Current Research in Food Science 8 (2024) 100779

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Quality changes of whitespotted conger (*Conger myriaster*) based physicochemical changes and label-free proteomics analysis during frozen storage

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A R T I C L E I N F O

Handling Editor: Xing Chen

Keywords:
Whitespotted conger
Frozen storage
Protein degradation
Quality deterioration
Proteomics analysis

A B S T R A C T

Whitespotted conger (*Conger myriaster*) muscle proteins were susceptible to oxidative denaturation during frozen storage. The objective of this study was to investigate the alterations in quality through physicochemical analysis and proteomics after whitespotted conger stored at temperatures of −18 °C and −60 °C. The microstructural observation revealed the noticeable variations such as increased interstitial space and fractured muscle fibre with extension of frozen storage time, and the muscle fibre of whitespotted conger stored at −60 °C were more intact than those stored at −18 °C. The raised TVB-N value indicated that the freshness of whitespotted conger decreased during 120-day frozen storage period. Analysis of myofibrillar protein content and SDS-PAGE demonstrated that compared to −18 °C, lower storage temperature (−60 °C) could better maintain the structure of whitespotted conger muscle by inhibiting protein degradation and oxidation. To reveal the mechanism of protein degradation, label-free quantitative proteomic analysis was performed through LC-MS/MS. The structural proteins including domain-associated proteins and actin-related proteins were up-regulated during frozen storage, but the phosphoglycerate kinase, phosphoglycerate mutase, and fructose-bisphosphate aldolase were down-regulated. Storage at −18 °C accelerated the up- or down-regulation of those differentially abundant proteins. According to KEGG analysis, up- or down-regulated pathways such as glycolysis/gluconeogenesis, carbon metabolism, biosynthesis of amino acids, and calcium signalling pathway mainly accounted for the protein degradation and quality reduction of whitespotted conger at low temperature. These results provided a theoretical basis for improving the quality stability of whitespotted conger during frozen storage.

1. Introduction

Whitespotted conger (*Conger myriaster*) is a crucial marine resource for the fish processing industry, which mainly distributed in the Yellow Sea, Bohai Sea, and East Sea of China, as well as the coast of Japan's Honshu island and the coast of the Korean Peninsula (Xiu et al., 2022; Kurogi et al., 2002). Whitespotted conger has received high attention due to its significant nutritional value, such as its rich protein content (approximately 17.0%) and substantial quantities of unsaturated fatty acids (EPA and DHA) (Oku et al., 2009). Nowadays, whitespotted conger is primarily harvested through fishing, but there will inevitably be death during the process from temporary rearing to live slaughter. These can lead to a severe loss in the quality of whitespotted conger in a short period because of the high content of unsaturated fatty acids and abundant endogenous enzymes in muscle tissue (Xie et al., 2020). Therefore, to prevent the degradation of quality has become the key to whitespotted conger prior to being processed into products.

Frozen storage as a widely used storage method for preserving aquatic products can effectively inhibit the microbial spoilage and enzymatic reactions, thereby preventing the deterioration of fish quality (Yuan et al., 2022). Nevertheless, the inhibitory effects of different lower temperatures on denaturation of muscle proteins varied in long term

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https://doi.org/10.1016/j.crfs.2024.100779
Received 24 February 2024; Received in revised form 13 May 2024; Accepted 4 June 2024
Available online 6 June 2024

storage. For instance, raw tuna that was ready-to-eat undergone ultra-freezing to attain elevated economic value and high quality (Jiang et al., 2019). In addition, even when subjected to low-temperature frozen storage, muscle protein denaturation and oxidation inevitably occurred (Yan et al., 2023). Hence, the alterations in physicochemical properties and protein degradation in the muscle tissue of whitespotted conger during frozen storage were the pivotal factors that affected the quality of fish (Wang et al., 2021). However, the proteins of whitespotted conger were influenced by various physiological and metabolic changes, including degradation of myofibril protein, protein oxidation, and modifications tacking place after translation. And conventional physical and chemical methods, such as TVB-N, myofibril protein, and SDS-PAGE, provided limited information on protein changes during frozen storage, which failed to elucidate the specific mechanisms underlying protein denaturation.

Proteomics is a kind of analytical chemistry technique that identifies the expression profiles, correlations, compositions, and functions of proteins in organisms with exceptional sensitivity, accuracy, and specificity (Zhang et al., 2023a). It possesses unparalleled advantages and serves as a pivotal tool for investigating the quality assessment of aquatic products in processing, transportation, and storage (Wang et al., 2023). Proteomics can elucidate the underlying molecular mechanisms and biological pathways that contribute to changes in quality by analysing protein metabolism and composition changes, thereby facilitating a deeper understanding of the regulatory patterns governing alterations in quality during the processing and storage of aquatic products. Previous proteomics studies have investigated the protein compositions to identify potential indicator proteins associated with the quality of *Penaeus vannamei* (Sun et al., 2023), elucidated the underlying mechanism of mitochondria in tilapia red meat discoloration (Xiang et al., 2023), and validated that pre-immersion in trimethyl phosphate sodium could effectively delay muscle protein degradation during frozen storage (Zhang et al., 2020b). Therefore, proteomic techniques offer a robust tool for investigating the underlying mechanisms of protein degradation.

Furthermore, the majority of studies pertaining to whitespotted conger have primarily focused on the nutritional composition (Zhang et al., 2023b) and processing technology (Jung et al., 2018). There were fewer physicochemical researches on the quality changes of whitespotted conger after storage at low-temperature, let alone its proteomics analysis. Therefore, whitespotted conger samples were stored at temperatures of −18 °C and −60 °C for 120 days in this study to research the physicochemical properties of whitespotted conger, including total volatile base nitrogen (TVB-N), myofibrillar protein (MP) content, and protein degradation. The key proteins and pathways of whitespotted conger muscles were studied through label-free liquid chromatography coupled with bioinformatics analysis. This study aimed to elucidate the denaturation mechanisms of whitespotted conger proteins during frozen storage using a label-free proteomics strategy, and established a fundamental basis for future investigations in quality changes of whitespotted conger during frozen storage.

2. Materials and methods

2.1. Chemical reagents

Trichloroacetic acid solution (TCA), iodoacetamide (IAA), dithiothreitol (DTT), urea, ethylene diamine tetraacetic acid (EDTA), formic acid, and triethylammonium bicarbonate (TEAB) were supplied by Sigma-Aldrich Trade Co., Ltd. (Shanghai, China). Acetonitrile was purchased from Thermo Fisher Scientific Inc. (Shanghai, China). Trypsin was obtained from Promega Biotech Co., Ltd. (Beijing, China). Protease inhibitor was bought from Calbiochem Biotechnology Co. Ltd. (Shanghai, China). The other chemicals were all analytical grade and supplied by Yingrui Chemical (Qingdao, China).

