, including inactive procathepsin L, active free cathepsin L, and a complex form with endogenous inhibitors, such as cystatins and α-cysteine proteinase inhibitors.[21–23] Cathepsin L is generally synthesized as procathepsin L, which can be further transformed to the mature form by acid treatment.[22] Cystatins and α-cysteine proteinase often complex with protease.[22–24] A cathepsin L-inhibitor complex was purified from chum salmon with a molecular mass of 50 kDa. It consisted of a 30 kDa and a 37 kDa form of cathepsin L and a 15 kDa endogenous cysteine protease inhibitor.[23] The molecular mass of the purified protease in this study was approximately 63 kDa, much larger than that of the reported free form. It is possible that the purified cathepsin L-like protease is part of the lysosomal proteinase in the tissues, leading to the autolysis of sea cucumber. But this kind of protease should exist in an inactive state in the sea cucumber under normal conditions without any stimulus. Hence, it was suggested that the purified cathepsin L-like enzyme might exist in the form of its enzyme-inhibitor complex or precursor. Cystatins are inhibitors of most cysteine proteinases of the papain type. The molecular weights of cystatins are always between 13 and 15 KD. The supposed enzyme-inhibitor complex is perhaps composed of a cysteine protease-like enzyme and two cystatins. Investigation of the correct form of this enzyme is underway.

