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Label-free based proteomics revealed the specific changes of muscle proteins in pike eel (*Muraenesox cinereus*) under cold stress

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ABSTRACT

Chemical- and liquid chromatography coupled with mass spectrometry (LC–MS) based proteomics strategies were executed to investigate the alterations of protein profiles in pike eel (*Muraenesox cinereus*) muscle during chilling (CPE) and frozen (FPE) storage. Chemical results indicated that springiness and myofibrillar protein (MP) content of muscle tissues decreased significantly during 6 days of chilled and 120 days of frozen storage. LC–MS-based proteomics analysis suggested that great alterations occurred in muscle proteins mainly induced by cold stress. The differentially abundant proteins (DAPs) with low abundances in CPE and FPE samples included the annexins, fibronectin, ribosomal proteins, *T*-complex proteins, tubulin beta chain, and histones, which were mostly associated with the membrane structural constituents, cytoskeleton, and binding functional proteins. Results of eukaryotic cluster of orthologous group (KOG) verified that these identified DAPs were mainly converged in the cytoskeleton function resulting from cold conditions, which in turn affected the physical structure and chemical performances of muscle tissues.

Introduction

Pike eel (*Muraenesox cinereus*) as one of the main valuable marine resources is widely distributed in the coastal areas of China, Japan, and South Korea as well as in the Arafura Sea and north Australia. Pike eel products have grown increasingly popular around the world because of the high nutritional benefits of their polyunsaturated fatty acids (PUFAs), proteins/peptides, mineral elements, essential amino acids, and other useful substances (Bi et al., 2018). They are commonly processed in large quantities into refrigerated, roasted, pickled, or semi-dried muscle products before being transported, exported, and consumed. Previous studies on the abundant nutritional compounds in fresh eel muscle, the drying process and its kinetics, quality changes during different drying processes, and roast cooking process have been conducted (Li et al., 2017; Bi et al., 2018). However, according to the literatures, no detailed investigations on the protein denaturation and the alterations of pike eel muscle tissues under cold stress or other conditions have been conducted in depth.

Fresh pike eel is very susceptible and tends toward undesirable deterioration after being captured, mainly due to its high unsaturated fatty acid content and abundant endogenous enzymes in its muscle tissues (Xie et al., 2020). Refrigerated and subsequent frozen storage as preferred processing methods can effectively retard biochemical variations and inhibit bacterial growth in the muscle tissues; however, denaturation and oxidation of the muscle proteins still occur in frozen tissues, especially during long-term storage, which lead to the deterioration of fish quality (i.e., off-flavors, texture degradation, drip loss, discoloration, and even rancidity of muscle products). More importantly, the development of protein denaturation/oxidation is rather complex, especially concerning numerous intermediates generated with different molecular weights, subunits, structures, and spatial conformations. Therefore, it is difficult to illustrate the denaturation processes and specific alterations of the target proteins, especially on the molecular level, occurring in the muscle tissues according to traditional physical and chemical procedures. Additionally, the current findings on the stability and functional properties of muscle proteins in the pike eel during cold storage have been inconclusive.

Proteomics is a very powerful strategy for describing thousands of protein profiles and their correlations, compositions, and functions within organisms while also identifying specific protein species that

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differ in the number of amino acids and peptide lengths (Picard & Gagaoua, 2020). Liquid chromatography coupled with mass spectrometry (LC–MS)-based proteomics studies have been comprehensively performed to investigate the changes in protein metabolism and its compositions to distinguish species, illuminate functions, and identify protein biomarkers in organisms (Zhou et al., 2021). Label-free proteomics strategy is a reliable, versatile, and cost-effective approach, which is performed to compare relative abundances of proteins across multiple LC–MS experiments without utilizing isotopic tags or stable isotope. It is directly based on the observation that the amount of protein correlates well with spectral counts or peak intensities of peptides unique to a specific protein. Previous proteomics studies have explored protein compositions to distinguish jumbo squid (*Dosidicus gigas*) from neon flying squid (*Ommastrephes bartramii*) (Shui et al., 2021), identify biomarkers in Alaska pollock, Atlantic cod, and Greenland halibut (Chien et al., 2021), and detect fish species in mixed samples (Varunjikar et al., 2022).

Although these reports contributed toward understanding the similarities and differences of the target proteins, the detailed associations between protein profiles and the denaturation occurring in muscle tissues still require further investigation. The present study was carried out to investigate in detail the effect of cold storage on the protein changes occurring in pike eel (*M. cinereus*) muscle by using chemical and proteomics analyses. This study provides a underlying database for the muscle protein compositions and alterations affected by cold stress and also provide fundamental basis for the denaturation mechanisms of muscle proteins in pike eel during cold storage.

Materials and methods

Chemical reagents

A bicinchoninic acid (BCA) protein assay kit and a protease inhibitor cocktail were obtained from Beijing Bio-Fly Biotech Co., Ltd. (Beijing, China). Carbamide, NH_4HCO_3, formic acid, acetonitrile, and urea, were supplied by Sigma-Aldrich Trade Co., Ltd. (Shanghai, China). Trypsin was purchased from Promega Biotech Co., Ltd. (Beijing, China). Tris (hydroxymethyl)aminomethane, dithiothreitol, and iodoacetamide were supplied by Gen-View Scientific Inc. (Beijing, China).

Fish samples and treatments

Fresh pike eel (*M. cinereus*) with a weight of 1.35 ± 0.16 kg and a body length of 68 ± 8 cm was commercially purchased from a local fish market in Zhoushan (Zhejiang, China; harvested in August 2021). The obtained fish samples (10 specimens; K-values were determined as 1.8–3.2%) were packaged in flake ice in a cooler (0–4 °C, at atmospheric pressure) and immediately transported to our lab in Zhejiang Ocean University within 30 min. Upon arrival, the fish were removed from the cooler and quickly washed with distilled water (0–4 °C). Next, the samples were manually prepared by removing the viscus, fins, and the head, but the samples were not deboned. Afterward, the processed samples were cut into sections 6 cm in length. The resulting fish pieces were placed onto plastic trays and packaged in polyethylene bags. Finally, the packaged samples were separately maintained at 0 °C for 6 days and at − 18 °C for 120 days (according to the previous experiment). At different periods, the fish samples were collected, thawed at 0–4 °C for 3 h, and then analyzed.

Texture profile and myofibrillar protein (MP) content analysis

The springiness of the fish muscle was determined using a TMS-Pro texture analyzer (VA, USA) according to the procedures previously reported by Ying et al. (2021). MPs extracted from pike eel were prepared, and their content was measured using the method described by Shui et al. (2021).