2.2. Samples preparation

Fresh whitespotted conger (weight: 0.5 ± 0.1 kg, body length: 38.0 ± 3.0 cm, and body width: 3.4 ± 0.2 cm) was purchased from Qingdao, China in April 2023 (average daytime temperature: 17 °C) and transported to laboratory via using a seawater storage tank. Then, the whitespotted conger was manually operated by removing tissues such as the viscus, bones, and head. After washed with distilled water (4 °C), the processed fish were cut into sections of 5.0 cm in length and packaged in closed polyethylene bags. Finally, the whitespotted conger samples were stored at temperatures of −18 °C and −60 °C for frozen storage, respectively. At the set time points of 0, 20, 40, 60, 80, 100, and 120 days, whitespotted conger samples were thawed at 4 °C for 3 h prior to being tested in this study.

2.3. Optical microscope analysis

The microstructure of the whitespotted conger was analysed according to the method of Bao et al. (2020). Whitespotted conger samples were cut into small pieces (5.0 × 5.0 × 20.0 mm) and fixed with 4.0% paraformaldehyde for 24 h at 4 °C. Through embedded in paraffin and stained with hematoxylin-eosin (HE), the variations of muscle fibril in whitespotted conger samples were observed under a light microscope.

2.4. TVB-N content analysis

The TVB-N values of whitespotted conger samples were analysed via an automatic Kjeldahl analyser (JK-9830, Jinan Jingrui Analytical Instruments Ltd, Jinan, China) (Hu et al., 2022). The minced whitespotted conger sample (20.0 g) was added to deionized water (100.0 mL) for 30 min extraction. After filtered, the filtrate (10.0 mL) was combined with a magnesium oxide suspension (1.0%, w/v, 5.0 mL) and deionized water (10.0 mL) in a distillation tube. Boric acid solution (10.0 mL) and mixed indicator (methyl red and bromocresol green, 100 μL) were added to the receiving bottle, which was titrated by a standard titration solution of hydrochloric acid (0.1 mol/L) until to the endpoint. The TVB-N values of whitespotted conger samples were calculated according to equation (1):

$$X = \frac{(V_1 - V_2) \times c \times 14}{m \times (V/V_0)} \times 100 \quad (1)$$

where X (mg/100 g) was the content of TVB-N in whitespotted conger sample; V_1 and V_2 were the volumes of HCl standard titration solution consumed in the experimental and blank control groups, respectively; c was the concentration of HCl standard titration solution; m was the sample weight; V was the filtrate volume (10.0 mL in this study); V_0 was the total volume of the sample liquid (100.0 mL in this study).

2.5. MP content and total sulfhydryl content analysis

According to a previously reported method (Zhan et al., 2022), the minced whitespotted conger meat (3.0 g) was added to buffer A (15.0 mL) containing Tris-HCl (10.0 mmol/L, pH 7.2) and homogenized (T18, IKA, Guangzhou, China) for 3 min with 30 s interval. After the mixture was centrifuged at 6000 rpm for 10min (4 °C), the supernatant was extracted again. The resulting precipitate was homogenized with 15.0 mL buffer B (10.0 mmol/L Tris-HCl, 0.6 mol/L NaCl, pH 7.2), and applied to extract the MP at 4 °C for 1 h. Finally, the prepared MP solution was stored at −80 °C and measured within 2 days.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

To investigate the changes of proteins in whitespotted conger samples during frozen storage at −18 °C and −60 °C, total proteins were extracted and analysed using SDS-PAGE (Tai et al., 2023). Whitespotted

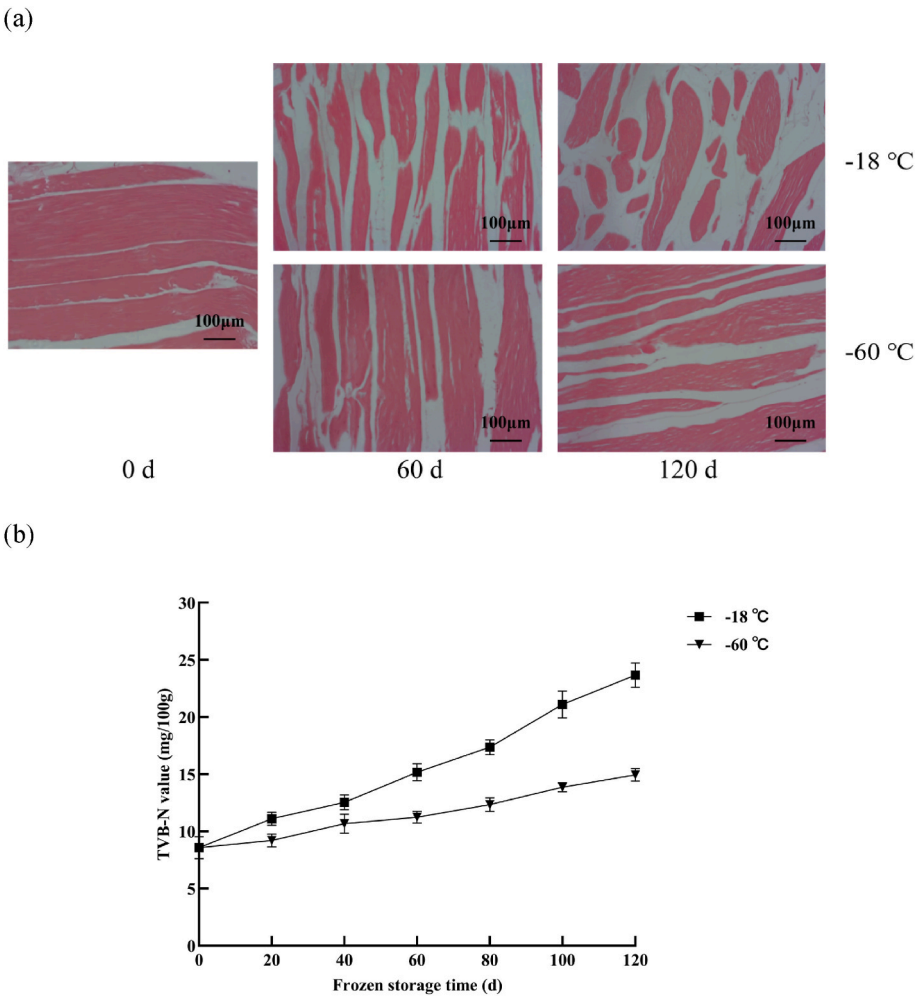

Fig. 1. Changes in the microstructure (a) and total volatile base nitrogen (TVB-N) content (b) of whitespotted conger samples during storage at −18 °C and −60 °C.

conger sample (2.0 g) was homogenized with 5.0% SDS solution (18.0 mL) to prepare the total protein solution. This mixture was then centrifuged at 10000 rpm for 30 min and incubated in a water bath at 95 °C for 10 min. Then, the mixture of 10.0 μL total protein solution and 10.0 μL 2 × SDS-PAGE loading buffer was heated in boiling water for 5 min. After cooling, the mixture was centrifuged at 12,000 rpm to obtain the supernatant, which was used for loading into the gradient gel (12.0% SDS-PAGE). During SDS-PAGE, a voltage of 80 V was set until the protein samples reached the separation gel (~30 min), followed by increasing the voltage to 120 V for another 60 min. Finally, Coomassie brilliant blue dye was utilized to stain the protein bands.

