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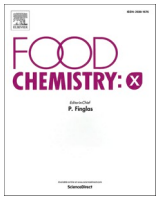

Proteomics reveals the mechanism of protein degradation and its relationship to sensorial and texture characteristics in dry-cured squid during processing

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ABSTRACT

Proteolysis in dry-cured squid contributes to the development of sensory and textural attributes. In this study, label-free quantitative proteomics was conducted to study the mechanism of proteolysis and its correlation with quality changes. The results showed that the protein profile of dry-cured squid changed markedly during processing, which was confirmed by the quantification of myofibrillar protein, amino nitrogen and total free acids, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Thirty-two key differentially abundant proteins were found to be correlated with sensory and texture characteristics, including myofibrillar protein, tubulin beta chain, collagens, heat shock proteins and cytochrome *c*. The correlation analysis indicated that myosin regulatory light chain and tubulin beta chain played the most important role in the development of texture and sensory attributes in squid samples during the dry-curing process. The results offered novel insights into proteolysis in dry-cured squid and its relationship to quality changes.

1. Introduction

The Jumbo squid, *Dosidicus gigas*, is among the most widely consumed and fastest-growing squid species. Its global production reached approximately 0.9 million tonnes in 2018 (Food and Agriculture Organization of the United Nations [FAO], 2020). Curing and drying methods are traditionally used for preservation; they can also endow the squid products with a unique flavour. Dry-cured squid is prepared by blending squid flesh with about 10% (*w*/w) salt and then drying it in the air or in an oven. A series of complex reactions involving protein degradation, lipolysis, carbohydrate degradation and nucleotide metabolism occur during the dry-curing process via synergistic effect of autochthonous microbiota and endogenous enzymes (Liao et al., 2022). It has been reported that the dry-curing process can affect the sensory qualities of dry-cured squid through protein degradation (Zhao, Hu, Zhou, & Chen, 2022).

Protein is an important nutritional and functional component that is responsible for organoleptic qualities and the texture of food products. Myofibrillar proteins, collagens and other structural proteins play a vital role in the textural stability of aquatic animal products during processing. For example, the degradation of titin, nebulin, desmin and troponin T contributes to the collapse of the Z and M lines and the disintegration of actin and myosin, changes that alter the texture (Yang et al., 2019). The structure and arrangement of collagen in tissue may lead to its tightness (Xiao et al., 2023). Various proteinases and peptidases lead to the release of large amounts of small peptides and individual amino acids, which contribute to the more intense taste profile of dry-cured fish products (Yang et al., 2021; Zhao et al., 2022). Peptides are important taste enhancers and exhibit bitter, salty, sweet, sour and kokumi taste (Fu, Amin, Li, Bak, & Lametsch, 2021). They also serve as essential precursors for the development of certain characteristic odours through the Maillard reactions with sugars (Xu et al., 2021). Taste-active amino acids and their derivatives greatly contribute to the formation of aromatic compounds, for examples, branched aldehydes, via amino acid

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degradation. Therefore, it is important to explore the mechanism of protein hydrolysis and its role in quality changes of dry-cured squid during processing.

Food processing technology influences protein composition and structure and affects food texture and sensory qualities (Liu et al., 2022). The structural and functional properties of food proteins can be changed by the interaction with other food components (e.g. water, fat and sugar) and processing conditions (e.g. temperature, pH and water activity; Amiri, Sharifian, & Soltanizadeh, 2018). During the dry-curing process, muscle proteins are degraded by endogenous acidic proteases, principally sarcoplasmic cathepsins, and microbial enzymes, including proteases, peptidases and lipases. As a result, investigating the proteolyic mechanisms in aquatic animal products during the dry-curing process is still challenging. Proteomics is a powerful method for uncovering the proteolytic changes of muscle proteins of dry-cured products during processing. Recent studies have explored the changes in protein components of dry-cured products (Zhou et al., 2019; Zhou et al., 2024), but few have reported on the overall protein composition in dry-cured squid. Moreover, how proteolysis affects the development of the flavour and texture of dry-cured squid is still unclear. Therefore, this study aimed to investigate the variations in the protein composition of squid samples during the dry-curing process and to reveal the relationship between the key proteins and texture and sensory attributes. We hypothesise that the protein composition of dry-cured squid changes under microbial activity during processing and then influences the development of the sensory and textural attributes.

2. Material and methods

2.1. Preparation of dry-cured squid samples

Frozen squid (*D. gigas*) was supplied by Ningbo Fei Run Marine Biological Polytron Technologies Inc. The squid was placed in polystyrene boxes and transferred to the lab on ice. After being thawed, the viscera, skin and tentacles of the squid were removed. The squid flesh was washed with cold water before processing. Fifty squid (about 585 ± 40 g in weight) were used. There were two steps to the dry-curing process. First, the flesh was cured with 10% (*w*/w) salt in a clean pot at 10 °C for 6 h. Then, the cured squid was dried at 45 °C for 24 h in a drying tunnel. Raw squid samples (M) and squid samples after salting (S) or dry-curing (D) were collected. There were six biological replicates at each time point. The samples were stored at −80 °C until analysis.

2.2. Squid protein extraction

Squid protein was extracted as described by Lin et al. (2020), with minor modifications. Briefly, squid flesh was homogenised in a blender (AUX, HX-PB9322, Ningbo, China) on ice. Next, 20 mg of each sample was rapidly powdered using the liquid nitrogen grounding method, mixed with lysis buffer (containing 100 M NH_4HCO_3 and 8 M urea; pH 8.0) and then disrupted by ultrasonication. After centrifugation (12,000*g*, 4 °C, 15 min), the pellet was discarded. The supernatants was treated with 10 mM DL-dithiothreitol (DTT; 56 °C, 1 h) and then alkylated with adequate iodoacetamide (IAM) in the dark (25 °C, 1 h). The mixture was mixed with cold acetone and incubated for 2 h at −20 °C. After centrifugation (12,000*g*, 4 °C, 15 min), the pellet was washed twice with acetone and then dissolved in buffer containing 100 mM triethylammonium bicarbonate (TEAB; pH 8.5) and 8 M urea to obtain squid protein. The Bradford protein assay was used to measure the protein concentration. Three replicates of each group were used for proteomic analysis.

