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Cold-induced denaturation of muscle proteins in hairtail (*Trichiurus lepturus*) during storage: Physicochemical and label-free based proteomics analyses

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ABSTRACT

Physicochemical, proteomics, and bioinformatics analyses were conducted to investigate protein profiles in *Trichiurus haumela* under frozen (120 d) and chilled (6 d) storage. Springiness, chewiness, myofibrillar active sulfhydryl content, and Ca^{2+}-ATPase activity significantly decreased, suggesting that cold stress altered muscle proteins. Compared with fresh hairtail (FH), 66 common differentially abundant proteins (DAPs) had lower abundances in chilled (3 d; CSH) and frozen (120 d; FSH) hairtail, including myosin binding proteins, filamins, actinin, troponin, and muscle-restricted coiled-coil protein. Gene Ontology (GO) annotation showed DAPs were mainly involved in cellular process, cellular anatomical entity, intracellular, and binding items. Eukaryotic orthologous group (KOG) analysis revealed that changes in cytoskeleton and energy production and conversion functions dominated during cold storage, degrading the myofibril and connective tissue structures and the physicochemical performance of muscle tissues. This study presents deep insights into the protein alternation mechanisms in hairtail muscle under cold stress.

1. Introduction

Hairtail (*Trichiurus lepturus*) is an important economic marine fish species that is widely found in the eastern Pacific Ocean and almost all coastal regions of China. Hairtails are rich in high-quality proteins, essential amino acids, mineral elements, and healthy lipid profiles, including a large volume of unsaturated fatty acids (FAs), e.g., n3 and n6 C20, C22 FAs, and α-linolenic acid. Commercially, they are commonly prepared as fresh products or processed into surimi, semi-dried, pickled, and frozen fillet products before transport, storage, and consumption. Importantly, hairtail products, even under chilled or frozen conditions, are also susceptible to environmental stress (e.g., low temperature, light, and oxygen) due to the activity of spoilage bacteria, the abundance of proteases (e.g., trypsin-like protease, cathepsins, and calpains), and the high content of unsaturated FAs (Hu et al., 2021). The deterioration of proteins and lipids in hairtail muscle tissues that unavoidably occurs during cold storage is undesirable (Yuan, Chen, Benjakul, Sun, & Zhang, 2022), resulting in decreased flavor quality, texture degradation, drip loss, discoloration, and even the rancidity of hairtail muscle products (Thanonkaew, Benjakul, Visessanguan, & Decker, 2008).

Myofibrillar proteins (MPs), as dominant components in fish muscle, mainly consist of myosin, actin, actinin, troponin, tropomyosin, and other minor proteins, which account for 66–77 % of the total muscle proteins (Adegoke & Tahergorabi, 2021). MP denaturation involves rather complex biochemical processes upon exposure to cold stress and storage, such as the formation of intermolecular aggregates, the dissociation of MPs, conformational changes, and the modification of peptides and/or amino acids. Large numbers of intermediates and end-products with different conformations, subunits, and molecular weights are generated during the development of the denaturation process, which significantly affects normal MP functions, e.g., turbidity, solubility, water holding capacity, sulfhydryl content, and Ca^{2+}-ATPase

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activity (Mohan, Ramachandran, & Sankar, 2006; Lorentzen et al., 2020). These properties are also used to evaluate the extent of protein denaturation in the muscle. However, these parameters only provide limited information on the degree of protein denaturation and are not suitable for analyzing the formed intermediaries, peptide subunits, or their alternations and compositions. It is also complicated to connect the physicochemical properties to changes in the protein molecules of the muscle tissues. Additionally, current studies reporting primarily on the stability properties and alternations of serial MPs in the hairtail products upon exposure to cold stress are still inconclusive. A label-free based proteomics approach is based on the relationship between protein concentration and mass spectrometer (MS) signal response, which has been widely performed to investigate the alternations of protein metabolism, identify protein biomarkers, distinguish species, and illuminate interactions between proteins in organisms (Zhou, Pan, Cao, & Zhou, 2021). Several reports have determined protein compositions to reveal homeostasis mechanism in shrimp (*L. vannamei*) (Fan, Wang, & Wang, 2019), identify fresh and frozen-thawed curled octopus (*E. cirrhosa*) (Guglielmetti et al., 2018), identify protein biomarkers of beef tenderness (Picard & Gagaoua, 2020), and distinguish between neon flying squid (*O. bartramii*) and jumbo squid (*D. gigas*) (Shui, Yao, Jiang, Benjakul, & Zhang, 2021).

Although these studies contributed toward understanding the protein functions and their associations with protein profiles in organisms, the proteomic analyses of fish and its products exposure to post-mortem conditions still requires further investigation. Therefore, the current work was performed to gain insight into the denaturation mechanisms related to the alternations of proteins in hairtail (*T. lepturus*) muscle upon exposure to chilled and frozen conditions. Specifically, traditional physicochemical and label-free based proteomics technologies were carried out to thoroughly describe the protein compositions and their alterations.

2. Materials and methods

2.1. Hairtail samples

Hairtail samples (20 specimens) were purchased from the Shenjiamen aquatic market in September 2021 (Zhoushan, China). Fresh hairtail individuals (K-values were measured as 1.4–2.6 % according to the report by Ocaño-Higuera, Maeda-Martínez, Marquez-Ríos, Canizales-Rodríguez, Castillo-Yáñez, Ruíz-Bustos, et al. (2011)) with an average length of 70 ± 4 cm and an average thickness of 1.8 ± 0.3 cm were placed on ice inside foam boxes and then transported to our laboratory. Then, the fish samples were removed and washed rapidly with distilled water (4 °C). After first removing the tail, head, and viscera, the samples were cut into 5–6 cm long pieces. The obtained hairtail fillets (3 pieces) were placed on plastic trays, which were additionally packaged in polyethylene bags. The resulting samples (42 bags) were separately maintained at −18 °C for 120 days and at 4 °C for 6 days in refrigerators. Finally, the frozen samples were removed and thawed at 4 °C for 3 h in a refrigerator before the following determinations.

