

Quality deterioration of *Litopenaeus vannamei* associated with protein changes during partial freezing storage

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Abstract: Proteins play a substantial role in the deterioration of partial freezing shrimp product quality. In this study, traditional protein indicators were used to determine changes in shrimp muscle quality during storage, and the changed proteins were identified using proteomic analysis. The decrease in total sulfhydryl (SH) content and the increase in carbonyl content indicate protein is denatured. The decrease in Ca²⁺-ATPase activity and the increase in surface hydrophobicity also indicate protein denatured. The increase of hydrophobic interaction and disulfide bonds suggest a larger and closer network among proteins. A total of eight changed protein bands were detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) diagram under partial freezing storage. Three of them were identified as α -actinin (97 kDa), invertebrate connectin (I-connectin (55 kDa)), and troponin I (30 kDa), respectively, which are essential components of myofibrillar protein. The results of the bioinformatic analysis showed that α -actinin and troponin I are unstable proteins with a secondary structure dominated by α -helix, while I-connectin is a stable protein with a secondary structure dominated by random coil. All three proteins are hydrophilic and predicted to be non-toxic on ToxinPreds.

Keywords: *Litopenaeus vannamei*; protein changes; partial freezing; quality deterioration; bioinformatic analysis

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1 Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important crustaceans with high farming production and considerable economic value [1]. Due to its high nutrients, including plentiful amino acids, peptides, polyunsaturated fatty acids, and other valuable substances, *L. vannamei* has high market potential and dominates the global value chain of the crustacean aquaculture market [1,2].

Partial freezing is the process of freezing or partially freezing food by lowering the temperature of the product to 1 or 2 °C below its initial freezing point. It inhibits microbial growth, slows enzymatic activity, and delays deterioration. However, undesirable protein changes will hurt the product quality during partial freezing [3]. Protein is a fundamental component of the aquatic product muscle. It will cause irreversible physical changes after the structure is destroyed [2,4]. Endogenous proteolytic enzymes degrade the protein to form tiny molecular peptides and non-protein nitrogen. Decomposition products are further decomposed to produce volatile products such as sulfides, resulting in unpleasant odors [5]. The freshness of aquatic products is closely related to the changes in muscle protein.

Proteomics is a powerful tool that helps to supplement the information of proteins by revealing the connections between muscle proteins and the quality traits of products. Traditional

gel-dependent proteomics is based on two-dimensional gel electrophoresis coupled with mass spectrometry [6]. Insights on protein changes during partial freezing storage by combining traditional quality studies and bioinformatic analysis is a method to analyze the quality during storage.

In a traditional index study, shrimp (*Metapenaeus ensis*) had reduced water retention capacity and protein denaturation and oxidation during frozen storage [7]. Also, the expression of ribosomal proteins (L44, L7a) and heat shock protein 21 were down-regulated as identified using the tandem mass tags (TMT) protein labeling technique [7]. In a study of *L. vannamei*, 83 shared differential proteins were identified through label-free proteomics. Shrimp muscle underwent significant quality changes during partial freezing storage, including protein denaturation, oxidation, and structural damage [6]. A previous study of *L. vannamei* frozen for 120 days showed that protein oxidation occurred during storage, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) results supported the observations regarding carbon groups, total sulfhydryl (SH) and bis-tyrosine content, and protein surface hydrophobicity [1].

The above studies on changes in shrimp product quality suggested that proteins play an essential role in quality deterioration. However, the relationship between specific changed proteins and shrimp quality altered under partial freezing storage were barely scarce. Therefore, the purpose of the present study was

to analyze, identify, and characterize the quality-related proteins in partial freezing stored shrimp by traditional quality study, proteomic analysis, and bioinformatic analysis.

2 Materials and methods

2.1 Materials

Fresh *L. vannamei* ((12.0 ± 1.0) g) were selected from a local shopping center in Guangzhou and transported to the laboratory to be cleaned. The heads and tails of shrimp were cut, muscle left behind was stored at -3°C refrigerator. Samples were taken for analysis at the indicated time periods.

2.2 Extraction of the total protein

The total protein was extracted from the muscle of the shrimp using the Total Protein Extraction Kit. Prepare a new lysis solution by adding 10 μL of phenylmethylsulfonyl fluoride (PMSF), 10 μL of protease inhibitor, and 10 μL of phosphatase inhibitor in 1 mL of lysis solution. According to the operating instructions for total tissue protein extraction, 1 g of tissue was added to the buffer with 5 mL of the new lysis solution and placed on ice for pulverization. The homogenized tissues were centrifuged in the condition of 10 000 r/min, 30 min at 4°C . The supernatant was collected, then protected in -80°C refrigerator for subsequent experiments. The Bradford protein assay kit was used to determine the protein concentration by an enzyme-labeling measuring instrument (Sunrise-basic Tacan, Tecan, Swiss). The subsequent experiments with the enzyme-labeling measuring instrument were all of this model.

2.3 Determination of protein indices

2.3.1 Total SH content

The total SH group was measured with the total SH group kit produced by Jiancheng Bioengineering Institute, Nanjing, China. To pre-configure the required reagents, 10 μL of protein solution was mixed with the liquid reagents required for the kit, reacted for 5 min and the absorbance at 405 nm was measured with an enzyme-labeling measuring instrument. Total SH content was denoted by $\mu\text{mol/g prot}$.