Label-free proteomics analysis

Label-free based proteomics analyses of fresh pike eel (0 days; PE), chilled pike eel (4 days, as a representative; CPE), and frozen pike eel (120 days, as a representative; FPE) were carried out to evaluate the effects of cold storage on the alterations of proteins in pike eel muscle. Three pike eel samples (mashed muscle; 4 °C) from each group were pooled together, and three replicates were collected from these pooled samples as the technical replicates in this experiment.

Protein extraction and digestion

Briefly, the fish muscle (100 mg) was homogenized in liquid nitrogen and then mixed with four volumes of buffer solutions containing 1% (v/v) protease inhibitor cocktail, 100 mmol/L Tris-HCl, and 8.0 mol/L urea in a 5-mL centrifuge tube. Afterward, the mixture was sonicated on ice using an ultrasonic processor (UP-400, Shanghai OuHor Mechanical Equiptment Co., Ltd., Shanghai, China), followed by centrifugation (Pico 17 Centrifuge, Thermo Scientific, Bremen, Germany) at 10,000 × g for 20 min (4 °C). The collected protein sediment was incubated in the buffer solutions (containing 1% (v/v) protease inhibitor cocktail, 100 mmol/L Tris-HCl, and 8.0 mol/L urea), homogenized, and extracted again. The protein concentration of the collected supernatant was then measured using a BCA kit according to its assay instructions. The protein extracts (including 20 μL of quality control (QC) samples) were used for the following LC–MS analysis.

The resulting extracted proteins (50 μg) were first reduced with 1 mol/L dithiothreitol in a water bath at 37 °C for 1 h. The proteins were then further alkylated with 1 mol/L iodoacetamide in a dark room at 25 °C for an additional hour. Next, 100 μL Tris-HCl buffer containing 8.0 mol/L urea was added to the protein solution. After centrifugation at 12,000 × g for 5 min, 50 mmol/L NH_4HCO_3 solution was added to the collected supernatant. Afterward, the obtained mixture was centrifuged (Pico 17 Centrifuge, Thermo Scientific, Bremen, Germany) at 12,000 × g for 5 min once again. The obtained proteins were digested using trypsin solutions at 50:1 protein-to-trypsin mass ratio, which was maintained in a water bath at 37 °C for 16 h. The obtained digested peptides were dissolved in 10% formic acid solution and desalted through a C18 ZipTip pipette tip. Finally, the resulting peptides were lyophilized and used for the following LC–MS/MS analysis.

LC–MS/MS analysis

The obtained peptides were separated and determined using an UltiMate 3000 RSLC nano-liquid chromatography machine coupled online with a high-resolution mass spectrometer system (Orbitrap Fusion Lumos, Thermo Scientific, Bremen, Germany) according to the procedures reported by Lin et al. (2021) with some modifications. Briefly, the samples were added to 0.1% (v/v) formic acid solution and then loaded into a self-made C_{18} reversed-phase column (50 cm × 75 μm). The concentrated peptides were further passed through a C_{18} analytical column (12 cm × 150 μm). Herein, a binary mobile phase was obtained at a flow rate of 600 nL/min, which included the mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A gradient elution was collected according to the following procedure: 93% A and 7% B, 0–11 min; 85% A and 15% B, 11–48 min; 75% A and 25% B, 48–68 min; 60% A and 40% B, 68–69 min; and held at 100% B until the 75th minute. Afterward, the target peptides were detected and analyzed on the MS/MS system. The parameters of the full-scan MS were as follows: orbitrap resolution, 120,000; maximum injection time, 50 ms; automatic gain control (AGC) target, 500,000; and scan range, 300–1400 *m/z*. Then, the dd-MS spectra were recorded using the following parameters: isolation mode, quadrupole; scan range mode, auto normal; first mass, 120; isolation window, 1.6; AGC target, 5,000; maximum injection time, 35 ms; and collision energy, 30%.

Protein identification

Proteome Discover (version 2.2; Thermo Fisher Scientific, Bremen,

Germany) software was used to identify the detected peptides according to its operational procedures. The parameters were as follows: false discovery rate (FDR), ≤ 0.01; peptide confidence, high; fragment mass tolerance, 0.6 Da; peptide mass tolerance, ± 10 ppm; variable modifications, methionine oxidation and acetylation on protein (*N*-term); fixed modifications, carbamidomethyl; max missed cleavages, 2; and the cleaving enzyme, trypsin. The identification of the target proteins was achieved through the analysis of MS/MS data matching to the Uniprot *Electrophorus electricus* database.

Bioinformatics analysis

Three comparison batches were used including CPE *vs*. PE, FPE *vs*. PE, and FPE *vs*. CPE. For the comparisons, proteins with the calculated fold changes (FCs) greater than 1.5 or less than 1/1.5 were considered as differentially abundant proteins (DAPs). Gene ontology (GO) annotation (including cellular component, molecular function, and biological process domains) of the target DAPs detected in the comparisons was performed using the GO database (http://geneontology.org/). Additionally, the functional classification of DAPs was analyzed using the eukaryotic cluster of the orthologous group (KOG) database (https://www.ncbi.nlm.nih.gov/research/cog-project/).

Data analysis

The determination of the springiness, chewiness, and MP content was executed in triplicate ($n = 3$). The Statistical Package for the Social Sciences SPSS v12.0 package (SPSS Inc., IL, USA) was used to evaluate the significant difference resulting from the protein alterations. Duncan's test was also carried out to identify significant differences (P less than 0.05).

Results and discussion

Springiness and MP content analysis

The changes in the springiness and MP content were evaluated in the pike eel muscle during 6 days of chilling and 120 days of frozen storage, and the determinations are shown in Fig. 1. The initial springiness and MP content of the fresh muscle (0 days) were 3.89 mm and 109.5 g·prot/L, respectively. The relatively high springiness and MP content denote the satisfactory fresh quality of the fish samples used in this study. During the subsequent storage period, the springiness and MP content of the eel muscle samples were similar. The springiness decreased significantly (P less than 0.05) to 2.01 mm after 6 days of chilled storage and to 1.50 mm after 120 days of frozen storage, while the MP content decreased to 81.6 g·prot/L and 69.8 g·prot/L, respectively. The decreased extracted MPs was closely associated with reductions in the muscular springiness. These results indicated that protein dissociation (forming small water-solubility molecules), aggregation (decreased salt solubility), and/or oxidation likely occurred to some extent in the muscle tissues after the cold storage. The current observations (springiness and MP content) were similar to previous findings in Atlantic salmon (*Salmo salar* L.) after 4 months of frozen storage (Pereira de Abreu et al., 2010) and in bighead carp (*Hypophthalmichthys nobilis*) after 9 months of frozen storage (Liu et al., 2022).