2.2. Chemical reagents

Trypsin was purchased from ProMag Biotechnology (Beijing, China). A protease inhibitor cocktail was supplied by Merck Drugs & Biotechnology (Darmstadt, Germany). Analytical grade ammonium bicarbonate (NH_4HCO_3), maleate, formic acid (FA), dithiothreitol (DTT), tris (hydroxymethyl)aminoethane (Tris), 2-nitro-5-thiosulfobenzoate (NTSB), ammonia, urea, iodoacetamide (IAA), and acetonitrile (ACN) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Springiness and chewiness analysis

The springiness (mm) and chewiness (mJ) of hairtail muscle were determined by using a TA.XT PlusC texture analyzer (Stable Micro Systems Ltd, Godalming, United Kingdom). The measurement procedures were carried out according to the following: holding time, 3 s; constant speed, 1.0 $mm \cdot s^{-1}$; compression, 50 %; trigger force, 0.6 N; and probe type, P/50.

2.4. Myofibrillar Ca^{2+}-ATPase activity analysis

Myofibrillar proteins (MPs) in the hairtail muscle tissues were extracted using Tris–maleate buffer solutions according to Zhang, Yan, Su, and Chen (2020). The obtained MP extractions (1.0–2.0 mg/mL protein, except where otherwise specified) were added to 0.6 mol/L KCl solution. Then, Ca^{2+}-ATPase activity (μmol Pi/mg/min) in the mixture was determined by using an ultratrace Ca^{2+}-ATPase activity assay kit (Wuhan Saipei Biotechnology Co., Ltd, Wuhan, China) according to the instructions.

2.5. Active sulfhydryl (A-SH) content analysis

The A-SH content of the MP extractions was determined by reaction with NTSB solution according to the procedure by Zhang, Xue, Li, Wang, Yang, and Xue (2016). The MP extraction was incubated with NTSB solution in a water bath (40 °C) for 30 min. After the reaction, the resulting solution was subjected to an absorbance determination at 412 nm (Uv1910, Shanghai Lingguang Technology Co., ltd., Shanghai, China). The A-SH content was calculated as mmol/g protein using an absorption coefficient of 13.9/(mmoL·cm).

2.6. HPLC-MS/MS analysis

In this experiment, the label-free proteomics analysis of fresh hairtail (0 d, FH), frozen hairtail (120 d, FSH), and chilled hairtail (3 d, CSH) samples were performed to investigate the influence of cold stress on changes in the protein profiles of hairtail muscle tissues. Three hairtail fillets from each group (FH, CSH, or FSH) were pooled together (mashed muscle collected from the same location of the hairtail as biological replicates), and then three replicates collected from these pooled samples were used as the technical replicates.

2.6.1. Protein extraction

Hairtail muscle proteins were extracted, digested, detected, and identified according to the method previously reported by Yuan et al. (2022). Briefly, the mashed samples (100 mg) were added to extraction buffer solutions (400 μL) containing 100 mmol/L Tris-HCl (4 °C, pH 8.0), 8.0 mol/L urea, 2.0 mol/L thiourea, 1 % (w/v) DTT, and 1 % (v/v) protease inhibitor. The mixture was homogenized in a homogenizer (BSH-24, Leopard scientific instruments Co., Ltd, Beijing, China). The resulting homogenate was centrifuged (JIDI-16D, Guangzhou Jidi Instrument Co., Ltd., Guangzhou, China) at 12,000×*g* for 15 min (4 °C). Afterward, the obtained precipitate was resuspended in the same extraction buffers, homogenized, centrifuged, and extracted. The combined supernatant was considered as the muscle protein extraction.

2.6.2. Trypsin digestion

The protein extraction was mixed with 1 mol/L DTT and incubated in a water bath at 37 °C for 60 min. After cooling to room temperature, 20 mol/L IAA was added to the solution and incubated in the dark at 25 °C for another 60 min. After centrifuging at 14,000×*g* for 3 min, 50 mmol/L NH_4HCO_3 was added to the resulting supernatant and then centrifuged at 14,000×*g* for another 3 min (JIDI-16D, Guangzhou Jidi Instrument Co., Ltd., Guangzhou, China). Subsequently, the collected precipitates (proteins) were further digested by using trypsin (protein to trypsin mass ratio, 1:50), and incubated in a water bath at 37 °C for 16 h. After digestion, 10 % (v/v) FA was added to the solution, followed by centrifugation at 14,000×*g* for 5 min. The obtained supernatant was desalted through C18 ZipTip pipette tips. The resulting peptides were

lyophilized and suspended in 0.2 % (v/v) FA using the following HPLC-MS/MS analysis.

2.6.3. HPLC-MS/MS analysis

Digested peptides were analyzed by using tandem mass spectrometer (MS/MS, Orbitrap Fusion™ Lumos™) in conjunction with high-performance liquid chromatography (HPLC, UltiMate 3000 Nano HPLC system) using instruments obtained from Thermo Fisher Science (Bremen, Germany). First, the peptides were dissolved in 0.1 % (v/v) FA solution, loaded into a C18 pre-column (3 μm, 75 μm × 50 cm) and subsequently separated by a C18 analytical column (1.93 μm, 150 μm × 12 cm) at a flow rate of 450 nL/min. The HPLC analysis was executed with a binary mobile phase containing 0.1 % (v/v) FA in H_2O (solvent A) and 0.1 % (v/v) FA in acetonitrile (solvent B). A gradient elution procedure was carried out according to the following guidelines: 0–11 min, 7 % B and 93 % A; 11–48 min, 15 % B and 85 % A; 48–68 min, 25 % B and 75 % A; 68–69 min, 40 % B and 60 % A; and 69–75 min, 100 % B. The following full-scan MS parameters were collected as follows: scan range, 350–2000 *m/z*; max injection time, 80 ms; automatic gain control (AGC) target, 3e6; and resolution, 120,000. Analysis of the subsequent mass spectra was performed as follows: first mass, 120; scan range mode, auto normal; maximum injection time, 35 ms; AGC target, 5000; isolation window, 1.6; and collision energy, 30 %.

