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Integrated 4D label-free proteomics and data mining to elucidate the effects of thermal processing on crisp grass carp protein profiles

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ABSTRACT

The crisp grass carp (CGC; *Ctenopharyngodon idellus* C. et V.), known for its unique texture and flavour, is a culinary delicacy whose quality is significantly influenced by thermal processing. This study employed 4D label-free proteomics and data mining techniques to investigate the proteomic changes in CGC muscle tissue induced by various heating temperatures. CGC samples were subjected to a series of heat treatments at increasing temperatures from 20 °C to 90 °C. Proteins were extracted, digested, and analysed using high-resolution mass spectrometry. The proteomic data were then subjected to extensive bioinformatics analysis, including GO and KEGG pathway enrichment. We identified a total of 1085 proteins, 516 of which were shared across all the temperature treatments, indicating a core proteome responsible for CGC textural properties. Differential expression analysis revealed temperature-dependent changes, with significant alterations observed at 90 °C, suggesting denaturation or aggregation of proteins at higher temperatures. Functional enrichment analysis indicated that proteins involved in amino acid metabolism, glutathione metabolism, and nucleotide metabolism were particularly affected by heat. Textural analysis correlated these proteomic changes with alterations in CGC quality attributes, pinpointing 70 °C as the optimum temperature for maintaining the desired texture. A strong positive correlation between specific upregulated proteins was identified, such as the tubulin alpha chain and collagen alpha-1(IV) chain, and the improved textural properties of CGC during thermal processing, suggesting their potential as the potential biomarkers. This study offers a comprehensive proteomic view of the thermal stability and functionality of CGC proteins, delivering invaluable insights for both the culinary processing and scientific management of CGC. Our findings not only deepen the understanding of the molecular mechanisms underpinning the textural alterations in CGC during thermal processing but also furnish practical insights for the aquaculture industry. These insights could be leveraged to optimize cooking techniques, thereby enhancing the quality and consumer appeal of CGC products.

1. Introduction

The crisp grass carp (CGC; *Ctenopharyngodon idellus* C. et V.), a cultivated variant of the common grass carp, was selectively bred to feed exclusively on faba beans (*Vicia faba* L.), resulting in a distinctive textural profile that is noticeably firmer and more brittle compared to its traditional counterpart (Li et al., 2022a). This specialized diet not only influences the growth patterns of the CGC but also enhances its muscle firmness, chewiness, and elasticity, leading to a product with increased market value and unique sensory attributes. The improved textural qualities of the CGC have garnered significant consumer appeal. Despite extensive research into the textural characteristics and gut microbiota of CGC throughout its farming cycle (Li et al., 2018), there is a notable lack of scientific understanding regarding how heat treatment affects CGC crispness. Thermal processing markedly affects the organoleptic properties of fish products, including texture, flavor, and aroma, thereby influencing consumer acceptance and preferences (Lin et al., 2016; Xu et al., 2020). Furthermore, it can modulate the nutritional value of fish,

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impacting the bioavailability of critical nutrients such as fatty acids and vitamins (Quitral et al., 2006; Tsoupras et al., 2022). This processing also prompts alterations in the tertiary and quaternary structures of proteins, which may affect their digestibility and the profile of available amino acids (Wang et al., 2021a). The implications of thermal processing for protein integrity represent a pivotal research domain with significant health-related consequences. Thermal-induced protein denaturation and aggregation during culinary preparation can substantially modify protein functionality. Therefore, elucidating the mechanisms by which thermal processing affects protein structures in fish is essential for ensuring food safety and for optimizing the nutritional quality of fish products. Unraveling the proteomic alterations postheating and their corresponding impact on CGC is vital to support the progression of processing techniques and the development of new products within the CGC sector. The investigation of these thermal-induced changes holds immense theoretical importance and offers practical insights for the scientific management of CGC. Proteomics, with its ability to analyse protein expression, posttranslational modifications, interactions, and functions, is an essential tool in this exploration (Forné et al., 2010). Employing proteomic approaches allows us to delve deeply into the biological characteristics of CGC, enriching our understanding of its unique textural attributes and advancing scientific knowledge in the field of fish science and technology.

Significant advances have been made in the field of fish proteomics, with many research findings emerging in succession. For example, proteomics analysis techniques have been used to investigate fish immune response mechanisms (Ye et al., 2018), monitor fish reactions to toxins and environmental stress (Karim et al., 2011), and decode the mechanisms of fish disease occurrence and the interactions between hosts and pathogens (Ahmed et al., 2019). Among these advancements, the development of 4D label-free proteomics technology has taken new research efforts. Due to the complexity of proteins and limitations of detection techniques, traditional proteomics methods often struggle to achieve comprehensive coverage of the proteome. However, 4D label-free proteomics technology, which combines liquid chromatography (LC), high-resolution mass spectrometry (MS), ion mobility spectrometry (IMS), and time-of-flight (TOF) mass spectrometry, has realized high-dimensional separation and precise quantification of proteins (Yu et al., 2020). The integrated application of this technology not only greatly expands the coverage and resolution of protein detection but also enhances the dynamic range of analysis through improvements in time and spatial resolution, allowing researchers to capture subtle changes in protein expression. These changes could be key indicators of environmental stress (Wang et al., 2021b), changes in nutritional status (Wang et al., 2023), or disease progression (Huo et al., 2023). 4D label-free proteomics technology provides us with an unprecedented macroscopic perspective to comprehensively understand the biological traits of CGC at the protein level. Currently, this technology has been widely applied in various fields, including fish studies. In silver carp (*Hypophthalmichthys molitrix*), 4D label-free proteomics has been used to analyse the changes in the physical, structural, and assembly characteristics of myofibrillar proteins (MPs) (Li et al., 2023). As a process of deeply exploring large datasets, data mining excels, particularly in revealing patterns and correlations within proteomics data. Machine learning algorithms, cluster analysis, and principal component analysis have become key tools for interpreting the vast amount of data generated from proteomic studies. Combining data mining with proteomics analysis can identify key proteins affected during thermal processing and the biological pathways involved (Haoudi and Bensmail, 2006). This integrated analytical approach can clearly define the critical temperature thresholds affecting the protein stability and functionality of CGC, providing a scientific basis for determining protein stability points and functionality transition points.