2.3.2 Determination of Ca^{2+} -ATPase activity

The Ca^{2+} -ATPase activity was measured with the Ca^{2+} -ATPase assay kit produced by Jiancheng Bioengineering Institute, Nanjing, China. After the enzymatic and phosphorus fixation reactions, the absorbance at 636 nm was measured with an enzyme-labeling measuring instrument. The Ca^{2+} -ATPase activity was denoted by U/mg prot ($\mu\text{mol Pi}/(\text{mg prot}\cdot\text{h})$), in addition Pi is inorganic phosphorus.

2.3.3 Determination of surface hydrophobicity

Surface hydrophobicity was prepared following the method described by Pan et al. [6]. The concentration of extracted protein was adjusted to 1 mg/mL, and 1 mL of protein solution was added to 200 μL of 1 mg/mL bromophenol blue solution and shaken for 15 min, then centrifuged in the condition of 3 000 r/min for 15 min at 4°C . The absorbance at 595 nm was measured with an enzyme-labeling measuring instrument. The surface hydrophobicity was expressed by the binding amount of bromophenol blue and calculated by the following formula:

$$\text{Bromophenol blue binding amount}/\mu\text{g} = 200 \mu\text{g} \times (A_c - A_s) / A_c$$

Where the A_c was control absorbance and A_s was sample absorbance.

2.3.4 Determination of carbonyl content

Carbonyl content was measured with the protein carbonyl assay kit produced by Jiancheng Bioengineering Institute, Nanjing, China. The reaction was carried out by adding the required reagents according to the instructions, and the supernatant was removed by successive centrifugation. The precipitate turned white by washing with a mixture of ethyl acetate and ethanol (1:1, V/V); the precipitate was dissolved and centrifuged at 12 000 r/min for 15 min at 4°C . The absorbance of the supernatant was measured at 370 nm with an enzyme-labeling measuring instrument. The carbonyl content was denoted by nmol/mg prot.

2.3.5 Determination of intermolecular bonds

The intermolecular bonds were prepared as the method described by Pan et al. [6]. Each tube with 2 g of shrimp muscle was added 10 mL of A (0.05 mol/L NaCl), B (0.6 mol/L NaCl), C (0.6 mol/L NaCl + 1.5 mol/L urea), D (0.6 mol/L NaCl + 8 mol/L urea), and E (0.6 mol/L NaCl + 8 mol/L urea + 1.5 mol/L β -mercaptoethanol) was added separately. After homogenization, the protein concentration was determined by centrifuging the supernatant at 10 000 r/min for 15 min at 4°C after 1 h of resting at 4°C . The differences between two adjacent tubes are the content of ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bonds.

2.4 SDS-PAGE

The molecular weights of total protein of shrimp muscle were determined using SDS-PAGE following the method of Dong et al. [4]. The total protein solution was diluted at a 1:1 (V/V) ratio using a $5 \times$ SDS-PAGE sample loading buffer, boiled for 5 min and cooled down at room temperature. Electrophoresis was performed with the Mini Gel Tank (Thermo Fisher Scientific) using a 12% precast gel (NuPAGE, Thermo Fisher Scientific, Shanghai, China). Electrophoretic loading volume was 15 μg , and the reference material was Prestained Color Protein Marker (6.5–270.0 kDa, Beyotime Biotechnology). After the electrophoresis was finished, the protein was stained with Coomassie Brilliant Blue (Beyotime Biotechnology) for 1 h and then decolorized, and the electrophoretic bands were scanned.

2.5 Identification of total protein

Manually cut off target spots on the SDS-PAGE gel. After destaining, dehydration, alkylation and enzyme digestion, the total proteins were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on the Q-Exactive mass spectrometer (Thermo Fisher Scientific). The columns were Acclaim PepMap RSLC C_{18} (300 $\mu\text{m} \times 5$ mm, 5 μm , 100 $\text{\AA}$, Thermo Fisher Scientific) and Acclaim PepMap C_{18} (75 $\mu\text{m} \times 150$ mm, 3 μm , 100 $\text{\AA}$, Thermo Fisher Scientific). Resolution of MS/MS was 17 500.

Search the Uniprot database through MASCOT (<http://www.matrixscience.com/>) to find amino acid sequences. The following restrictions were set: fixed modification, carbamidomethylation (C); variable modification, methionine, oxidation (M); enzyme, trypsin; maximum missed cleavages, 1; peptide mass tolerance, 20 ppm; fragment mass tolerance, 0.6 Da; mass values, monoisotopic; significance threshold, 0.05.

2.6 Bioinformatic analysis

The instability coefficients of the proteins were obtained using ProtParam (<http://web.expasy.org/protparam/>). The hydrophilicity and hydrophobicity of the proteins were predicted using Protscale (<https://web.expasy.org/protscale/>). The protein secondary structure was predicted by SOPMA (https://npsa-prabi.ibcp.fr/NPSA/npsa_sopma.html). Functional domains of protein sequences were predicted using NCBI (<https://www.ncbi.nlm.nih.gov/guide/domains-structures/>). Proteins were scanned using ToxinPred2 (<https://webs.iitd.edu.in/raghava/toxinpred2/index.html>).

