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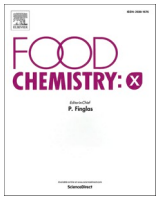

Investigating the migration hypothesis: Effects of trypsin-like protease on the quality of muscle proteins of red shrimp (*Solenocera crassicornis*) during cold storage

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Bromophenol blue (PubChem CID: 8272)
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Disodium hydrogen phosphate (PubChem CID: 24203)
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ABSTRACT

This study aimed to investigate the effect of trypsin-like protease (TLP) on the quality of muscle proteins in red shrimp (*Solenocera crassicornis*) during cold storage. The results indicated that the activity of TLP decreased significantly in the head of shrimp but increased significantly in the muscle tissues during the cold storage. The myofibril fragmentation index (MFI) value of intact shrimp was significantly higher than that of beheaded shrimp, while the Ca^{2+}-ATPase activity of intact shrimp was significantly lower than that of beheaded shrimp. SDS-PAGE analysis showed that the molecular weight of purified TLP from the shrimp head was about 24 kDa, and the TLP showed high activity at 50 °C and pH 8, indicating that the TLP belongs to the trypsin family. Results from *in vitro* simulation experiments indicated that the process of TLP incubation significantly reduced the particle size and enlarged the distribution of myofibrillar proteins (MPs) in shrimp muscle tissues. The comparisons were made with respect to the control samples. It can be inferred that TLP migrated from the shrimp head to the muscle tissues during storage and thus promoted the degradation of MPs in red shrimp. The beheading treatment could be an effective mean to maintain better quality and extend the commercialization of shrimp products.

1. Introduction

Red shrimp (*Solenocera crassicornis*), one of the most valuable marine resources, is primarily harvested in the oceans of India, Malaysia, Indonesia, Japan, and China. Red shrimp products have become increasingly popular among consumers due to their high-quality proteins, minerals, nutritional benefits, and delicious taste. The annual yield of red shrimp in China was 336,421 tons according to the China fisheries statistical Yearbook (Yearbook, 2020). In recent years, research on keeping shrimp fresh has become of great interest to aquatic product researchers. Preservation techniques such as low-temperature refrigeration, modified atmosphere packaging, active packaging, and irradiation preservations, among others, have been studied (Peng et al., 2022). Commercially, traditional preservation techniques such as freezing are widely used in shrimp preservation. Low temperatures have been shown to significantly weaken the activity of microorganisms and endogenous/exogenous enzymes, effectively extending the shelf life of shrimp products (Peng, Chen, Ji, & Liu, 2019). However, the deterioration of shrimp muscle still occurs during cold storage, majorly induced by the formation of irregular ice crystals and the degradation of proteins resulting from endogenous protease (Rahman et al., 2021).

Autolysis occurred relatively quickly in the head of shrimp after body fishing (Zheng, Ping, Xu, Li, & Guo, 2022), causing the texture of muscle tissues to become softer, the elasticity to decrease, and the head of shrimp to turn black during cold storage (Wang, Lei, Ma, Yuan, & Sun, 2018). Shrimp contains various endogenous enzymes, such as trypsin, cathepsin B, D, H, L, and calpains (Chen et al., 2022). Wang et al. (2014) pointed out that cathepsin B, D, and L are involved in the decomposition of myofibrillar, actin, and myosin during the degradation of muscle proteins (Wang et al., 2014). Recent evidence suggests that autolysis is

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the key factor in the textural deterioration of shrimp, which is closely related to the degradation of myofibrillar proteins induced by endogenous trypsin-like proteases (TLPs) (Peng et al., 2019). Previous studies have indicated that the major endogenous enzyme causing the degradation of muscle proteins in freshwater prawn (*Macrobrachium rosenbergii*) is TLP (Sriket, Benjakul, & Visessanguan, 2011). Tang et al. (2022) found that the softening of Pacific white shrimp (*Litopenaeus vannamei*) muscle during cold storage was mainly due to the degradation of fibrin and collagen resulting from the actions of TLP. It is noteworthy that the TLP could promote the degradation of shrimp muscle proteins, leading to the deterioration of muscle quality (Zhuang, Hong, Zhang, & Luo, 2021). Therefore, the roles of TLP in shrimp have received increased attention in the aquatic processing field in recent years.

TLP, a pancreatic serine protease, is an important enzyme of the digestive system, which is mainly located in the hepatopancreas. It specifically hydrolyzes the carboxyl-terminal side of the positively charged amino acids, thus cleaving the protein molecules into smaller peptides (Kaur & Singh, 2022). The hepatopancreas contains an abundance of endogenous enzymes that are concentrated in the head of the shrimp, among which TLP is the most important endogenous protease in red shrimp (Nikoo, Xu, Regenstein, & Noori, 2021). Sea-leaw et al. (2019) reported that the blackening process of the shrimp (*L. vannamei*) body occurs step by step, with the head turning black first and the tail turning black last. TLP might promote the blackening of shrimp (*L. vannamei*) by activating the activity of phenol oxidase (Sae-leaw & Benjakul, 2019). Peng et al. (2019) suggested that TLP might migrate from the head to the tail. The intensity of fluorescence-labeled TLP was strongest at the first abdominal segment of the shrimp (*L. vannamei*) body, then it decreased in the muscle of the first abdominal segment but gradually increased in the second abdominal segment with prolonged storage time (Peng et al., 2019). The studies reviewed here support the hypothesis that TLP transfer from the head to the body of shrimp.