2.7. Protein extraction and digestion

The minced whitespotted conger sample (100.0 mg) was mixed with four volumes of buffer solution containing protease inhibitor (1.0%, v/v), Tris-HCl (100.0 mmol/L), and urea (8.0 mol/L) in a centrifuge tube. Subsequently, the mixture was sonicated for 2 min using an ultrasonic processor (JP-080ST, Skymen Technology Co., Ltd., Shenzhen, China). After centrifuged at 12,000 g (4 °C for 10 min), the supernatant was taken to obtain protein. Finally, the protein concentrations of whitespotted conger samples were determined by a bicinchoninic acid (BCA) kit (Beyotime Biotechnology, Shanghai, China) according to the instructions.

The digestion reaction was performed with the total protein and reducing agent buffer at 37 °C for 1 h. IAA with a final concentration of 50.0 mmol/L was added for protein denaturation in the dark for 15 min, which was ceased by 1.0 mol/L DTT solution. 50.0 mmol/L NH_4HCO_3 solution was employed to achieve the final concentration of urea below 1.0 mol/L. Subsequently, sequencing-grade trypsin solution was added, followed by overnight incubation at 37 °C, and the resulting peptides were then desalted using C18 solid phase extraction cartridges. Finally, the obtained peptide solution was freeze-dried in a lyophilizer in vacuo.

2.8. LC-MS/MS analysis

LC-MS/MS analysis was performed by an ultra-high performance liquid system (EASY-nLC 1200, Thermo Fisher Scientific, Waters, USA), which was used to separate the peptides dissolved in phase A (0.1% (v/v) formic acid solution) of the liquid chromatography mobile phase. The analysis time was set at 120 min, and the experimental gradient B phase (acetonitrile with 0.1% (v/v) formic acid solution) increased from 8.0% to 100.0%. The flow rate was maintained at 300.0 nL/min.

The separated peptides were injected into an NSI ion source for ionization and then detected by mass spectrometer (Q Exactive HF, Thermo Fisher Scientific, Waters, USA). The ion source voltage was set at 2.3 kV, and both peptide precursor ions and secondary fragments were tested and analysed using a high-resolution Orbitrap in Q Exactive HF. The primary mass spectrometer scanning range was set from 400 to 1800 m/z with a resolution of 60,000, and the secondary scan had a resolution of 15,000. In the data acquisition mode, the top 20 peptide parent ions with highest signal intensity were selected and entered into the higher energy collision dissociation (HCD) collision cell for fragmentation with a fragmentation energy of 28 ev, and the second-level

mass spectrometry was also performed sequentially. To enhance the effective utilization of mass spectrometry, the automatic gain control (AGC) was set at 3E6, 10000 ions signal threshold, and the maximum ion injection time at 50 ms. Meanwhile, 45 s dynamic exclusion time for tandem mass spectrometry scanning was set in this research to prevent repeated scans of precursor ions.

2.9. Protein identification

After searched and analysed via the software Proteome Discoverer 2.5, the obtained protein list was conducted to match with various databases. The specific analysis parameters were as follows: false discovery rate (FDR), ≤0.01; peptide confidence, high; fragment mass tolerance, 0.02 Da; peptide mass tolerance, 10 ppm; variable modifications, oxidation (M), acetylation on protein (N-terminus), met-loss/−131.040 Da (M) (N-terminus), and met-loss + acetyl/−89.030 Da (M) (N-terminus); fixed modifications, carbamidomethyl (C); max missed cleavages, 2; and the cleaving enzyme, trypsin. The target proteins were identified through the analysis of MS/MS data matched with the UniProt database.

2.10. Bioinformatics analysis

During the process of protein identification, the proteins with calculated fold changes (FC) greater than 1.2 or less than 0.83 ($P < 0.05$) were considered as the differentially abundant proteins (DAP). Cluster of Orthologous Groups (COG) protein database (including 26 categories) (http://www.ncbi.nlm.nih.gov/COG/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (www.kegg.jp/kegg/pathway.html) were used for annotation and functional classification analysis of DAP.

2.11. Statistical analysis

All measurements were performed independently in triplicate and the experimental data collected in this study were expressed as mean ± standard deviation (SD). The statistical analysis was calculated by One-Way ANOVA test with SPSS 26.0 (IBM SPSS Statistics, Chicago, IL, USA). Duncan's test was performed to identify the significant differences ($P < 0.05$). Figures were drawn via Origin 2022 (OriginLab, Northampton, MA, USA) and GraphPad Prism 9.5 (GraphPad Software, SD, USA).

3. Results and discussion

3.1. Microstructural characteristics of whitespotted conger

The microstructural variations of whitespotted conger samples in longitudinal sections after frozen storage at −18 °C and −60 °C were observed by optical microscopy and shown in Fig. 1a. Generally speaking, the whitespotted conger muscle is composed of connective tissue and muscle fibre. The muscle fibre of fresh whitespotted conger (0 d) were intact and closely arranged due to suffering fewer effects of internal and external factors such as protease, oxidation, and other factors. However, after being stored at −18 °C for 60 days, the phenomenon of increased gap and fracture between muscle fibre appeared in the microstructures of whitespotted conger samples, which was owing to the degradation effects occurred in connective tissue and muscle fibre reduced the elasticity and extensibility of whitespotted conger muscle (Zhang et al., 2020a). With the frozen storage time increasing, the micromorphology of muscle fibre gradually became irregular. Until to 120 days, the fracture between muscle fibre in −18 °C group was more significant than those in fresh group, resulted in only a small proportion of muscle fibre maintaining the original morphology. Compared to −18 °C group, the degradation degree of muscle fibre in −60 °C group was relatively lower at 60 days, and a slight fibre fracture occurred at 120 days. These results showed that the muscle structure of whitespotted conger could be better maintained in −60 °C frozen storage due to the lower degradation level of muscle fibre.