2.7 Statistical analysis

All experiments were conducted in triplicate and the results were expressed as mean $\pm$ standard deviation. The data analysis was subjected to One-Way ANOVA, tested by the Duncan's method using the software SPSS (IBM SPSS Version 25.0). Differences were considered statistically significant at $P < 0.05$.

3 Results and discussion

3.1 Changes of total SH content

Protein contains total SH content which reflects protein oxidation [5]. The total SH content of shrimp muscle at -3°C significantly decreased with the prolongation of storage time (Figure 1(A)). In this study, the initial value was $33.785\ \mu\text{mol/g prot}$, which decreased to $19.718\ \mu\text{mol/g prot}$ after 4 weeks. Zhang et al. [8] studied the change of total SH content of skipjack tuna (*Katsuwonus pelamis*) during storage, and found that the decrease of total SH content was due to SH oxidation or disulfide bond exchange.

At low temperatures, SH groups were easily oxidized to disulfide groups, resulting in protein aggregation, denaturation and a decrease in the total SH content [5]. The decrease in total SH content is a result of cross-link formation between SH groups of proteins and interactions between exposed SH groups and other compounds [9,10]. It was reported that the main component of muscle protein is myofibrillar protein with massive SH groups, so the low SH content means high protein oxidation. The head portion of myosin existed two types of SH groups, which was involved in the oxidation and ATPase activity [1].

3.2 Changes of Ca^{2+} -ATPase activity

Ca^{2+} -ATPase activity is an index of protein denaturation in aquatic products. It was reported that the loss of Ca^{2+} -ATPase activity in the myosin head region would affect myosin and actomyosin integrity, which further leads to protein denaturation [11]. As shown in Figure 1(B), the Ca^{2+} -ATPase of shrimp muscle at -3°C significantly decreased with the prolongation of storage time.

The protein rearrangement by protein-protein interactions and the formation of ice crystals destroyed water retention ability and protein integrity, which is supposed to be responsible for reducing Ca^{2+} -ATPase activity during -3°C [6]. The oxidation of SH groups on the myosin globular head was presumed to influence the changes in Ca^{2+} -ATPase activity [12]. The activity of Ca^{2+} -ATPase is influenced by the integrity of myogenic fibronectin, and small microstructural changes can affect its changes [8].

3.3 Changes of surface hydrophobicity

The surface hydrophobicity is one of the protein indicators to

measure the degree of protein denaturation, which is determined by the relative content of hydrophobic amino acids [13]. The change of surface hydrophobic storage over 4 weeks were shown in Figure 1(C). The value of surface hydrophobicity of shrimp samples showed an increasing trend during 4 weeks of storage, increasing noticeably from 12.243 to $16.351\ \mu\text{g}$ ($P < 0.05$). The increasing trend indicated the exposure of hydrophobic groups in the side chains of protein and protein degradation at -3°C , suggesting the changes in protein properties.

The exposed hydrophobic residues may lead to the aggregation of protein through hydrophobic interactions [13]. It was supposed that the partial freezing temperature would unfold myofibrillar proteins, expose hydrophobic aliphatic and aromatic amino acids, and change the position of the hydrophilic and hydrophobic groups, leading to the conformation changes of proteins [14].

3.4 Changes of carbonyl content

Protein carbonyl content is one of the indicators widely used to assess the degree of oxidation of protein oxidation [15]. Figure 1(D) showed the changes in the carbonyl content of myofibrillar protein in the shrimp samples. The value increased significantly ($P < 0.05$) with partial freezing storage time in all batches in this study. The initial carbonyl content was $0.175\ \text{nmol/mg prot}$; the value increased to $0.649\ \text{nmol/mg prot}$ after 4 weeks of storage.

The carbonyl group is mainly generated by the oxidation of the side chain of the amino acid with NH_2 by the attack of free radicals and the scission of the peptide chain [16]. The formation of protein carbonyls from particular amino acid side chains was related to the conformational changes of myofibrillar proteins, probably leading to fragmentation, aggregation, loss of solubility and functionality [15]. Regarding protein carbonylation, Mario [17] pointed out that the formation of protein carbonyls might affect muscle quality, including water-holding capacity, texture and flavor.

3.5 Changes of intermolecular bonds

Intermolecular bonds include ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bonds. As shown in Figure 1(E), the content of hydrophobic interactions showed no significant change from 0 to 1 week of storage ($P > 0.05$), and the relative content of disulfide bonds showed no significant change from 1 to 2 weeks ($P > 0.05$). However, in terms of the overall trend of change, disulfide bonds and hydrophobic interactions increased significantly ($P < 0.05$), while the strength of ionic bonds and hydrogen bonds showed a significant decrease ($P < 0.05$).

The increase in disulfide bonds is mainly due to the oxidation of SH groups, which is negatively correlated with the content of SH groups [6]. Conformational changes in muscle proteins during storage result in exposed hydrophobic groups. The increase in hydrophobic interactions and disulfide bonds suggest a more extensive, tighter network between proteins [18].