2.6.4. Protein identification and bioinformatics analysis

HPLC-MS/MS data were searched using Proteome Discoverer™ version 2.2 software (Thermo Fisher Science, Bremen, Germany) based on the UniProt database (Nov. 16, 2021; https://www.uniprot.org/). The operating procedures were performed as follows: peptide confidence, high; fragment mass tolerance, 0.6 Da; variable modifications, methionine oxidation and acetylation on protein (*N*-term); max missed cleavages, 2; protein cleaving enzyme, trypsin; peptide mass tolerance, ± 10 ppm; fixed modifications, carbamidomethyl; and false discovery rate (FDR), ≤ 0.01. Three comparison batches were made, i.e., CSH vs FH, FSH vs FH, and FSH vs CSH comparisons, and differentially abundant proteins (DAPs) were identified in each comparison according to the calculated fold change (FC; <1/1.5 or >1.5) values for the proteins. Moreover, Gene Ontology (GO) and eukaryotic orthologous group (KOG) analyses were carried out to explore the functional annotations and categories of the target DAPs in the comparisons.

2.7. Data analysis

Data were expressed as the mean ± standard deviation (SD). All determinations were carried out in triplicate ($n = 3$) except where otherwise specified. SPSS software (version 20.0, SPSS Inc., Chicago, USA) was used to analyze the significant differences calculated from the determinations. Duncan's multiple range tests were conducted for the comparisons to determine these significant differences at $P < 0.05$.

3. Results and discussion

3.1. Springiness and chewiness analysis

The springiness and chewiness of hairtail muscle (Fig. 1A and 1B) were measured during chilled (6 d) and frozen (120 d) storage. According to the results, similar decreasing trends in the springiness and chewiness parameters were observed in both types of hairtail samples during chilled and frozen storage. However, the decreasing trend observed for the chilled hairtail samples increased remarkably with the storage time. Compared with chilled treatment, the hairtail muscle tissues showed comparatively higher springiness and chewiness when these samples were frozen. The adverse effects on the textural properties caused by the temperature and storage period appeared to be retarded

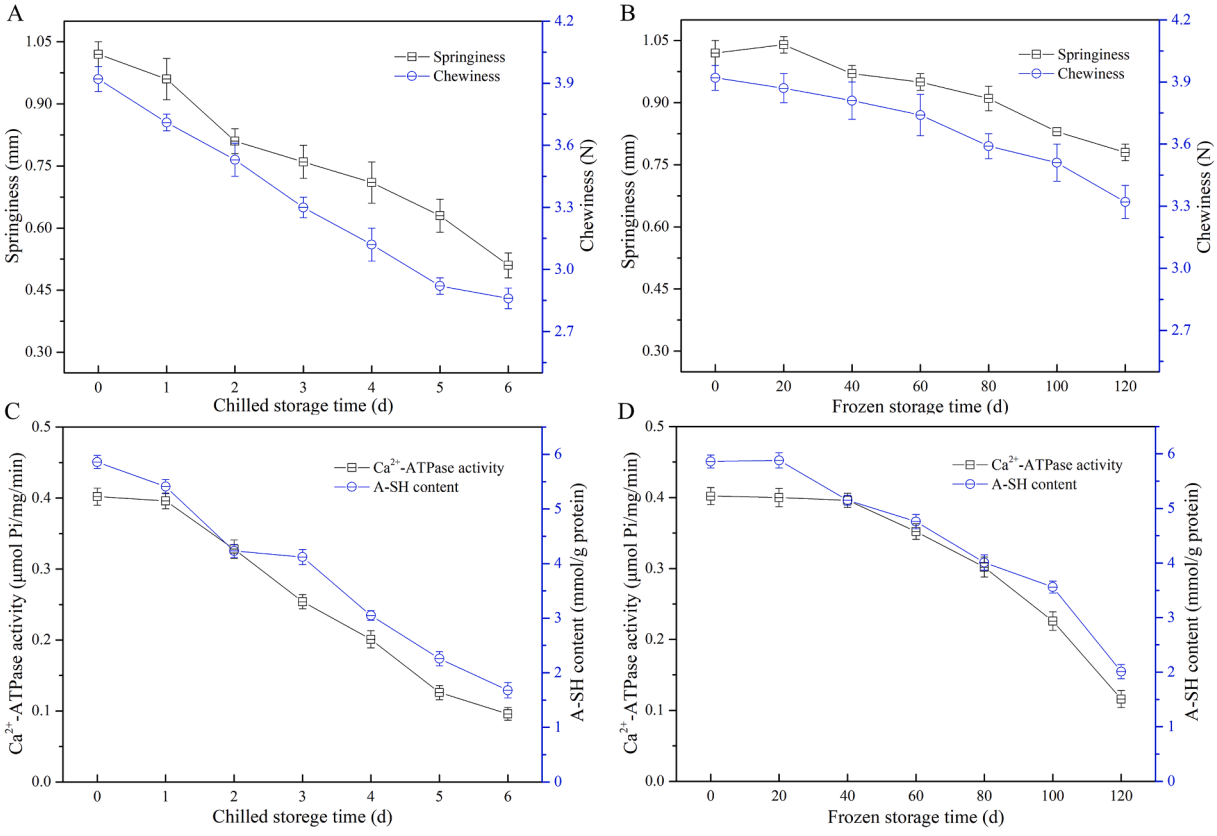

Fig. 1. Changes in the springiness, chewiness, myofibrillar Ca^{2+}-ATPase activity, and myofibrillar A-SH content in the hairtail muscle during 6 days of chilled and 120 days of frozen storage.

during frozen storage.

The stability and function of myofibrillar proteins (MPs) and connective tissues are closely related to the alternations in the springiness and chewiness of the hairtail muscle. The deterioration of the textural properties can be attributed to several factors during processing and storage. During frozen storage, ice crystals grow continuously, their shapes become irregular, and their size increases remarkably, which leads to considerably mechanical damage to the MPs, fiber bundles, and connective tissues (Zhang, Cao, Wei, & Ying, 2020). Besides, the dissociation and fragmentation of MPs also occur in the muscle tissues, especially under chilled conditions, which is mainly caused by endogenous cathepsins, calpains, and several serine proteinases as well as bacterial activities (Xie, Wang, Wang, & Qian, 2020). Moreover, protein/lipid oxidation is generally unavoidable and unpredictable during cold storage, resulting in the formation of active carbonyls, tyrosine, and several oxidation products, which all show adverse effects on the stability of MPs and the textural properties of the muscle tissues (Liu et al., 2021).

