Characterization of proteolysis in muscle tissues of sea cucumber *Stichopus japonicus*

Chen-Chen Zhao¹, Yang Yang¹, Hai-Tao Wu^{1,2,*}, Zhi-Mo Zhu¹, Yue Tang^{1,2}, Cui-Ping Yu^{1,2}, Na Sun^{1,2}, Qiang Lv¹, Jia-Run Han¹, Ao-Ting Li¹, Jia-Nan Yan¹, and Yue Cha¹

¹School of Food Science and Technology, Dalian Polytechnic University, Dalian 116034, China

²National Engineering Research Center of Seafood, Dalian 116034, China

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*Corresponding Author
Tel: +86-411-86318731
Fax: 86-411-86318655
E-mail: wht205@163.com

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Abstract The proteolysis in muscle tissues of sea cucumber *Stichopus japonicus* (sjMTs) was characterized. The proteins from sjMTs were primarily myosin heavy chains (MHCs), paramyosin (Pm), and actin (Ac) having a molecular mass of approximately 200, 98, and 42 kDa, respectively. Based on SDS-PAGE analysis and quantification of trichloroacetic acid (TCA)-soluble peptides released, degradation of muscle proteins from sjMTs was favorable at pH 5 and 50°C. Proteolysis of MHCs was mostly inhibited by cysteine protease inhibitors, including *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) and antipain (AP). E-64 and AP completely inhibited the degradation of Pm and Ac, while iodoacetic acid showed a partially inhibitory effect. These results indicated that the proteolysis of sjMTs was mainly attributed to cysteine proteases. Avoidance of setting the tissues at 40–50°C and slightly acidic condition and inhibition of cysteine proteases are helpful for decreasing sea cucumber autolysis.

Keywords: sea cucumber, muscle tissues, autolysis, proteolysis, cysteine protease

Introduction

In recent years, sea cucumber fisheries have expanded in catch and value in Asia (1). The mariculture production of sea cucumbers has exceeded 200,000 tons in China (FAO 2014). However, sea cucumber *Stichopus japonicus* is easily subjected to autolysis, which seriously affects the quality of sea cucumbers, consequently inducing economic losses.

Autolysis is a common phenomenon for some aquatic animals that leads to tissue proteolysis. Proteolysis of fish muscle has been associated with the softening of gel products and the quality loss of fish flesh (2). Proteolysis of muscle tissues has been extensively investigated in fish and shrimp (2–4), but little is known regarding the same in sea cucumbers. The external character of *S. japonicus* is typically an oblate cylinder tapering toward both ends, encircled by variously shaped tentacles; they are mostly of dull coloration, present in shades of gray, brown, up to black. The main parts of *S. japonicus* include body wall, mouth, calcareous ring, digestive tract, coelom, etc, with complex internal structures (5). The body wall is the major edible part of *S. japonicus*, which is composed of cuticle, dermis, outer circular, and inner longitudinal muscle tissues (6).

The dermis constitutes the greater part of the body wall and determines its thickness. A thick dermis comprises collagen fibers

arranged in an orthogonal fashion, ground substance, and reticular fibers. The dermis is followed by a layer of circular muscle fibers that may form a complete cylinder but usually is interrupted at five points by the five longitudinal muscle bands that course along the inner surface of the ambulacra (5). Muscle tissues are key organs and are closely related to the sea cucumber's movement. The outer circular and inner longitudinal muscle tissues lay between the dermis and coelarium to support the body wall of sea cucumber. Cuticle and dermis tissues of sea cucumber contain collagen and noncollagenous proteins (7,8). Proteins from muscle tissues of sea cucumber are mainly myosin heavy chains (MHCs), paramyosin (Pm), and actin (Ac) (8). MHCs and Ac are primary myofibrillar proteins in muscle tissues of aquatic animals, and Pm is abundant in most invertebrate muscles (9–12). Our previous study reported that the noncollagenous proteins in *S. japonicus* body wall are more sensitive to autolysis than collagen (8). Thus, a more particular knowledge of autolysis in muscle tissues is necessary to provide information for preventing sea cucumber autolysis.