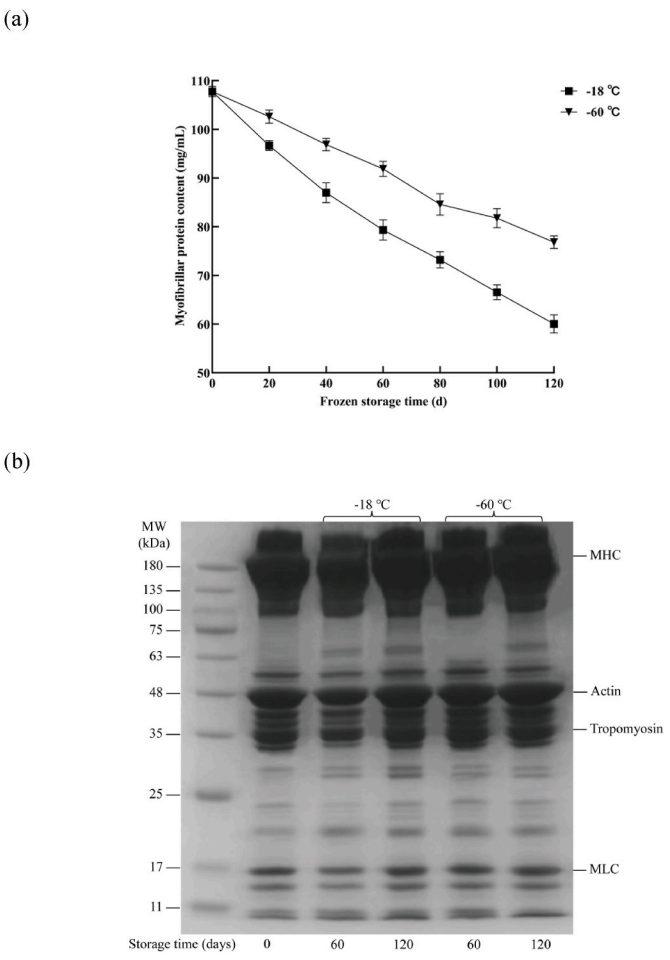

Fig. 2. Changes in myofibrillar proteins contents (a) and SDS-PAGE profile (b) of whitespotted conger samples during storage at −18 °C and −60 °C. MHC: myosin heavy chain. MLC: myosin light chain.

3.2. TVB-N analysis

TVB-N value as an indicator of freshness level reflects the degradation degree of proteins in aquatic products. With the action of endogenous enzymes and microorganisms, proteins were decomposed into volatile basic nitrogen-containing substances such as ammonia and amines, which could cause TVB-N values increase (Qian et al., 2023). According to the Chinese Aquatic Standard (ST3106-2010) protocol, TVB-N value should be no more than 15.0 or 30.0 mg/100 g for first-grade or qualified frozen whitespotted conger, respectively. In this study, the initial TVB-N value of fresh whitespotted conger (0 d) was 8.57 ± 0.96 mg/100 g, and increased to 15.17 ± 0.74 mg/100 g at −18 °C and 11.23 ± 0.51 mg/100 g at −60 °C after a storage period of 60 days (Fig. 1b). At the end of frozen storage (120 days), the TVB-N values of whitespotted conger in −60 °C group was 14.93 ± 0.55 mg/100 g which approached the first-grade of Chinese Aquatic Standard (15.0 mg/100 g), while the TVB-N values of whitespotted conger sample in −18 °C group raised to 23.67 ± 1.07 mg/100 g. Thus, the whitespotted conger samples stored at −18 °C could not reach the first-grade level of the Chinese Aquatic Standard (ST3106-2010) during 60 and 120 days frozen period. It was indicated that the relatively lower storage temperature (−60 °C) could significantly slow down the degradation degree of proteins in whitespotted conger samples by reducing the speed of enzymatic reactions and microbial growth, but not completely inhibited the vital activities involved in degraded proteins (Ye et al.,

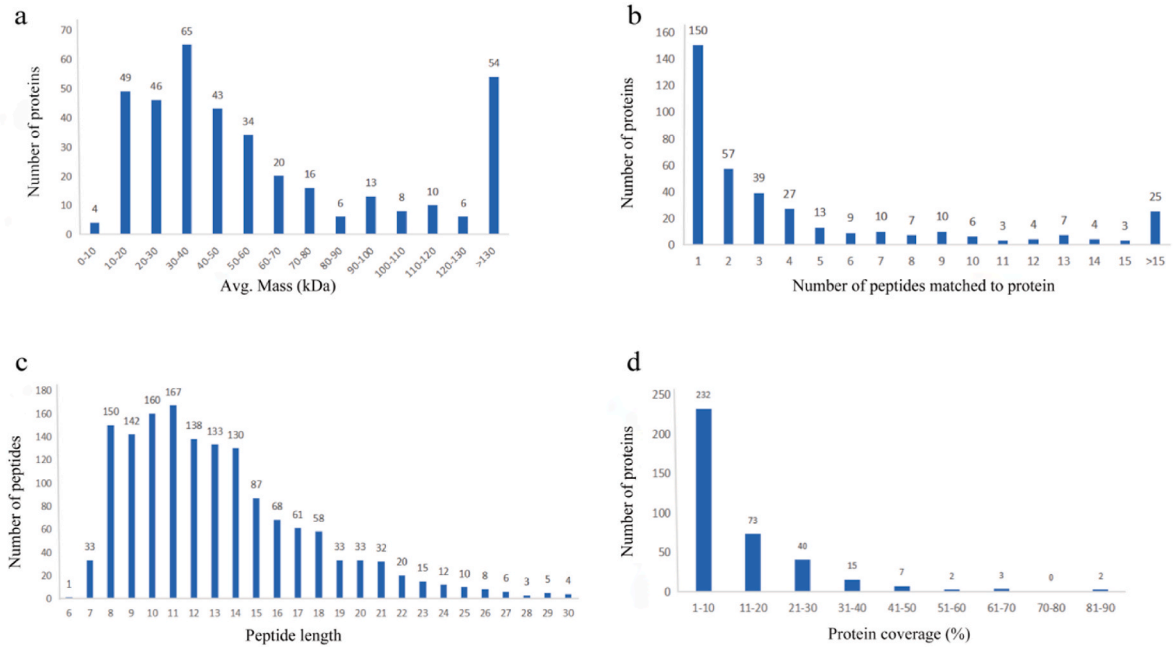

Fig. 3. (a) Molecular weight, (b) peptide count, (c) lengths of peptides in the amino acids, and (d) protein coverage of the detected proteins in the whitespotted conger muscle identified by label-free proteomics analysis.

2021).