Our previous research suggested that muscle softening in red shrimp easily occurs during cold storage, which may be related to TLP participating in the degradation of shrimp muscle proteins (Li et al., 2022; Zhou, Ding, Benjakul, Shui, & Zhang, 2023). However, little is currently known about the effects of TLP on shrimp muscle proteins. Therefore, the goal of this study was to investigate the effects of TLP in red shrimp on the quality of muscle proteins during cold storage. Additionally, this study aimed to explore the possible transfer mechanism of TLP from the head to the body of shrimp. The results of this study provide a basis for understanding the effects of TLP on protein changes in red shrimp during cold storage.

2. Materials and methods

2.1. Chemical reagents

The reagents used in this experiment, including sodium chloride, ammonium sulfate, formaldehyde, bromophenol blue, trichloroacetic acid, disodium hydrogen phosphate, sodium dodecylsulfate (SDS), methyl red, sodium acetate, and ammonium persulfate (APS) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). A bicinchoninic acid (BCA) protein assay kit and a trypsin assay kit were purchased from the Nanjing Jian Cheng Institute of Bioengineering (Nanjing, China). Glucan gel G-50 and Q-agarose gel FF were purchased from Shanghai Cable Bridge Biotechnology Co., Ltd. (Shanghai, China). All chemical reagents were of analytical grade. The RSD of chemical reagents was less than 1 %.

2.2. Shrimp processing and treatment methods

Fresh red shrimp (200 pieces) with a length between 12 and 14 cm and weighing between 25 and 28 g were purchased from an international aquatic market in Zhoushan, Zhejiang Province, China, in August 2021. The shrimp samples (TVBN content of fresh shrimp was determined to be 7.08 ± 0.12 mg/100 g) were placed in a cooler filled with slurry ice and transported to the laboratory within 30 min. Upon arriving at the laboratory, the shrimp were thoroughly washed with distilled water (0–4 °C). The samples were divided into two groups: intact shrimp (without any treatments) and beheaded shrimp (shrimp were beheaded but not peeled and deveined). Both groups were refrigerated at 4 °C for 8 days and − 18 °C for 120 days, and samples were measured every 2 and 30 days during the storage period, respectively.

2.3. Extraction of myofibrillar proteins

Myofibrillar proteins (MPs) were prepared and extracted as described by Zhang et al. (2018a) with minor modifications. Briefly, 5 volumes (v/v) of ice-cold isolation buffer solution (pH 7.2, 10 mmol/L Tris-HCl) was added to the shrimp muscle tissues (second segment). The obtained mixture was homogenized (XHF-DY, Scientz, Zhenjiang, China) at 10,000 rpm for 30 s in an ice-water bath. This was followed by sample centrifugation at 1000 × *g* for 10 min (CF16RN, Hitachi Corporation, Japan). The supernatant was discarded and the extraction process was repeated three times. Subsequently, the connective tissue was removed through a 40-mesh filter cloth. The obtained supernatant was mixed with 5 volumes (v/v) of ice-cold isolation buffer solution (pH 7.2, 10 mmol/L Tris-HCl containing 0.6 mol/L NaCl). The mixture was homogenized and centrifuged at 6000 × *g* for 15 min at 4 °C. Finally, the resulting supernatant was collected, and this was the extracted MP solution. The initial concentration of MPs was 57.0 mg/mL, and the concentrations were measured using the method described by Chen et al. (2023). The extraction of MPs was carried out at 4 °C.

2.4. Extraction and purification of trypsin-like protease

The extraction and purification of TLP were realized following a process described by Pan, Chen, Hao, and Yang (2019). The reported method was slightly modified to conduct the studies. The hepatopancreas was collected as the raw materials for TLP extraction. The dissection of the hepatopancreas was done under cold conditions. Next, 3 volumes (w/v) of ice-cold isolation buffer solution (0.01 mol/L Tris-HCl, pH 7.5) were mixed with the hepatopancreas samples. Subsequently, the samples were mashed and homogenized at 10,000 rpm for 30 s (SHA-B, Liang You experimental instrument factory, Shanghai, China). The mixture was centrifuged at 1000 × *g* for 30 min following extraction (4 °C, 4 h). The supernatant was precipitated using a saturated solution of ammonium sulfate. Next, the buffer solution (0.01 mol/L Tris-HCl, pH 7.5) was added to the obtained sediment and centrifuged at 1000 × *g* for 30 min (4 °C). The crude TLP was successively passed through SephadexG-50 (column size, 3 × 100 cm) and Q-Sepharose FF columns (column size, 5.4 × 20 cm). A buffer solution (pH 7.2, 0.05 mol/L Tris-HCl containing 0.2 mol/L NaCl) was used to elute the Sephadex-G 50 column. The flow rate was maintained at 0.4 ml/min. The linear gradient elution (pH 8.6, 0.03 mol/L Tris-HCl containing 0–1.0 mol/L NaCl) method was subsequently used for purification. Finally, the eluted samples (identified by the peak reflecting TLP activity) were collected and analyzed following the method of SDS-PAGE (Wang et al., 2022). Finally, the purified TLP was stored at − 80 °C in a refrigerator (MDF-U53V, Sanyo, Corporation, Japan) after lyophilization until use.