2.3. Protein digestion

One hundred and twenty-five milligrams of extracted protein was dissolved in 100 μL of lysis buffer (Zhao, Chong, Hu, et al., 2022). Sequencing-Grade Modified Trypsin (Promega, Madison, WI, USA) in 100 mM TEAB was added to the protein solution at a trypsin-to-protein ratio of 1:50. The digestion proceeded for 16 h at 37 °C. After digestion, the samples were acidified with formic acid. The supernatant was collected and desalted with a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) (centrifugation at 12000*g*, 25 °C, 5 min). The samples were freeze-dried and stored at −20 °C for later use.

2.4. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis

The analysis was conducted on a Thermo Scientific Q Extractive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Waltham, MA, USA) combined with an ultra high–performance liquid chromatography system (Evosep One; Gainesville, FL, USA). For analysis, the peptides were dissolved in formic acid solution (1 mL/L) and then centrifugated (14,000 *g*, 4 °C, 20 min) (Zhao, Chong, Hu, et al., 2022). The supernatant (1 μL) was taken and separated with a 15 cm × 150 μm × 1.9 μm column at 200 nL/min. Water containing 1 mL/L formic acid (A) and acetonitrile–water (8:2, *v*/v) containing 1 mL/L formic acid (B) were used as the elution solvents. Electrospray ionisation (ESI) employed a spray voltage of 2.1 kV and a heated capillary temperature of 320 °C. The parameters of full-scan MS were set as follows: automatic gain control (AGC) target, 3×10^6; resolution, 60,000 ($m/z = 200$); scan range, 350–1500 m/z; and maximum injection time, 20 ms. Then, the top 40 precursors with the highest abundance in the full scan were fragmented with the higher-energy C-trap dissociation (HCD) method. The parameters of the dd-MS scan procedure were set as follows: normalised collision energy, 27%; resolution 15,000 ($m/z = 200$); maximum injection time, 45 ms; AGC target, 1×10^5; dynamic exclusion, 20 s; and intensity threshold, 2.2×10^4.

2.5. Protein identification and quantification

The Proteome Discover 2.0 software was used to identify and quantify proteins against a database (979226-uniProt. fasta) by using the following parameters: peptide mass tolerance, 10 ppm; fragment ions mass tolerance, 0.02 Da; fixed modifications, carbamidomethyl; variable modifications, methionine oxidation and N-terminal acetylation; missed cleavages, 2; and false discovery rate (FDR) < 1%. Three comparisons were made: S versus M, D versus S, and D versus M. Student's *t*-test was used for statistical analysis of the protein quantification results. Differentially abundant proteins (DAPs) were identified by calculating the fold-change (FC; $P < 0.05$, ratio < 0.67 or > 1.5) of proteins.

2.6. Bioinformatics analysis of proteins

Gene Ontology (GO) analysis was performed to assign the DAPs to the biological process (BP), molecular function (MF) and cellular component (CC) groups with the InterProScan tool against non-redundant protein databases.

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

For total protein extraction, tissue (300 mg) was ground with lysis buffer containing 8 M urea, 100 mM Tris-HCl, 10 mM DTT and 1 mM phenylmethanesulfonyl fluoride (pH 8.5). After centrifugation (14,000*g*, 4 °C, 30 min), the supernatant was collected. The protein concentration were determined with a BCA Protein Assay Kit (TaKaRa, Beijing, China). SDS-PAGE analysis of the extracted protein was conducted with a Bio-Rad protein electrophoresis system (Hercules, CA, USA). A separation gel containing 12% polyacrylamide and a stacking gel containing 5% polyacrylamide were used (Xu, Cao, Zhang, & Yao, 2020). Ten microliters of the samples was loaded onto the gel, and the protein was

separated at 120 V for 1 h. Then, the gel was stained with Coomassie Brilliant Blue R-250 (0.1%, *w/v*) for 20 min and destained in methanol (25%, *v*/v) containing acetic acid (8%, v/v) for 1 day.

2.8. Quantification of myofibrillar protein, amino nitrogen and free amino acids (FAAs)

Myofibrillar protein was extracted from squid samples according to the protocol described by Shui et al. (2021), with some modification. In brief, the muscle was homogenised in 20 mM Tris-maleate buffer (containing 0.05 M KCl; pH 7.0) with a blender. After centrifugation (10,000*g*, 4 °C, 10 min), the pellet was mixed with buffer and then extracted for the second time. The myofibrillar protein content was assessed with a fish myofibrillar protein enzyme-linked immunosorbent assay (ELISA) kit (PYRAM, Shanghai, China). The amino acid nitrogen of squid samples was measured by using the Chinese standard GB 5009.235–2016 for food. FAA analysis was conducted according to a pervious study (Zhao, Hu, & Chen, 2022). In brief, 2 g of minced sample was blended with sulphosalicylic acid. After centrifugation (6300*g*, 4 °C, 10 min), 100 μL of supernatants was loaded into an amino acid analyser (S—433D, Sykam, Eresing, Germany) coupled with a Sykam ion-exchanger resin (LCA K06/Na, 4.6 mm × 150 mm × 7 μm; Sykam). The taste activity value (TAV) was determined to identify taste-active FAAs. Each experiment was performed in triplicate.

2.9. Analysis of volatile substances

The experiment was conducted based on the study by Zhao, Hu, Zhou, and Chen (2022). In brief, volatile compounds were extracted by using headspace solid-phase microextraction gas chromatography (HS-SPME-GC). Minced squid (2 g) with 6 mg/L of 2,4,6-trimethylpyridine solution applied as an internal standard was incubated in a water bath at 60 °C for 50 min. There were three replicates for each group. Gas chromatography–mass spectrometry (GC–MS) used a gas chromatograph (Agilent, Palo Alto, CA, USA), a 5975C liquid chromatograph (Agilent) and a DB-5MS capillary column (60 m × 0.32 mm × 1 μm; Agilent). The temperature of the transfer line was 250 °C. The column temperature increased from 30 to 92 °C at 4 °C/min. After holding for 2 min, the temperature rose to 120 °C at 5 °C/min; then it rose to 240 °C at 6 °C/min and was kept at that temperature for 6 min. High-purity helium was applied as the carrier gas. The flow rate was set as 1.2 mL/min. Splitless injection was carried out at 250 °C. The following MS conditions were used: ion source temperature, 200 °C; detector interface temperature, 280 °C; ionisation energy, 70 eV; mass range, 35–350 *m/z*. The NIST 14.0 database was used to identify of volatile substances. The relative concentration of volatile substances was determined. The relative odour activity value (ROAV) was calculated based on the thresholds of volatile compounds refer to van Gemert (2011).