3.3. Protein degradation analysis

MP plays a pivotal role in the structure and function of muscle tissue. The MP contents of whitespotted conger muscle showed a declining trend with the extension of frozen storage time at −18 °C and −60 °C (Fig. 2a). After 120 days frozen storage, the MP contents in −18 °C group and −60 °C group decreased to 44.28% and 28.72%, respectively, which were significantly lower than that in fresh whitespotted conger (107.76 mg/mL). This might be due to the destruction of protein peptide bonds and protein structure by endogenous enzymes hydrolysis and ice crystal growth during the low-temperature storage process, which leaded to the changes in physicochemical properties and physiological function of MP (Gómez-Estaca et al., 2020). Previous studies have also reported the similar changes in MP contents of hairtail during frozen storage (Yan et al., 2023). Therefore, the MP contents of whitespotted conger samples in −60 °C group were significantly higher than that in −18 °C group ($P < 0.05$). These results of MP contents variations were also consistent with those of TVB-N, which further supported that lower temperature (−60 °C) could slow down the protein loss and oxidation of whitespotted conger samples during frozen storage.

Fig. 2b showed the SDS-PAGE analysis of proteins extracted from whitespotted conger muscles stored at −18 °C and −60 °C. Myosin heavy chain (MHC), actin, and tropomyosin were the main proteins of the fish muscle fibre. With frozen storage time extension, the proteins of whitespotted conger samples stored at −18 °C and −60 °C degraded in varying degrees and appeared the notable shift on 120 days. For the whitespotted conger samples stored at −18 °C for 120 days, the bind of MHC and tropomyosin exhibited an indistinct strip. In addition, the bands between 17 and 25 kDa have increased and deepened in colour, which implied that a protein was degraded into peptides or other low molecular weight compounds (Xiong et al., 2020). In contrast, the MHC with a molecular weight of about 200 kDa was more complete in the whitespotted conger stored at −60 °C than stored at −18 °C, indicated that −60 °C frozen storage could effectively reduce the degree of protein degradation in whitespotted conger. Besides, the decrease of myofibrillar proteins was attributed to the degradation of MHC and tropomyosin (Fig. 2b). Therefore, myofibrils, MHC, actin, and tropomyosin played a major role in maintaining the cytoskeleton, whose degradation could significantly impact the texture of whitespotted conger muscle.

3.4. Label-free proteomic analysis

3.4.1. Proteins identification

The proteomic analysis of whitespotted conger samples was carried out via LC-MS/MS after stored at −18 °C and −60 °C for 0 days, 60 days, and 120 days. A total of 1519 peptides and 353 proteins (including at least one unique peptide) were identified with an FDR confidence interval of ≤0.01 through label-free proteomic analysis. Then, mass spectrometry data were subjected to quality control assessments, encompassing protein mass, number of peptides matched to protein, peptide length, and protein coverage (Fig. 3).

As shown in Figs. 3a and 63.0% of identified proteins (237 proteins) had a molecular weight ranging from 10 kDa to 60 kDa, and the proportion of proteins with a molecular weight exceeding 130 kDa was 14.0% (54 proteins), which represented the natural distribution of molecular weights in fresh whitespotted conger muscle. Apart from that, less than 7.0% of the identified proteins (25 proteins) contained more than 15 peptides, and fewer than 10 peptides were found in 93.0% of the identified proteins (328 proteins). The relative molecular weights of peptides were determined with minimal error, ensuring reliable results (Fig. 3b). Additionally, 94.0% of the detected peptides (1425 peptides) ranged from 7 to 21 amino acids in length, and less than 6.0% of detected peptides had more than 21 amino acids in length, which coincided with the effect of trypsin digestion on the proteins (Fig. 3c) (Chu et al., 2019). Furthermore, the identified proteins showed relatively lower protein coverage, 38.0% (142 proteins) of which had more than 10.0% sequence coverage (Fig. 3d). This might be owing to the limited whitespotted conger proteins information available in the UniProt database. Therefore, the results verified that trypsin digestion and LC-MS/MS detection with high reliability and feasibility could be employed in Label-free proteomic analysis of whitespotted conger samples. Moreover, it demonstrated the appropriateness of this approach for subsequent bioinformatics analysis.