2.5. Analysis of trypsin-like protease activity and stability

TLP activity was determined using a trypsin activity assay kit (Nanjing Jian Cheng Institute of Bioengineering, Nanjing, China). The process outlined in the operation manual was used to conduct the studies. Briefly, the reaction system (pH 8, 0.05 mol/L Tris-HCl) was incubated at 37 °C for 10 min in a water bath. Next, 50 μL of a solution of TLP was added to the reaction system, and the mixture was allowed to stand for 20 min. The 5 % (w/v) trichloroacetic acid solution was added

to the system, and the solution was then centrifuged at 4000 × *g* for 10 min (4 °C). The OD values of the supernatant were recorded at 280 nm using a spectrophotometer. TLP activity was expressed as the tyrosine equivalent in the supernatant: an enzyme activity unit is defined as the amount of enzyme (U/mg) required to produce 1 μmol tyrosine per minute (Zhu et al., 2021).

The pH and temperature stability of TLPs were determined according to the method of Peng et al. (2019) with minor modifications. Purified TLP was incubated in a buffer solution (0.05 mol/L Tris-HCl). The pH was varied to analyze the samples (pH 2–11). The experiments were conducted for 30 min at room temperature. Tris-HCl (0.05 mol/L) was added to the purified TLP solution (pH 8), which was incubated at different temperatures (0–100 °C) for 30 min. The activity of TLP (stability) was investigated by measuring the enzyme activity at different pH (pH 2–11) and temperature (15–80 °C) conditions following these procedures.

2.6. SDS-PAGE analysis

SDS-PAGE analysis was performed by the method of Wang et al. (2022) on 12 % resolving gel with 5 % stacking gel. Briefly, 5 % (w/v) SDS solution was added to the purified TLP sample, and the mixture was incubated in a water bath (85 °C, 1 h). The mixture was dispersed over 2 min at a speed of 4000 r/min using an oscillator (SHA-B, SHA-B, Liang You experimental instrument factory, Shanghai, China). Following this, the sample was centrifuged at 5000 × *g* for 5 min (4 °C). The resulting supernatant (15 uL) was subjected to the conditions of electrophoretic separation. A Mini Protean cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA) was used to conduct these experiments. The gel was stained with 0.25 % (w/v) Coomassie Brilliant Blue R-250 and destained in 25 % (v/v) methanol solution containing 7.5 % (v/v) acetic acid. The gel was photographed following the processes of staining and destaining using a gel imager (GE Healthcare, UK).

2.7. Mixture of trypsin-like protease and shrimp muscle in vitro

Briefly, the peeled shrimp muscle samples were immersed into 5 volumes of the purified TLP (30 U/mL) solution, and the samples were incubated in vitro. The obtained system (pH 8.0) was maintained at 35 °C for 6 h and sampled every 1 h. After incubation, the myofibril fragmentation index (MFI), Ca^{2+}-ATPase activity, and particle size of MPs in the muscle tissues were studied according to the methods presented in the following sections. The peeled shrimp muscle immersed in distilled water (devoid of TLP) were the control samples.

2.8. Analysis of the myofibril fragmentation index

The MFI values of the MPs in the muscle tissues were determined according to the method of Xu, Cao, Zhang, and Yao (2020) with slight modifications. Briefly, the shrimp muscle (second segment) was mixed with 10 volumes (v/v) of the MFI buffer solutions (pH 7, containing 100 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L NaN_3, 1 mmol/L $CaCl_2$, and 20 mmol/L KH_2PO_4/K_2HPO_4), and the mixture was homogenized for 30 s. The sediment obtained following centrifugation at 2000 × *g* for 10 min (4 °C) was re-suspended in the same buffer solution. Subsequently, the samples were extracted again. The absorption of the resulting supernatant sample was recorded at 540 nm using a visible spectrophotometer. The method reported by Bai et al. (2022) was followed to arrive at the results.

2.9. Analysis of myofibril proteins particle size

The particle size of MPs in the muscle tissues was analyzed using a Zeta sizer Nano (NanoZS90, Malvern Instruments, Worcestershire, UK) according to the report by Chen, Xu, and Zhou (2016). The MPs extracted from shrimp muscles were placed in a quartz cuvette and analyzed using the dynamic light scattering (DLS) technique (90°, 25 °C, wavelength at 632.8).

2.10. Analysis of myofibril protein Ca^{2+}-ATPase activity

The MP Ca^{2+}-ATPase activity in the muscle tissues of shrimps was determined according to the method of Zhang et al. (2018b). The reported method was slightly modified to conduct the experiments. The extracted MPs (1–2 mg/mL) were dissolved in a Tris–maleate buffer solution (pH 7.0, containing 0.1 mol/L $CaCl_2$ and 20 mmol/L adenosine 5́-triphosphate). The mixture was then incubated at 30 °C in a water bath (5 min). Then, 15 % (w/v) trichloroacetic acid (0 °C) was added to the mixture, and the mixture was centrifugated at 4000 × *g* for 5 min (4 °C). Ca^{2+}-ATPase activity was assayed by measuring the release of inorganic phosphate from the supernatant. The method reported by Zhou et al. (2023) was followed to conduct the experiments.