In this study, 4D label-free proteomics coupled with data mining analysis was employed to elucidate the complexity of protein composition and functionality during the thermal processing of CGC. We particularly examined the alterations in protein structures and properties induced by heat treatment and their subsequent impact on the texture and flavor of the CGC. These insights not only advance our understanding of the implications of thermal processing in food technology but also provide a scientific foundation for enhancing the quality of CGC. Through this approach, we not only revealed the molecular mechanism behind the tenderness of CGC flesh but also provided robust molecular biological support for the aquaculture industry, aiding in scientific management and genetic improvement. Furthermore, the findings of this study also have significant implications and a broad impact on the research of aquatic food science, nutrition, and even environmental ecology, opening up new perspectives and research avenues for academic studies and industrial development in related fields.

2. Materials and methods

2.1. CGC sample preparation

All CGC were cultured from the same batch of fry under the same conditions at the Huangsha Aquatic Products Market, Guangzhou City, Guangdong Province, China. Fresh CGC weighing approximately 4.5 kg were screened, stunned, decapitated, scaled, gutted and washed under running cool water to remove mucus, blood and other impurities. Finally, blocks with 3 cm × 3 cm × 2 cm were carefully removed from the dorsal muscles. To ensure uniformity, each sample was taken from a predetermined position and conformed to a specified size. Precise thermal control was employed in a water bath to subject the samples to heat treatment at various temperatures: 20 °C, 40 °C, 50 °C, 70 °C, 80 °C, and 90 °C. A uniform heating duration of 30 min was maintained for each temperature setting to ensure thorough thermal penetration of the samples. External thermocouples (Testo SE & Co. KGaA, Lenzkirch, Germany) with a measurement accuracy of ±0.5 °C were used to continuously record the core temperature of the samples throughout the heat treatment process. Regular calibration was performed prior to each experimental run based on a certified reference thermometer. The samples were categorized into groups based on the treatment temperatures as follows: T0 (untreated fresh CGC), T20 (CGC heated at 20 °C), T40 (CGC heated at 40 °C), T50 (CGC heated at 50 °C), T70 (CGC heated at 70 °C), T80 (CGC heated at 80 °C), and T90 (CGC heated at 90 °C). After heat treatment, the samples were cooled and stored at -40 °C until use. The same parts of three CGCs were selected for treatment in each group to minimize the effect of individual differences on the results.

2.2. Protein extraction and digestion

Upon the addition of liquid nitrogen, the CGC tissue samples were thoroughly ground into a fine powder in a prechilled mortar. Each sample was lysed with four volumes (relative to the tissue powder) of SDT buffer (containing 4% [w/v] SDS, 100 mM Tris/HCl [pH 7.6], 0.1 M DTT). After centrifugation at 4 °C, 12,000 g for 10 min, the supernatant was carefully transferred to a fresh sterile centrifuge tube. The protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). A 20 μg of protein for each sample was mixed with 5X loading buffer respectively and boiled for 5 min. The proteins were separated on a precast 4%-20% SDS-PAGE gel at a constant voltage of 180 V for 45 min and stained with Coomassie Blue R-250. Following the filter-aided sample preparation (FASP) method, proteins were digested with trypsin (Wiśniewski, 2018). The digested peptides from each sample were desalted using C18 cartridges (Empore™ SPE Cartridges C18, standard density, bed I.D. 7 mm, volume 3 ml, Sigma), concentrated by vacuum centrifugation, and reconstituted in 40 μl of 0.1% (v/v) formic acid. The peptide content was estimated by the UV light spectral density at 280 nm (Huo et al., 2023).

2.3. LC–MS/MS data acquisition

Each sample was separated with a nanoliter flow rate HPLC liquid system Easy nLC (Thermo Scientific, USA) (buffer solution A: 0.1% formic acid aqueous solution, buffer solution B: 0.1% formic acid acetonitrile aqueous solution (84% acetonitrile)). The product was loaded from the autosampler onto a loading column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18) and separated on an analytical column (Thermo Scientific EASY column, 10 cm, ID75 μm, 3 μm, C18-A2) at a flow rate of 300 nL/min. The separated samples were analysed by mass spectrometry using a timsTOF Pro mass spectrometer. MS and MS/MS were detected and analysed using TOF in positive ion detection mode with the ion source voltage set to 1.5 kV. The mass spectral scanning range was set from 100 to 1700 m/z. The data acquisition mode was performed with a parallel accumulation-serial fragmentation (PASEF) mode.

2.4. Protein identification and bioinformatics analysis

Protein identification: The raw LC–MS/MS data were analysed with MaxQuant software (version 1.6.14, https://www.maxquant.org/) for library identification and quantitative analysis (Prianichnikov et al., 2020).

Subcellular localization analysis: Protein sequences were subcellularly localized using the online software CELLO (version 2.5, http://cello.life.nctu.edu.tw/) (Yu et al., 2014).

Protein structural domain analysis: The InterProScan software package was utilized to run scanning algorithms from the InterPro database for functional characterization of sequences, thus obtaining structural domain annotations of the target protein sequences in the Pfam database (Jones et al., 2014).

GO functional annotation: The process of GO annotation of a target protein collection using Blast2GO (https://www.blast2go.com/) can be summarized into four steps: sequence comparison (Blast), GO term extraction (Mapping), GO annotation (Annotation) and InterProScan supplemental annotation (Annotation Augmentation) (Conesa et al., 2005).

KEGG pathway annotation: KEGG pathway annotation was performed on the target protein collection using KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/tools/kaas/) software (Moriya et al., 2007).

Enrichment analysis: Enrichment analysis was performed by comparing the distribution of individual GO classifications (or KEGG pathways, domains, or others) in the target protein set and the overall protein set using Fisher's exact test (FET)(Ji et al., 2011).

Cluster analysis: Prior to the initiation of clustering analysis, extensive preprocessing of the dataset was conducted, encompassing standardization of the data to normalize the range of variable scales and imputation of missing values to maintain the integrity of the clustering process. The refined dataset was then subjected to a dual-phase clustering approach, employing both the Mfuzz package (version 2.32.0) for soft clustering of time series expression data and hierarchical clustering algorithms to delineate and discern intrinsic data groupings (Kumar et al., 2007; Zou et al., 2021). The clustering outcomes were visually represented through heatmaps constructed using the Complexheatmap R software package (R version 3.4)(Tian et al., 2022).

Correlation analysis: Pearson correlation coefficients were calculated to assess the relationship between differentially expressed proteins and quality attributes of CGC during thermal processing. Data normalization was performed to ensure comparability, and a significance threshold was established at $p < 0.05$ with Bonferroni correction for multiple testing. Heatmaps were generated to visualize the correlations (Schober et al., 2018).