2.10. Texture profile analysis (TPA)

The texture profile of squid samples was determined by using a Texture Measurement System Pro (TMS-Pro) equipped with a TMS 75 mm Diameter Platen (Food Technology Corporation, Sterling, VA, USA) according to the method published by Xu et al. (2020) with some modification. In brief, there were six replicates per sample (2 × 2 cm) for the analysis. The parameters for TPA were set as follows: working distance, 50%; trigger force, 0.2 N; machine crosshead speed, 50.0 mm/min; pause time between the two circles, 2.0 s.

2.11. Statistical analysis

SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. The Shapiro–Wilk test was used to assess the normality of the distribution of the experimental data. Then, one-way analysis of variance (ANOVA) and Duncan's test were conducted. R 3.6.3 (R Core Team, Vienna, Austria) and OriginLab Origin 2019b (Northampton, MA, USA) were used to generate heat maps. Pearson's correlation analysis and average-linkage clustering were performed to evaluate the relationship between the protein profile and the quality attributes of the squid samples.

3. Results and discussion

3.1. Qualitative and quantitative analysis of dry-cured squid proteins

There were 1148 proteins identified in the various squid samples during the dry-curing process. Fig. 1a presents the molecular weight of these identified proteins. The most abundant proteins in the squid samples were 20–30 kDa, representing 17.9% of the identified proteins. Most of the peptides were found from 7 to 23 amino acids long (Fig. 1b), consistent with the peculiarity of trypsin digestion (Zhang, Yao, & Aubourg, 2020). As displayed in principal component analysis (PCA) score plot (Fig. 1c), PC1 and PC2 accounted for 48.56% and 13.84%, respectively, of the total variability. There were 268 DAPs in the M, S and D groups (Fig. 1d), including 205 proteins with a FC < 0.67 or > 1.5 and $P < 0.05$, and 63 unique proteins. Forty-three DAPs were upregulated and 23 were downregulated in the squid samples after salting, suggesting that salting promoted mild proteolysis. Apparently, more low-molecular-weight proteins were produced in cured squid samples. The salt content plays an important role in microbial metabolism and enzyme activity in dry-cured fish products (Zhao, Hu, Zhou, & Chen, 2022). Besides, protein aggregation might occur at high NaCl concentrations (Zhang et al., 2022), which could result in low quantity of proteins observed than expected. More squid samples proteins were degraded during the drying process, since 43 were upregulated and 23 were downregulated. Compared with raw squid samples, there were 81 upregulated DAPs and 123 downregulated DAPs in dry-cured squid samples, suggesting marked protein degradation during the processing. A previous report suggested that the dry-curing process had a great effect on the microbial community and flavour development in dry-cured squid samples (Zhao, Hu, Zhou, & Chen, 2022); these factors are strongly linked to protein hydrolysis.

Fig. 1e shows the relative abundance of the top 15 DAPs identified in the squid samples. Of these top 15 DAPs, myosin regulatory light chain LC-2 (A0A6P7T4N8), myosin heavy chain isoform B (G4V4Y7) and NADH: ubiquinone oxidoreductase core subunit S1 (A0A812DE87) were the most abundant DAPs in squid samples. Myosin heavy chain isoform B (G4V4Y7, 221.1 kDa) was dominant in the raw squid samples; its intensity showed a marked decrease after the dry-curing process. The relative abundance of myosin regulatory light chain LC-2 (A0A6P7T4N8, 19.9 kDa) significantly increased during the dry-curing process and it became the dominant DAP in the dry-cured squid samples. These results revealed the variety of the protein composition in the squid samples and the degradation of large proteins into low-molecular-weight proteins during processing. The breakdown of muscle proteins contributes significantly to the development of the flavour and textural properties of dry-cured fish products (Zhao, Chong, Hu, et al., 2022). As previously reported, intense degradation of myosin heavy chain increases the bitterness and adhesiveness of the products (Zhou et al., 2019).

The variation in the DAP profiles of the squid samples represents the changes in characteristics of sensory and textural characteristics during processing. The key DAPs in the squid samples that were correlated with changes in the sensory and textural characteristics are shown in a heat map (Fig. 2); additional information is presented in Table S1. There were 32 key DAPs, including 27 cytoskeletal proteins, 2 heat shock proteins, 2 cytochrome *c* isoforms and 1 collagen. There were 11 types of key DAPs (10 upregulated and 1 downregulated) identified in the cured squid samples when compared with the raw squid samples. The molecular weight of most DAPs with increased abundance was <50 kDa, suggesting that low-molecular-weight proteins play an important role in the