2.11. Data analysis

All determinations were carried out in triplicate except where otherwise specified. The data were presented as means ± standard deviation (SD). The analysis of variance (ANOVA) method was used to evaluate the significant difference between the two samples using *SPSS* software (Version 27, IBM, Chicago, USA). Duncan's multiple-range test was executed, and the statistical difference was significant at $\alpha = 0.05$ level.

3. Results and discussion

3.1. TLP activity in shrimp muscles

TLP is an important endogenous enzyme mainly located in the hepatopancreas of shrimp heads (Zhuang et al., 2021). Fig. 1 illustrates the changes in TLP activity in both intact (including head and muscle tissues) and beheaded shrimp during 8 days of chilled and 120 days of frozen storage. The TLP activity in the muscle tissues of beheaded shrimp remained lower (15–27 U/mg) throughout the storage period compared to intact shrimp samples. Conversely, TLP in the head of intact shrimp exhibited significantly ($P < 0.05$) higher activity levels than those in the muscle tissues of beheaded shrimp. Notably, the TLP activity in the head of intact shrimp decreased significantly with increasing storage time. Remarkably, the activity of TLP in the intact shrimp muscle increased initially during the early period and then decreased in subsequent storage periods. This suggests that the migration of TLP from shrimp heads to muscle tissues may have contributed to these results. These observations are consistent with previous reports by Zhou et al. (2023) and Sriket et al. (2012), which suggested that changes in trypsin activity in freshwater prawn (*M. rosenbergii*) and sword prawn (*Parapenaeopsis hardwickii*) heads were likely related to its diffusion (or migration) from the head to muscle tissues.

Additionally, the TLP activity in the head and muscle of intact shrimp exhibited similar decreasing tendencies and significantly decreased during 4–6 and 60–120 days of storage ($P < 0.05$). This may be due to the denaturation of muscle proteins (including TLP) induced by the cold temperature and prolonged storage, resulting in the inactivation of TLP in the Pacific white shrimp (*L. vannamei*) (Sun et al., 2023). During cold storage, the decreased water-retention ability, the migration of water into extracellular spaces, the formation and growth of ice crystals, and the protein oxidation might promote the degradation and/or aggregation of muscle proteins (Zhang et al., 2018a; Zhang et al., 2018b). These findings indicated that TLP might transfer or diffuse from the shrimp head (hepatopancreas) to muscle tissues. However, there are currently no relevant studies on the migration or diffusion mechanisms of TLP in shrimp samples.

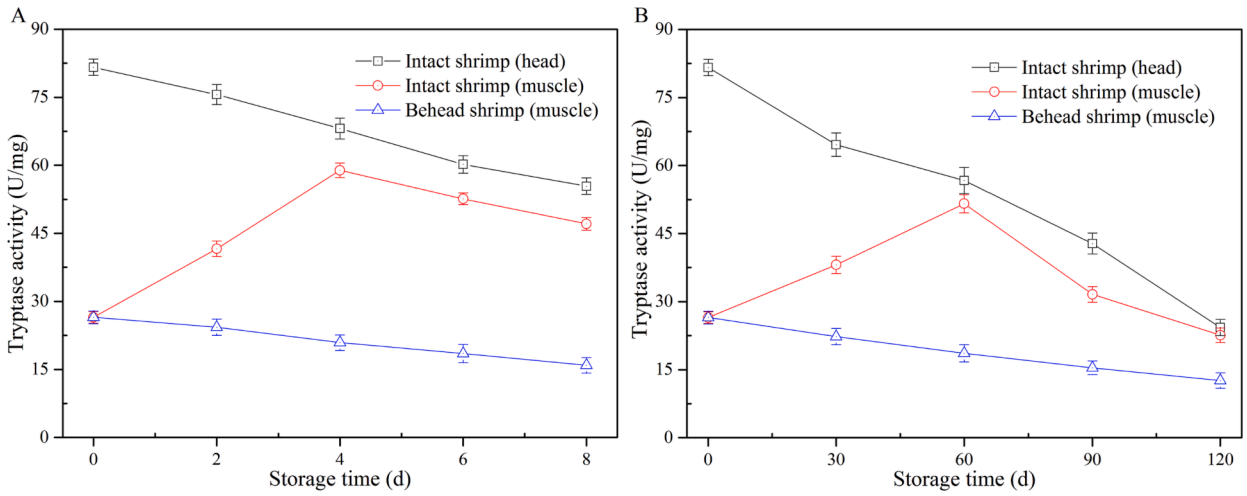

Fig. 1. Changes in the activities of trypsin-like protease (TLP) in the intact and behead shrimp muscle tissues during chilled (4 °C; A) and frozen (−18 °C; B) storage. Values are means ± standard deviation.