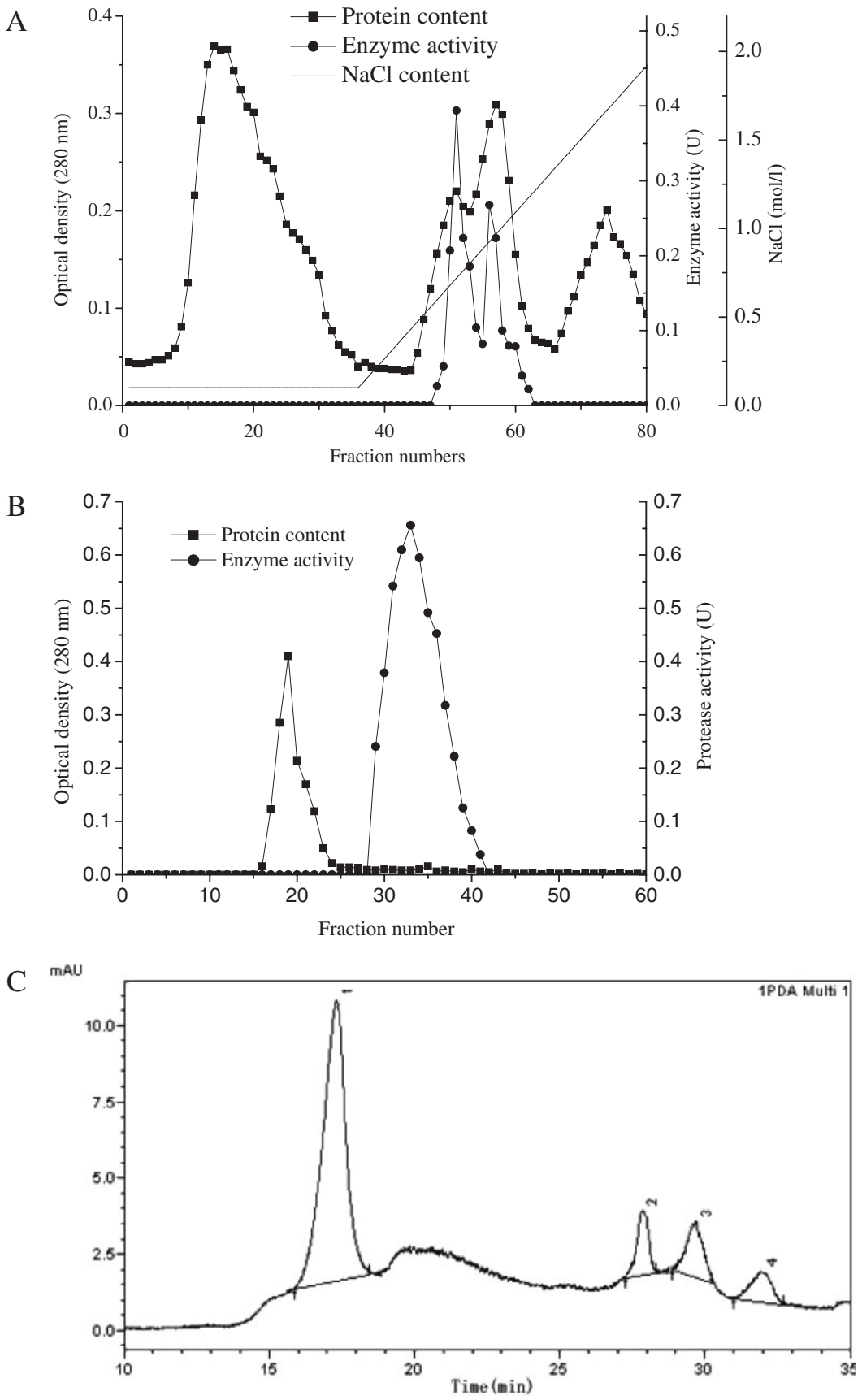

Fig. 1. Purification of the Protease from the Body Wall of the Sea Cucumber *Stichopus japonicus*.

A, Ion-exchange chromatography on a DEAE Sepharose CL-6B column; B, Gel filtration on a Sephadex G-75 column; C, Size exclusion chromatography on a TSK-GEL column.

Table 1. Purification Scheme for the Protease from the Sea Cucumber

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2,700.00	1,928.00	1.40	1.00	100.00
$(NH_4)_2SO_4$ Fraction	2,285.00	1,145.00	2.00	1.43	84.63
DEAE Sepharose CL-6B	1,992.00	810.00	2.46	1.76	73.78
Sephadex G-75	501.00	58.00	8.64	6.17	18.56
TSK-GEL	172.00	2.87	59.93	42.81	6.37

Unit (U), One unit of the enzyme activity was defined as 1 μM of NMec released per min.

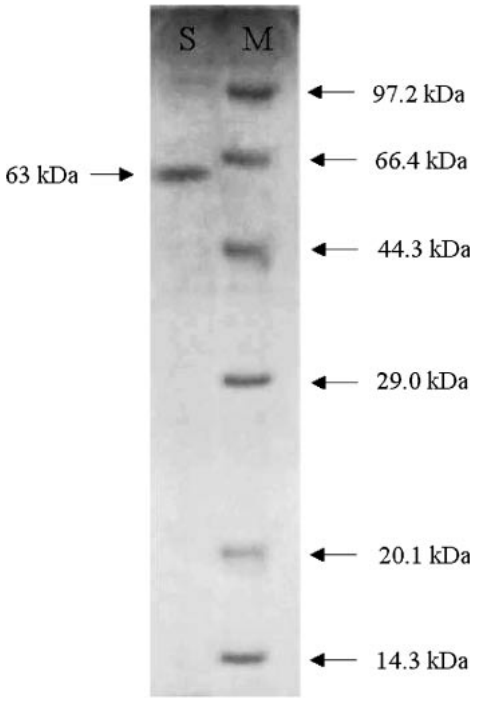

Fig. 2. SDS–PAGE of the Purified Protease.

Lane S, purified protease; lane M, low molecular weight markers (lysozyme, 14.3 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 29.0 kDa; ovalbumin, 44.3 kDa; albumin, 66.4 kDa; phosphorylase, 97.2 kDa).

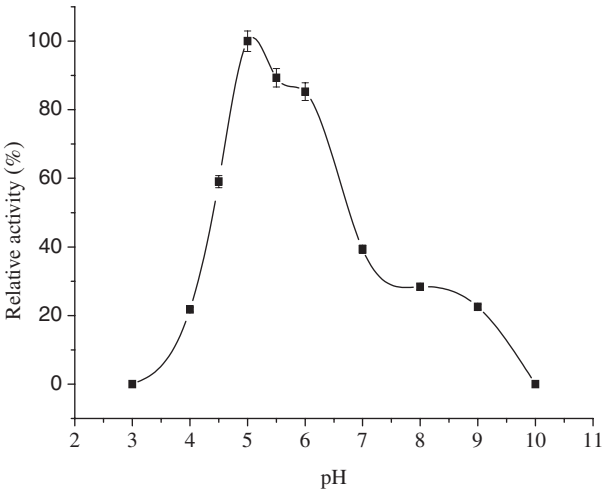

Fig. 3. Effect of pH on the Activity of the Purified Protease.