2.5. Texture and nutritional analysis of CGC

The textural properties of CGC were analysed using a TA-XT2i texture analyzer (Stable Micro Systems Ltd., UK) equipped with a 1 kg load cell and a 35 mm diameter cylindrical probe. The testing protocol involved a double compression test at 30% strain with pre- and post-compression speeds set at 1 mm/s and 5 mm/s, respectively, with a 5-s interval between compressions. The textural parameters measured included hardness, adhensiveness, chewiness, and elasticity, with definitions for each parameter following the methodology described by Bourne (1978). Nutritional composition was determined according to the methods of the China national standard, with moisture (GB/T 5009.3—2003), protein (GB/T 6432—1994), ash (GB/T 5009.4—2003), and crude fat content (GB5009.6-2016) being quantified. The amino acid profile was assessed using the PICO TAG method, whereby samples were hydrolyzed in 6 N HCl for 24 h, followed by neutralization, dilution, and derivatization, and analysed using high-performance liquid chromatography (Lin et al., 2016). All experiments were replicated at each temperature point to ensure the reliability of the data.

3. Results

3.1. Identification and quantitative analysis of the CGC proteome

The project identified the structure of the metabolites of CGC under different conditions by matching the retention time, fragmentation spectra, collision energy and other information. The results of the identification were rigorously manually checked and confirmed. Finally, 123,596 matched spectra, 8,921 peptides, 1,085 identified proteins, and 1,071 quantifiable proteins were obtained. Most protein groups consisted of 1 protein with a molecular weight mainly between 10 and 500 kDa. The number and coverage of peptides in the protein group were distributed as 0%-85% and 1-100, respectively. Moreover, the peptide mass ranged from 700 to 4000 Da, and the peptide length ranged from 7 to 45 amino acids. There were 856 overlapping proteins in the three replicate groups of untreated CGC (T0). There were 881, 812, 860, 777, 773, and 714 overlapping proteins in the replicate groups of CGC treated at different temperatures of 20, 40, 50, 70, 80, and 90, respectively. Intersection of these seven groups of overlapping proteins revealed that there were 516 shared proteins (Fig. 1).

3.2. Differential analysis of protein expression in CGC treated with different temperatures

Differences in CGC protein expression in response to different temperatures were analysed by comparing each treated sample (Tn, n: the treatment temperature) with fresh CGC. The results showed that a heating temperature of 90 °C resulted in the greatest number of protein differences compared with 316, while a heating temperature of 20 °C resulted in a relatively low number of 197 proteins. The other four intermediate temperatures (40 °C, 50 °C, 70 °C, 80 °C) exhibited similar numbers of different proteins. Among the differential proteins identified by the six temperature treatments, 41 were shared differential proteins, of which six were upregulated proteins (FC>2, A0A3N0XU78 (Tubulin alpha chain), A0A3N0YC21(Collagen alpha-1(IV) chain), A0A3N0YEG7 (Medium-chain specific acyl-CoA dehydrogenase), A0A3N0YG26 (Pre-B-cell leukemia transcription factor-interacting protein 1), A0A3N0YVF2 (Protein MEMO1), A0A3N0YY23 (DNA damage-binding protein 1)), nine were downregulated proteins (FC<0.5, Q76CD7 (MHC class I antigen), A0A3N0XIA3 (Serine/arginine-rich splicing factor 5), A0A3N0XQ52 (Gelsolin), A0A3N0YEC1 (Junctophilin), A0A3N0YI36 (Interferon-inducible GTPase 5), A0A3N0YY54 (Creatine kinase), A0A3N0Z1T0 (Afadin), Q6T3V0 (Dolichyl-diphosphooligosaccharide), E9JM91 (Beta-actin)), eighteen more proteins, and the other 8 differential proteins are expressed in different groups with