3.2. Purification and stability of trypsin-like protease

To investigate the possible migration of TLP from the head to muscle tissues of shrimp, TLP was extracted and purified from the hepatopancreas, and the results are presented in Fig. 2. As shown in Fig. 2A, three protein peaks were obtained, and the TLP activity was mainly detected around the second peak. The collected crude TLP was further eluted using a 0.3 M (molar) NaCl solution, and the purity of the obtained TLP was verified using SDS-PAGE (Fig. 2B, inset). The purified TLP exhibited a single band with a molecular weight (MW) of 24 kDa, confirming that the obtained TLP had good purity. This result is consistent with previous studies that reported the MW of serine protease to be commonly between 20 and 40 kDa (Song, Shi, Meng, Wu, & Zhang, 2020). Salamanca, Barría, Asenjo, and Andrews (2001) reported the MW of TLP in the Antarctic krill (*Euphrasia superba*) as ~ 30 kDa (Salamanca et al., 2001). Tang et al. (2022) isolated and purified trypsin from white shrimp (*L. vannamei*) and found its MW to be ~ 33 kDa (Tang et al., 2022). Ren, Zhao, and Wang (2011) discovered a TLP from the prawn (*Penaeus chinensis*) with an enzyme MW of 27 kDa. Literature reports suggest that TLPs, as important serine protease, vary significantly in molecular weight across different shrimp species.

The activity of TLP is easily affected by pH and temperature, as shown in Fig. 3. The relative activity of purified TLP increased initially, then significantly decreased ($P < 0.05$) when pH and temperature varied between 2 and 7 and 15–80 °C, respectively. The optimum pH and temperature of TLP were determined to be 8.0 and 50 °C, respectively. Tang et al. (2022) observed that a TLP extracted from whiteleg shrimp (*L. vanname*) had an optimum pH of around 7.0. Nikoo et al. (2021) found that trypsin had high activity in the head of pacific white shrimp (*L. vanname*) under conditions of 40 °C and pH = 8.0. In the current study, TLP showed typical enzymatic characteristics of trypsin. It is essential to prevent pH and temperature from deviating from their optimum levels to maintain the quality of shrimp during storage.

3.3. Analysis of myofibril fragmentation index

MFI reflects the degree of MP degradation during process and storage. A high MFI value indicates significant damage to the integrity and stability of myofibers (Zhang et al., 2018a). As shown in Fig. 4A, the MFI values corresponding to the beheaded and intact shrimp samples increased significantly ($P < 0.05$) during the freeze–storing period. The degradation of MPs in the shrimp muscles can be potentially attributed to the migration from the muscle proteins. This was caused by the growth of ice crystals (Peng et al., 2022). The MFI values of intact shrimp samples were higher than those of beheaded shrimps. This suggests that TLP might slowly migrate from the shrimp head to muscle tissues through capillary channels formed during the growth of ice crystals. This process promotes the degradation of MPs in the muscle tissues (Bhat et al., 2019; Zhou et al., 2023). The MFI values of MPs mixed (*in vitro*) with purified TLPs also increased significantly ($P < 0.05$)

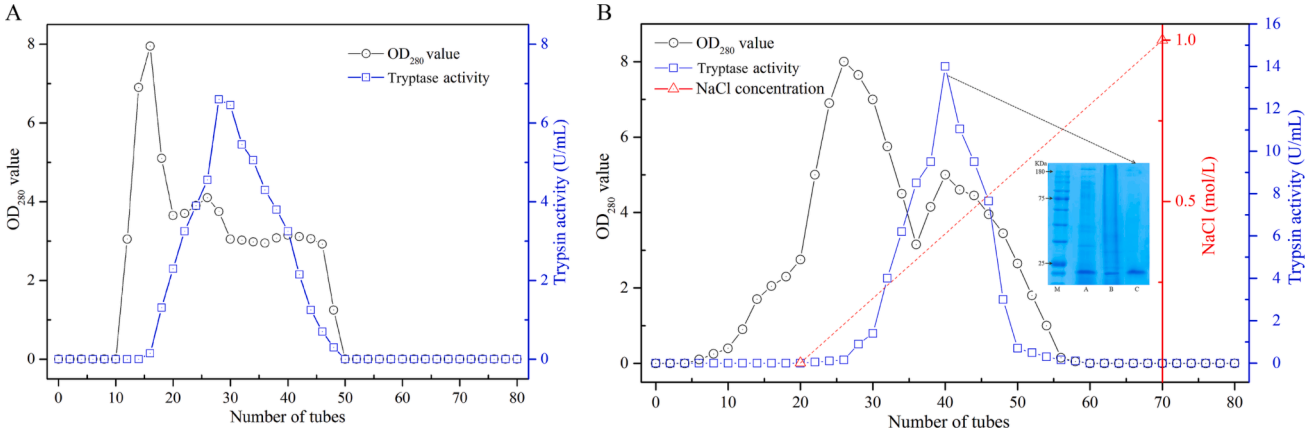

Fig. 2. Purification of trypsin-like protease by A, Q-Sepharose FF ion exchange chromatography; B, Sephadex G-50 gel chromatography. Inset photograph, the purified TLP was analyzed by SDS-PAGE.