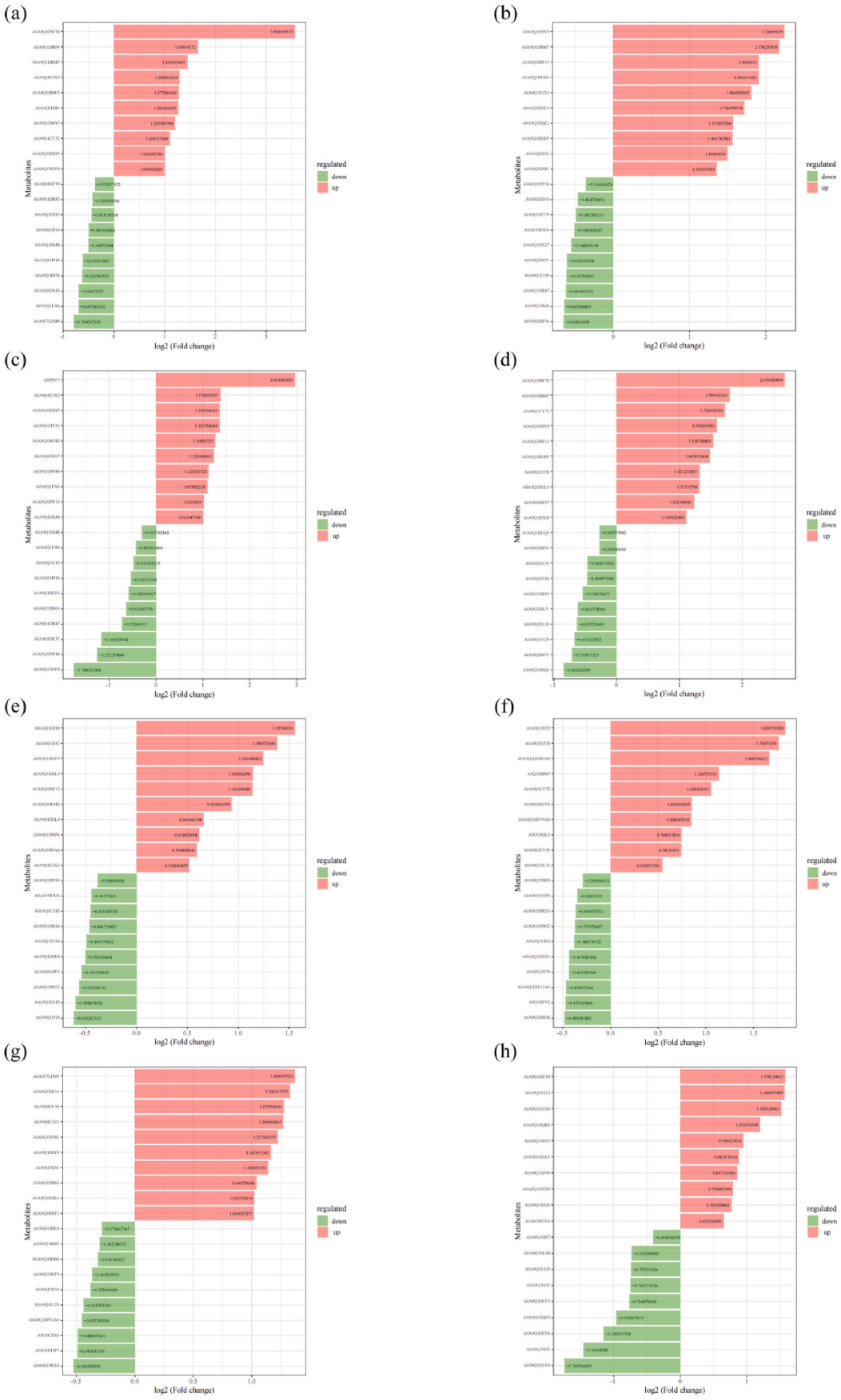

Fig. 4. The difference multiples bar charts of the DAP in the −18 °C-60 d *vs.* 0 d (a), −60 °C-60 d *vs.* 0 d (b), −18 °C-120 d *vs.* 0 d (c), −60 °C-120 d *vs.* 0 d (d), −60 °C-60 d *vs.* −18 °C-60 d (e), −60 °C-120 d *vs.* −18 °C-120 d (f), −18 °C-120 d *vs.* −18 °C-60 d (g), and −60 °C-120 d *vs.* −60 °C-60 d (h) comparisons.

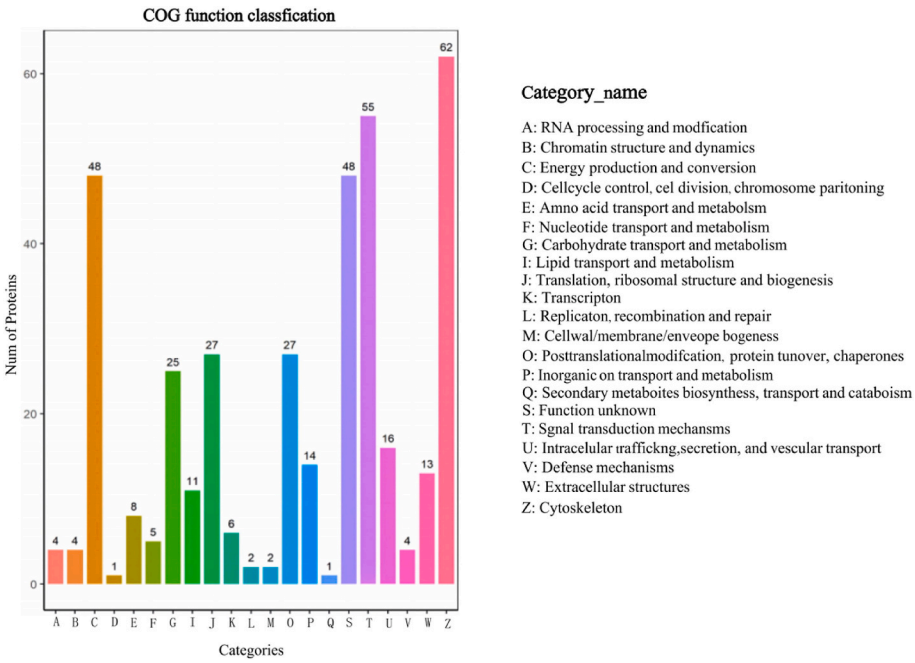

Fig. 5. DAP clarified by COG annotation.

3.4.2. *DAP analysis*

Label-free proteomics was used to reveal the compositions and alterations of proteins in whitespotted conger after frozen storage with different temperatures (−18 °C and −60 °C) during 60 days and 120 days. DAP were identified according to FC > 1.2 or FC < 0.83 ($P < 0.05$). Compared with the fresh group, 24, 25 proteins were up-regulated, and 16, 24 proteins were down-regulated in the samples stored at −18 °C and −60 °C for 60 days, respectively. At 120 days, 29 up-regulated proteins and 11 down-regulated proteins occurred in −18 °C group, while there were 23 up-regulated proteins and 18 down-regulated proteins in −60 °C group. The results showed that there were more DAP in −60 °C group than that in −18 °C group. This might be attributed to the activation of proteins that inhibited the apoptosis and maintained the normal cell function after death, which caused an increase in the number of DAP (Zimmermann et al., 2001). Moreover, protein degradation occurred at a faster rate in −18 °C group compared to the −60 °C group, leaded to a smaller number of identified DAP. What's more, 11 proteins were up-regulated and 30 proteins were down-regulated in the samples stored at −60 °C for 60 days compared to those stored at −18 °C (Fig. 4). In the comparison of −60 °C-120 d *vs.* −18 °C-120 d, 16 proteins were up-regulated while 50 proteins were down-regulated. With the extension of frozen storage time, the up-regulated proteins and down-regulated proteins significantly rose, which suggested that the increased frozen storage time could amplify the impacts of frozen storage temperature on protein compositions of whitespotted conger. In two comparison groups of −60 °C-60 d *vs.* −18 °C-60 d, and −60 °C-120 d *vs.* −18 °C-120 d, 64 and 34 DAP were identified at −18 °C and −60 °C, respectively (Fig. 4). So the number of DAP would decrease with the frozen temperature falling. These results suggested that a lower frozen storage temperature could diminish the impact of frozen storage time on the protein compositions of whitespotted conger.

Besides, all DAP changes of whitespotted conger samples mainly focused on structural domain proteins, muscle-related proteins, and metabolic enzymes (Table S1 S2). In the −18 °C-60 d *vs.* 0 d, −60 °C-60 d *vs.* 0 d, −18 °C-120 d *vs.* 0 d, and −60 °C-120 d *vs.* 0 d groups, large amounts of domain-containing proteins (A0A9Q1HSV9, A0A9Q1DBM7, A0A9Q1HW15, A0A9Q1E1X3, A0A9Q1DQZ2, A0A9Q1I452, A0A9Q1D981, A0A9Q1HW78, A0A9Q1DBP4, A0A9Q1D6N7, A0A9Q1E378, A0A9Q1CY72, A0A9Q1DF07) and ribosomal proteins (A0A9Q1DUH3, A0A9Q1DGL0) were significantly up-regulated, which might be caused by the growth and recrystallization of ice crystals in muscle tissue during frozen storage (Ma et al., 2015). Compared with fresh samples, the energy metabolism enzymes (A0A9Q1HT79, A0A9Q1DR87, AOA9Q1DZI5, A0A9Q1HP34, A0A9Q1I010, AOA9Q1DS33, A0A9Q1E190, and AOA9Q1D6J8) and muscle-related proteins (A0A9Q1DI74, A0A455R7U6, A0A9Q1HX27, A0A9Q1D3V9) were significantly down-regulated in −18 °C and −60 °C groups. In the frozen storage groups, the degradation of metabolic enzymes mainly appeared in phosphoglycerate kinase, fructose-bisphosphate aldolase, glycerol 3-phosphate dehydrogenase, and some other glycolysis/gluconeogenesis, enzymes, which was consistent with previous studies on bighead carp (Gao et al., 2024). The ice crystal formation during frozen storage exerted a significant destructive effect on the structures of metabolic enzymes, and leaded to the degradation of polypeptides. In addition, the degradation of myofibrillar composition related proteins such as troponin I, tropomodulin, and myosin-binding protein H also occurred during frozen storage, which resulted in the alterations of muscle structure (Bao et al., 2020; Gao et al., 2023).