MPs play important roles in muscle structure, function, and integrity, which have a positive correlation with the textural properties (such as the springiness, chewiness, and hardness) of muscle tissues. In the current study, significant decreases in the springiness and MP content mainly resulted from either the damage/degradation of muscle proteins or the partial crosslinking/aggregation of proteins into macromolecular polymers. This was likely due to microorganism growth and its metabolite actions, endogenous enzyme hydrolysis, and/or ice crystal growth/recrystallization and their physical damage effects (during freezing conditions) (Ramírez-Guerra et al., 2012; Zhang et al., 2020a; Zhang et al., 2020b). More importantly, the alterations of muscle proteins occurring in muscle tissues involved several intricate biochemical processes (e.g., protein dehydration, myofibril degradation, ice crystal growth, and protein/lipid oxidation), and their denaturation products were also extremely complex, including numerous compounds with different abundance levels, conformational structures, reactive intermediaries, and functional stabilities. Thus, the associations between different cold treatments and protein alterations including their resulting products still need to be explored.

Quantification validation of proteomics

The protein molecular weight, peptide count, sequence coverage distribution, and the length of peptides in the amino acids (Fig. 2) of the detected target proteins in pike eel muscle were evaluated through the validity and reliability of the obtained MS/MS data, analytical procedures, and database search results. In total, 1690 peptides were detected in the pike eel muscle and 537 proteins were further identified with at least one unique peptide and an FDR confidence interval of ≤ 0.01. As shown in Fig. 2A, 67% (360 proteins) of the identified proteins had a molecular weight ranging from 10 kDa to 60 kDa, while 6% (35 proteins) of the proteins exceeded 200 kDa, presenting a natural molecular weight distribution in the fish muscle. Similar results were also found in the yellow croaker (*Larimichthys polyactis*) (Wang et al., 2022) and sea bass (*Lateolabrax japonicus*) (Xiang et al., 2022). Additionally, less than 4% (21 proteins) of the identified proteins contained more than 20 peptides, and fewer than 18 peptides were found in the remaining 96% (516 proteins) (Fig. 2B). Additionally, 96% (516 proteins) of the detected proteins ranged from 7 to 21 amino acids in length (Fig. 2C),

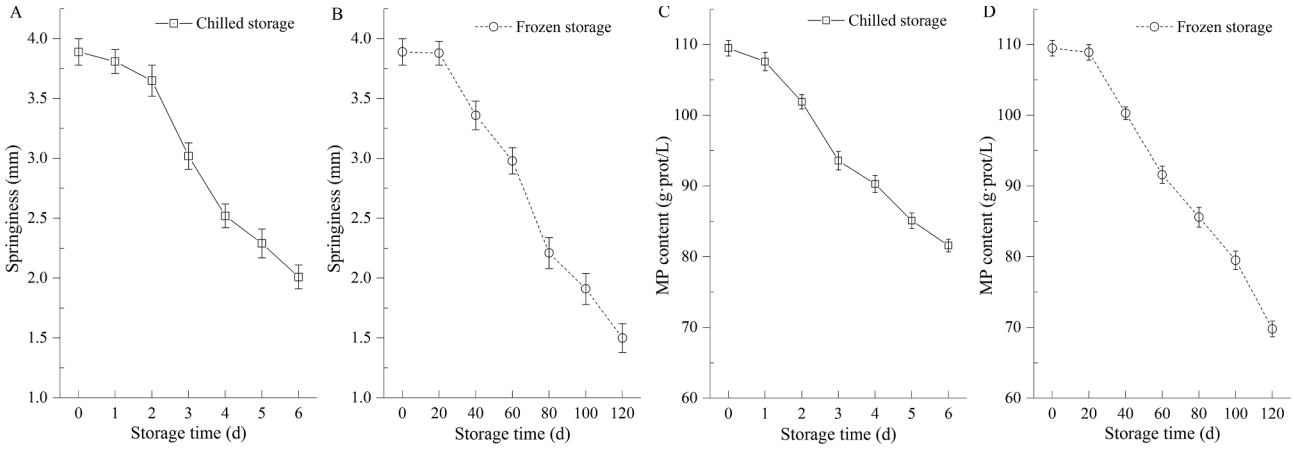

Fig. 1. Changes in the (A, B) springiness and (C, D) MP content in the pike eel muscle during chilled and frozen storage.