3.2. Myofibrillar Ca^{2+}-ATPase activity and A-SH content analysis

Changes in the myofibrillar Ca^{2+}-ATPase activity and A-SH content were investigated in the hairtail muscle samples during both types of storage, as presented in Fig. 1C and D. The initial fresh hairtail had relatively high Ca^{2+}-ATPase activity and A-SH content, indicating the good protein quality of the hairtail samples. Due to cold stress, endogenous enzymes, oxidative actions, and spoilage bacteria were promoted during storage, and Ca^{2+}-ATPase activity and A-SH content both decreased markedly in the hairtail samples after 6 days (4 °C) and 120 days (−18 °C) of storage. Although both types of hairtail samples showed similar decreasing trends during storage, the values for the frozen hairtail were significantly higher than those of the chilled samples.

The development of protein denaturation in the muscle products is generally evaluated by the parameters of the MP content, surface hydrophobicity, Ca^{2+}-ATPase activity, and total/active-SH content. In the current study, significant decreases in the myofibrillar Ca^{2+}-ATPase activity indicated that conformational changes located in the myosin globular head and/or the aggregation/rearrangement of this protein likely occurred in the hairtail muscle tissues during cold storage (Sun, Zhang, Bhandari, & Yang, 2019). Moreover, the active-SH groups buried in the MPs and on their surface were vulnerable to attack during this process and/or cold storage, resulting in the formation of disulfide groups and rapid reductions in MP A-SH content (Gao, Hou, & Zeng, 2019). It was previously noted that reductions in the A-SH content of MPs (especially for the myosin globular head) were commonly accompanied by a decrease in myofibrillar Ca^{2+}-ATPase activity (Shao et al., 2018). Additionally, compared with the chilled conditions, freezing conditions were found to be effective in preventing myofibrillar Ca^{2+}-ATPase activity and A-SH content from sharply decreasing in the hairtail muscle tissues during storage. These findings were also in agreement with the above texture (springiness and chewiness) determinations.

Notably, the development of protein denaturation involved numerous conformational changes, modification, dissociation, and/or oxidation, forming incredibly intricate protein polymers, polypeptides, and final products (Walayat, Xiong, Xiong, Moreno, Niaz, Ahmad, et al., 2020). Thus, the associations between cold stress and alterations in the protein profiles need to be further explored.

3.3. Validation of label-free MS/MS approaches

Qualification and validation checking was executed on MS/MS data to evaluate the reliability of the analytical process and the used instruments. The results found 3020 peptides detected in hairtail muscle based on the obtained spectral data. A total of 718 proteins containing at least one unique peptide were further identified with FDR values of less than 0.01. Regarding the molecular weight (MW) distribution (Fig. 2A), there were 466 identified proteins (64.9 %) with a MW of 10–60 kDa, 152 proteins (21.2 %) of 60–140 kDa, and 58 proteins (8.1 %) with a MW above 200 kDa, providing a natural MW range and distribution similar to the previous findings in bighead carp (*Hypophthalmichthys nobilis*) and sea bass (*Lateolabrax japonicus*) muscle (Liu et al., 2022; Xiang et al., 2022). For the peptide count analysis (Fig. 2B), 666 identified proteins (92.8 %) were composed of 1–20 peptides, while 52 proteins (7.2 %) contained more than 20 peptides. Additionally, the length (in amino acids) of the peptides mainly ranged from 7 to 21 amino acids, with less than 3 % of detected peptides being more than 23 amino acids in length (Fig. 2C), which was beneficial for detection by MS/MS analysis. The digested peptides composed of fewer than 5 amino acids or more than 20 amino acids could not be effectively trapped by the MS/MS spectrometer, mainly owing to their low/high electric charges and incompatible MWs (Yuan et al., 2022). These observations were in accordance with the digestion characteristics of trypsin in the muscle proteins. Moreover, a relatively high protein sequence coverage was also observed in this study, of which 100 identified proteins (13.9 %) had $\geq$ 30 % sequence coverage, and 396 identified proteins (55.2 %) showed $\geq$ 10 % sequence coverage that matched the reported proteins in the Uniprot database. Collectively, the current findings on the distribution of MWs, sequence coverages, and the peptide counts and lengths of the identified proteins suggested that the obtained MS/MS data were adequate for the subsequent bioinformatics analysis and further demonstrated the validity and reliability of the operating procedures executed during the trypsin digestion, HPLC separation, and MS/MS detection.

3.4. Differentially abundant protein (DAP) analysis

From the DAP analysis, many DAPs were detected and identified in the CSH vs FS and FSH vs FH comparisons (Supplementary Table S1, S2, and S3), which verified that significant changes in the hairtail muscle proteins most likely resulted from the cold temperatures and storage stress. Sixty-six common DAPs (Supplementary Table S3) accumulated at lower abundances in the CSH and FSH samples when compared to the FH samples, which included myosin binding protein C (slow type), filamin A (alpha), actinin (alpha 2b), calcium-transporting ATPase, troponin I type 1a (skeletal, slow), myosin (heavy chain b), myosin-binding protein Ha, muscle-restricted coiled-coil protein. These structural proteins are considered critically important components of the myofiber membrane in the muscle tissues, which mainly contain myosin and its binding proteins, filamins (actin-binding proteins), actinin, troponin, and nebulin (Yuan et al., 2022). The alternations, degradation, modification, and/or dissociation of these proteins, even to a small extent, are closely associated with the structural changes in skeletal muscle. This resulted in tremendous changes in the muscle property and quality, such as texture deterioration and juice loss (Wu, Clerens, & Farouk, 2014; Zhang, Mao, Yao, & Aubourg, 2020). In the current work, the lower abundance of structural proteins identified in CSH and FSH samples was most likely owing to the denaturation of MPs in the muscle tissues, which mainly resulted from the mechanical damage induced by ice crystal growth and recrystallization, endogenous protease actions, bacterial growth and reproduction, and/or biochemical processes occurring during chilled and frozen storage (Purslow, Gagaoua, & Warner, 2021; Yuan et al., 2022).