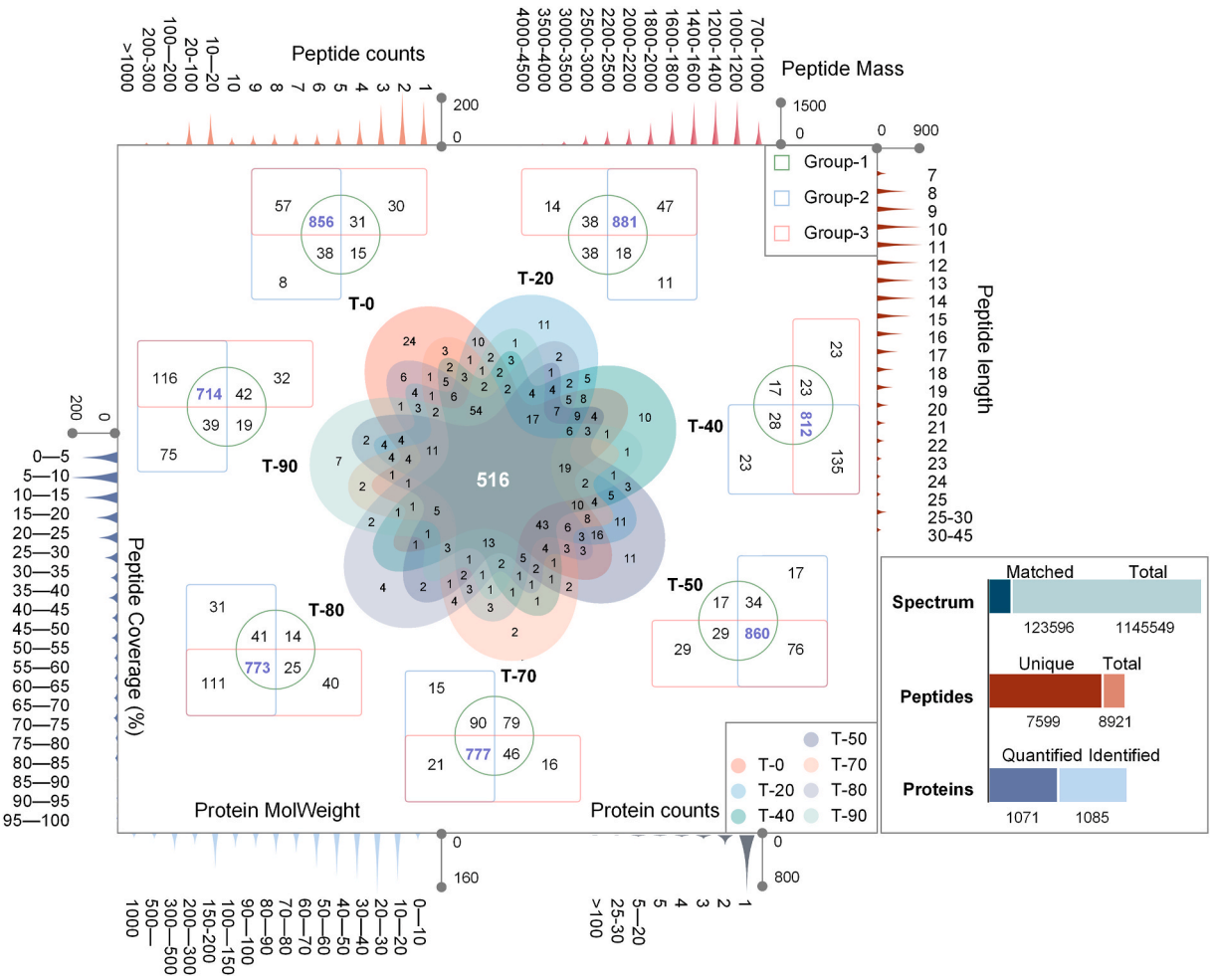

Fig. 1. Quantitative proteome analysis of CGC. Left center: analysis of protein overlap: Edwards-Venn plots: intragroup; Petal-Venn plot: intergroup. Left outside: Characterization of identified proteins and peptides: Left: peptide coverage; Bottom: protein molecular weight and protein number; Right: peptide length; Top: peptide number and mass. Right: Statistical chart of the identification and quantification data.

inconsistent trends. Among these differential proteins, the most common of which were downregulated as the number of proteins increased with temperature. Notably, the number of proteins whose expression was significantly downregulated (fold change (FC) < 0.1) was also consistent with this pattern (Fig. 2).

The effect of gradual warming on CGC proteins was further explored

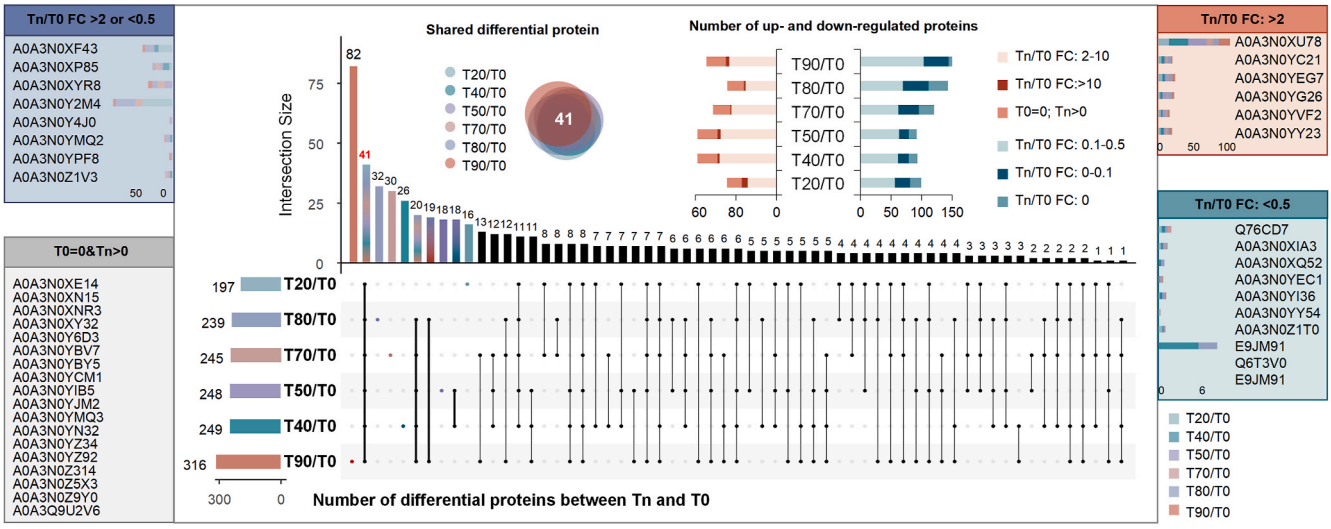

Fig. 2. Analysis of differential proteins in CGC treated with different temperatures. Center: Intersection and numbers of differential proteins. Outside: Shared differential proteins. Tn: CGC treated at 20 °C, 40 °C, 50 °C, 70 °C, 80 °C and 90 °C. T0: fresh CGC. FC: fold change.

by comparing neighboring low-temperature-treated and high-temperature-treated groups (20 °C treatment group vs. fresh CGC, 40 °C treatment group vs. 20 °C treatment group, 50 °C treatment group vs. 40 °C treatment group, 70 °C treatment group vs. 50 °C treatment group, 80 °C treatment group vs. 70 °C treatment group and 90 °C treatment group vs. 80 °C treatment group) as a means of observing the progressive alterations in proteins under heating conditions. The results showed that the treatment temperatures of 80 °C to 90 °C and 20 °C to 40 °C produced the highest number of changes in CGC proteins (238 and 219, respectively). The temperature range from 40 °C to 50 °C had the least change, with only 139 differential proteins, and the number of upregulated and downregulated proteins also varied the least at the same time. Six (A0A3N0XZJ4 (Laminin subunit gamma-1), S4U1R3 (Actin alpha 1b), A0A3N0XK60 (NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial), A0A3N0XXN4 (Alpha-2-macroglobulin), A0A3N0YGC6 (Tubulin beta chain) and A0A3N0ZAF7 (THAP-type domain-containing protein)) of these differential proteins were shared among the six comparison groups, but no proteins were simultaneously upregulated or downregulated across the comparison groups (Fig. 3).

To further clarify the interrelationship between temperature and CGC proteins, we used Mfuzz and hierarchical clustering to classify the expression patterns of proteins according to whether they exhibited the same change in expression at different temperatures. Mfuzz analysis yielded 10 expression patterns, two of which (cluster 1 with 135 proteins and cluster 2 with 100 proteins) showed an increasing trend with increasing temperature, and two of which (cluster 3 with 153 proteins and cluster 4 with 123 proteins) showed a decreasing trend with increasing temperature, as well as a nodal trend at 20 °C, 40 °C, 50 °C and 70 °C. The other two patterns were less regular and were not as randomly distributed, with fewer associated proteins (only 47 and 77, respectively). The results of hierarchical clustering revealed that the proteins were mainly clustered into three clusters during heat treatment (fresh CGC and 20 °C treatment group, 40 °C treatment group vs. 50 °C treatment, and 70 °C treatment group vs. 80 °C treatment group), while the 90 °C group was closer to the 80 °C treatment group (Fig. 4).