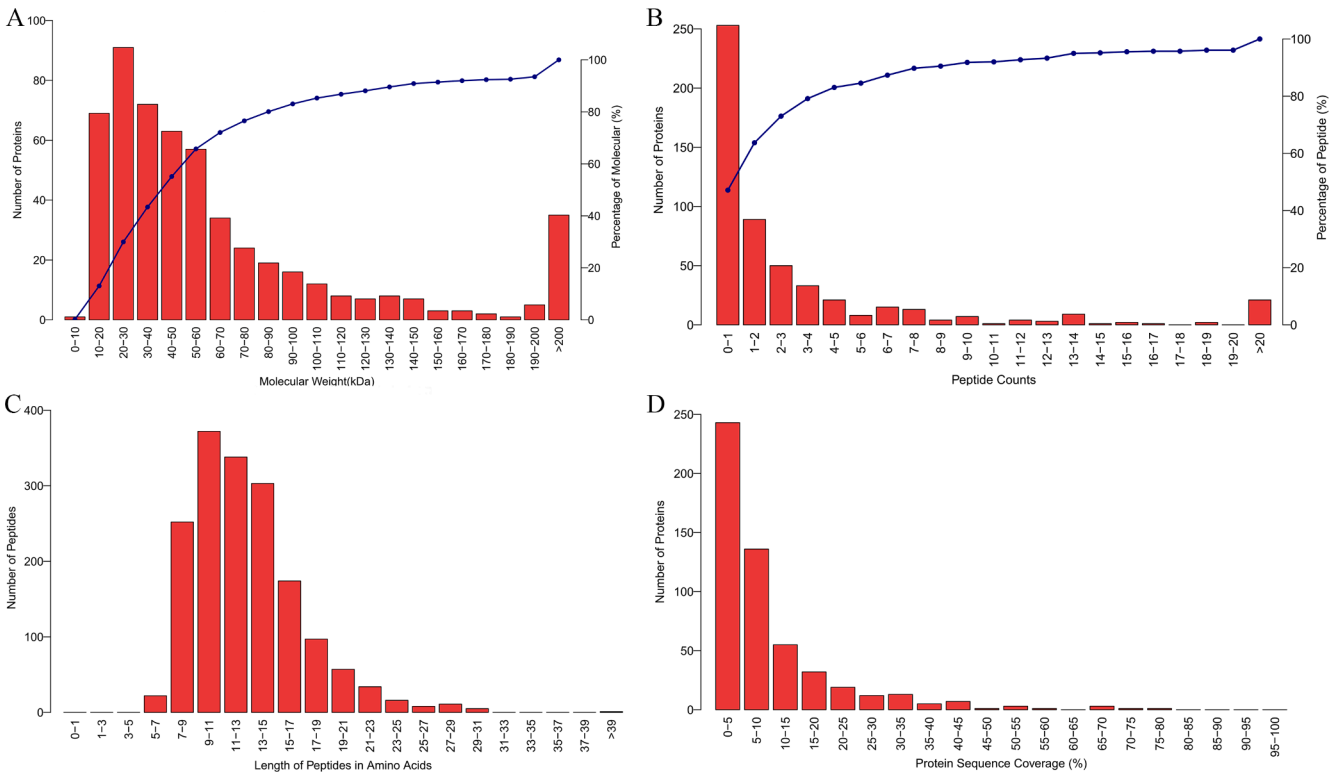

Fig. 2. (A) Molecular weight, (B) peptide count, (C) lengths of peptides in the amino acids, and (D) protein sequence coverage of the detected proteins in the pike eel muscle identified by label-free proteomics analysis.

while less than 4% were more than 20 amino acids in length, which coincided with the effect of trypsin digestion on the proteins. It was considered that most muscle proteins were 2–20 amino acids in length, although several large polyproteins were found in the muscle (Méndez & Pazos, 2017). For the MS/MS analysis, the digested target peptides of more than 20 amino acids or fewer than 5 amino acids in length could not be effectively detected by the MS equipment, mainly due to their high/low molecular weights and incompatible electric charges (Chu et al., 2019). In addition, the identified proteins showed relatively low proteome sequence coverage (Fig. 2D), of which 32% (169 proteins) had more than 10% sequence coverage that matched the published *E. electricus* proteins. This may be due to the limited eel protein information available in the UniProt database. Taken together, the obtained results of the peptide lengths (in amino acids), peptide count, and molecular weight of identified proteins verified the reliability and feasibility of trypsin digestion and LC–MS/MS detection, also meeting the required qualifications and demonstrating the suitability of the approach for subsequent bioinformatics analysis.

DAP analysis

Label-free proteomics was used to reveal the compositions and alterations of muscle proteins in pike eel after chilled (CPE) and frozen (FPE) storage, compared with the fresh (PE) samples. A large number of DAPs (137) were identified in the CPE samples, including 82 differentially abundant proteins (DAPs) accumulated at lower abundances and 55 DAPs accumulated at higher abundances (Supplementary Table S1; excluding uncharacterized proteins). Meanwhile, 148 DAPs were identified in the FPE and PE comparison, including 131 DAPs accumulated at lower abundances and 17 DAPs accumulated at higher abundances in the FPE samples (Supplementary Table S2; excluding uncharacterized proteins). Thus, the numbers of DAPs identified in the CPE *vs.* PE and FPE *vs.* PE comparisons also confirmed that significant alterations induced by cold stress had occurred in the pike eel muscle proteins when compared with the PE samples.

Collectively, the DAPs (Supplementary Tables S1 and S2) identified in the CPF and FPE treatments showed similar distributions and variations after cold-temperature storage when compared to the fresh samples. Common lower-abundance DAPs in the CPE and FPE samples included aldedh (A0A4W4GD12) and amidohydro-rel (A0A4W4EBS4) domain-containing protein, annexin (A0A4W4DQ99), calponin (A0A4W4GN60), FABP domain-containing proteins (A0A4W4F6E5), fibronectin (A0A4W4GJE6), fibronectin (A0A4W4GJE6), GLOBIN domain-containing protein (A0A4W4F7B0), histone H2A (A0A4W4FIB8), kinesin light chain (A0A4W4FT15), myosin motor domain-containing protein (A0A4W4FD66), proline-rich transmembrane protein 4 (A0A4W4FBM6), serotransferrin (A0A4W4G4R6), and tubulin beta chain (A0A4W4GQD8), among others. There were also several common DAPs with higher abundance in the CPE and FPE samples including aspartate transaminase (A0A4W4E3K0), cytochrome *c* oxidase polypeptide (A0A4W4GSM3), and small monomeric GTPase (A0A4W4DML2).

In the current study, the lower-abundance DAPs detected in the CPE and FPE samples were mainly due to the denaturation, oxidation, and/or degradation of proteins resulting from the psychrophilic microorganism growth, endogenous enzyme actions, moisture migration/solute concentration, and/or biochemical processes occurring in muscle tissues during cold storage (Lin et al., 2021; Purslow et al., 2021). Annexins are considered as the phospholipid-, calcium-, and cytoskeletal-binding proteins, the functions of which are mainly associated with cellular membrane-related events. They are sensitive to cold stress, reactive oxygen and nitrogen species, physical damage, and other conditions, which easily lead to a lower abundance found in the muscle tissues (Zhang et al., 2020a; Zhang et al., 2020b). Calponin as a thin filament-associated protein is capable of binding calmodulin, actin, and tropomyosin in muscle tissues, accounting for the regulation of smooth muscle contraction and its physical strength. The lower abundance of this protein is consistent with the decreased springiness and chewiness

properties in the CPE and FPE samples, which is also in agreement with previous findings in frozen/thawed curled octopus (*Eledone cirrhosa*) (Guglielmetti et al., 2018). Fibronectin is a multifunctional glycoprotein (including adhesion and cytoskeletal functions) providing stability to various organs and muscle tissues. It can be rapidly degraded by many different types of endogenous and exogenous proteinases (Feist & Hiepe, 2007), resulting in its decreased abundance in muscle tissues.