Studies have indicated that cysteine cathepsin is mainly involved in degradation of both intracellular and extracellular proteins (13). Cysteine proteases have been recognized to hydrolyze muscular proteins in sea bass (9) and arrowtooth flounder (10). The activities of cathepsin L and cathepsin B are enhanced in the UV-induced

autolysis of *S. japonicus* body wall (14). The degradation of noncollagenous proteins in cuticle and dermis tissues from *S. japonicus* is partly attributed to cysteine proteases (8). However, there is little information available in the literature regarding the autolysis of muscle tissues in sea cucumbers.

To characterize the autolysis of sea cucumbers, several cysteine protease have been purified from sea cucumbers, including cysteine-like protease (15), cathepsin L-like enzyme (16,17), and cathepsin B (18). Via an autolysis reaction, three antioxidant peptides were obtained from the sea cucumber guts (19). As a continuation of previous studies, in the present study, the proteolysis in muscle tissues of *S. japonicus* was characterized. The proteolysis of sea cucumber muscle tissues was studied via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and trichloroacetic acid (TCA)-soluble oligopeptide assay. Moreover, the contribution of cysteine proteases to the muscle proteolysis of *S. japonicus* was also reported.

Materials and Methods

Animals and chemicals Fresh sea cucumbers *S. japonicus* were supplied by Dalian Qianri Seafood Co., Ltd. (Dalian, China). Both outer circular and inner longitudinal muscles were immediately removed by avulsion from the sea cucumber body wall. The muscle tissues were frozen and stored at 80°C.

Coomassie Brilliant Blue (CBB) R-250, *N,N,N,N*-tetramethylethylenediamine, and sodium dodecyl sulfate (SDS) were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Antipain (AP), iodoacetic acid (IA), and *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Protein molecular weight markers were obtained from Takara Biotechnology Co., Ltd. (Kusatsu, Japan). All other chemicals were of analytical grade.

Characterization of autolysis of sea cucumber muscle tissues

Autolysis of sea cucumber was conducted as previously described (8). Briefly, the muscle tissues from sea cucumber were homogenized, irradiated by a UV lamp, and added with 0.2-M McIlvaines buffer with desired sample/buffer ratio (4:1, 2:1, 1:1, 1:2, 1:4) (w/v) for autolysis. The sample further was incubated at the desired temperature (30, 40, 50, 60, 70°C) and pH (4, 5, 6, 7, 8) for desired period (1, 2, 3, 4, 5, 6 h). The autolyzed muscle tissues were compared with the controls (the unirradiated or irradiated muscle tissues without further incubation).

As for the effect of various protease inhibitors, the samples of muscle tissues mince were mixed thoroughly with IA (2 µmol/g sample), E-64 (0.2 µmol/g sample) or AP (0.2 µmol/g sample) in 0.2 M McIlvaines buffer (pH 5.0), and incubated at 50°C for 3 h.

SDS-PAGE assay After autolysis, all samples were added with SDS

and urea solution containing 0.1 M Tris-HCl (pH 8.0), 8 M Urea, 4% SDS, 2% mercaptoethanol, and 20% glycerol at a ratio of 1:2 (w/v). The samples were boiled for 5 min and further shaken overnight. After centrifugation at 10,000× *g* for 10 min, the supernatant (10 µL) was examined by SDS-PAGE (10% running gel, 5% stacking gel, ATTO, Tokyo, Japan). The gels were stained with 0.05% (w/v) CBB R-250 in 9% (v/v) acetic acid and 45% (v/v) ethanol. After complete destaining of CBB R-250, the gels were imaged and analyzed via GelQuant 2.7 software (DNR Bio-Imaging Systems, Jerusalem, Israel). As for the effect of various protease inhibitors, the percentage of inhibitory rate was calculated by the changes of band intensities as follows:

$$\text{Inhibition (\%)} = \frac{BI_I - BI_C}{BI_{R1} - BI_C} \times 100\%$$

where BI_I is the band intensity of sample (with inhibitor) incubated at pH 5 and 50°C for 3 h, BI_C is the band intensity of control (without inhibitor) incubated at pH 5 and 50°C for 3 h, and BI_{R1} is the band intensity of raw material without any treatment.