3.3. Functional enrichment analysis of proteins in CGC treated with different temperatures

Differential proteins from neighboring temperature treatment groups were enriched by GO and KEGG pathways to analyse the biological characteristics of differentially expressed proteins during temperature evolution. Among the downregulated proteins, BP, MF and CC were most enriched (65 BP, 82 MF and 43 CC) when the treatment temperature was increased from 80 °C to 90 °C, whereas they were least enriched (33 BP, 55 MF and 28 CC) by the treatment temperature of 50 °C compared to 40 °C (Fig. 5A). The main terms included translation, microtubule-based process, methylation, etc., for BP, ATP binding, GTP binding, structural constituent of ribosome, etc., for MF, and integral component of membrane, ribosome, cytoplasm, etc., for CC (Fig. 5D). For upregulated proteins, the highest enrichment of BP, MF and CC (41 BP, 69 MF and 44 CC) was achieved when the treatment temperature was increased from 20 °C to 40 °C, while the lowest enrichment of all three was observed when the treatment temperature was from 40 °C to 50 °C (37 BP, 39 MF and 26 CC) (Fig. 5C). The main terms of BPs included microtubule-based process, translation, immune response, etc.; the MFs included metal ion binding, ATP binding, calcium ion binding, etc.; and the CCs included integral components of the membrane, cytoplasm, cell, etc. (Fig. 5F). GO term differences during the variation in the seven temperatures in the enrichment of both downregulated and upregulated proteins were predominant in the MF and BP categories, with fewer alterations in the CC category (Fig. 5B). GO crossover analysis of upregulated and downregulated proteins revealed that the downregulated proteins were enriched with more and almost all shared GO terms than the upregulated proteins, with actin filament binding (MF) and nucleus (CC) being specific to the upregulated proteins and carbohydrate metabolic process, cell redox homeostasis (BP), zinc ion binding, translation initiation factor activity, DNA binding (MF), and microtubule, cytoskeleton (CC) being unique among the downregulated proteins (Fig. 5E). Global analysis revealed that KEGG mainly focuses on the amino acid metabolism pathway, glutathione metabolism, nucleotide metabolism pathway, ribosome, carbohydrate metabolism pathway and lipid metabolism pathway, with amino acid, carbohydrate, and lipid metabolism accounting for the majority. There were more downregulated proteins enriched in the KEGG pathway than upregulated proteins (Fig. 5G). Further crossover analysis via KEGG pathway analysis revealed that the upregulated and downregulated proteins were mainly associated with the metabolic category, while the downregulated proteins were involved in genetic information processing, cellular processes, and organismal systems categories (Fig. 5H).

To gain a multidimensional perspective on the effect of temperature change on CGC, we examined the clustering classes (Cluster 1&2: upregulated clusters from T0 to T90; Cluster 3&4: downregulated

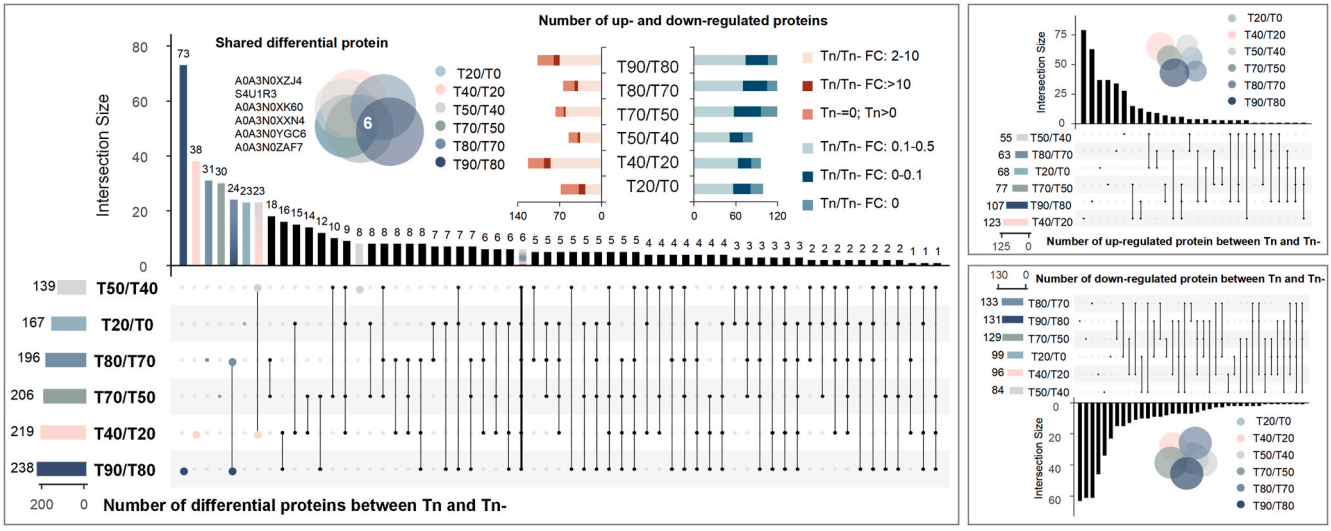

Fig. 3. Analysis of the gradual changes in CGC protein expression induced by heating treatments at neighboring temperatures. Left: Intersection and numbers of differential proteins. Right: Intersection and numbers of upregulated and downregulated proteins. Tn and Tn-: neighboring low-temperature-treated groups and high-temperature-treated groups. T0: fresh CGC.