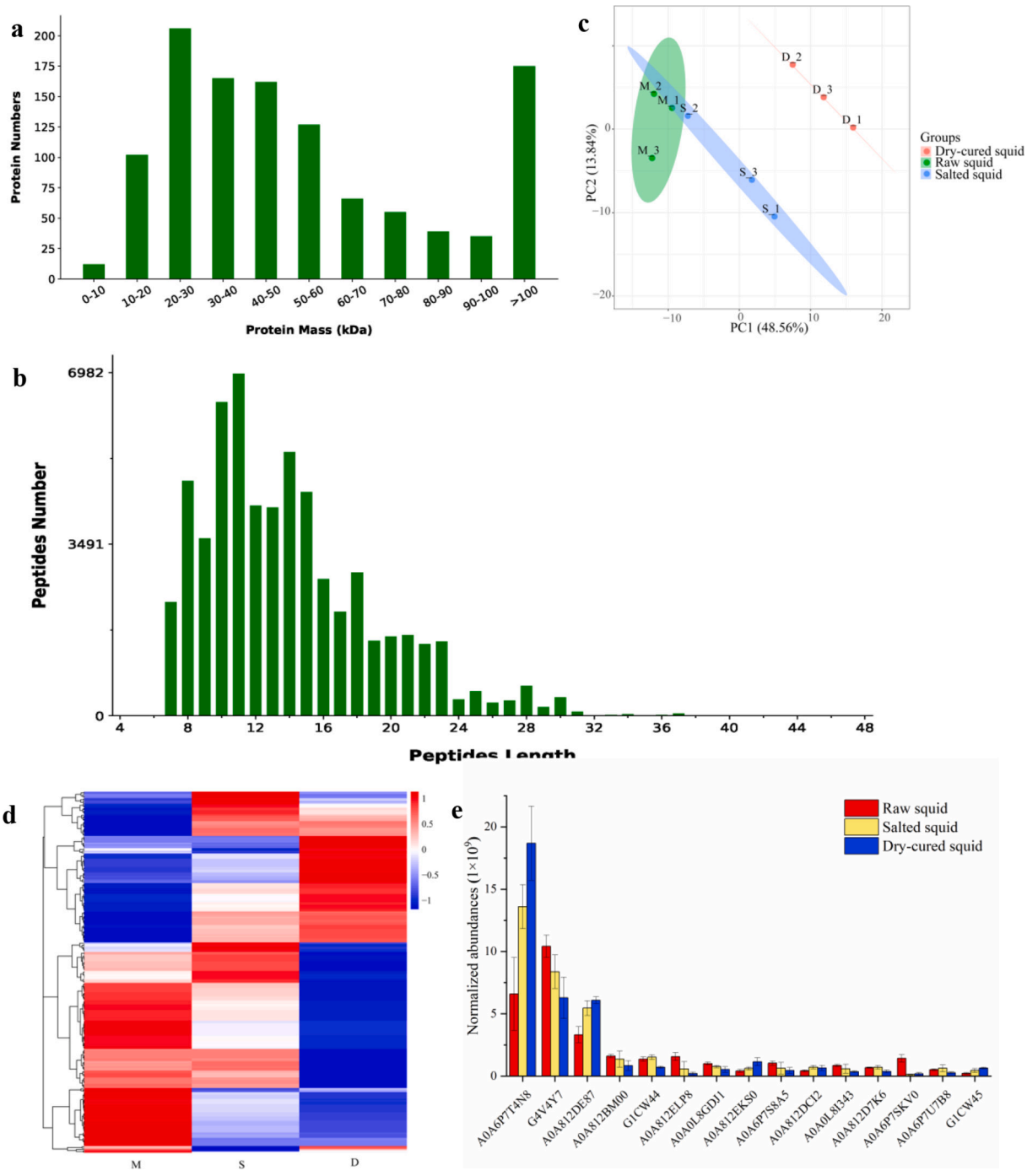

Fig. 1. Proteomic analysis of various squid samples. (a) Molecular weight and (b) pepitides number of all identified proteins. (c) Heat map visualisation of the differentially abundant proteins. Red indicates higher relative abundance of the protein, while blue indicates lower relative abundance. (d) Intensities of the top 15 differentially abundant proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quality changes of squid samples. In the D versus S comparison, only 6 key DAPs were identified in dry-cured squid samples (4 downregulated and 2 upregulated). The relative abundance of muscle myosin heavy chain (B2ZTQ5), actin (Q9NBZ7, Q9NC50) and myosin light chain (A0A6P7T5S9) declined markedly after drying, suggesting the degradation of cytoskeletal proteins and the breakdown of the muscle structure. Compared with the raw squid samples, there were 23 key DAPs (12 downregulated and 11 upregulated) in the dry-cured squid samples. Myosin heavy chain (G4V4Y7, B2ZTQ5), actin (Q9NBY3, A0A812CKE2, Q9NC46) and filamin A (A0A812BM00) showed significantly decreased expression. The relative abundance of myosin light chain (A0A6P7T4N8), tubulin chain (I0JGU7, A0A812DI02, A0A6P7TE58, A0A812DIH7) and hypothetical protein (A0A812EKS0) was enhanced after processing. Heat shock proteins, which are induced in response to environment stress, are closely correlated with muscle structure (Malheiros et al., 2018). Yan, Li, Hu, Ma, and Lu (2021) suggested that heat shock proteins are tenderness markers in yak meat. The release of cytochrome *c* could activate caspases-3 and accelerate the degradation of myofibrillar proteins (Ding, Wei, Zhang, Zhang, & Huang, 2021). After the dry-curing process, the relative abundance of heat shock proteins (A0A812CBL4, A0A812BR06) and cytochrome *c*1 (A0A812BXV5) in the squid samples was highly increased, suggesting the great variation in

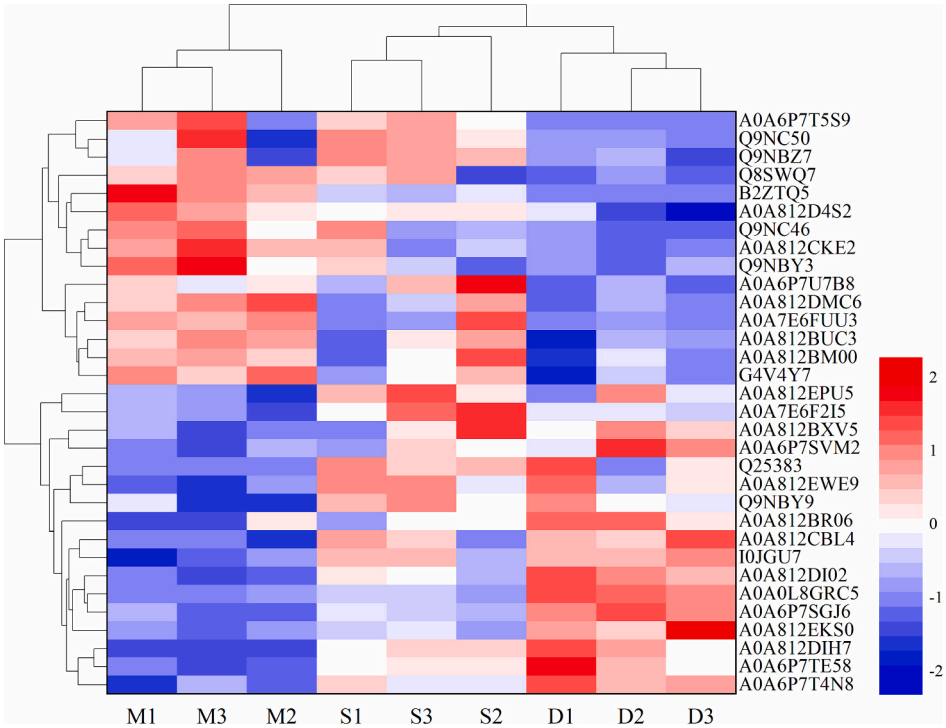

Fig. 2. Heat map visualisation of 32 differentially abundant proteins related to flavour and texture development in various squid samples. Red indicates a higher relative abundance of the protein, while blue indicates a lower relative abundance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