TCA-soluble oligopeptide assay The samples of both sea cucumber muscle tissues mince and autolyzed muscle tissues mince were directly added to the same volume of 20% TCA. After the mixtures were centrifuged at 12,000× *g* for 20 min, the TCA-soluble oligopeptides released from muscle tissues mince were examined by the Lowry method (20). TCA-soluble oligopeptides content was calculated as microgram of tyrosine per gram muscle tissues. As for the effect of various protease inhibitors, the percentage of inhibitory rate was calculated by the changes of TCA-soluble oligopeptides content as follows:

$$\text{Inhibition (\%)} = \frac{T_C - T_I}{T_C - T_{R1}} \times 100\%$$

where T_C is the TCA-soluble oligopeptides of control (without inhibitor) incubated at pH 5 and 50°C for 3 h, T_I is the TCA-soluble oligopeptides of the sample (with inhibitor) incubated at pH 5 and 50°C for 3 h, and T_{R1} is the TCA-soluble oligopeptides of raw material without any treatment.

Amino acid analysis The amino acid compositions of sea cucumber muscle tissues were determined via HPLC equipped with a ODS amino acid column (4.6 mm× 250 mm), Elite-AAK (Dalian Elite Analytical Instrumental Co. Ltd., Dalian, China), according to our previous reports (21,22). Briefly, the basic amino acid residues were determined by hydrolysis at 110°C for 24 h using 6-M HCl prior to the derivatization with 2,4-dinitro-fluorobenzene. The specific amino acid concentrations were determined from the respective absorption intensities, which were calibrated to amino acid standards (Dalian Elite Analytical Instrumental Co. Ltd.). Alkaline hydrolysis was also conducted for determining the tryptophane level.

Statistical analysis All values were expressed as mean±SD, and

statistical analysis was performed using Student's *t*-test.

Results and Discussion

Proteins in muscle tissues of sea cucumber *S. japonicus* As shown in Fig. 1, proteins extracted from *S. japonicus* muscle tissues (sjMTs) were primarily MHCs, Pm, and Ac having a molecular mass of approximately 200, 98, and 42 kDa, respectively, which is in agreement with our previous study (8). Paramyosin is abundant in most invertebrate muscles, including those from Mollusca and Arthropoda, such as squid (11), octopus (23), and crayfish (12) matching the molecular mass of 97–110 kDa (24).

Literatures have been reported that collagen and acidic polysaccharides are the main components in sea cucumber body wall (25,26). However, our previous study indicated that the proteins extracted from the cuticle and dermis of *S. japonicus* are primarily noncollagenous proteins, with a molecular mass of approximately 200 and 44 kDa, respectively, confirming the high insolubility of collagen in *S. japonicus* body wall. The degradation of these two noncollagenous proteins during autolysis of the cuticle and dermis tissues of sea cucumbers has also been reported (8). But, little is known regarding the changes in MHCs, Pm, or Ac in muscle tissues during the autolysis of sea cucumber. Thus, as a continuation of our previous study, was the proteolysis in sjMTs further investigated in the present study.

Effect of sample to buffer ratio on the proteolysis in muscle tissues of *S. japonicus* As protease is usually involved in the autolysis of marine animals, enzymological factors have a significant effect on proteolysis in muscle tissues of *S. japonicus*. The key enzymological factors usually include the ratio of enzyme to substrate (E:S), concentration of substrate (S%), concentration of enzyme (E%), pH, temperature, inhibitor, and activator. The autolysis of muscle tissues was conducted by homogenizing, adding with buffer, and incubating at the desired temperature and pH for desired period. Therefore, the homogenized muscle tissues had a constant E:S, and the value of S% and E% could be adjusted using different ratios of sample to buffer. As shown in Fig. 1, after incubation at 50°C and pH 5 for 3 h, muscle protein degradation was more pronounced with a sample-to-buffer ratio of 1:1, 1:2, and 1:4 (w/v) as confirmed by the obviously decreased band intensities of MHCs, Pm, or Ac. The reports have been reported that both goatfish (*Mulloidichthys martinicus*) mince (2) and Pacific white shrimp (*Litopenaeus vannamei*) meat (4) were treated with a sample-to-buffer ratio of 1:3 (w/v) for autolysis induction. To avoid the effect of over dilution on the subsequent analysis, 1:1 (w/v) was selected as the ratio of sample to buffer for autolysis process of sjMTs.