Purified protease was assayed in a pH range of 3.0–10.0 with 8 mM DTT at 37 °C for 10 min. Activity was determined using Z-Phe-Arg-NMec as a substrate in triplicate at each pH level.

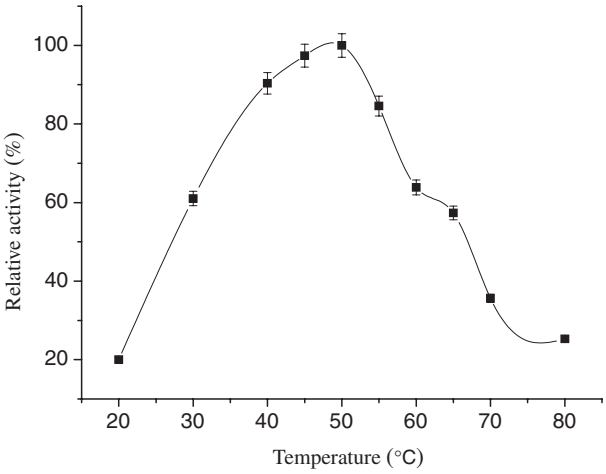

Fig. 4. Effects of Temperature on the Activity of the Purified Protease.

Purified protease was assayed at various temperatures for 10 min in sodium acetate buffer (pH 5.0) containing 8 mM DTT. Activity was determined using Z-Phe-Arg-NMec as a substrate in triplicate.

Characterization of the purified protease

As Fig. 3 shows, the optimum pH for the hydrolysis of Z-Phe-Arg-NMec was 5.0. This result is similar to those for cathepsin L from mackerel,[6] arrowtooth flounder,[20] and muscle of anchovy.[7] However, the protease activity rapidly decreased above the optimal pH. This result indicates the susceptibility of the purified protease in the neutral-alkaline region. The optimal temperature and thermal stability of the purified protease were examined between 20 °C and 80 °C. Maximum activity was observed at 50 °C (Fig. 4). Thermal stability gradually decreased toward 40 °C and activity was largely lost above 50 °C (Fig. 5). These results suggest that the purified protease had relatively good thermal stability. This thermal stability of the protease is assumed to be one of the crucial factors causing autolysis or tissue degradation in sea cucumber processing.

Partial or complete inhibition was observed in the presence of thiol-blocking agents E-64 and indoacetic acid, but thiol-activating agents DTT and EDTA enhanced activity, which confirmed that the purified protease was a thiol-protease (Table 2). Both serine-(PMSF, TI) and metallo-(1, 10-phenanthroline) protease inhibitors partially inhibited activity, but cysteine protease inhibitors, antipain, and leupeptin evidently inhibited the purified protease. These results suggest that the purified protease belonged to cysteine protease and contained SH groups. The effect of various metal ions,

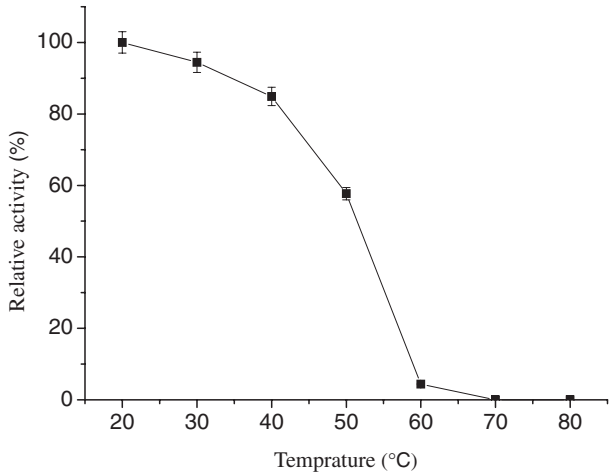

Fig. 5. Thermal Stability of the Purified Protease.

After the purified protease was incubated at various temperatures for 30 min, activity was determined at 50 °C for 10 min in sodium acetate buffer (pH 5.0) containing 8 mM DTT using Z-Phe-Arg-NMec as a substrate in triplicate.