Forty-seven common DAPs (Supplementary Table S2) accumulated at higher abundances in the CSH and FSH samples when compared to the FH samples, which mainly included adenylate kinase isoenzyme 1, ribosomal protein S5, glycerol-3-phosphate dehydrogenase [NAD(+)], 40S ribosomal protein S2 and S7, 60S ribosomal protein L7a, triosephosphate isomerase, alpha-1,4 glucan phosphorylase. Among these, adenylate kinase catalyzes the transfer of the phosphate group between ATP and AMP in muscle tissues and plays essential roles in adenine nucleotide metabolism and cellular energy homeostasis. The action of

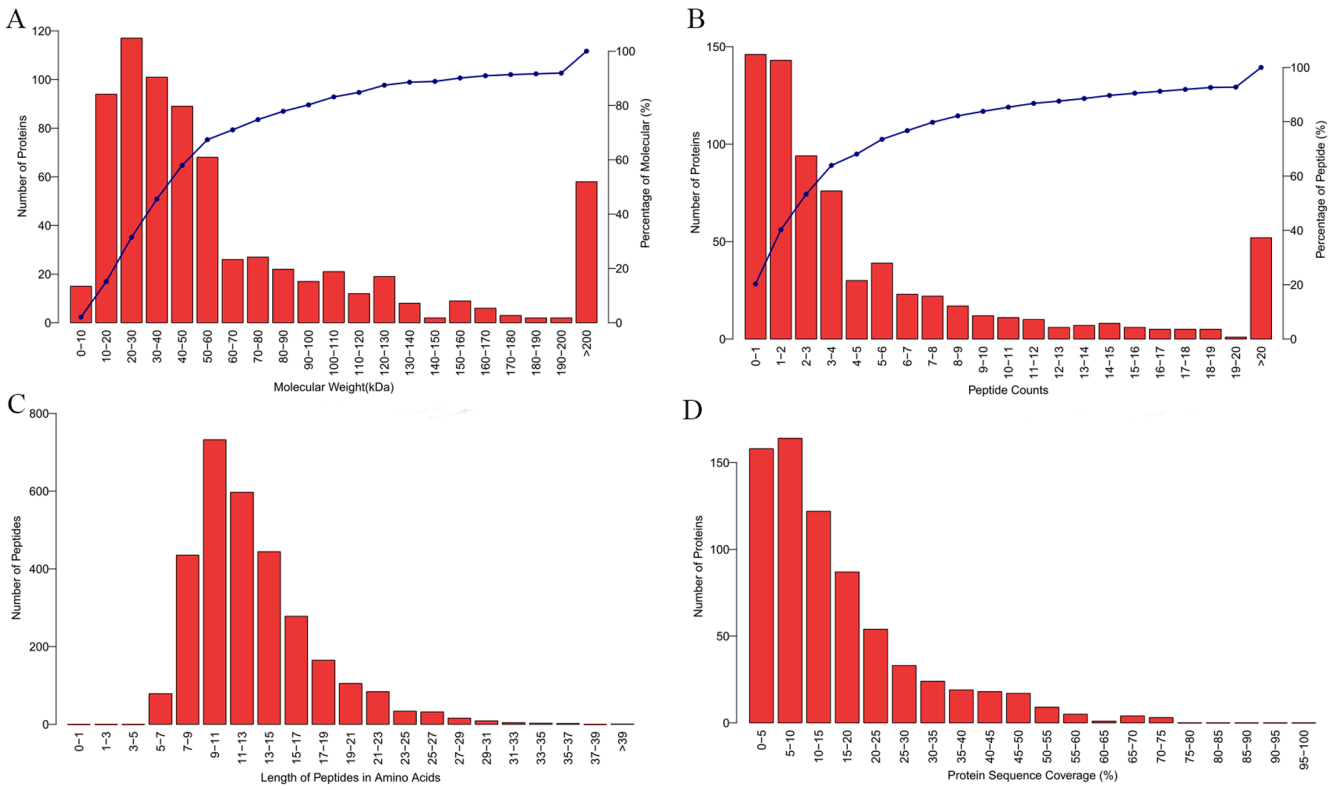

Fig. 2. (A) Molecular weight, (B) peptide count, (C) peptide length, and (D) sequence coverage of the proteins in the hairtail muscle detected by label-free based HPLC-MS/MS measurements.

adenylate kinase promoted the conversion of ATP to AMP and further into IMP and NH_3 molecules (Longo, Lana, Bottero, & Zolla, 2015), which were negatively correlated with the freshness and quality of the muscle products. The GPDH enzyme located at the mitochondrial membrane in muscle tissues has been reported to produce $O_2^{\cdot-}$, a reactive oxygen species (ROS), during the transport of electrons from NADH to the electron transport chain (Zelickson, Ballinger, Dell'Italia, Zhang, & Darleyusmar, 2013). This presumably accelerated the development of protein/lipid oxidation occurring in the high-fat hairtail muscle during cold storage. In addition, the ribosome in the myofibroblast was mainly composed of many ribosomal protein subunits, which in turn provide crucial structural protection effects against external stress. However, ribosome organelles and their proteins/subunits are also vulnerable to physical damage, cold stress, radical attack, and other conditions, leading to the degradation, dissociation, and/or oxidation of these proteins (Gu, Wei, Zhang, & Liu, 2020; Zhang, Li, Hong, Luo, & Lametsch, 2020). These actions presumably influenced the subsequent trypsin enzymolysis on the ribosomal proteins and the following detected DAP compositions (identified in high quantities in the CSH and FSH samples).