Effect of pH on the proteolysis in muscle tissues of sea cucumber *S. japonicus* The proteolysis in sjMTs was investigated in the pH range

Fig. 1. Effect of sample to buffer ratio on the proteolysis in muscle tissues of sea cucumber *Stichopus japonicus*. Autolysis of muscle tissues mince was carried out at 50°C, pH 5 with a sample-to-buffer ratio from 4:1 to 1:4 for 3 h after UV irradiation. The raw material of sea cucumber muscle tissues without any treatment (R1) was used as negative control. HM: high-molecular weight markers; LM: low molecular weight markers; (1) Myosin heavy chains; (2) Paramyosin; (3) Actin.

from 4 to 8. After incubation at 50°C for 3 h, muscle protein degradation was more pronounced at pH 4, 5, and 6, as confirmed by the obviously decreased band intensities of MHCs, Pm, or Ac. The band intensity of MHCs, Pm, and Ac decreased by 91.8, 82.9, and 66.1%, respectively, at pH 5 by comparing with that of the control sample (R1) (Fig. 2). These results suggest that MHCs and Pm were more susceptible to degradation than Ac during autolysis in *S. japonicus*, which is consistent with the other reports indicating that MHCs is favorable to proteolysis in fish mince (2,27).

Reports have shown that actin dramatically degrades in the autolysis of arrowtooth flounder mince (10) and Pacific white shrimp mince (4), which is in agreement with our findings. Nevertheless, other literatures have demonstrated that actin is known to be proteolytically resistant in comparison to MHCs, demonstrating no degradation in proteolysis of goatfish (2) or bigeye snapper (3). These differences in actin degradation degree may be attributed to the proteases specificity in various aquatic species.

Muscle protein degradation varies with species. Pacific white shrimp mince showed the highest autolytic activity in the pH range of 2 to 4 as confirmed by the maximum content of TCA-soluble peptide and the greatest degradation of MHCs (4). Proteolysis of goatfish muscle was mainly induced by thermally activated and acidic proteases at pH under 6 (2). Our previous study indicated that the optimal pH for the proteolysis of noncollagenous proteins was pH 6.1 in tissues of cuticle and dermis from the sea cucumber body wall (8). Thus, the proteolysis in muscle tissues was characterized at both pH 5 and 6 in further studies.

Effect of temperature on the proteolysis in muscle tissues of sea cucumber *S. japonicus* Temperature is one of the key factors

Fig. 2. Effect of pH on the proteolysis in muscle tissues of *Stichopus japonicus*. Autolysis of muscle tissues mince was carried out at 50°C, pH 4–8 for 3 h after UV irradiation. The raw material of sea cucumber muscle tissues without any treatment (R1) was used as negative control. HM: high-molecular weight markers; LM: low molecular weight markers; (1) Myosin heavy chains; (2) Paramyosin; (3) Actin.

affecting proteolysis. The degradation of muscle proteins in sjMTs was investigated at 30, 40, 50, 60, and 70°C for 3 or 6 h at pH 5 and 6, respectively. As shown in Fig. 3, the SDS-PAGE results indicated that the strongest proteolysis was observed at 50°C for both pH 5 and 6 as confirmed by the greatest decline in band intensities of MHCs, Pm, and Ac.

It has been reported that deterioration of surimi gels is attributed to the severe degradation of myosin when the temperature is higher than 50°C (28–30). The maximum autolysis of mince from bigeye snapper (*Priacanthus macracanthus* and *Priacanthus tayenus*) and goatfish (*Mulloidichthys vanicolensis*) was found at 60°C (3,31). An increase in degradation of squid mantle muscle was found at a temperature of up to 40°C, and an abrupt decrease was shown above 40°C (32). Autolysis in Pacific white shrimp meat was enhanced in the temperature range of 35 to 40°C. The autolytic activity in Pacific white shrimp mince was marked at 40 and 35°C in the presence and absence of 2.5% NaCl, respectively (4). Our previous study has indicated that the optimal temperature for the degradation of noncollagenous proteins was 46.3°C in the cuticle and dermis tissues from the body wall of sea cucumbers (8). Thus, the characterization of proteolysis in muscle tissues was further investigated at 50°C.