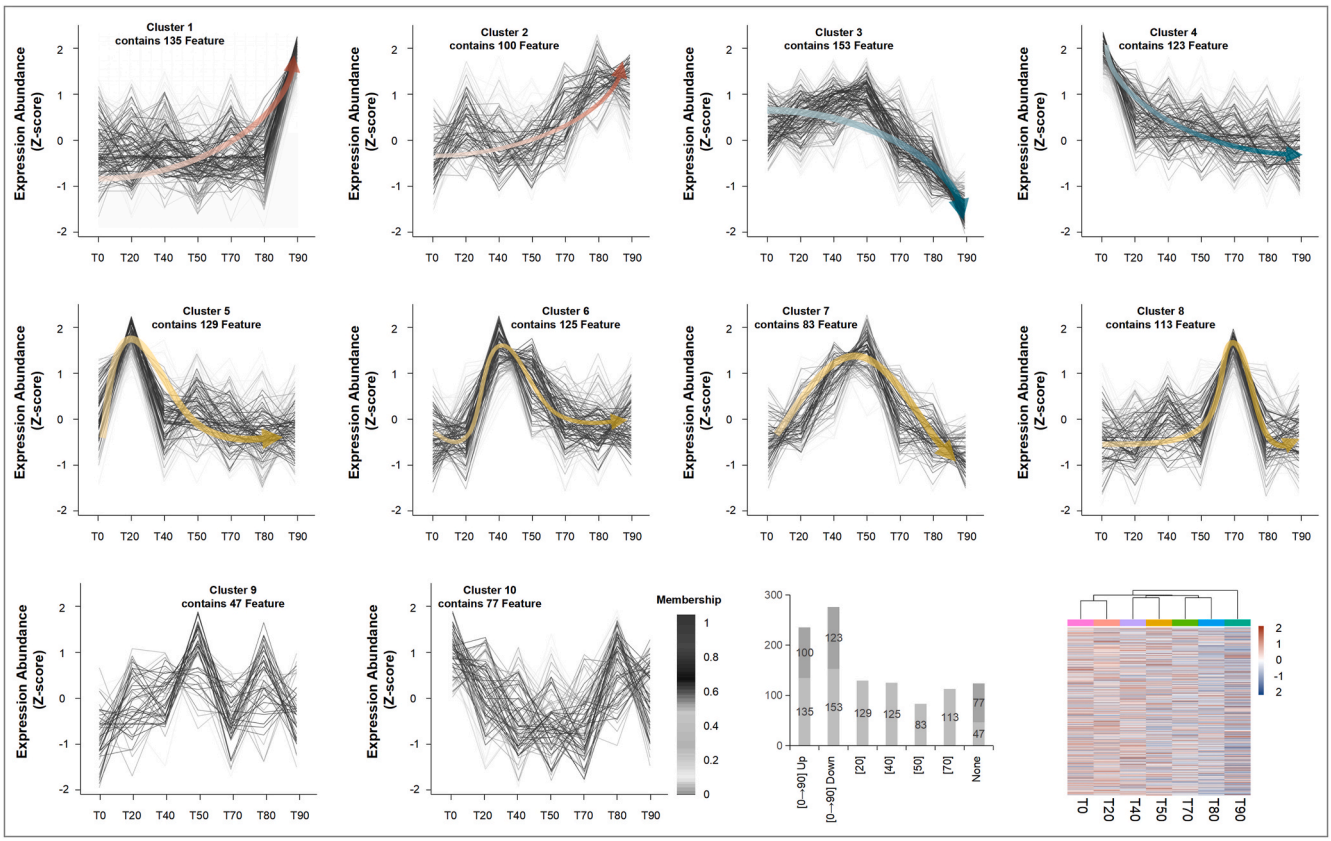

Fig. 4. Cluster analysis of all proteins based on their expression level change patterns using Mfuzz and hierarchical clustering.

clusters from T0 to T90; Cluster 5, T20 loci; Cluster 6 T40 loci; Cluster 7: T50 loci; Cluster 8: T70 loci) of the preceding differential proteins. With the change in temperature, different clusters indeed showed corresponding assemblies at the corresponding temperature intervals or loci, where the upregulated clusters at T0 to T90 included proteins such as A0A059PBU4 (myosin heavy chain A), A0A059PBX8 (myosin heavy chain B) and A0A141B1 W3 (glutathione transferase), and the downregulated clusters at T0 to T90 included proteins such as Q6IT78 (calmodulin), A0A3N0YRP4 (Ras-related protein rab7) and A0A3N0YC50 (ubiquitin-conjugating enzyme E2 R1) (Fig. 6A). Analysis of the GO results revealed that Clusters 3 and 4 were enriched with the most GO terms (98 BP, 136 MF, and 70 CC), followed by Clusters 1 and 2 (93BP, 118 MF, and 65CC) and Cluster 6 (37 BP, 65 MF, and 35 CC) (Fig. 6B). There are four BP terms (translation, protein folding, microtubule-based process, etc.).), eleven MF terms (ATP binding, actin filament binding, calcium ion binding, etc.).), and five CC terms (integral component of membrane, cytoplasm, nucleus, etc.) for all six clusters. Almost all shared terms were more enriched in Clusters 1 & 2 than in Clusters 3 & 4 and the other clusters, except for three terms, carbohydrate metabolic processes, metal ion binding and actin binding, which were significantly more enriched in Clusters 3 & 4 (Fig. 6C). Protein upregulated clusters of Cluster 1 & 2 in KEGG pathway are mainly targeted on signaling pathways (cGMP-PKG signaling pathway, PI3K-Akt signaling pathway, cAMP signaling pathway, etc.) and hormonal synthesis (steroid hormone biosynthesis, betalain biosynthesis, biosynthesis of unsaturated fatty acids, etc.) and protein downregulated clusters of Cluster 3 & 4 are mainly linked to lipid metabolism (glycosaminoglycan biosynthesis, steroid biosynthesis, glycosphingolipid biosynthesis, etc.), amino acid metabolism (glycine, serine and threonine metabolism, cysteine and methionine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, etc.) and carbohydrate metabolism (fructose and mannose metabolism, porphyrin metabolism, beta-alanine metabolism, etc.) (Fig. 6D). Of these, 25 pathways, such as the regulation of actin cytoskeleton, tight junction and PI3K-Akt signaling pathways, were common, 14 pathways, such as the hedgehog signaling pathway, steroid hormone biosynthesis and biosynthesis of unsaturated fatty acids, were specific to clusters 1 and 2, and 11 pathways, such as isoquinoline alkaloid biosynthesis, tropane, piperidine and pyridine alkaloid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, were specific to clusters 3 and 4 (Fig. 6E).

3.4. Analysis of the change rule of CGC quality characteristics during the heating process

The systematic assessment of the quality characteristics of CGC at varying heating temperatures (20 °C, 40 °C, 50 °C, 70 °C, 80 °C, and 90 °C) revealed the following patterns. The results showed that as the heating temperature increased, the crude protein and total amino acid contents of the CGC exhibited a slight upwards trend, while the contents of ash and crude fat remained relatively stable throughout the heating process. The moisture content gradually decreased with increasing heating temperature. Notably, significant parameter changes were observed at the 50 °C and 70 °C temperature points. At 50 °C, the parameters peaked and then declined at 70 °C. As the temperature continued to increase, the parameters underwent further alterations (Fig. 7B). Amino acid analysis revealed that the levels of glutamic acid, aspartic acid, lysine, leucine, and arginine, which are relatively abundant in CGC, exhibited small changes during the heating process but still peaked and then decreased at 50 °C and 70 °C (Fig. 7A). In terms of texture analysis, indices such as the hardness, adhesion, chewiness and elasticity of the CGC were greatest at 70 °C, indicating that the textural properties of the CGC were optimal at this temperature (Fig. 7C).