3.6 SDS-PAGE and identification of the target proteins

SDS-PAGE is the tool that can determine molecular protein weight and identify biological macromolecules by separating biological macromolecules of different molecular weights. The molecular weights of the proteins of shrimp muscle were dominantly located from 17 to 200 kDa (Figure 1(F)). The electrophoretic profiles of shrimp showed myosin heavy chain (MHC, approximately 200 kDa), and myosin light chain (MLC, approximately 17–23 kDa). Actin (approximately 45 kDa) and tropomyosin (approximately

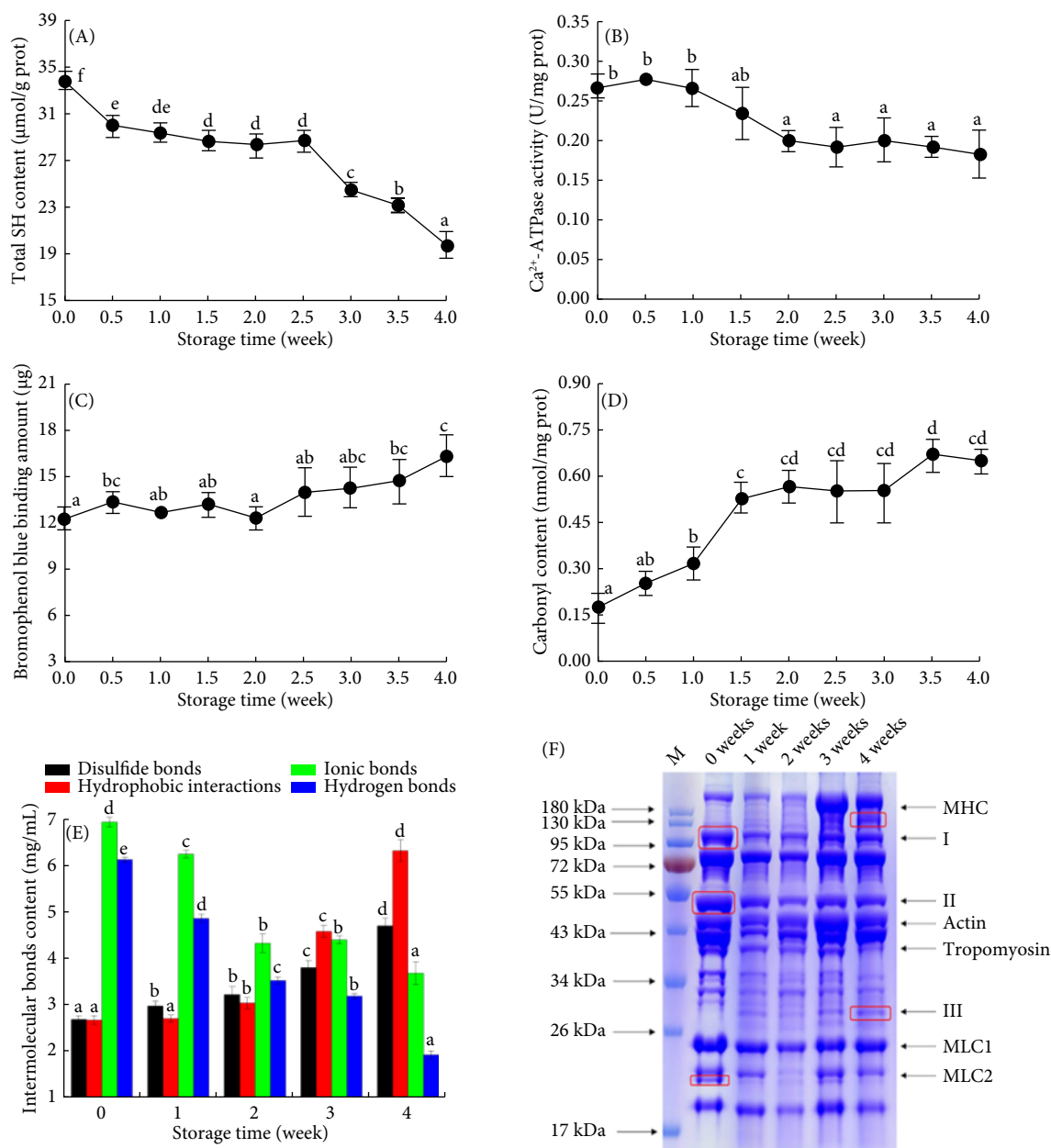


Figure 1 Changes in protein indicators of shrimp at -3 °C. (A) Total SH content; (B) Ca²⁺-ATPase activity; (C) Surface hydrophobicity; (D) Carbonyl content; (E) Intermolecular bonds content; Different lowercase letters indicate significant differences ($P < 0.05$). (F) SDS-PAGE of protein extract; Lane M, protein marker; Lanes 1 to 5, extracts from 0, 1, 2, 3, and 4 weeks, respectively. Red marked bands were considered to be changed obviously during storage. The bands I, II and III were selected for further protein identification.