myofibrillar structure and textural quality. As structural constituents of the extracellular matrix, collagens are considered to be responsible for the textural characteristics of fish muscles (Wu et al., 2023). A higher density of collagen was related to more intense hardness and chewiness and increased shear force (Jia et al., 2022). The relative abundance of collagen type V/XI/XXIV/XXVII alpha (A0A812EPU5) in squid samples increased significantly after curing, suggesting a change in the textural quality of the salted squid samples.

The results from SDS-PAGE analysis showed that there was marked variation in the protein profile of the squid samples during processing (Fig. 3). Based on the literature, myosin heavy chain, paramyosin, actin, tropomysin, troponin T and myosin light chain were identified in the electrophoretic gel of total protein extracted from various squid samples (Nyaisaba et al., 2019; Xu et al., 2020). The intensity of the myosin heavy chain band apparently decreased after the dry-curing process, a change that is consistent with the proteomics analysis regarding muscle myosin heavy chain (G4V4Y7, B2ZTQ5). There was a slight decrease in the actin band, which was in line with the changes in relative abundance of actin (A0A6P7U7B8, A0A812CKE2, Q9NC46, Q9NBZ7, Q9NC50). The degradation of tubulin (~50 kDa) of squid samples during processing was also observed. Tubulin, formed by polymerisation of alpha and beta tubulins, is one of the proteins that composes microtubules, which are key structural components of the cytoskeleton. The native α/β-tubulin heterodimer can be degraded by E3 ubiquitin ligases (Lundin, Leroux, & Stirling, 2010). Proteolysis in dry-cured seafood products during processing is attributed to the joint influence of microbial enzymes (Zhao, Chong, Hu, et al., 2022) as well as endogenous enzymes. Calpains and cathepsin are the main endogenous enzymes that lead to the degradation of myofibrillar protein (Yang et al., 2019).

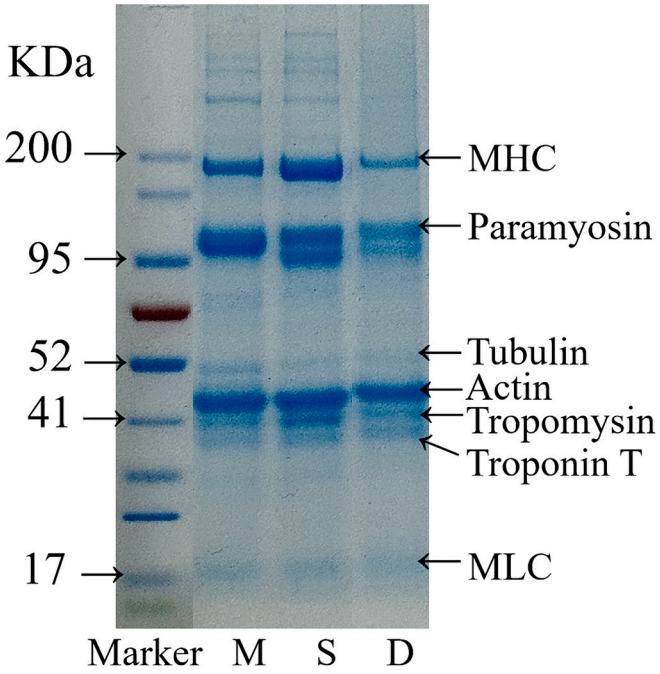

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of various squid samples (total proteins). M, raw squid samples; S, salted squid samples; D, dry-cured squid samples.

3.2. Bioinformatics analysis of DAPs

The functions of the DAPs were evaluated with GO analysis, which categorised these proteins into three groups: BP, MF and CC. Fig. 4 demonstrates the GO enrichment analysis of DAPs in the S versus M, D versus S and D versus M comparisons. In the S versus M comparison (Fig. 4a), there were 47 DAPs in the MF group ($P < 0.05$), including 31 upregulated and 16 downregulated proteins. Most of the DAPs are associated with ion binding (GO:0043167), hydrolase activity (GO:0016787) and metal ion binding (GO:0046872). Muscle myosin