In the comparison of FPE *vs*. CPF, a large number of lower-abundance of DAPs (90; details were not provided) were found in the FPE samples, including 40S ribosomal proteins S18, S2, S4, S8, and SA, 60S ribosomal proteins L11 and L23, calcium-transporting ATPase, calponin, histones H2A and H4, myosin motor domain-containing protein, and *T*-complex protein 1, among others. Only 16 high-abundance DAPs were identified in the FPE samples. These results indicated that more alterations had occurred in the frozen muscle proteins when compared with the chilled fish samples, which was mainly due to how the long-term frozen storage and the ice crystals formed caused the degradation and denaturation of muscle proteins in the pike eel tissues. Ribosomal proteins are important structural components in the ribosome. The decreased abundance levels of these proteins were generally related to the reduced stability of the ribosome and its deteriorated structural framework (He et al., 2018). Histone proteins were found to correlate with the responses of muscle tissues to environmental stresses including freezing treatment and frozen storage (Fan et al., 2019). The low abundance of histones in the FPE samples decreased the resistance of muscle tissues to stress induced by frozen storage. These findings were also in agreement with our previous studies on frozen whiteleg shrimp muscle proteins (Ying et al., 2021).

Gene ontology (GO) analysis

GO annotations of the DAPs in the PE, CPE, and FPE samples were collected to explore the effects of low temperatures and subsequent storage on the variations of proteins in the pike eel muscle. The classification and annotation results are shown in Fig. 3. Although the identified DAPs in the CPE *vs*. PE comparison (Fig. 3A) showed similar annotations and distributions when compared with the FPE *vs*. PE comparison (Fig. 3B), there were also clear alterations in the GO categories between the two comparisons, especially for high-abundance DAPs associated with the biological process domain in the FPE samples. It was further confirmed that the low-temperature and long-term storage had much greater effects on the muscle proteins compared with the chilled samples.