38 kDa) were also clearly visible in this electrophoresis pattern. During 4 weeks of storage, the total protein of the sample changed to a certain extent. The optical density of the MHC at approximately 180 kDa decreased with the partial freezing storage period. The MLC with a molecular mass of 20 kDa gradually weakened. Our results showed a considerable decrease in the intensity of the band at approximately 97 and 50 kDa in the samples stored 4 weeks as compared with the fresh samples. This decrease was likely caused by the denaturation, proteolysis, and/or oxidation during low temperature storage [3]. In addition, a new lighter band was found in the region between 30 kDa onward during storage. This may be attributed to the breakdown of higher molecular weight proteins [19]. The changes of these proteins above also support the previous results regarding total SH content,

Ca²⁺-ATPase activity, carbonyl content, and the protein surface hydrophobicity. These reflect protein content, structure and functional properties changes and indicate proteins' oxidative degradation and denaturation [20].

In verify the proteins related to the quality of *L. vannamei*, the SDS-PAGE bands were cut off and further identified by LC-MS/MS. The obtained scores were significant ($P < 0.05$) under this search condition and the identification results were considered valid. Our results showed that 28, 39, and 5 of the digested peptide fragments (fragments were numbered in the order of observed peptide mass numbers/charge numbers) were sequenced, respectively (Table 1). Toxicity analysis of the peptide fragments showed that the identified peptides were all non-toxic. The protein is named α -actinin (GenBank accession number: ROT76585.1),

Table 1 Amino acid sequences of fragments in α -actinin, invertebrate connectin (I-connectin), and troponin I identified by LC-MS/MS.

Proteins	No.	Observed peptide mass	Calculated peptide mass	Peptide sequence	Corresponding position
α -Actinin	1	342.231 9	682.449 0	KLVPAR	488–493
	2	375.716 7	749.418 4	HLDIPR	94–99
	3	424.745 4	847.476 3	LGTLTTSR	365–372
	4	485.221 3	968.427 3	LMEEYER	667–673
	5	552.787 8	1 103.561 1	SVTDIANAWK	752–761
	6	380.893 0	1 139.656 3	RTLPWLQAR	171–179
	7	580.301 4	1 158.588 0	DNTLQAEQK	494–503
	8	587.803 6	1 173.596 0	EGLLLWCQR	27–35
	9	614.801 6	1 227.588 4	AEIHESWTAGK	282–292
	10	636.830 1	1 271.644 5	DGLAFCALIHR	54–64, 567–577
	11	649.787 7	1 297.560 9	EEMLSQDFR	293–302, 806–815
	12	676.280 0	1 350.544 0	CQAICDQWDR	355–364
	13	679.335 8	1 356.659 5	MLDPDDLVTNPK	613–624
	14	457.258 2	1 368.751 4	IDQLHLEFAKR	386–396
	15	701.370 2	1 400.725 9	QQDNTLADLQKK	693–704
	16	701.397 0	1 400.777 0	LTSDLLEWIRR	161–171, 674–684
	17	708.883 5	1 415.750 7	NALEETKLEK	374–385
	18	716.383 1	1 430.751 7	LSNRPAYLPSEK	226–238
	19	717.859 7	1 433.705 2	NVNVQNFTSFK	42–53, 555–566
	20	747.377 7	1 492.742 2	HKAEIHESWTAGK	793–805
	21	512.611 4	1 534.810 3	ARLETNFTNLQTK	211–223, 724–736
	22	786.412 4	1 570.810 3	HRPDLLDYSQSK	65–77
	23	795.352 6	1 588.690 1	SFEWLLSEMMR	256–267
	24	799.393 1	1 596.770 5	FAIQDISVEEMTAK	13–26, 526–539
	25	848.473 9	1 694.931 5	LVPARDNTLQAEQK	1 002–1 016
	26	585.280 0	1 752.817 9	KHEAFESDLAAHQDR	315–329
	27	836.721 1	2 507.138 7	AEIHESWTAGKEEMLSQDFR	282–302, 795–815
	28	1 309.919 0	5 235.628 5	ATLGEADKEYQAIMS LVTEVQHIAQQYQIPG GLDNPYTTLTSQVISSK	435–482
I-Connectin	1	509.264 6	1 016.513 8	DIDIVEGTR	572–580
	2	530.803 2	1 059.592 4	LGQAVTSASVK	1 317–1 327
	3	548.767 7	1 095.519 6	LQYLDSSR	1 347–1 355
	4	588.275 4	1 174.536 2	LEPMNDPTMK	1 127–1 136
	5	588.782 9	1 175.553 2	LEPYDPSPMK	1 794–1 803
	6	615.250 0	1 228.485 2	ADDEGIYMCR	2 469–2 478
	7	654.830 9	1 307.647 0	QSDAGQYTVLAR	1 037–1 048
	8	660.355 0	1 318.692 1	VEWVFVNGVEIK	2 068–2 078
	9	684.352 8	1 366.691 5	IEVSDVPMQPPR	2 157–2 168
	10	696.364 0	1 390.712 6	ASLSDAGVITCVAK	370–393
	11	702.864 9	1 403.714 4	STTEETVITQAPK	1 759–1 771
	12	736.339 7	1 470.667 4	LWEGQSAHVECR	1 782–1 793
	13	738.390 5	1 474.764 3	AVGTPMPFLTQWK	439–451
	14	739.866 4	1 477.719 5	ATNAAGQAVTSCSIK	1 979–1 993
	15	776.896 7	1 551.778 0	AYNDLGEAVTTASIK	2 379–2 393