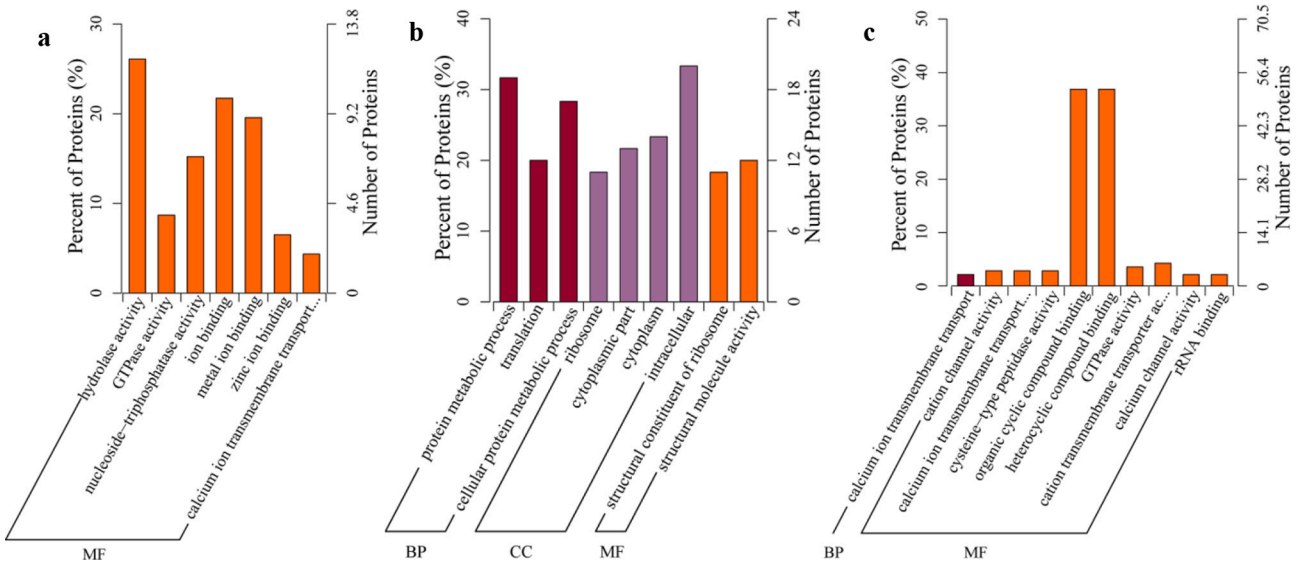

Fig. 4. Gene Ontology (GO) functional annotation classification of differentially abundant proteins in the salted (S) versus raw (M) (a), dry-cured (D) versus S (b) and D versus M (c) comparisons.

heavy chain (B2ZTQ5), tubulin beta chain (I0JGU7), tubulin beta (A0A812DI02), tubulin alpha (A0A812DIH7) and tubulin beta-2 chain like (A0A6P7TE58) play a dominant role in hydrolase activity. The increased relative abundance of these DAPs indicates the increased hydrolase activity in squid muscle. Myosin regulatory light chain LC-2 (A0A6P7T4N8) was related to (metal) ion binding; its increased relative abundance suggests an increase in (metal) ion binding.

In the D versus S comparison (Fig. 4b), 129 DAPs were associated with BP (37.2%), CC (45.0%) and MF (17.8%) ($P < 0.05$), comprising 22 upregulated proteins and 107 downregulated proteins. In BP, the DAPs are related to affect protein metabolic process (GO:0019538), translation (GO:0006412) and cellular protein metabolic process (GO:0044267). In CC, the DAPs are distributed in ribosome (GO:0005840), cytoplasmic part (GO:0044444), cytoplasm (GO:0005737) and intracellular (GO:0005622), which play important roles in cellular structure. In MF, the DAPs are related to the structural constituent of the ribosome (GO:0003735) and structural molecule activity (GO:0005198). As the major ribosomal components, ribosomal proteins are responsible for preserving the stability of transfer RNA (tRNA) and are considered to be indicators of food textural characteristics (Song et al., 2022; Zhang et al., 2020). The downregulation of ribosomal proteins suggests the breakdown of structure proteins. It can be inferred that drying has a vital effect on muscle stability of squid samples.

In the D versus M comparison, there were 136 DAPs related to MF (97.8%) and BP (2.2%) ($P < 0.05$), specifically 91 downregulated and 45 upregulated proteins. Most of the DAPs, including myosin heavy chains (G4V4Y7, B2ZTQ5), heat shock protein 90 alpha (A0A812BR06) and ribosomal proteins (A0A812DHQ6, A0A812ALH6, A0A6P7SPU5, A0A0L8GSW6, A0A0L8I1J6), are involved in heterocyclic compound binding (GO:1901363) and organic cyclic compound binding (GO:0097159). Food processing technology, such as the heating process, can induce changes in protein composition and structure, and thus affect food flavour and flavour binding characteristics (Liu et al., 2022). The binding abilities of myofibrillar protein to flavours compounds such as aldehydes and ketones decreases after oxidation (Shen, Stephen Elmore, Zhao, & Sun, 2020). The release of flavour compounds is affected by the variations of myofibrillar protein structure via secondary structure conformation alteration and disruption of interior binding sites of the hydrophobic and reactive sulfhydryl area (Han, Cai, Cheng, & Sun, 2021; Lou, Yang, Sun, Pan, & Cao, 2017). Based on the GO analysis, the changes in the structure and binding abilities of muscle proteins had a great influence on the sensory characteristics of the squid samples.

3.3. Analysis of myofibrillar protein, amino nitrogen and FAAs

The variations in the myofibrillar protein, amino nitrogen and FAAs concentrations were investigated (Fig. 5a–e). The myofibrillar protein content in the squid samples decreased significantly from 188.93 to 178.61 μg/g after the dry-curing process; this finding is consistent with the variation in the relative abundance of myofibrillar proteins from the proteomics data. The amino nitrogen concentration in the squid samples decreased during the curing period but ultimately increased after drying. The FAA concentration showed a similar trend. The umami, sweet and bitter FAA concentrations were 45.31, 608.30 and 830.90 mg/100 g, respectively after processing. There were 6 taste-active FAAs (TAVs ≥1) in the squid samples, including glutamic acid, alanine, methionine, arginine and histidine (Table S2). These FAAs could contribute greatly to the taste characteristics of dry-cured squid. The increase in the amino nitrogen and total FAA concentrations indicates the dramatic protein hydrolysis in the squid samples.