In −60 °C-60 d *vs.* −18 °C-60 d and −60 °C-120 d *vs.* −18 °C-120 d groups, differentially up-regulated proteins included structural domain proteins (A0A9Q1I452, A0A9Q1HSV9, A0A9Q1HW15, A0A9Q1HNA6, A0A9Q1E1X3, A0A9Q1I972, A0A9Q1E378, A0A9Q1DBM7), ribosomal proteins (A0A9Q1DGL0, A0A9Q1DUH3), and muscle-related proteins (A0A9Q1CY72, A0A9Q1D3V9, A0A9Q1D3L0, A0A9Q1DNP6, A0A9Q1CY45). These results indicated that lower storage temperature (−60 °C) was conducive to inhibiting the degradation of myofibrillar proteins. Ribosomes are composed of a smaller 40S subunit and a larger 60S subunit due to the intricate nature of organelles. Ribosomal proteins play a crucial role in facilitating protein synthesis and ensuring the stability of tRNA molecules during transcription. Prior research showed that the 40S and 60S ribosomal proteins of shrimp soaked in distilled water were down-regulated during storage at −18 °C for 30 days, which was related to the decrease in extraction rate owing to protein denaturation (Zhang et al., 2020b). Energy metabolic enzymes (A0A9Q1E190, A0A9Q1I510, A0A9Q1I779, A0A9Q1HPV2) were significantly down-regulated, which was caused by the loss of energy at lower temperatures. Previous studies have shown that phosphoglycerate kinase was related to the softening of fish flesh

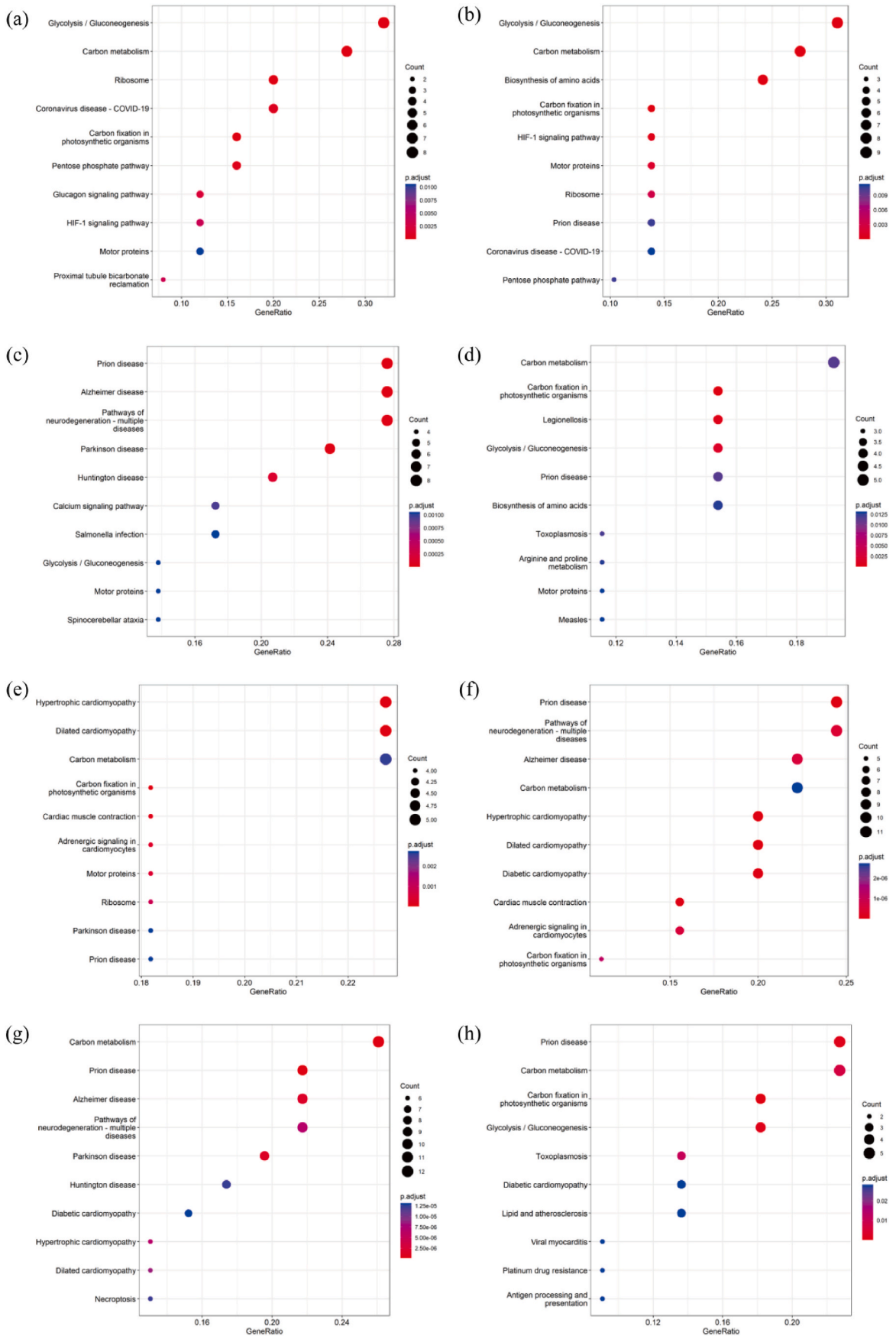

Fig. 6. KEGG pathway enrichment analysis of the DAP in the −18 °C-60 d *vs.* 0 d (a), −60 °C-60 d *vs.* 0 d (b), −18 °C-120 d *vs.* 0 d (c), −60 °C-120 d *vs.* 0 d (d), −60 °C-60 d *vs.* −18 °C-60 d (e), −60 °C-120 d *vs.* −18 °C-120 d (f), −18 °C-120 d *vs.* −18 °C-60 d (g), and −60 °C-120 d *vs.* −60 °C-60 d (h) comparisons.