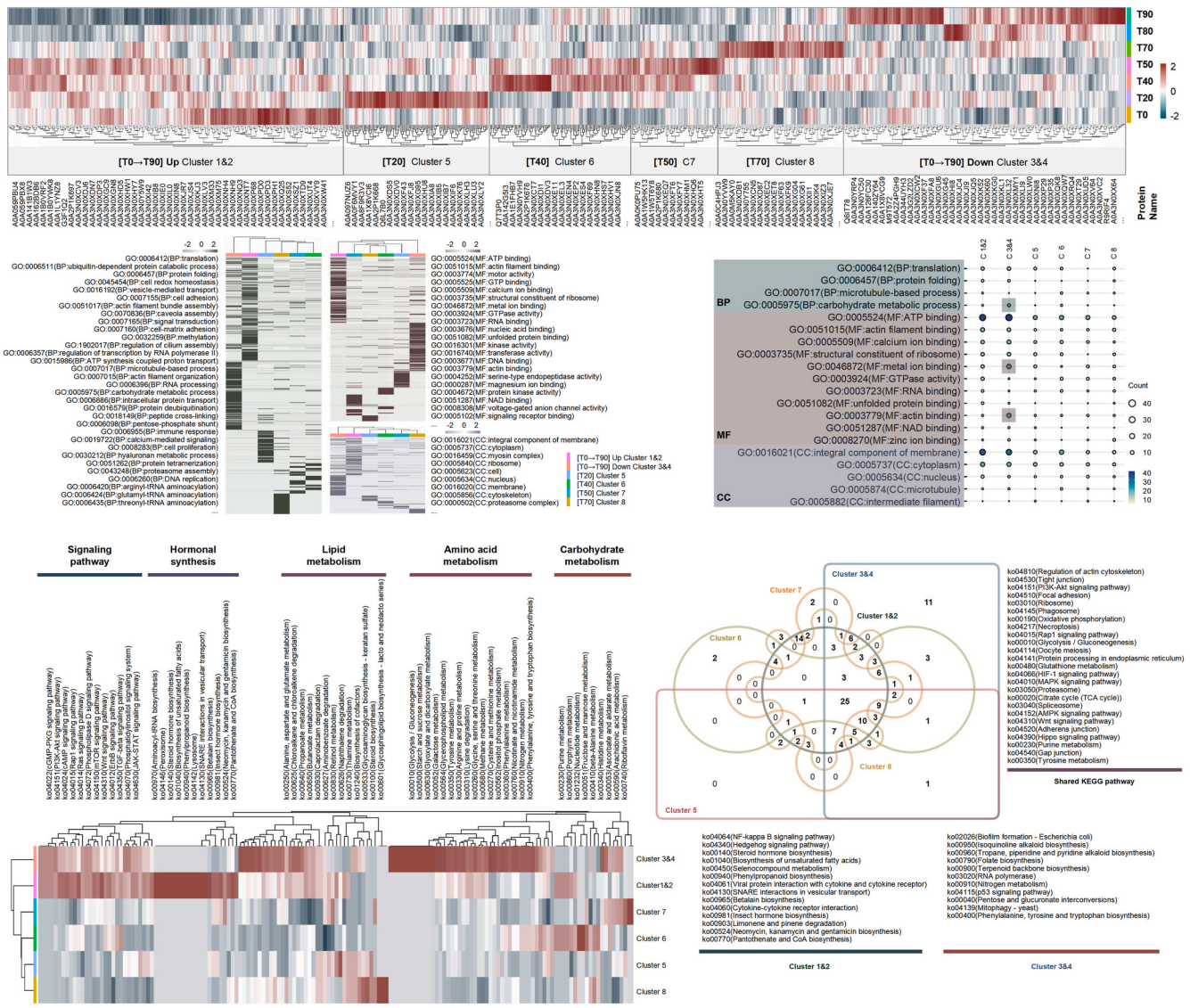

Fig. 5. Functional enrichment analysis of GO and KEGG for differential proteins of neighboring temperature treatment groups. (A) GO enrichment analysis of downregulated proteins. (B) Number analysis of BP, MF and CC. (C) GO enrichment analysis of upregulated proteins. (D) Top 10 terms in BP, CC and MF of downregulated proteins. (E) Shared GO analysis of differentially expressed proteins. (F) Top 10 terms in BP, CC and MF of upregulated proteins. (G) KEGG enrichment analysis of differentially expressed proteins. (H) Shared KEGG analysis of differential proteins.

3.5. Correlation analysis of protein properties and quality changes in CGC during heating processing

In addition to comprehensive proteomic analysis and functional enrichment studies, correlation analyses were performed to elucidate the relationship between the characteristics of CGC proteins and quality changes during thermal processing. The correlation coefficients between the expression levels of proteins differentially expressed at various heating temperatures and the measured quality parameters were assessed. The results indicated a strong positive correlation between the upregulated proteins and the textural properties of the CGC samples. Specifically, the tubulin alpha chain (A0A3N0XU78) exhibited a strong correlation with CGC hardness, while the collagen alpha-1(IV) chain (A0A3N0YC21) was strongly correlated with both elasticity and chewiness of CGC. Pre-B-cell leukemia transcription factor-interacting protein 1 (A0A3N0YG26) and DNA damage-binding protein 1 (A0A3N0YY23) exhibited significant positive correlations with all four textural parameters: hardness, elasticity, chewiness, and adhesiveness. These findings suggest that these four proteins may serve as potential biomarkers for the thermal processing of CGC (Fig. 8).