3.4. Analysis of volatile substances by GC–MS

Fig. 5f–j shows the variation in the volatile compound composition of the dry-cured squid samples. There were 44 volatile substances identified, and the variety and concentrations of aroma substances increased during processing. After processing, the concentrations of flavour compounds increased dramatically from 164.23 to 1165.69 μg/kg. Both curing and drying have a great influence on the content of volatile substances. The increase in the amount of volatile substances are connected with the treatment conditions, for example, the salt content, time and temperature. Alcohols and carbonyl compounds are the main aroma compounds of dry-cured seafood products due to their low threshold values (Moretti, Vasconi, Caprino, & Bellagamba, 2017). Ketones, alcohols and aldehydes were dominant in the dry-cured squid, of which the relative concentrations reached 393.71, 379.68 and 224.61 μg/kg, respectively, after dry-curing. Among these compounds, 2,3-butanedione was the most abundant, which imparted a delightful butter and popcorn smell to the squid products. The ROAV of the volatile compounds is shown in Table S3. Eleven compounds played a crucial role in the aroma characteristics of the products, including 2,3-butanedione, methional (roast), nonanal (fatty, green), hexanal (grass, fat), 3-methylbutanal (caramel), heptanal (fat), (E)-2-octenal (roast), decanal (soap,

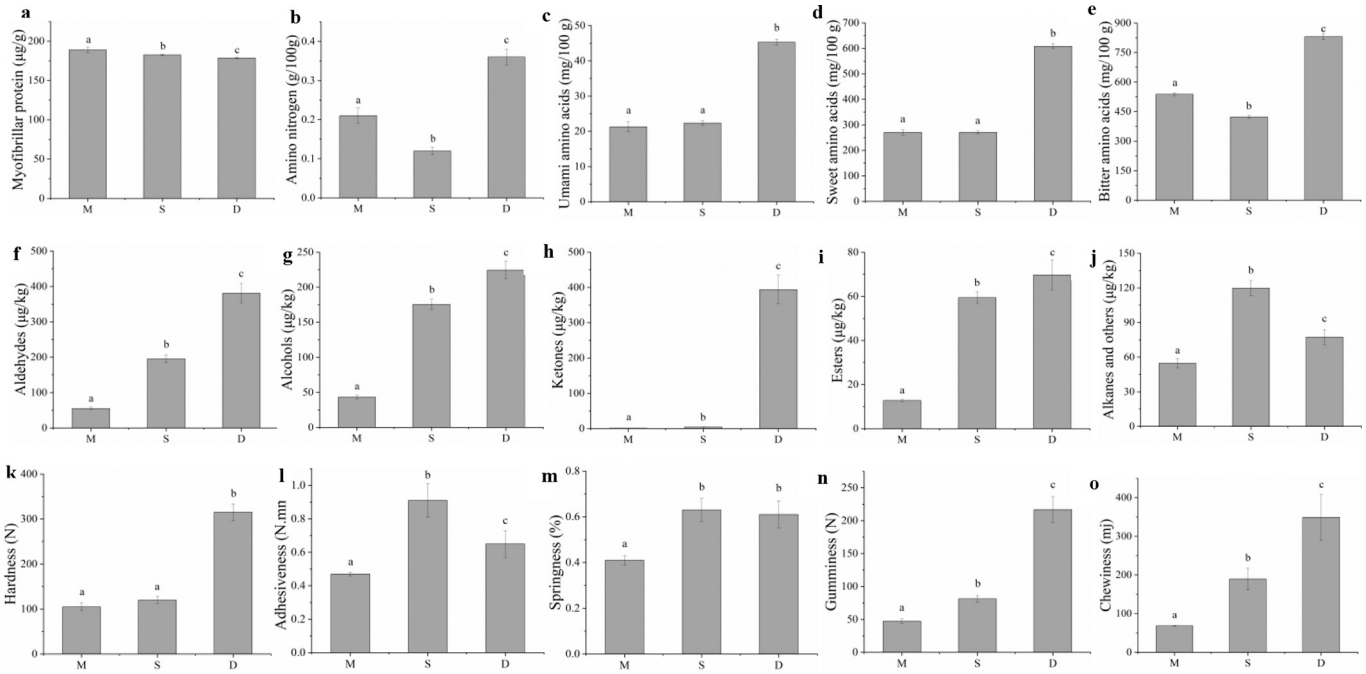

Fig. 5. Changes in concentrations of (a) myofibrillar protein, (b) amino nitrogen, (c–e) free amino acids and (f–j) volatile compounds and (k–o) texture profile of dry-cured squid samples during processing. Different letters in the same subgraph indicate a significant difference ($p < 0.05$).

tallow), 1-octen-3-ol (fatty, fishy), trimethylamine (fermented fish-like aroma) and dimethyl trisulfide (fishy, fried garlic, onion). The production of volatile compounds is attributed to the biochemical reactions of aroma precursors – for example, FAAs and unsaturated fatty acids (Zhao, Hu, Zhou, & Chen, 2022).

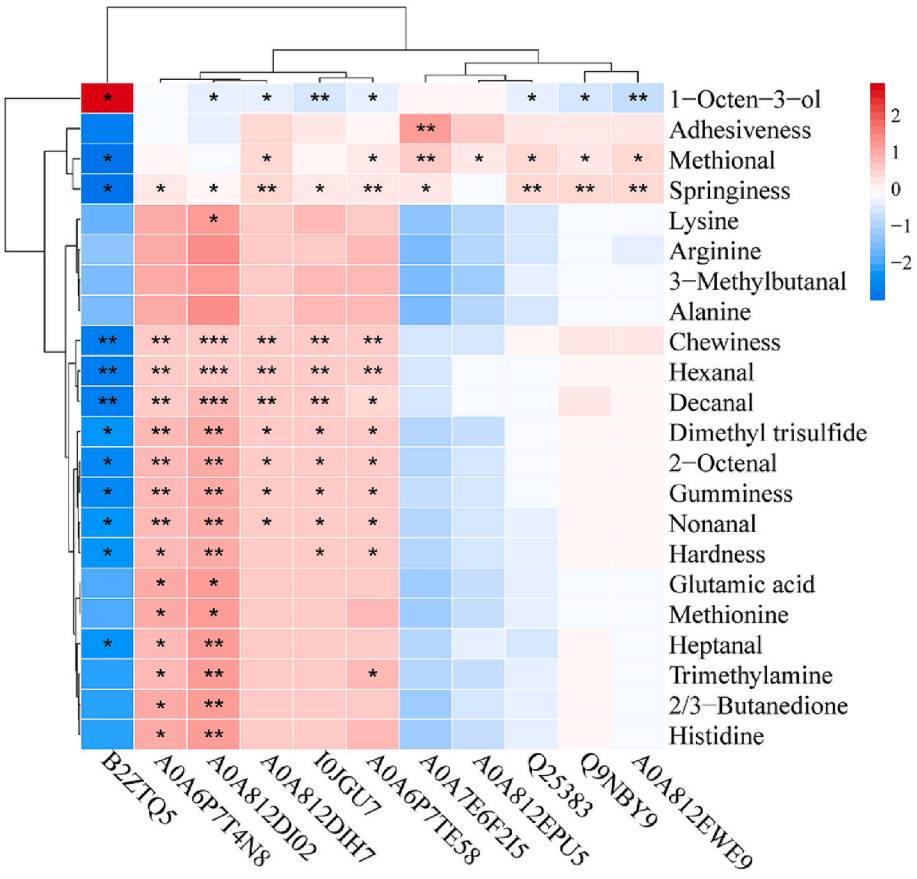

Fig. 6. Heat map of Pearson's correlation coefficients demonstrating the correlation between the differentially abundant proteins (DAPs) and texture and sensorial characteristics.