Biological process annotation analysis

For biological process annotation, the DAPs in the CPE *vs*. PE and FPE

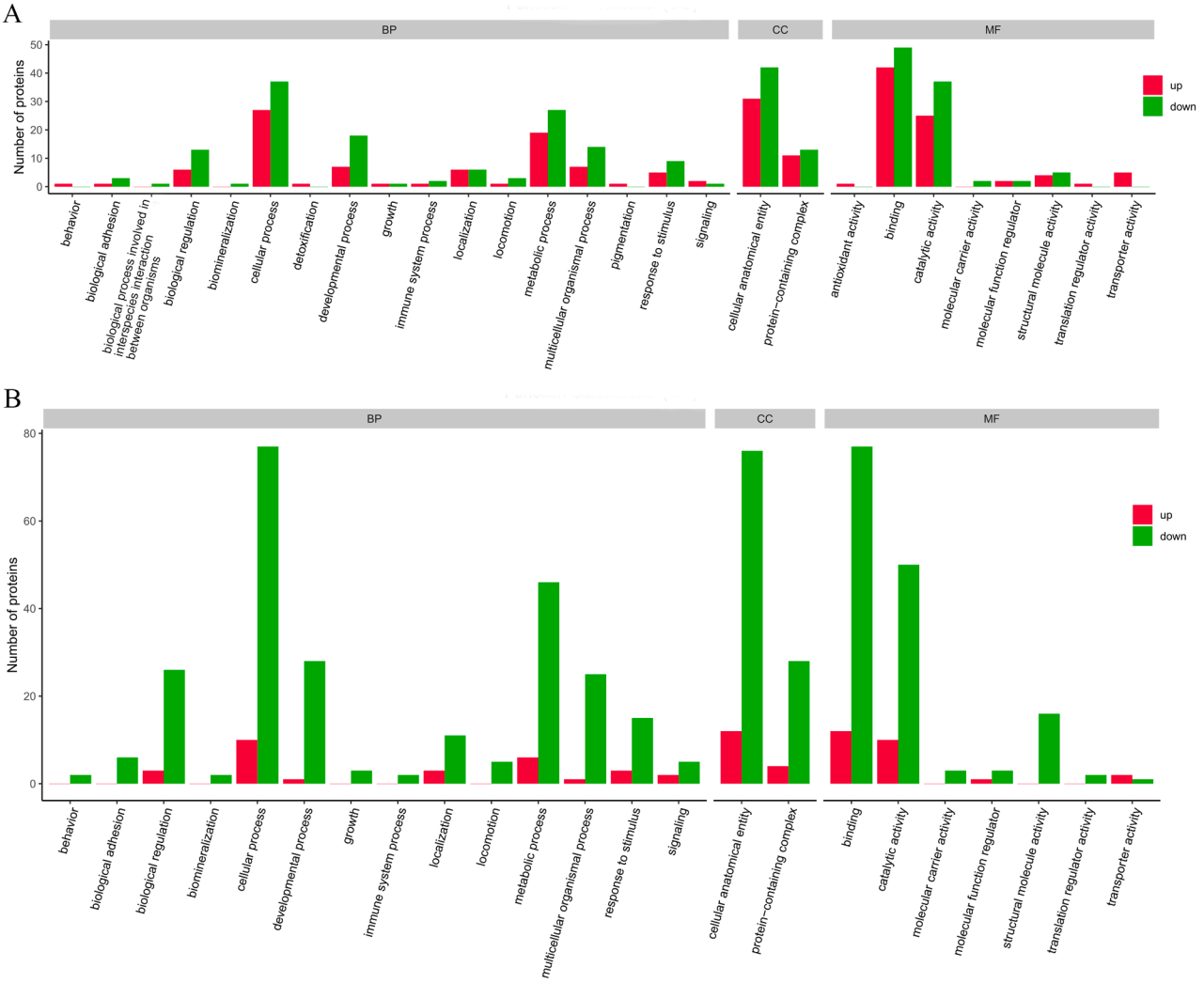

Fig. 3. GO annotations including the cellular component (CC), molecular function (MF), and biological process (BP) domains of the DAPs identified in the (A) chilled (4 days) *vs*. fresh pike eel and (B) frozen (120 days) *vs*. fresh pike eel comparisons. Green, lower abundance levels of DAPs (downregulated); red, higher abundance levels of DAPs (upregulated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vs. PE comparisons were mainly involved in the cellular (GO:0009987), metabolic (GO:0008152), multicellular organismal (GO:0032501), and developmental (GO:0032502) processes. Compared with the PE samples, calponin, amidohydro-rel domain-containing protein, spectrin beta chain, and tubulin beta chain involved in the cellular process showed significantly lower abundance in both CPE and FPE samples, which were noted as the structural constituents of actomyosin, actin filament bundle, or cytoskeleton in the muscle tissues. The obtained results confirmed the loose and disordered structural and functional properties of the cytoskeleton/nucleoskeleton proteins and their weak resistance to cold stress. These findings were consistent with the altered proteins detected in the swamp crayfish (*Procambarus clarkii*) under cold stress (Li et al., 2022) and in silver carp (*Hypophthalmichthys molitrix*) under mild oxidation conditions (Fang et al., 2021). For the metabolic process, the DAPs in the CPE and FPE samples were associated with the 3-hydroxyanthranilate-3,4-dioxygenase, formyltetrahydrofolate dehydrogenase, multifunctional fusion protein, homogentisate 1,2-dioxygenase, glyceraldehyde-3-phosphate dehydrogenase, and ATP-citrate synthase, which were related to the oxidation–reduction, lipid metabolic, and carbohydrate metabolic processes occurring in the tissues. Protein/lipid oxidation takes place unavoidably in muscle tissues even under freezing conditions, the degree of which is closely related to muscle quality (Tan et al., 2021). The protein/lipid oxidation process is extremely complicated and leads to the formation of a series of oxidation peptides/subunits in the muscle tissues. Thus, several proteins involved in the oxidizable process and metabolism are susceptible to alteration during processing, transportation, and frozen storage.

Cellular component annotation analysis

For the cellular component annotation, all the DAPs were converged in the cellular anatomical entity (GO:0110165) and protein-containing complex (GO:0032991). Compared to the PE samples, the DAPs in the CPE and FPE samples, including histones H2A, H2B, and H4, 40S ribosomal proteins S20 and SA, 60S ribosomal protein L23, *C*2 domain-containing protein, calcium-transporting ATPase, dynein light chain and roadblock, fibronectin, IF rod domain-containing protein, kinesin light chain, and tubulin alpha and beta chains, showed lower abundances after storage, likely due to the cold stress–induced denaturation and the oxidation of proteins/lipids. The current findings were partially consistent with the previous reports on sea bass (*Lateolabrax japonicus*) fillets after chilled storage (Xiang et al., 2022) and mud shrimp (*Solenocera melantho*) after frozen storage (Shi et al., 2018). Histone proteins involved in the protein–DNA complexes were related to the muscle cells responding to environmental stresses including freezing, frozen storage, and oxidative processes (Fan et al., 2019). Additionally, *C*2 domain-containing protein, IF rod domain-containing protein, calcium-transporting ATPase, dynein light chain, fibronectin, and tubulin chains as important cytoskeleton, microtubule, or dynein complex components were embedded in the cell membrane. The decreased abundance of these DAPs suggested that the stability and integrity of the cell membrane and its organelles may be damaged during low-temperature storage (Grasmeijer et al., 2013).

Molecular function annotation analysis

As described in molecular function annotation, the identified DAPs in the PE, CPE, and FPE samples mainly belonged to the binding (GO:0005488), catalytic (GO:0003824), and structural molecular (GO:0005198) activity. Among the DAPs, the ADF-H domain-containing protein, actin-depolymerizing factor, and metavinculin were classified into the actin/actin filament binding function. These detected low-abundance proteins are the components of actin and play important roles in the natural function, structure, and organization of the muscle proteins and tissues (Zhang et al., 2020a; Zhang et al., 2020b). Additionally, the myosin motor domain-containing protein, *T*-complex protein 1 subunit, ATP-citrate classified into ATP- and nucleotide-binding functions, calcium-transporting ATPase, annexin, calponin classified into the calmodulin-binding function, and LIM domain-containing protein, serotransferrin, GLOBIN domain-containing protein, and TGc domain-containing protein classified into the metal ion binding function all showed lower-abundance in the CPE and FPE samples. These observations were in accordance with the variations in stress-induced silver carp (*Hypophthalmichthys molitrix*) muscles (Zhang et al., 2020a; Zhang et al., 2020b). Binding proteins were considered to have important roles in the muscle organizational framework, such as in promoting the assembly of myosin bundles, maintaining homeostasis via binding to structural proteins, regulating protein contraction and relaxation, and preserving physical strength by binding against environmental stresses (Purslow et al., 2021). Clearly, the freezing and subsequent frozen storage in the current study greatly damaged the binding functions of the above DAPs and weakened the connecting strength in the muscle tissues, which were consistent with the TPA and MP content results.

Eukaryotic cluster of the orthologous group (KOG) analysis

KOG analysis of the DAPs identified in the CPE *vs*. PE and FPE *vs*. PE comparisons is depicted in Fig. 4, which was divided into 25 function categories. According to the KOG results, the identified DAPs mainly belonged to the following functional categories in both the comparisons, mainly including the cytoskeleton; posttranslational modification, protein turnover, and chaperones; signal transduction mechanisms; energy production and conversion; translation, ribosomal structure, and biogenesis; extracellular structures; and carbohydrate transport and metabolism categories.

Among these, the cytoskeleton function played the primary role in the normal structure and function of muscle tissues, the DAPs of which mainly involved actin-binding protein (KOG0518), talin (KOG4261), beta-spectrin (KOG0517), calponin (KOG2046), beta-tubulin (KOG1375), kinesin light chain (KOG1840), Ca^{2+}-binding actin-bundling protein (KOG0046), and the actin filament-coating protein tropomyosin (KOG1003), among others. During the chilled storage, the microorganisms grew continuously in the samples, and their numbers greatly increased over the prolonged period. The microbial metabolites and endogenous enzymes in the muscle tissues synergistically promoted the hydrolysis and/or degradation of the protein and peptide bonds, leading to the subsequent cross-linking, aggregation, rearrangement, and/or irreversible denaturation of proteins (Szymczak, 2016; Qiu et al., 2020), rupturing the natural structure of the myofibrils in the muscle tissues (causing decreased cytoskeleton function). In the case of the frozen samples, the growth and recrystallization of ice crystals occurred in the tissues, especially during long-term storage and under temperature fluctuation. This destroyed the normal structure of the connective tissues and myofibrils, resulting in serious mechanical damage to the tissues (Zhang et al., 2020a; Zhang et al., 2020b). Taken together, the detected DAPs involved in the cytoskeleton function of the pike eel muscle were highly affected by the low-temperature–induced changes during storage, which directly affected the physical structure and chemical performances of the muscle tissues.