Table 1 (Continued)

Proteins	No.	Observed peptide mass	Calculated peptide mass	Peptide sequence	Corresponding position
I-Connectin	16	781.333 6	1 560.651 5	YVNPEDSGTYTCR	1 700–1 712
	17	788.915 0	1 575.814 4	TAQEIAEATTAPVFK	825–839
	18	797.869 5	1 593.723 9	MGEAVNSCNVTVSAR	2 115–2 129
	19	537.956 2	1 610.845 0	VLVNEAGNHSLMISK	365–379
	20	807.920 6	1 613.826 0	EGVITESARPEGLEK	2 264–2 278
	21	810.365 9	1 618.719 2	AENMLGSAHTSSCVR	1 180–1 194
	22	540.603 3	1 618.787 2	SAIDTTSMHEVTLISK	1 066–1080
	23	815.392 3	1 628.768 2	DLVFDSEHPDSLQK	1 333–1346
	24	820.380 9	1 638.746 0	AFNHMGAEQSSVSFK	2 245–2259
	25	848.940 9	1 695.867 9	EALLDQTQHSEALEK	1 732–1746
	26	849.390 0	1 696.764 8	ETILSDTMNQEAMSK	1 600–1 614
	27	851.910 7	1 701.803 2	SDFIVETQHPEAMAK	1 866–1 880
	28	854.395 8	1 706.778 1	VEGTGSMQLGTVDEER	1 994–2 009
	29	871.408 8	1 740.805 4	TDTEVETQYQETVAK	931–945
	30	879.442 3	1 756.863 2	KDLVFDSEHPDSLQK	1 332–1 346
	31	890.953 6	1 779.890 4	DQQLIENAPLHFEAR	1 645–1 659
	32	600.627 8	1 798.859 8	RHQEPQYDIEEISR	1 087–1 100
	33	620.973 3	1 859.898 8	RSIYLDQTQHPMGWEK	2 130–2 144
	34	934.440 7	1 866.866 9	SMQEQTINEAPTQVK	1 226–1 241
	35	951.484 0	1 900.950 3	ANNLLNEGQHVHLEGR	2 175–2 191
	36	505.512 5	2 018.022 1	AHHVPKAPPEEQPVEPPK	548–565
	37	684.053 8	2 049.137 1	LPEDAVVIEKPNFGRPLR	1 892–1 909
	38	701.027 4	2 100.064 0	APEEQPVEPPFTHPLR	554–571
	39	1 056.073 5	2 110.131 0	SKPEFTQLLSDPKPLPEGK	1 101–1 119
Troponin I	1	419.199 8	836.385 1	GFMTPER	35–41
	2	491.759 2	981.503 2	KAAEFNFR	157–164
	3	443.218 2	1 326.631 1	YDLEMEIMRK	109–118
	4	693.851 6	1 385.689 9	AKNLDGANEDALR	79–91
	5	490.241 8	1 956.936 3	AICKEYHDHIASIESGK	92–108

I-connectin (GenBank accession number: ROT65894.1) and troponin I (GenBank accession number: AFW99840.1) matched these corresponding fragments, respectively. The identified proteins including α -actinin, I-connectin, and troponin I are essential components of the myofibrillar protein. Myofibrillar protein accounts for 50%–70% of muscle tissue protein and is responsible for physiological activities such as muscle contraction [1].

3.7 Bioinformatic analysis of the target proteins

α -Actinin, troponin and I-connectin account for a significant part of the structural proteins. α -Actinin is the major component of the Z-disk, which helps stabilize the structure of sarcomere. Based on the previous study, it has been suggested that m-calpain could release and degrade α -actinin from the Z-disk [21]. I-connectin is found in thick filaments and troponin exists in thin filaments of myofibers. They both have important roles in organizing sarcomeres. Degradation of these filamentous components or proteolysis could gradually lead to loss of structural integrity, weakening the myofibers [22]. It was reported that many proteins

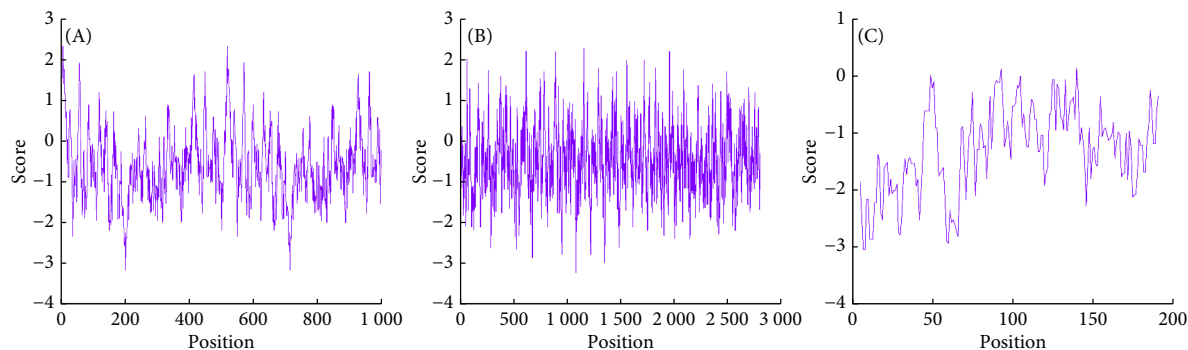
in crustaceans are degraded by endogenous enzymes and microbial activity during post-mortem storage [23]. The quality of the product is degraded by the degradation and denaturation of the structural proteins resulting in changes in the structure of the muscle and thus deterioration in quality [23].