In addition, a total of 101 DAPs were identified in the FSH vs CSH comparison, of which 57 DAPs and 44 DAPs were accumulated at higher and lower abundances, respectively, in the FSH sample. The higher abundances of DAPs in the FSH sample are shown in Supplementary Table S3 and included collagen proteins (type XII alpha 1b, VI alpha alpha 1, and VI alpha 3), annexin, tubulin beta chain, and myosin components (light chain 13; heavy polypeptide 1.3; heavy chain 6; and light chain 10). Collagens, as one of two types of fibrous connective proteins, provide critical mechanical strength and support to fish muscle tissues, which are comparatively more stable than other muscle proteins (Wang, Yan, Ding, & Ma, 2022). The degradation and/or damage of the muscle collagens weakened the binding force and enlarged the intercellular space between the muscle fibers. In the current study, the frozen muscle samples showed a higher collagen abundance than the chilled models, which indicated that freezing temperatures may be beneficial for retarding the hydrolysis and the degradation of collagen in the muscle tissues during storage. These results were similar to the findings for *Coregonus peled*, where collagen digestion was considered the primary reason for the degraded textural properties of the fish muscle. Significantly inhibition effects on collagen degradation were also found in the *C. peled* muscle during 6 days of super-chilled (-2°C) storage (Fan et al., 2021). Annexins are calcium-, phospholipid-, and cytoskeleton-binding proteins, and their functions are mainly linked to membrane-related events occurring in muscle tissues. They are considered vulnerable to radical species, cold conditions, endogenous enzymes, and environmental stress, especially under relatively high-temperature conditions (Sayd, Chambon, Laville, Lebret, Gilbert, & Gatellier, 2012). Thus, annexins showed a relatively stable property in the muscle tissues under freezing conditions in the current study due to the decreased oxidative deterioration of proteins/lipids and the inhibited growth of spoilage microorganisms.

3.5. Gene Ontology (GO) annotation analysis

Functional annotations of the identified DAPs in hairtail muscle were collected based on the GO knowledge base. Overall, the GO annotation (Fig. 3) results showed similar distributions and convergences of the DAPs between the CSH vs FH and FSH vs FH comparisons. It was suggested that similar alternations in the DAP profiles might have occurred in the CSH and FSH samples when they were exposed to chilled and frozen stress.

Biological process annotation revealed that some commonly identified DAPs in the CSH and FSH had converged in the cellular process (GO: 0009987) compared with the FH samples, which can be related to several structural proteins, e.g., myosin binding protein C (muscle fiber component), actinin alpha 2b (myofibril assembly), metavinculin (cell adhesion), myosin heavy polypeptide 1.1 (somite specification), and collagen type VI alpha 1 (muscle structure development). These lower-