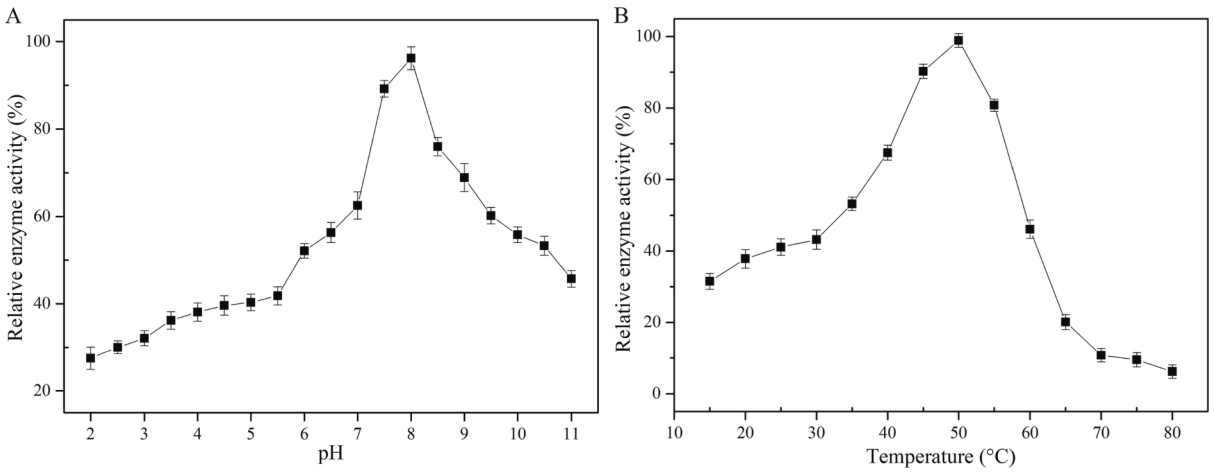

Fig. 3. Effects of pH (A; 2–11) and temperature (B; 15–85 °C) on the activity of purified TLP. Values are means ± standard deviation.

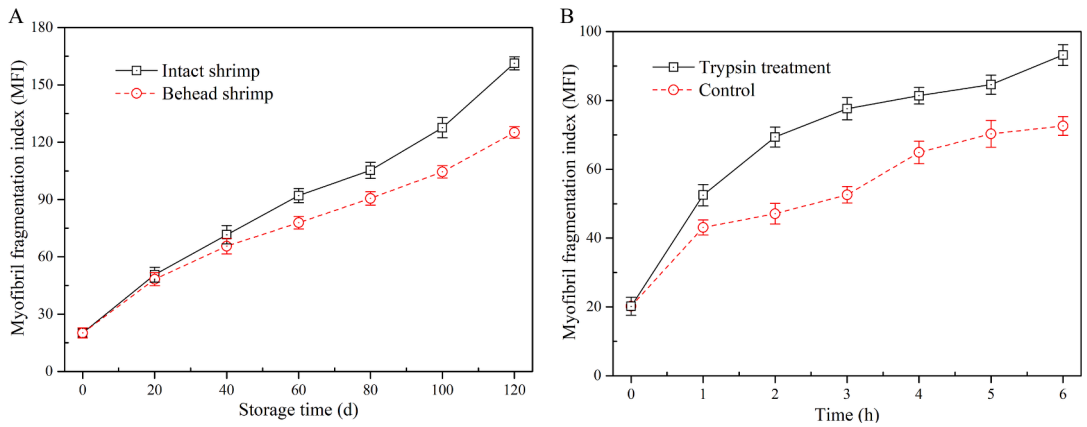

Fig. 4. Analysis of MFIs recorded for MPs in the shrimp samples. A, myofibril proteins corresponding to the intact and beheaded shrimp samples frozen and stored for 120 d; B, extracted myofibril proteins mixed with purified TLP *in vitro* during 6 h of incubation. Values are means ± standard deviation.

during 6 h of incubation. The values were significantly ($P < 0.05$) higher than those of the control samples. These validation results obtained from *in vitro* studies confirmed that TLP promoted the degradation of MPs in the shrimp muscles.

3.4. Particle size of myofibril proteins

The particle size of MPs is commonly used to reflect the degree of protein degradation and aggregation in the samples (Tang et al., 2022). The change in the particle size of MPs was determined to evaluate (in vitro) the effect of TLP treatment on the efficiency of degradation of muscle proteins. Fig. 5A shows that the MPs in fresh shrimp samples were large and presented a narrow size distribution. The MPs extracted from peeled shrimps were incubated in distilled water for 6 h (Fig. 5E), and these presented reduced particle sizes (<100 μm). These small particles accounted for more than 20 % of all the particles. This size was significantly smaller than the sizes of the particles incubated in distilled water for 3 h (Fig. 5C) and fresh samples. These observations may be attributed to the fact that cathepsin and troponin promote the degradation of MPs, resulting in the extension of polypeptides and molecules formed in the muscle tissues (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). The size of the MPs of the peeled shrimp samples incubated with TLP in *vitro* for 6 h (Fig. 5D) was less than 100 μm, and these particles accounted for > 35 % of the particles. Notably, the window of the size distribution of MPs was significantly wider than that of the fresh samples and the samples incubated in distilled water. The results indicated that TLP promoted the degradation of MPs, resulting in reduced particle size and widened size distribution of MPs under conditions of prolonged incubation time.

It is noteworthy that the degradation of MPs in shrimp was considerably more pronounced upon the addition of TLP compared to immersion in water alone. Peng et al. (2019) found that TLP could hydrolyze the myofibrillar proteins of white shrimps (especially MHC) to form small molecular peptides. The results indicated that TLP played an important role in the degradation of MPs. It was indirectly verified that the TLPs in shrimp muscles increased during cold storage, and this resulted in the deterioration of muscle proteins. This phenomenon can be potentially attributed to the migration of TLPs located in the hepatopancreas region to the muscle tissues of shrimps.