3.5. TPA analysis of dry-cured squid

Fig. 5k–o present the variations in the texture profile of the squid samples during processing. The adhesiveness, hardness, gumminess, chewiness and springiness of the squid samples were enhanced to different extents after processing. The processing parameters, such as salt and temperature, have a great effect on the textural quality of processed meat by affecting its water distribution and mobility (Zhang, Yin, & Wang, 2021). Denaturation of various muscle structural proteins such as myosin, actin and collagens in processing lead to changes in muscle texture during heat treatment (Ishiwatari, Fukuoka, & Sakai, 2013). The proteins that are connected to muscle texture are principally involved in oxidative stress and protein structure (Song et al., 2022).

3.6. The relationship between DAPs and the quality traits of the squid samples

Pearson's correlation analysis was performed to evaluate the relationship between the DAPs and quality attributes of dry-cured squid during processing; the results are presented in a heat map (Fig. 6). Eleven DAPs were highly correlated with the sensory and textual qualities of the squid samples. Most of these DAPs were correlated with the springiness of the dry-cured squid during processing. The development of adhesiveness was connected with changes in the relative abundance of titin (A0A7E6F2I5), a giant protein that is located near myosin. The degradation of titin has been reported to be related with fish softening (Yang et al., 2019). In the present study, the relative abundance of titin (A0A7E6F2I5), with a molecular weight of 1262.8 kDa, increased remarkably after the curing process ($P < 0.05$), which may have resulted in the enhanced adhesiveness. The increased relative abundance of myosin regulatory light chain LC-2 (A0A6P7T4N8), tubulin beta chain (I0JGU7), tubulin beta (A0A812DI02) and tubulin beta-2 chain-like (A0A6P7TE58) was positively related with the variation in hardness, springiness, gumminess and chewiness of squid samples during the dry-curing process. Comparatively, the relative abundance of muscle myosin heavy chain (B2ZTQ5) decreased during processing; this reduction was negatively correlated with these textural changes. Myosin is one of the most abundant myofilaments and contributes to the the cytoskeletal structure (Frontera & Ochala, 2015). Myosin regulatory light chain LC-2 (A0A6P7T4N8), the most abundant DAP identified in the squid samples, has a greater contribution to the textural changes of dry-cured squid during processing. According to Ge, Xu, Xia, Zhao, and Jiang (2018), the degree of myosin regulatory light chain phosphorylation has a marked effect on the structural integrity of thick filaments and their interactions with thin filaments. Tubulin, as a key cytoskeleton protein, plays a vital role in muscle textural characteristics. Xu, Mao, Deng, Xie, and Luo (2023) demonstrated that the colour and textural properties of shrimp during storage are closely associated with tubulin. Zhang et al. (2021) regarded tubulin as one of the potential structural protein markers that influence the tenderness of cooked meat. The high correlation between tubulin beta chain and the textural properties suggests the important role of tubulin in the textural changes of squid samples during the dry-curing process.

Based on the results, muscle myosin heavy chain (B2ZTQ5), the myosin regulatory light chain LC-2 (A0A6P7T4N8), tubulin beta chain (I0JGU7), tubulin beta (A0A812DI02), tubulin beta-2 chain-like (A0A6P7TE58) and tubulin alpha (A0A812DIH7) were closely connected with the flavour of squid samples. The increased relative abundance of myosin regulatory light chain LC-2 (A0A6P7T4N8) and tubulin beta (A0A812DI02) correlated positively with the formation of hexanal (grass, tallow, fat), 2-octenal (roast, fatty), nonanal (fatty) and decanal (soap, tallow) ($P < 0.01$), which were the main aldehydes in the squid samples. The development of trimethylamine and dimethyl trisulfide, which play important roles in fermented fish aroma formation (Song et al., 2021), was significantly correlated with the increased relative abundance of myosin regulatory light chain LC-2 (A0A6P7T4N8), tubulin beta (A0A812DI02) and tubulin beta (A0A812DI02) ($P < 0.05$). Of the taste-active FAAs in dry-cured squid, glutamic acid and histidine were positively correlated with tubulin beta (A0A812DI02) ($P < 0.05$). These results suggested that myosin regulatory light chain and tubulin had a remarkable contribution to sensory attributes of dry-cured squid samples.

4. Conclusions

Variations in the protein profile were analysed to investigate proteolysis in dry-cured squid products and its connection to the change in sensory and textural attributes during the dry-curing process. The protein composition changed remarkably during processing: there were a total of 268 DAPs in the various dry-cured squid samples. Muscle proteolysis occurred in dry-cured squid as more low-molecular-weight substances were produced. Quantification of myofibrillar protein, amino nitrogen and total FAAs confirmed the breakdown of muscle proteins. Thirty-two DAPs were identified as key proteins that were correlated with the development of the sensory and textural attributes, of which myosin regulatory light chain and tubulin beta chain played important roles. These findings contribute to revealing the changes in protein profile and its connection to the sensory and texture properties of squid products during the dry-curing process.

CRediT authorship contribution statement

Dandan Zhao: Writing – original draft, Funding acquisition, Conceptualization. **Yizhou Fang:** Formal analysis, Data curation, Methodology. **Zhengxun Wei:** Methodology, Investigation. **Wenkai Duan:** Software. **Yu Chen:** Writing – review & editing. **Xuxia Zhou:** Validation, Supervision. **Chaogeng Xiao:** Validation. **Wenxuan Chen:** Writing – review & editing, Visualization, Resources.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101409.

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