3.7.1 α -Actinin

α -Actinin is a cytoskeletal protein responsible for maintaining the structural and mechanical integrity of actin filaments in the Z-disk [24]. Exploit α -actinin GenBank accession number and find the protein entry identifier on Uniprot as A0A423TJG5 (Table 2). From the physicochemical properties and hydrophobicity analysis of α -actinin by online tools. The results showed that α -actinin contains 1 081 amino acids. The instability coefficient of α -actinin was 40.73, which was determined as an unstable protein, indicating that it is unstable *in vitro* and prone to degradation (standard < 40 is a stable protein). The hydrophobic of α -actinin was analyzed by ProtScale. Amino acid scores greater than 0 are hydrophobic proteins and less than 0 are hydrophilic proteins. As shown in Figure 2(A), the

Table 2 Target proteins related information and protein prediction.

Bands	Description	Accession	Number of amino acids	Instability index	Predicted sequence ID	Occurrence	Prediction
I	Putative α -actinin	A0A423TJG5	1 081	40.73	Seq_1-16	No hits found	Non-toxin
II	I-Connectin	A0A3R7NSY1	2 811	39.26	Seq_1-41	No hits found	Non-toxin
III	Troponin I	K7WDK6	195	48.82	Seq_1-3	No hits found	Non-toxin

**Figure 2** Changes in hydrophilicity diagram of protein at -3°C . (A) α -Actinin; (B) I-Connectin; (C) Troponin I.

amino acid sequences of α -actinin were primarily distributed in the hydrophilic region, presumably as a hydrophilic protein. The hydrophobic interaction between hydrophobic amino acids is the main force to maintain the structural stability of proteins, and the hydrophilic distribution of proteins can reflect the folding of proteins. In addition, hydrophobicity has a subtle control not only on the stability of the protein but also on the conformation adopted by the α -helix [25].

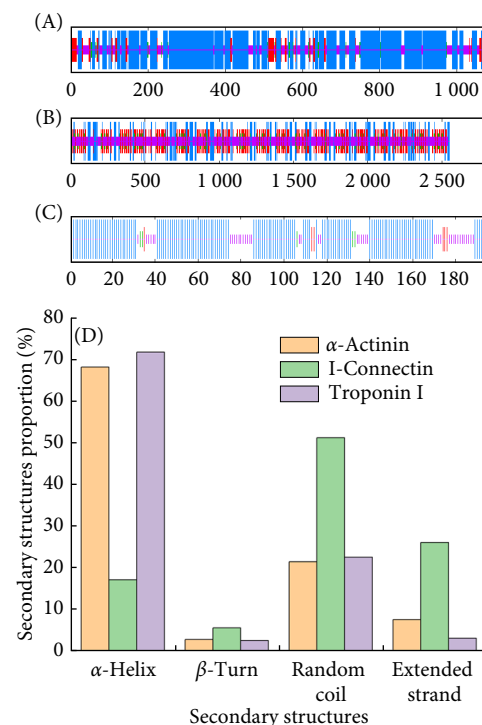
In the secondary structure of α -actinin through SOPMA online software, the results exhibited that α -helix and random coils have a more comprehensive distribution range, accounting for 68.18% and 21.46%, respectively (Figures 3(A) and 3(D)). This structural distribution means the secondary structure is relatively stable, but there is a possibility of binding to other structural proteins. The functional domains of the protein sequences were determined by NCBI, and the results showed that the whole sequences were functional domain sequences. The BLAST search function was exploited in the ToxinPred2 site to query protein sequences against the identified toxic and non-toxic proteins database. An identical sequence in the database query indicates that the sequence is predicted to be toxic, if there are no hits then it is non-toxic. A0A423TJG5 sequences had search sequences 1–16. No hits were found, showing that the protein is non-toxic (Table 2). Prediction of protein sequences in proteins can predict which peptides will be toxic in advance, and these peptides may be further removed or altered.

Related proteomics has reported that changes in the amount of α -actinin can reflect changes in the degree of postmortem protein hydrolysis [26]. The results of SDS-PAGE in this study also showed that band I was relatively thinner with prolonging storage time. However, consistently predicting quality traits using current biomarkers remains a challenge [26].