For the "posttranslational modification, protein turnover, and chaperones" function category, the involved proteins mainly included cytosolic Ca^{2+}-dependent cysteine protease (KOG0045), molecular chaperones HSP70/HSC70 (KOG0101), 20S proteasome, regulatory subunits (KOG0175, KOG0176, and KOG0182), chaperonin complex component subunits (KOG0361 and KOG0362), FKBP-type peptidyl-prolyl cis–trans isomerase (KOG0549), thiol-disulfide isomerase and thioredoxin (KOG0912), and 26S proteasome regulatory complex subunit (KOG2062). Several protein subunits were identified in the chilled and frozen samples, which seemed to be more vulnerable to cold stress compared with those in the fresh samples. The low-temperature conditions may induce conformational variation, decomposition, and/or depolymerization of the proteins, which likely influenced the trypsin digestion of muscle proteins and the subsequently detected subunit compositions (Shi et al., 2018; Lin et al., 2021). Specifically, the proteins

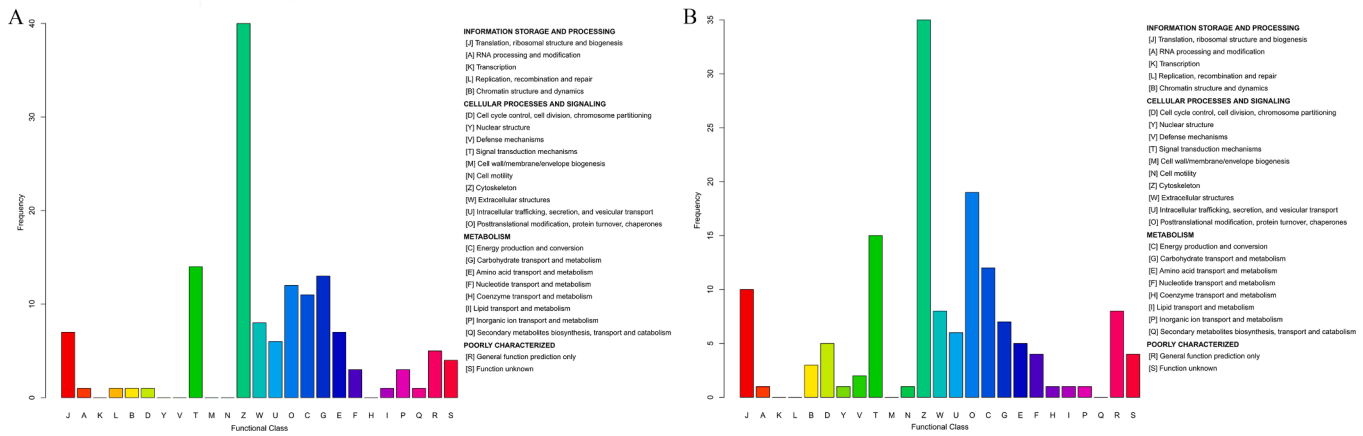

Fig. 4. KOG classifications (including 25 categories) of the DAPs identified in the (A) chilled (4 days) *vs*. fresh pike eel and (B) frozen (120 days) *vs*. fresh pike eel comparisons.

composed of multiple subunits may be easily altered or disintegrated in the pike eel muscle under low-temperature and long-term storage conditions.

Conclusion

In this study, the specific changes of protein profiles were determined in the pike eel muscle tissues during 6 days of chilling and 120 days of frozen storage using chemical- and LC–MS-based proteomics analyses. Chemical results indicated that cold stress and storage significantly decreased the springiness and MP content in the fish muscle, suggesting that significant changes in the protein profiles had occurred between the chilled/frozen and fresh fish samples. Label-free proteomics analysis identified 137 and 148 DAPs in the CPE and FPE samples, respectively, compared with the PE samples. Among these DAPs, annexins, calponin, fibronectin, ribosomal proteins, *T*-complex proteins, tubulin beta chain, histones, and calcium-transporting ATPase were more prone to denaturation/oxidation mainly resulting from the cold conditions and subsequent storage. This study provided a basic database to understand the alterations of protein profiles in pike eel muscle during chilling and frozen storage.

CRediT authorship contribution statement

Pengxiang Yuan: Investigation, Methodology, Formal analysis. **Xiaonan Chen:** Investigation, Methodology, Formal analysis. **Soottawat Benjakul:** Validation, Writing – review & editing. **Jipeng Sun:** Investigation, Supervision, Validation. **Bin Zhang:** Data curation, Project administration, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100275.

References

Bi, J., Li, Y., Wang, H., Song, Y., Cong, S., Yu, C., et al. (2018). Presence and formation mechanism of foodborne carbonaceous nanostructures from roasted pike eel (*Muraenesox cinereus*). *Journal of Agricultural and Food Chemistry, 11*, 2862–2869.

Chien, H. J., Huang, Y. H., Zheng, Y. F., Wang, W. C., Luo, C. Y., Wei, G. J., et al. (2021). Proteomics for species authentication of cod and corresponding fishery products. *Food Chemistry, 19*, Article 131631.

Chu, C., Yan, N., Du, Y., Liu, X., Chu, M., Shi, J., et al. (2019). iTRAQ-based proteomic analysis reveals the accumulation of bioactive compounds in Chinese wild rice (*Zizania latifolia*) during germination. *Food Chemistry, 289*, 635–644.

Fan, L., Wang, L., & Wang, Z. (2019). Proteomic characterization of the hepatopancreas in the Pacific white shrimp *Litopenaeus vannamei* under cold stress: Revealing the organism homeostasis mechanism. *Fish & Shellfish Immunology, 92*, 438–449.

Fang, M., Xiong, S., Yin, T., Hu, Y., Liu, R., Du, H., et al. (2021). Proteomic profiling and oxidation site analysis of gaseous ozone oxidized myosin from silver carp (*Hypophthalmichthys molitrix*) with different oxidation degrees. *Food Chemistry, 363*, Article 130307.

Feist, E., & Hiepe, F. (2007). Fibronectin autoantibodies. In *Autoantibodies* (Second Edition). Shoenfeld, Y., Gershwin, M. E., & Meroni, P. L., Ed., Elsevier Inc., 325-329.

Grasmeijer, N., Stankovic, M., De Waard, H., Frijlink, H. W., & Hinrichs, W. L. J. (2013). Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system. *Biochimica et Biophysica Acta, 1834*, 763–769.

Guglielmetti, C., Manfredi, M., Brusadore, S., Sciuto, S., Esposito, G., Ubaldi, P. G., et al. (2018). Two-dimensional gel and shotgun proteomics approaches to distinguish fresh and frozen-thawed curled octopus (*Eledone cirrhosa*). *Journal of Proteomics, 186*, 1–7.

He, Y., Huang, H., Li, L. H., & Yang, X. (2018). Label-free proteomics of Tilapia fillets and their relationship with meat texture during post-mortem storage. *Food Analytical Methods, 11*(11), 3023–3033.