3.6. Ca^{2+}-ATPase activity of myofibril proteins

The Ca^{2+}-ATPase activity is commonly used to determine the integrity of actomyosin. Changes in the MPs can result in a decrease in Ca^{2+}-ATPase activity (Nikoo et al., 2021). As shown in Fig. 6A, the Ca^{2+}-ATPase activity of MPs of the beheaded and intact shrimp samples decreased significantly during the frozen storage stage ($P < 0.05$). This can be potentially attributed to the formation of ice crystals that promote the destruction and degradation of MPs during this period (Peng et al., 2019). Moreover, the Ca^{2+}-ATPase activity of the intact shrimp sample was significantly ($P < 0.05$) lower than that of the beheaded shrimp sample. The results illustrated that TLP could be potentially

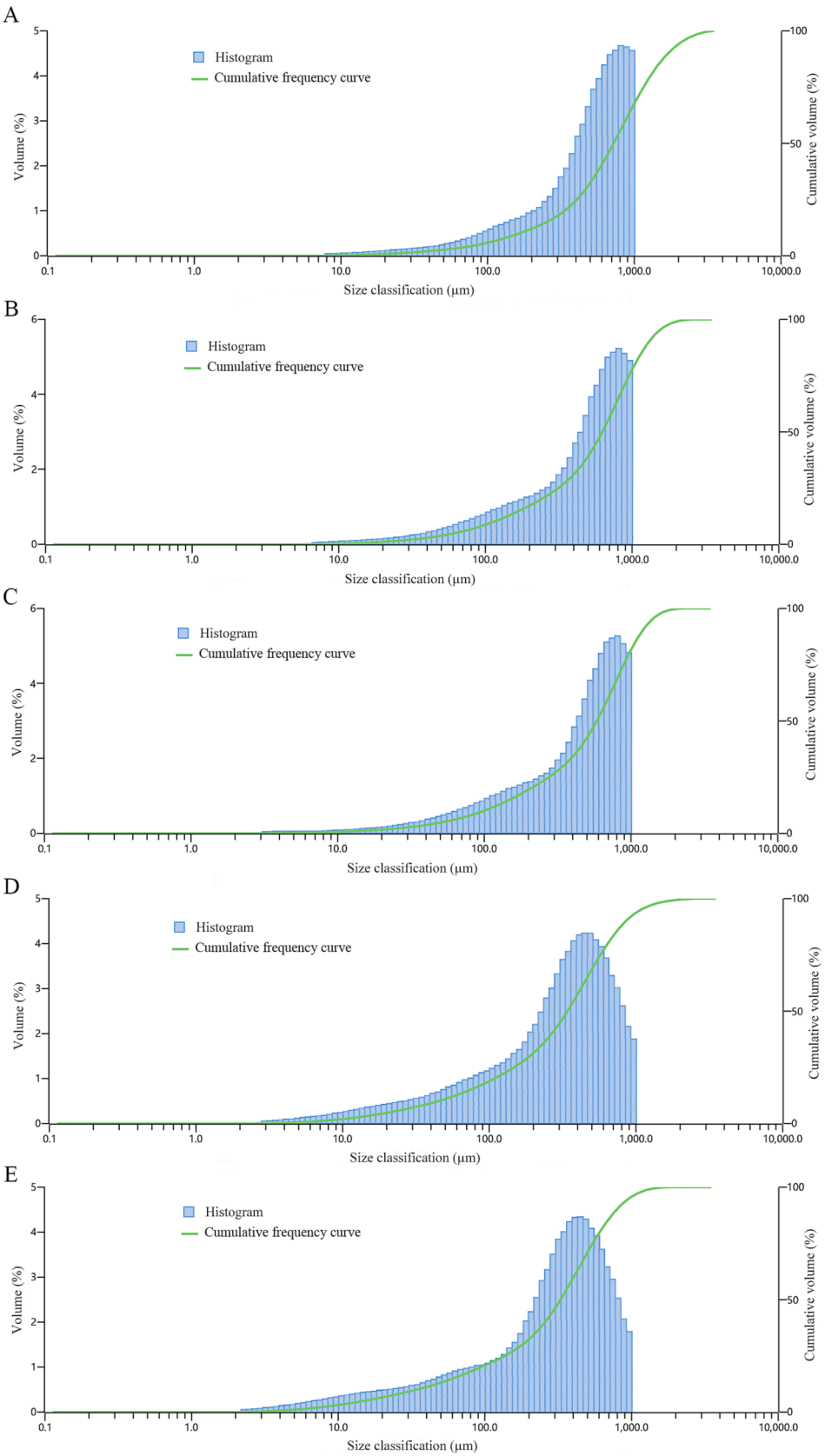

Fig. 5. Particle size and distribution of MPs in the shrimp samples. A, myofibril proteins extracted from fresh and peeled shrimp; B and D, myofibril proteins extracted from peeled shrimp incubated in *vitro* with TLP for 3 h and 6 h, respectively; C and E, myofibril proteins extracted from peeled shrimp incubated in *vitro* with distilled water for 3 h and 6 h, respectively.