3.7.2 I-Connectin

The I-connectin is a giant spring-like protein that anchors the A-band to the center of the myosin, revealing a possible contribution of electrostatic interactions in the I-connectin protein molecule [27]. I-Connectin GenBank accession number was used to

find the protein entry identifier on Uniprot as A0A3R7NSY1 (Table 2). The results showed that I-connectin contains 2 811 amino acids. The predicted value of I-connectin instability coefficient was 39.26, which was below the threshold value and presumably belonged to stable proteins. I-Connectin also belongs to hydrophilic protein because the number of hydrophobic amino acid residues is less than that of hydrophilic amino acids

**Figure 3** Changes in secondary structure of protein at -3°C . Brief plot of the secondary structure of (A) α -Actinin, (B) I-Connectin, and (C) Troponin I. The blue, green, red and purple represent α -helix, β -turn, extended strand, and random coil, respectively. The horizontal axis indicates the position of amino acids in this protein. (D) Secondary structure distribution of α -actinin, I-connectin, and troponin I.

(Figure 2(B)). The hydrophilic zone is denser, with the lowest peak of -3.233 at positions 1 089 and 1 090; the highest hydrophobic peak occurs at position 1 162 with a score of 2.278 .

The secondary structure of I-connectin was dominated by the random coil (51.25%) and extended strand (26.08%), while α -helix and β -turn accounted for only 17.10% and 5.57%, respectively (Figures 3(B) and 3(D)). The lipid and water binding affinity of these molecules changes because protein-protein interactions lead to changes in the secondary of protein molecules [28]. The same approach contracted the functional domain of the protein, and the results showed that the entire sequence of A0A3R7NSY1 is a functional domain sequence. A BLAST search of the ToxinPred2 website compared 41 known sequences in the database and showed that the protein was non-toxic (Table 2).

Connectin is strongly sensitive to proteolysis, and in a study on fish it was found that about 80% of the connectin was reduced after 24 h of storage [29]. The results of this study showed that band II, which has been identified as I-connectin became progressively thinner with storage time and protein degradation occurred. Biochemical analysis of known proteins is essential for applying proteins as potential biomarkers in food quality control [26].

3.7.3 Troponin I

Troponin is present at fixed intervals along the entire length of actin filaments, and it consists of troponin C (18 kDa), troponin I (20 kDa), and troponin T (30 kDa) [23]. The changes in troponin I, like those in α -actinin, are mainly related to the degradation of myogenic fibronectin [30]. Troponin I GenBank accession number was exploited to find the protein entry identifier on Uniprot as K7WDK6 (Table 2). The results showed that troponin I contains 195 amino acids with an instability coefficient of 48.82. Prediction data indicate that troponin I is an unstable protein. The amino acid at position 140 is the most hydrophobic with the highest score of 0.144, and positions 7 and 8 are the most hydrophilic with a score of -3.044 . The amino acid sequence almost covers the hydrophilic region and is a hydrophilic protein (Figure 2(C)).

The secondary structure of troponin I was dominated by α -helix (71.79%) and random coil (22.56%), while β -turn and extended strands accounted for only 2.56% and 3.08% (Figures 3(C) and 3(D)). The secondary structures of troponin I and α -actinin, as two protein instability proteins, had the same distribution trend; both were dominated by α -helix and random coil. I-Connectin is a stable protein that is dominated by the random coil and extended strands. The protein sequences (MADEEAKKKQEEIDRKKAEVRKRLEE-QSAKKQKKGFMTPERKKKLRLRLRKKAAEELKKEQERKAAE RRRIIDRCGKAKNLDGANEDALRAICKEYHDHIASIESGKYD LEMEIRKDYEINELNIQVNDLRGKFIKPTLKKVSKYENKFAK LQKKADEFNFRNQLKTVKKKEFELEDDKGAAEGDAPAEAAAA) were predicted to be complete sequences with all functional domains. Similarly, the toxicity of this protein sequence was predicted and compared with the three sets of sequences in the database, and the results showed that the protein was non-toxic.

Troponin I is a well-known substrate for calmodulin, and it has been demonstrated that caseinase activity decreases with storage time and troponin T is partially degraded [29]. Through the Z-disks weakened, costameres released, desmin and dystrophin breakdowned, α -actinin lossed, and nebulin proteolysed, titin and troponin I, calpains play a leading role in early storage [18]. Therefore, the quality deterioration of shrimp muscle during storage is mainly due to the degradation of low-abundance proteins. The appearance of low molecular weight band III during

storage is presumed to be due to degradation products by protease action.

4 Conclusion

The present study evaluated protein changes in shrimp muscle protein during 4 weeks of partial freezing storage. Compared with the fresh shrimp, shrimp with storage for 4 weeks at -3°C had a significantly quality deterioration, including an increase in carbonyl content and surface hydrophobicity, a decrease in total SH content and Ca^{2+} -ATPase activity. Similarly, shrimp muscle showed a significant increase in disulfide bonds and hydrophobic interactions and a significant decrease in ionic bonds and hydrogen bonds after 4 weeks of storage.

SDS-PAGE analysis and LC-MS/MS identification showed that some proteins had changed during storage. They then were identified as α -actinin, I-connectin, and troponin I. α -Actinin and troponin I are unstable proteins, the secondary structure is mainly composed of α -helix and random coil. However, I-connectin is a stable protein which secondary structure is mainly composed of random coil and extended strand. Further biochemical analysis of these proteins provides advanced knowledge of protein properties and toxicity to ensure quality and safety.

Conflict of interest

The authors have no conflict of interest to declare.

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