Li, J., Wan, J. Q., Bian, H., Zhong, Y. J., Cai, L. Y., Zou, L., et al. (2017). Effect of controlled freezing-point vacuum drying on color and flavor of *Muraenesox cinereus* fillets. *Journal of Food Process Engineering, 40*, Article e12273.

Li, X., Li, S., Shi, G., Xiong, G., Shi, L., Kang, J., et al. (2022). Quantitative proteomics insights into gel properties changes of myofibrillar protein from *Procambarus clarkii* under cold stress. *Food Chemistry, 372*, Article 130935.

Lin, H. M., Qi, X. E., Shui, S. S., Benjakul, S., Aubourg, S. P., & Zhang, B. (2021). Label-Free proteomic analysis revealed the mechanisms of protein oxidation induced by hydroxyl radicals in whiteleg shrimp (*Litopenaeus vannamei*) muscle. *Food & Function, 12*, 4337–4348.

Liu, Y., Zhang, L., Gao, S., Tan, Y., Luo, Y., Hong, H., et al. (2022). Effect of protein oxidation in meat and exudates on the water holding capacity in bighead carp (*Hypophthalmichthys nobilis*) subjected to frozen storage. *Food Chemistry, 370*, Article 131079.

Méndez, M., & Pazos, M. (2017). In *Proteomics to assess fish quality and bioactivity* (pp. 297–316). Elsevier Inc.

Pereira de Abreu, D. A., Paseiro Losada, P., Maroto, J., & Cruz, J. M. (2010). Evaluation of the effectiveness of a new active packaging film containing natural antioxidants (from barley husks) that retard lipid damage in frozen Atlantic salmon (*Salmo salar* L.). *Food Research International, 43*, 1277–1282.

Picard, B., & Gagaoua, M. (2020). Meta-proteomics for the discovery of protein biomarkers of beef tenderness: An overview of integrated studies. *Food Research International, 127*, Article 108739.

Purslow, P. P., Gagaoua, M., & Warner, R. D. (2021). Insights on meat quality from combining traditional studies and proteomics. *Meat Science, 174*, Article 108423.
Qiu, H., Guo, X., Deng, X., Guo, X., Mao, X., Xu, C., et al. (2020). The influence of endogenous cathepsin in different subcellular fractions on the quality deterioration of Northern pike (*Esox lucius*) fillets during refrigeration and partial freezing storage. *Food Science and Biotechnology, 29*(10), 1331–1341.
Ramírez-Guerra, H. E., García-Sifuentes, C. O., Pacheco-Aguilar, R., Ramirez-Suarez, J. C., Lugo-Sánchez, M. E., & Scheuren-Acevedo, S. M. (2012). The influence of ante-mortem hypoxia on the physicochemical stability of myofibrillar proteins in the muscle tissue of white shrimp (*Litopenaeus vannamei*) exposed to multiple freeze-thaw cycles. *European Food Research and Technology, 235*, 37–45.
Shi, J., Zhang, L., Lei, Y., Shen, H., Yu, X., & Luo, Y. (2018). Differential proteomic analysis to identify proteins associated with quality traits of frozen mud shrimp (*Solenocera melantho*) using an iTRAQ-based strategy. *Food Chemistry, 251*, 25–32.
Shui, S. S., Yao, H., Jiang, Z. D., Benjakul, S., & Zhang, B. (2021). The differences of muscle proteins between neon flying squid (*Ommastrephes bartramii*) and jumbo squid (*Dosidicus gigas*) mantles via physicochemical and proteomic analyses. *Food Chemistry, 364*, Article 130374.
Szymczak, M. (2016). Distribution of cathepsin D activity between lysosomes and a soluble fraction of marinating brine. *Journal of Food Science, 81*(8), 1966–1970.
Tan, M., Ding, Z., Yang, D., & Xie, J. (2021). The quality properties of frozen large yellow croaker fillets during temperature fluctuation cycles: Improvement by cellobiose and carboxylated cellulose nanofibers. *International Journal of Biological Macromolecules, 194*, 499–509.
Varunjikar, M. S., Moreno-Ibarguen, C., Andrade-Martinez, J. S., Tung, H. S., Belghit, I., Palmblad, M., et al. (2022). Comparing novel shotgun DNA sequencing and state-of-the-art proteomics approaches for authentication of fish species in mixed samples. *Food Control, 131*, Article 108417.
Wang, Y. Y., Yan, J. K., Ding, Y., & Ma, H. (2022). Effects of ultrasound on the thawing of quick-frozen small yellow croaker (*Larimichthys polyactis*) based on TMT-labeled quantitative proteomic. *Food Chemistry, 366*, Article 130600.
Xiang, Y., Sun, C., Zhao, Y., Li, L., Yang, X., Wu, Y., et al. (2022). Label-free proteomic analysis reveals freshness-related proteins in sea bass (*Lateolabrax japonicus*) fillets stored on ice. *LWT-Food Science and Technology, 155*, Article 112885.
Xie, X., Liu, X., Fei, Y., Zhao, Q., & Jin, S. (2020). Single and combined effects of tea polyphenols and edible chitosan coating on the shelf life improvement of refrigerated dagger-tooth pike conger (*Muraenesox cinereus*). *International Journal of Food Properties, 23*, 2110–2122.
Ying, X. G., Wu, Q. J., Shui, S. S., Zhang, B., & Benjakul, S. (2021). Insights into the similarities and differences of whiteleg shrimp pre-soaked with sodium tripolyphosphate and sodium trimetaphosphate during frozen storage. *Food Chemistry, 348*, Article 129134.
Zhang, B., Cao, H., Wei, W., & Ying, X. (2020a). Influence of temperature fluctuations on growth and recrystallization of ice crystals in frozen peeled shrimp (*Litopenaeus vannamei*) pre-soaked with carrageenan oligosaccharide and xylooligosaccharide. *Food Chemistry, 306*, Article 125641.
Zhang, L., Li, Q., Hong, H., Luo, Y., & Lametsch, R. (2020b). Search for proteomic markers for stunning stress and stress-induced textural tenderization in silver carp (*Hypophthalmichthys molitrix*) fillets using label-free strategy. *Food Research International, 137*, Article 109678.
Zhou, C. Y., Pan, D. D., Cao, J. X., & Zhou, G. H. (2021). A comprehensive review on molecular mechanism of defective dry-cured ham with excessive pastiness, adhesiveness, and bitterness by proteomics insights. *Comprehensive Reviews in Food Science and Food Safety, 20*, 3838–3857.