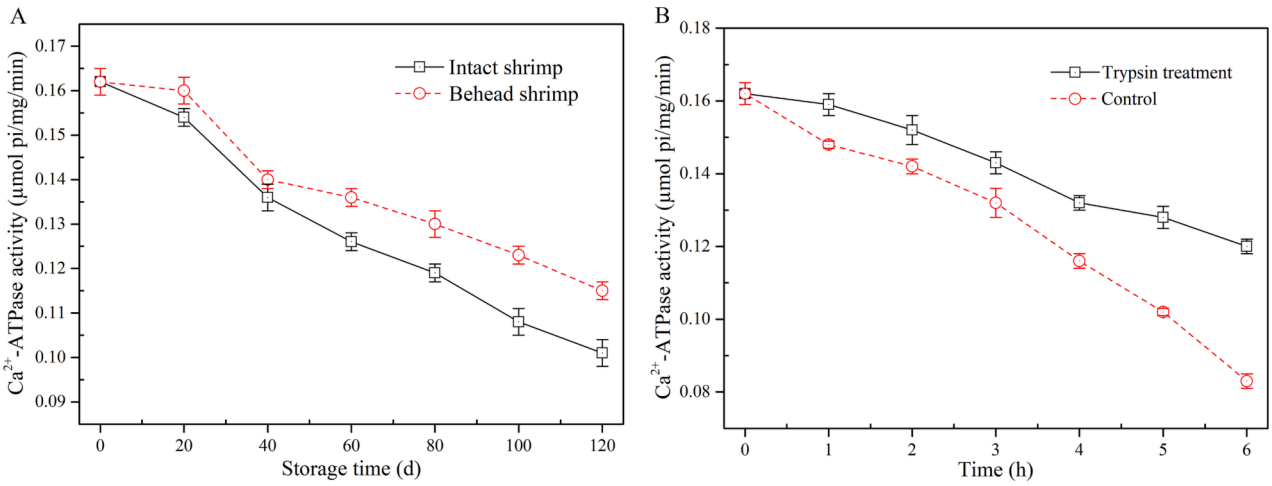

Fig. 6. MP Ca^{2+}-ATPase activity recorded for the shrimp samples. A, myofibril proteins extracted from intact and beheaded shrimps during 120 d of frozen storage; B, myofibril proteins extracted (in *vitro*; 6 h) from peeled shrimp incubated with purified TLP. The control was incubated with distilled water for 6 h (35 °C, pH 8). Values are means ± standard deviation.

transferred from the head to the muscle of shrimps. This promoted the degradation of MPs (Zhou et al., 2023).

Fig. 6B showed that the Ca^{2+}-ATPase activity of MPs reduced significantly during *in vitro* simulation experiments, and the activity of the TLP-treated group was significantly lower than that of the control ($P < 0.05$). The Ca^{2+}-ATPase activity recorded for the TLP-treated samples and the control samples decreased from 0.16 (0 h) to 0.08 μmol Pi/mg/min and 0.12 μmol Pi/mg/min (6 h), respectively. This result was consistent with that reported by Ma, Zhang, Deng, and Xie (2015), who also indicated the Ca^{2+}-ATPase activity in the peeled shrimp (*L. vannamei*) decreased significantly during frozen storage. They reported that the decrease in the Ca^{2+}-ATPase activity could be potentially attributed to the damage of myosin caused by the action of endogenous digestive protease. The results obtained from *in vitro* experiments confirmed that the addition of TLP promoted the decrease of Ca^{2+}-ATPase activity in the shrimp muscles. This indicated that TLP played an important role in degrading MPs in shrimp muscles.

Importantly, the MPs in the intact shrimps degraded by a higher degree than those corresponding to the beheaded shrimps. The addition of purified TLP further aggravated the decrease in Ca^{2+}-ATPase activity in shrimp muscles. Therefore, it could be speculated that TLP could migrate or get transferred from the head to the muscle of shrimps and promote the degradation of MPs in shrimp muscles.

4. Conclusion

In this study, we investigated the impact of TLP on the quality of muscle proteins in red shrimp by assessing the physicochemical properties of myofibrillar proteins, with particular attention to changes in enzyme activity during cold storage. Our findings showed that the intact shrimp had significantly higher myofibril fragmentation index (MFI) values and significantly lower Ca^{2+}-ATPase activity ($P < 0.05$) than the beheaded shrimp during storage. The activity of TLP decreased significantly in the intact shrimp (head) but increased significantly in the muscle tissues during the chilled and frozen stages ($P < 0.05$). The particle size of MPs was smaller in the TLP-treated samples than in the control. These results suggest that TLP may migrate from the head to the muscle tissues of shrimp and promote the degradation of MPs during cold storage. Our study enhanced our understanding of the effects of TLP on the muscle quality of shrimp samples. However, the migration mechanism of TLP requires further investigation.

Ethical approval

This study did not involve any animal or human experiments.

CRediT authorship contribution statement

Feili Zhan: Formal analysis, Writing – original draft, Validation. **Zhipeng Li:** Methodology. **Daodong Pan:** Writing – review & editing. **Soottawat Benjakul:** Writing – review & editing. **Xuepeng Li:** Writing – review & editing, Data curation. **Bin Zhang:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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