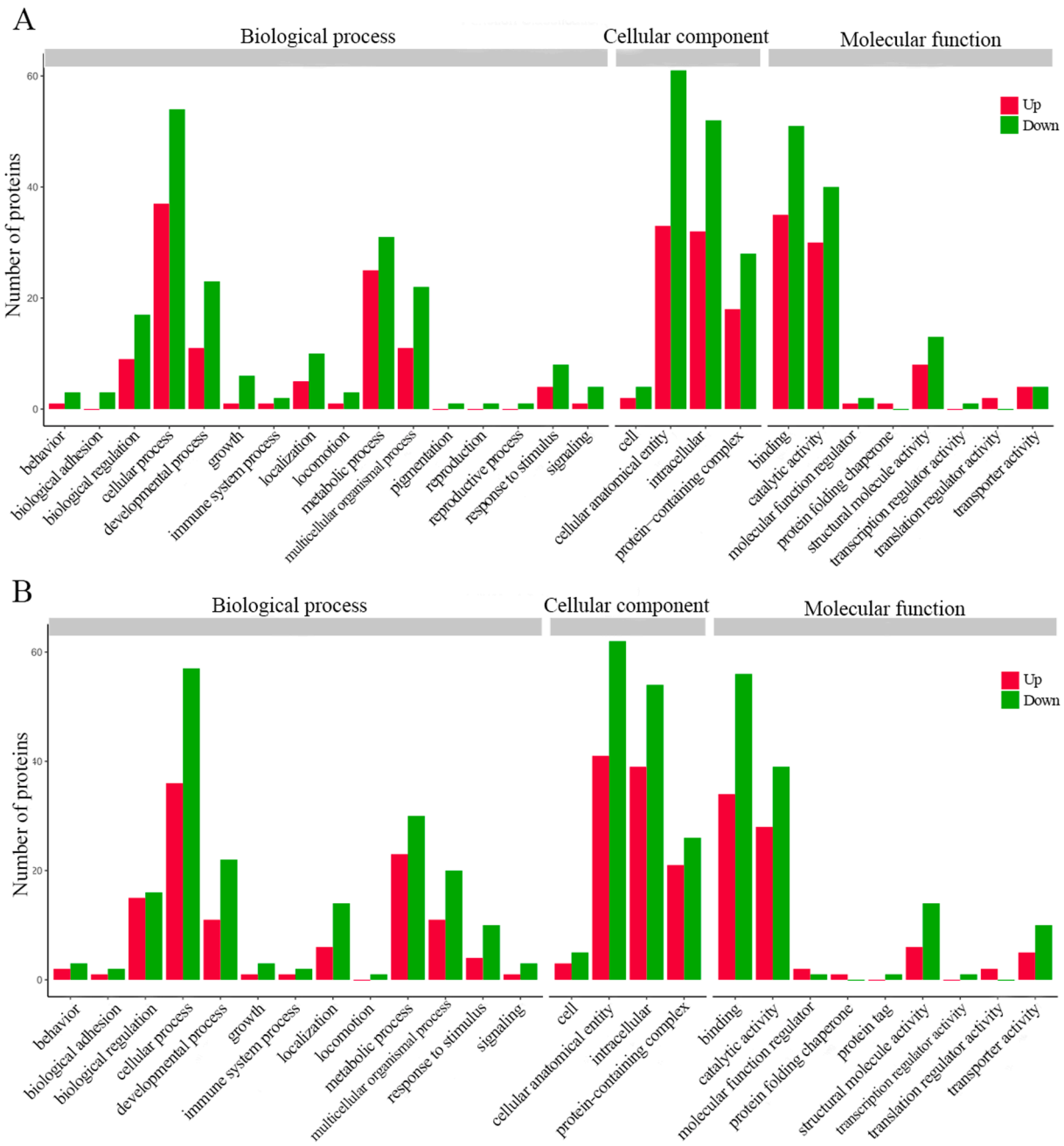

Fig. 3. GO annotation (including three domains) analysis of the DAPs identified by label-free based proteomics in the (A) CSH vs FH and (B) FSH vs FH comparisons. Red and green colors respectively represent the higher and lower abundance levels of the identified DAPs in the hairtail muscle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abundance DAPs in the CSH and FSH muscle were mainly induced by cold stress, indicative of the reduced structural integrity and stability of muscle tissues upon exposure to cold storage (Fan et al., 2021). In addition, the annotated multicellular organismal (GO:0032501), developmental (GO: 0032502), and metabolic (GO: 0008152) processes also covered several lower abundance DAPs in the CSH and FSH samples, including HATPase_c domain-containing protein (response to cold), calcium-transporting ATPase (calcium ion transport), and ATP synthase subunit alpha (ATP metabolic process), which were related to the proteolysis and/or destruction of proteins and the degree of quality deterioration occurring in muscle tissues (Lin, Qi, Shui, Benjakul, Aubourg, & Zhang, 2021).

As determined by cellular component annotation, most lower-abundance DAPs identified in the CSH and FSH samples, when compared to the FH samples, were classified as intracellular (GO:0005622) or cellular (GO:0110165) anatomical entities. Compared with the FH samples, the involved DAPs with lower abundances in the CSH and FSH samples, including myomesin 1a, A-band titin (as muscle thin filament, Z disc, and M band components), myosin heavy chain a/b/7-like/7B (myosin complex components), proton-translocating NAD(P)(+) transhydrogenase, NADH-ubiquinone oxidoreductase 75 kDa subunit, electron transfer flavoprotein-ubiquinone oxidoreductase, calcium-transporting ATPase (membrane components), and troponin I type 1a (troponin complex component), exhibited degradation-induced cold stress (e.g., ice crystals), enzymatic actions caused by proteases, and oxidation resulting from reactive radicals during storage. These findings were in partial agreement with the DAPs detected in frozen shrimp (*Solenocera melantho*) during 6 months of (at −20 °C and −40 °C)

storage (Shi et al., 2018) and in pike eel (*Muraenesox cinereus*) during chilled (at 0 °C, 6 d) and frozen (−18 °C, 120 d) storage (Yuan et al., 2022).

Within the molecular function annotation, the main DAPs were categorized as catalytic activity (GO:0005198) and binding (GO:0003824) items. For the CSH/FSH vs FH comparisons, the observed DAPs, such as filamin A (alpha) and C (gamma a), actinin alpha 2b, metavinculin (actin binding function) and ATP synthase subunit alpha, calcium-transporting ATPase, creatine kinase, and the ADP/GDP-forming subunit alpha (nucleotide binding, ATP binding, and calcium transmembrane transporter functions), were enriched in the binding annotation. These binding DAPs, which had essential roles in maintaining the typical structure, framework, homeostasis, and organization of muscle (protein) tissues, showed lower abundances in the muscle tissues after cold storage. In the present work, cold stress caused by chilled storage most likely dissociated the binding proteins (complexes), affecting the normal conformational structure and weakening its connecting strength. This would have led to the decreased binding functions of the muscle proteins and their biological tissues (Purslow et al., 2021), which is in accordance with the texture results. In addition, several DAPs with higher abundances belonging to the binding annotation were identified in the CSH and FSH samples, including adenylate kinase isoenzyme 1 and nucleoside diphosphate kinase (nucleotide binding), Zgc:110679 and parvalbumin 3 and 4 (calcium ion binding), and 2-phospho-D-glycerate hydro-lyase (magnesium ion binding). Meanwhile, the nucleoprotein, malate dehydrogenase, and triosephosphate isomerase (catalytic and oxidoreductase activities), considered to negatively effects the textural properties of the muscle proteins, were also identified with higher abundances in the CSH and FSH samples.

3.6. Eukaryotic orthologous group (KOG) functional analysis

The KOG functions of the DAPs identified in the CSH vs FH and CSH vs FH comparisons are presented in Fig. 4. The functions were separated into 25 classifications based on the KOG database. According to the results, the DAP functional classifications showed a similar distribution and convergence between the CSH vs FH and FSH vs FH comparisons, which confirmed identical changes in the available variations of the chilled and frozen treatments (after storage) in contrast to the fresh hairtail samples. The DAPs in both comparisons were mainly converged in 6 functional classifications, namely the cytoskeleton (Z); translation, ribosomal structure, and biogenesis (J); energy production and conversion (C); posttranslational modification, protein turnover, and chaperones (O); carbohydrate transport and metabolism (G); and signal transduction mechanisms (T) classifications.

The cytoskeleton functions as a basic structural framework for normal hairtail muscle tissues. The converged target proteins in this classification mainly included the myosin essential light chain (KOG0030), troponin (KOG3634), projectin/twitchin and related proteins (KOG0613), actin and related proteins (KOG0676), actin filament-coating protein (KOG1003), the myosin class II heavy chain (KOG0161), beta-tubulin (KOG1375), alpha-tubulin (KOG1376), and the Ca^{2+}-binding actin-bundling protein (KOG0035). The cytoskeletal proteins play a multitude of roles, such as maintaining structural integrity and stability, providing physical links between the membrane and organelles, and providing physical resistance to environmental stress in the muscle tissues (Yang, Chen, Li, Li, & Yang, 2021). In the current study, cold stress caused by chilled storage induced damage to the cytoskeletal proteins in the hairtail muscle. The microbial metabolites, exogenous and endogenous enzymes (especially under chilled conditions), ice crystal growth and recrystallization (especially under freezing conditions), and oxidative actions synergistically accelerated the degradation and/or hydrolysis of the cytoskeletal proteins and their peptide bonds, leading to series conformational changes, structural modifications, dissociation, and the irreversible denaturation of these proteins (Zhang, Mao, Yao, & Aubourg, 2020; Qiu et al., 2020). These actions in muscle tissues exerted adverse effects on the natural structure of the myofibrils and connective tissues, hindering the performance of the muscle tissues.