(Santos et al., 2019). The experimental results showed that −60 °C inhibited the activities of phosphoglycerate kinase and other metabolic enzymes in whitespotted conger samples, which indicated that the activity of muscle mitochondria was inhibited at low temperature, thereby reducing energy supply and glycolysis to produce lactic acid, and finally slowing the decline of fish quality (Nakazawa et al., 2022). However, with the extension of frozen storage time, the difference between metabolic enzymes decreased due to enzymes degradation (Fontanesi et al., 2008). Moreover, in the comparison of −18 °C-120 d *vs.* −18 °C-60 d and −60 °C-120 d *vs.* −60 °C-60 d groups, domain-containing proteins (A0A9Q1D130, A0A9Q1DV81, A0A9Q1E213, A0A9Q1HQR3), energy and nucleotide metabolic enzymes (A0A0F7LFM9, A0A9Q1DIV8, A0A9Q1I261, A0A9Q1DBS8, A0A9Q1HZK5, A0A9Q1HNF1) were significantly down-regulated. These results demonstrated that the extension of frozen storage time could exacerbate the myofibril protein degradation and energy loss.

3.4.3. Bioinformatics analysis of DAP

3.4.3.1. Cluster of Orthologous Groups (COG) annotation analysis. According to the function analysis, COG annotation could be divided into a total of 26 distinct categories (http://www.ncbi.nlm.nih.gov/COG/). Based on COG annotation analysis, the DAP identified in whitespotted conger samples were classified into 21 distinct functional categories, which were associated with the metabolic pathways such as cytoskeleton, posttranslational modification, protein turnover, chaperones, signal transduction mechanisms, energy generation and conversion, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism (Fig. 5).

During frozen storage, cytoskeletal functional proteins would be destroyed, which were crucial in the structure and function of muscle tissues. Simultaneously, microbial metabolites and endogenous enzymes could facilitate protein hydrolysis or degradation, disrupting the natural myofibril structures in muscle tissue and resulting in a decrease of cytoskeletal functional proteins in whitespotted conger samples. Therefore, the number of cytoskeleton functions in annotated DAP was the largest, accounting for 16.6% of total DAP. Signal transduction refers to all biochemical processes by which cells translate extracellular signals originating from the environment into specific responses. Metabolic enzymes mentioned above were participated in this process, such as phospholipases, kinases, and phosphatases.

3.4.3.2. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis. KEGG pathway enrichment analysis was applied to identify the key metabolic pathways and signal transduction pathways associated with the DAP. All DAP were annotated and mapped to pathways in the KEGG database, and the top 10 pathways were selected for analysis (Fig. 6).

In the comparisons of −18 °C-60 d *vs.* 0 d, −60 °C-60 d *vs.* 0 d, −18 °C-120 d *vs.* 0 d, and −60 °C-120 d *vs.* 0 d, KEGG pathway analysis revealed that the DAP pathways were primarily focused on glycolysis/gluconeogenesis, carbon metabolism, ribosome, and biosynthesis of amino acids (Fig. 6abcd). It was due to the cells entered a state of hypoxia and needed to provide enough energy for metabolic activities after organism death, and all cellular components (sugars, amino acids, and lipids) were potentially consumed for energy production (Gagaoua et al., 2021). When the whitespotted conger samples were subjected to a frozen storage temperature of −60 °C for 60 days, the KEGG pathway analysis displayed that the carbon metabolism was significantly altered compared with that in −18 °C (Fig. 6e). After being frozen at −60 °C for 120 days, the comparative analysis with the −18 °C-120 d group showed the significant variations in proteins primarily associated with carbon metabolism and calcium signalling pathways (Fig. 6f). Normally, calcium signalling is an intracellular signal transduction pathway that involves changes in intracellular calcium ion concentration and plays a important role in the regulation of cell growth, differentiation, and death. Therefore, the variations in calcium signalling pathways disclosed the rising degradation of proteins and the quality deterioration in whitespotted conger samples during frozen storage at −18 °C. However, the main pathway of DAP was carbon metabolism when whitespotted conger stored at −60 °C for 60 and 120 days. These results certified that the frozen storage temperature of −60 °C inhibited the quality deterioration of whitespotted conger samples during frozen storage. And carbon metabolism and glycolysis/gluconeogenesis were the main metabolic pathways of DAP after stored at −18 °C or −60 °C frozen storage. In addition, the changes in DAP numbers were less in −60 °C group than that in −18 °C group after frozen storage, which indicated that various metabolic processes progressed slowly and the quality loss of whitespotted conger was effectively delayed at −60 °C frozen storage (Fig. 6g and h).

4. Conclusion

During frozen storage, the muscle fibre of whitespotted conger were degraded and gradually fractured or vanished. The TVB-N values exhibited an increase, which indicated a decline in freshness for whitespotted conger. The reduction in MP content was attributed to the progressive degradation of proteins during frozen storage. Actin and tropomyosin were also degraded that resulted in the lighter bands observed in SDS-PAGE. Label-free proteomics analysis identified 353 DAP in whitespotted conger samples. Among these DAP, domain-containing proteins, ribosomal proteins, histones, and metabolic enzymes were more prone to denaturation/oxidation mainly resulted from the frozen storage temperature and time. This study provided a basic database to understand the alterations of protein profiles in whitespotted conger muscle during frozen storage.

KEGG analysis revealed the DAP were mainly distributed in glycolysis/gluconeogenesis, carbon metabolism, amino acid biosynthesis, and calcium signalling pathway. Lower frozen storage temperatures inhibited the above pathways and protected the integrity of muscle proteins. The findings of this study provided insight into the impact of frozen storage on the protein changes in whitespotted conger and revealed the deeper mechanisms of protein degradation and oxidation during long-term frozen storage. However, the specific effects of those degradation and oxidation products on whitespotted conger quality and nutritional attributes need to be further focused on in future studies.

CRediT authorship contribution statement

Hui Chen: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Data curation. **Yinggang Ge:** Investigation, Validation, Methodology. **Ting Yang:** Methodology, Writing – review & editing. **Shanshan Wang:** Supervision. **Nan Liu:** Software, Validation. **Yong Sun:** Writing – review & editing. **Deqing Zhou:** Project administration, Funding acquisition. **Rui Xi:** Project administration. **Guohui Sun:** Conceptualization, Supervision, Project administration, Validation, Writing – review & editing, Funding acquisition.

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.

Acknowledgment

This work was supported by the financial support of the Central Public-interest Scientific Institution Basal Research Fund, YSFRI, CAFS (NO. 20603022022016), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO. 2023TD72) and National Key R&D Program of China (Grant No. 2019YFD901703).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100779.

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