Time course for the proteolysis in muscle tissues of *S. japonicus* As shown in Fig. 4, the time course for proteolysis in sjMTs was investigated by incubation at 50°C and pH 5 or 6 for 1–6 h. Autolysis at pH 5 caused degradation of all major muscle proteins within 1 h, and the band intensities of MHCs, Pm, and Ac were markedly decreased in comparison to that of the control sample (R1) (Fig. 4A). By 3 h, the proteolysis at pH 5 was near completion accompanied by the appearance of degradation products with lower molecular

Fig. 3. Effect of temperature on the proteolysis in muscle tissues of *Stichopus japonicus*. Autolysis of muscle tissues mince was carried out at 30–70°C and pH 5 for 3 h (A), or at 30–70°C and pH 6 for 6 h (B) after UV irradiation. The raw material of sea cucumber muscle tissues without any treatment (R1) was used as negative control. HM, high-molecular weight markers; LM, low molecular weight markers; (1) Myosin heavy chains; (2) Paramyosin; (3) Actin.

weights. At pH 6, the proteolysis in sjMTs was noticeable after autolysis for 3 h, as confirmed by the obvious decrease in band intensities of MHCs, Pm, and Ac (Fig. 4B).

Determination of the released TCA-soluble oligopeptide is widely adopted to investigate the proteolysis of many aquatic resources, such as fish mince (2), fish skin (33), surimi (34), and shrimp mince (4). TCA-soluble oligopeptide released from muscle protein hydrolysis as a function of time at different pH values is shown in Fig. 5. The trend of increase in TCA-soluble oligopeptide content was in agreement with the decrease in band intensities of muscular proteins. As expected from the data in Fig. 2 and 4, the rate of muscle protein degradation was higher at pH 5 with a progressive increase in accumulation of TCA-soluble oligopeptides than that at pH 6. Therefore, the desirable condition for the proteolysis of sjMTs was 50°C and pH 5.

In many aquatic species, autolysis is concerned with endogenous proteases with the strongest capacity at a desirable temperature and pH. As mentioned above, the optimum autolytic condition for sjMTs

Fig. 4. Time course for the proteolysis in muscle tissues of *Stichopus japonicus*. Autolysis of muscle tissues mince was performed at 50°C and pH 5 (A) or 6 (B) for 1–6 h after UV irradiation. Raw materials of sea cucumber muscle tissue, without any treatment (R1) or without further incubation after UV irradiation (R2), were used as negative controls. HM: high-molecular weight markers; LM: low molecular weight markers; (1) Myosin heavy chains; (2) Paramyosin; (3) Actin.

Fig. 5. TCA-soluble oligopeptides released during the proteolysis of muscle tissues in sea cucumber *Stichopus japonicus*. Autolysis of muscle tissues mince was performed at 50°C and pH 5 or 6 for 1–6 h after UV irradiation. Data are expressed as means±SD from triplicate or quadruplicate determinations. *, #*p*<0.05 as compared with control R1 (assigned to 1) at pH 5 and 6, respectively.

Fig. 6. Effect of protease inhibitors on the proteolysis in muscle tissues of *Stichopus japonicus*. Autolysis of muscle tissues mince was performed at 50°C and pH 5 for 3 h after UV irradiation in the absence (positive control, C) or presence of iodoacetic acid (IA), *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) and antipain (AP) at desired doses as mentioned in materials and methods. HM, high-molecular weight markers; LM, low molecular weight markers. The raw material of sea cucumber muscle tissues without any treatment (R1) was used as negative control. (1) Myosin heavy chains; (2) Paramyosin; (3) Actin.

was different to those for muscle tissues from fish and shrimp, but near to the optimal temperature and pH for cysteine proteases in *S. japonicus*. A cathepsin-L like enzyme from *S. japonicus* body wall had an optimal temperature 45–50°C and pH stability of 5–6 (16). A cysteine-like protease from *S. japonicus* body wall showed an optimal temperature of 50°C and pH stability of 4–7 (15). Thus, the potential contribution of cysteine proteases to the proteolysis in sjTMs was further investigated.

Table 1. Effect of protease inhibitors on TCA-soluble oligopeptides released in proteolysis of *Stichopus japonicus* muscle tissues at pH 5

Inhibitors	Final concentration (μmol/g sample)	Inhibitory rate (%)
IA	2	71.9±2.9 ^{A1}
E-64	0.2	83.0±1.8 ^B
AP	0.2	78.7±2.7 ^B

¹Data are expressed as means±SD from triplicate determinations. For each inhibitory rate (%), different capital letters indicate significant differences among different treatments by addition with inhibitors at desired concentration ($p<0.05$).