Regarding the energy production and conversion KOG classification, the involved proteins mainly involved pyruvate dehydrogenase E1 (alpha subunit; KOG0225), dihydrolipoamide acetyltransferase (KOG0557), lactate dehydrogenase (KOG1495), $NADP^{+}$-dependent malic enzyme (KOG1257), malate dehydrogenase (KOG1496), and creatine kinases (KOG3581). In the present study, energy expenditure still occurred in muscle tissues during cold storage, and the cold conditions influenced the rate of energy metabolism (Scheffler & Gerrard, 2007). This can be related to the alternations of energy metabolism-related proteins, especially for several dehydrogenases, kinases, and transferases. It was considered that the KOG functions of the cytoskeleton and energy metabolism and their related proteins greatly affected muscle stability and function during processing and storage (Xu et al., 2021). These altered proteins were considered the leading causes of the development of muscle color and texture, myofibril structure, proteolysis, and oxidative stress (Purslow et al., 2021; Zhang, Li, Hong, Luo, & Lametsch, 2020).

Regarding translation, ribosomal structure, and biogenesis classification, the proteins identified in the comparisons included series 40S ribosomal proteins (e.g., S17, KOG0187; S2, KOG0886; S3A, KOG1628; S11, KOG1728; S16, KOG1753; S25, KOG1767; S8, KOG3283) and 60s ribosomal proteins (e.g., L10, KOG0857; L14/L17/L23, KOG0901;

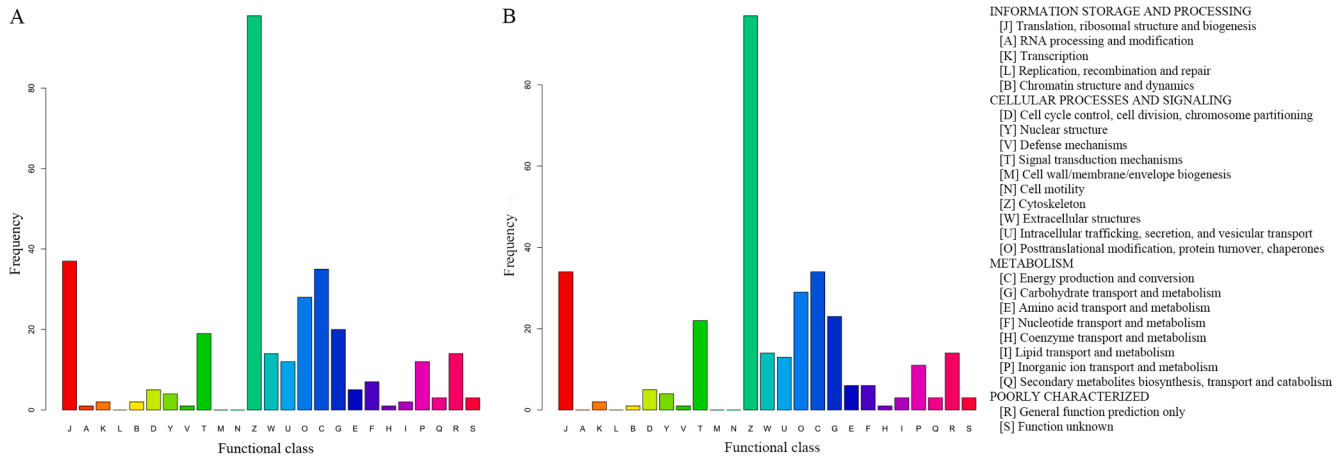

Fig. 4. KOG function (including 25 classifications) analysis of the DAPs in the hairtail muscle identified by label-free based proteomics in the (A) CSH vs FH and (B) FSH vs FH comparisons.

L10A, KOG1570; L23, KOG1751). The ribosome is structurally composed of 40S and 60S ribosomal proteins that provide an intracellular framework for RNA compounds in muscle tissues. The functions of these ribosomal proteins, representing about 10 % of all cellular proteins, are important in the compactness and integrity of the whole muscle tissues (Shui, Yao, Jiang, Benjakul, & Zhang, 2021). Alreshidi, Dunstan, Macdonald, Smith, Gottfries, & Roberts (2015) suggested that several ribosomal proteins likely played an additional role as a temperature sensor to acclimatize to cold stress. Additionally, ribosomal proteins were also reported to be closely correlated with the accelerated softening in stunning-stressed silver carp (*Hypophthalmichthys molitrix*) fillets (Zhang, Li, Hong, Luo, & Lametsch, 2020). In the current study, the alterations in the translation, ribosomal structure, and biogenesis functions might be a ribosomal response to cold exposure, which thus affected the chemical properties of the proteins and the physical performance of the muscle tissues.

4. Conclusion

In this work, the physicochemical properties of hairtail muscle and their protein alternations were determined during chilled (CSH) and frozen (FSH) storage and compared to the fresh hairtail samples (FH). The results showed that cold stress significantly affected the texture, myofibrillar Ca^{2+}-ATPase activity, and A-SH content of hairtail muscle tissues. It was considered that significant changes in the proteins occurred between the FSH and CSH/FSH samples. Moreover, the label-free proteomic analysis revealed 66 and 47 common DAPs accumulated at lower and higher abundances, respectively, in the CSH and FSH samples compared with the FH samples. These identified lower DAPs were mainly related to several structural proteins (e.g., myosin-binding proteins, actinin, metavinculin, myosin heavy polypeptides, and collagens) and likely resulted from the mechanical damage induced by cold stress, endogenous protease actions, and/or bacterial activity. This study provides further understanding of the physicochemical properties of muscle tissues associated with the protein alternations occurring in hairtail during cold storage.

CRediT authorship contribution statement

Shanshan Shui: Investigation, Methodology, Project administration, Resources, Software. **Hongbo Yan:** Conceptualization, Data curation, Formal analysis, Investigation. **Chuanhai Tu:** Supervision, Validation, Visualization. **Soottawat Benjakul:** Supervision, Validation, Visualization. **Santiago P. Aubourg:** Supervision, Validation, Visualization. **Bin Zhang:** Funding acquisition, Writing – original draft, Writing – review & editing.

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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Appendix A. Supplementary data

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