Table 2. Effect of Inhibitors and Activators on the Activity of the Purified Protease

Inhibitor/activator	Concentration	Relative activity (%)
Control		100
E-64	0.1 mM	0
Indoacetic acid	1 mM	0
PMSF	1 mM	80.07
TI	0.1 g/l	51.53
1,10-phenanthroline	1 mM	83.14
Antipain	0.1 mM	0
Leupeptin	0.1 mM	31.25
EDTA	1 mM	105.64
DTT	1 mM	110.15

Table 3. Effect of Metal Ions on the Activity of the Purified Protease

Metal ion	Concentration	Relative activity (%)
Control		100
Ca^{2+}	1 mM	92.33
Mg^{2+}	1 mM	96.50
K^{+}	1 mM	89.04
Fe^{2+}	1 mM	84.37
Mn^{2+}	1 mM	83.90
Zn^{2+}	1 mM	0
Cu^{2+}	1 mM	18.77

viz., K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}, on the purified protease was examined. As shown in Table 3, the protease was scarcely inhibited by Ca^{2+} and Mg^{2+} ions, moderately inhibited by K^{+}, Mn^{2+}, and Fe^{2+} ions, and completely inhibited by Cu^{2+} and Zn^{2+} ions. These results are similar to those for cathepsin L from Silver Carp.[25)]

Thus, the clear profile of enzyme activity, including pH and temperature optima, and response to inhibitors and activators, suggested that the purified protease was cathepsin L-like enzyme.

Table 4. Substrate Specificity of the Purified Protease

Substrate	Relative activity (%)
Z-Phe-Arg-NMec	100
Z-Arg-Arg-NMec	7.00
L-Arg-NMec	0

Table 5. Kinetic Constants for the Hydrolysis of Synthetic Substrates by the Purified Protease

Substrate	K_m (μM)	k_{cat} (1/s)	k_{cat}/K_m (1/mM/s)
Z-Phe-Arg-NMec	69.92	12.80	183.00
Z-Arg-Arg-NMec	ND	ND	ND

Substrate specificity and kinetic studies

As Table 4 shows, the cathepsin L-like enzyme had a potent action on Z-Phe-Arg-NMec, a specific substrate commonly used to assay the activity of cathepsin L. However, it hardly hydrolyzed Z-Arg-Arg-NMec or L-Arg-NMec, because cathepsin L preferentially cleaved peptide bonds with hydrophobic amino acid residues in P2 and P3.[26)] Z-Arg-Arg-NMec and L-Arg-NMe are ordinarily used as specific substrates of cathepsin B and H respectively.[27–30)] Therefore, Z-Phe-Arg-NMec was considered to be the best substrate for the assay of cathepsin L-like enzyme from the sea cucumber.

Kinetic constants for the hydrolysis of Z-Phe-Arg-NMec and Z-Arg-Arg-NMec were determined (Table 5). The *k*m value for sea cucumber cathepsin L-like enzyme (69.92 μM) was higher than that for spotted mackerel (3.42 μM)[6)] and chum salmon (1.7 μM),[31)] but similar to that for anchovy (73.4 μM).[7)] The different kinetic constants of cathepsin L-like enzyme in those marine animals result from the diversity of their living environments and physiological functions. The experimental conditions different researchers used, such as pH and temperature, might also induce this disparity.[32)]

In our lab, Qi *et al.* found that there was a kind of cysteine protease-like protease in the body wall of the sea cucumber *Stichopus japonicus*. They also found that the enzymatic characteristics of the cysteine protease-like protease they purified were very similar to cathepsin S in carp and cathepsin L in arrowtooth flounder, but they did not further identify the exact type of that protease.[15)] Accordingly, in this study, with some modifications, we further purified and characterized the cysteine protease-like protease in the body wall of this sea cucumber. The cysteine protease-like enzyme in sea cucumber was considered to be of more than one type. We also found the activity of cathepsin-B like protease in the body wall of sea cucumber *Chopus japonicus* (data not shown). The results in this study, especially the substrate specificity assay, showed that

one of the cysteine protease-like proteases in the body wall of *Chopus japonicus* may be the cathepsin L-like protease we purified. The type of the cysteine protease-like protease Qi *et al.* found and its relationship to the cathepsin L-like protease we purified is under investigation now.

These results provide some hints for processing. Autolysis can be controlled if the activity of the cathepsin L-like enzyme is effectively inhibited. Further studies related to the physiological function of cathepsin L-like enzyme and other proteases in sea cucumber are in progress. Kariya *et al.* found that structural changes in glycosaminoglycan (GAG) had a close relation with the toughness of sea cucumber.[33–36] All the research on protease and GAG should provide valuable information on the cause of autolysis.

Acknowledgments

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