Table 2. Eighteen basic amino acid compositions of *Stichopus japonicus* muscle tissues

Amino acids	Relative content (%)
Asp	10.23±0.13
Glu	16.85±0.31
Ser	4.57±0.10
Arg	9.33±0.12
Gly	6.29±1.14
Thr	5.11±0.15
Pro	4.68±0.49
Ala	5.44±0.13
Val	4.74±0.04
Met	2.00±0.58
Cys	0.48±0.23
Ile	4.39±0.08
Leu	7.31±0.30
Trp	0.13±0.00
Phe	3.48±0.24
His	1.61±0.33
Lys	9.04±0.65
Tyr	4.32±0.29

Contribution of cysteine proteases to the proteolysis in muscle tissues of *S. japonicus* To investigate the contribution of cysteine proteases to the proteolysis in sjMTs, cysteine protease inhibitors were adopted in the present study. E-64 and IA are well-known cysteine protease inhibitors (35), having marked inhibitory effects on cathepsin L-like enzyme from both the body wall and guts of sea cucumber (16,17). The activities of cathepsin B and cathepsin L-like enzyme from sea cucumber are totally suppressed by the addition of AP (16-18). As shown in Table 1, all the protease inhibitors, including IA, E-64, and AP, demonstrated obvious inhibitory actions with an inhibitory rate of more than 70% on TCA-soluble oligopeptide released in sjMTs. The inhibitory effect of these inhibitors was in the following order: E-64 AP>IA based on the inhibitory rate (Table 1). The inhibitory effects of IA, E-64, and AP on proteolysis in sjMTs were investigated at 50°C and pH 5 by SDS-PAGE. As shown in Fig. 6, the lowest bands intensity of MHCs, Pm, and Ac was found after autolysis for 3 h without the addition of protease inhibitors. MHCs partly remained by addition of IA, E-64, and AP, and the proteolysis degree of MHCs was suppressed by 18.5, 88.1, and 72.4%, respectively.

In the cases of Pm and Ac, E-64 and AP completely inhibited the degradation of these two muscle tissues, and IA showed partially inhibitory effect with an inhibition rate of 50.2–86.6%. The degradation products with a broad molecular mass were generated from muscle proteins during the autolysis of sjMTs. The TCA-soluble oligopeptide usually has a molecular mass of lower than 10 kDa forming one portion of degradation products from muscle proteins during autolysis of sjMTs, which results in the differences of inhibitory rate values between SDS-PAGE assay and TCA-soluble oligopeptide assay. The stronger effects of E-64 and AP on proteolysis of muscle proteins in sjMTs were consistent with the results of the TCA-soluble oligopeptide assay. These results indicate that cysteine proteases contribute to the proteolysis in sjMTs.

As the highest autolytic activity was observed at acidic pH and the proteolysis was strongly suppressed by E-64 and AP, cysteine proteases may be primary in sjMTs. In general, cysteine proteases catalyze the hydrolysis of peptide bonds related to thiol deprotonation in the enzyme's active site by a histidine residue. It has been reported that cysteine proteases display a preference for arginine, lysine, or glutamic acid at the P1 position and arginine, phenylalanine, tyrosine, proline, leucine, or valine well at the P2 position (36,37). As shown in Table 2, muscle tissues of *S. japonicus* contained all the above catalytic mechanisms and substrate specificity-related amino acid residues with a total relative content of more than 50% in 18 basic amino-acid residues, suggesting that cysteine proteases may play an important role in proteolysis of sjMTs. It has been reported that cathepsin L induced severe degradation of texture in both Pacific whiting (38) and arrowtooth flounder (39). Cathepsin L was also attributed to the degradation of actin in white muscle of sea bass (*Dicentrarchus labrax* L.) (9), which is similar to our results. Our previous study indicated that cysteine proteases partly contribute to the degradation of noncollagenous proteins in cuticle and dermis tissues from the sea cucumber body wall (8), which is consistent the results of our present study.

In the present study, the proteolysis of sjMTs was favorable at pH 5.0 and 50°C and cysteine proteases are mainly involved in the proteolysis. Since sjMTs are important parts in the body wall, the changes in sjMTs proteins have a great influence on product quality during processing of sea cucumber. Therefore, avoidance of setting the tissues at 40–50°C and slightly acidic condition and inhibition of cysteine proteases are helpful to decrease sea cucumber autolysis.

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