4. Discussion

Thermal processing is crucial for the food processing of aquatic products, as it not only ensures the safety of the product but also has a significant impact on the sensory characteristics of the product. CGC, a fish species widely popular in the culinary world, is renowned for its unique texture and flavour. Heat treatment during cooking can alter its texture and taste (Lin et al., 2016). Thus, a deep understanding of the changes in the protein components and structure of CGC during heating is essential for optimizing processing conditions and improving product quality. Although existing research has extensively discussed the thermal denaturation of fish proteins, it has predominantly concentrated on protein changes at a single temperature, and a comprehensive analysis of the detailed alterations within the fish proteome during the gradual heating process is lacking. This study used cutting-edge proteomics technology to reveal the specific effects of different heat treatment conditions on the proteome of CGC to fill this research gap. By integrating retention time matching, mass spectrometric fragment analysis, and collision energy data, this study successfully mapped the metabolite profiles of CGC at various temperatures. After precise data verification

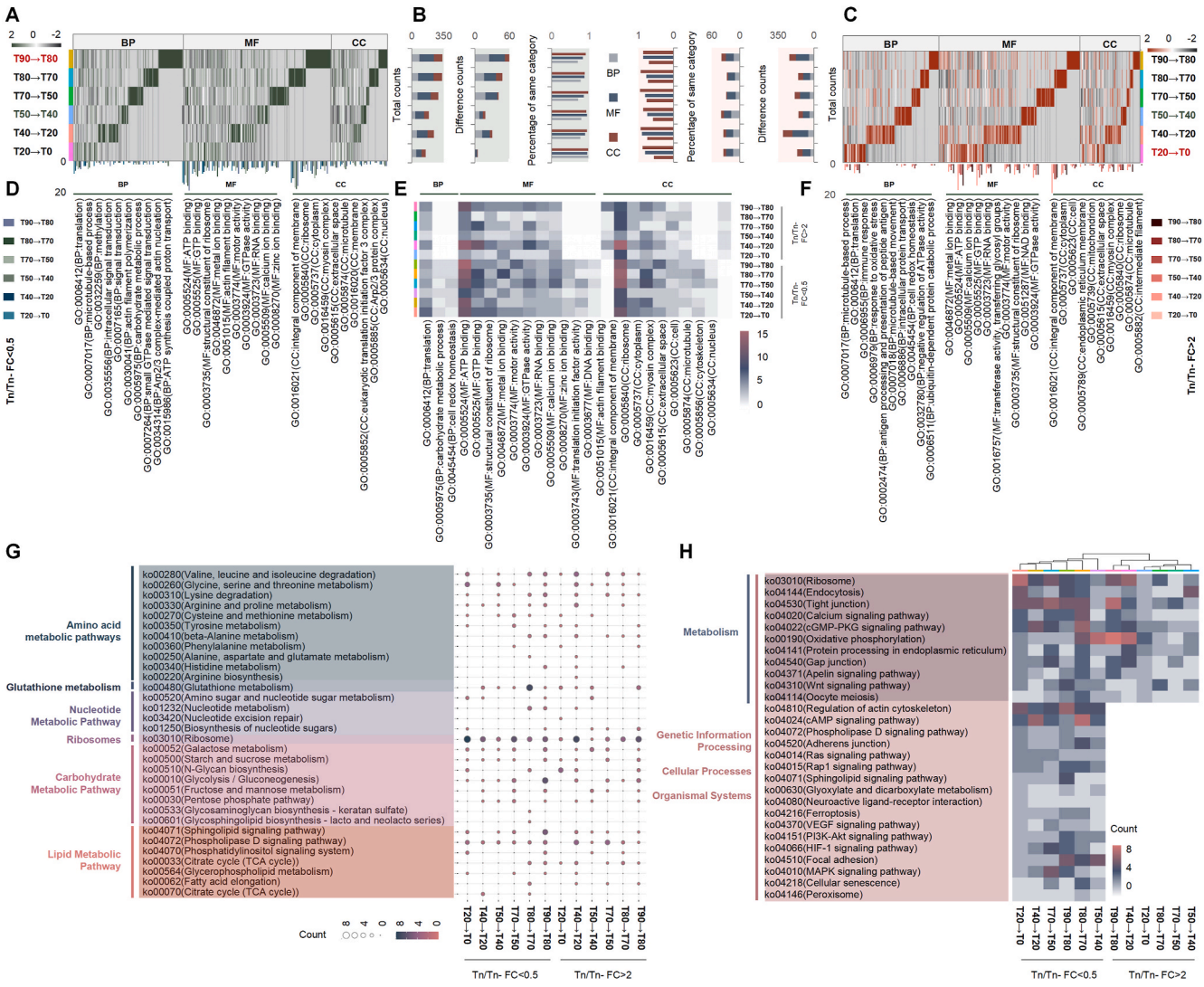

Fig. 6. GO and KEGG enrichment analysis of differentially clustered differential proteins. (A) Distribution of differential proteins in six clusters. (B) Enrichment analysis of GO (green: BP; red: MF; blue: CC). (C) Shared GO terms in six clusters. (D) Enrichment analysis of KEGG. (E) Shared KEGG pathways in the six clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

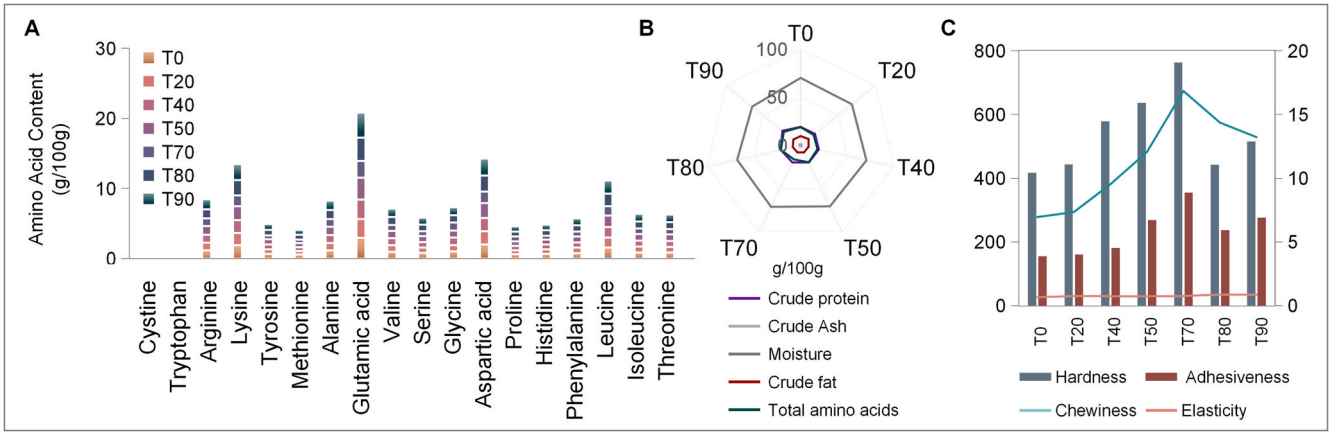

Fig. 7. Quality transformations in CGC at different heating temperatures. (A) Changes in the amino acid profiles of CGC at various heating temperatures. (B) Dynamics of crude protein, crude ash, moisture, crude fat, and total amino acid quantities in CGC at different heating temperatures. (C) Evolution in textural attributes of CGC (hardness, adhesiveness, chewiness, elasticity) at varying heating temperatures.

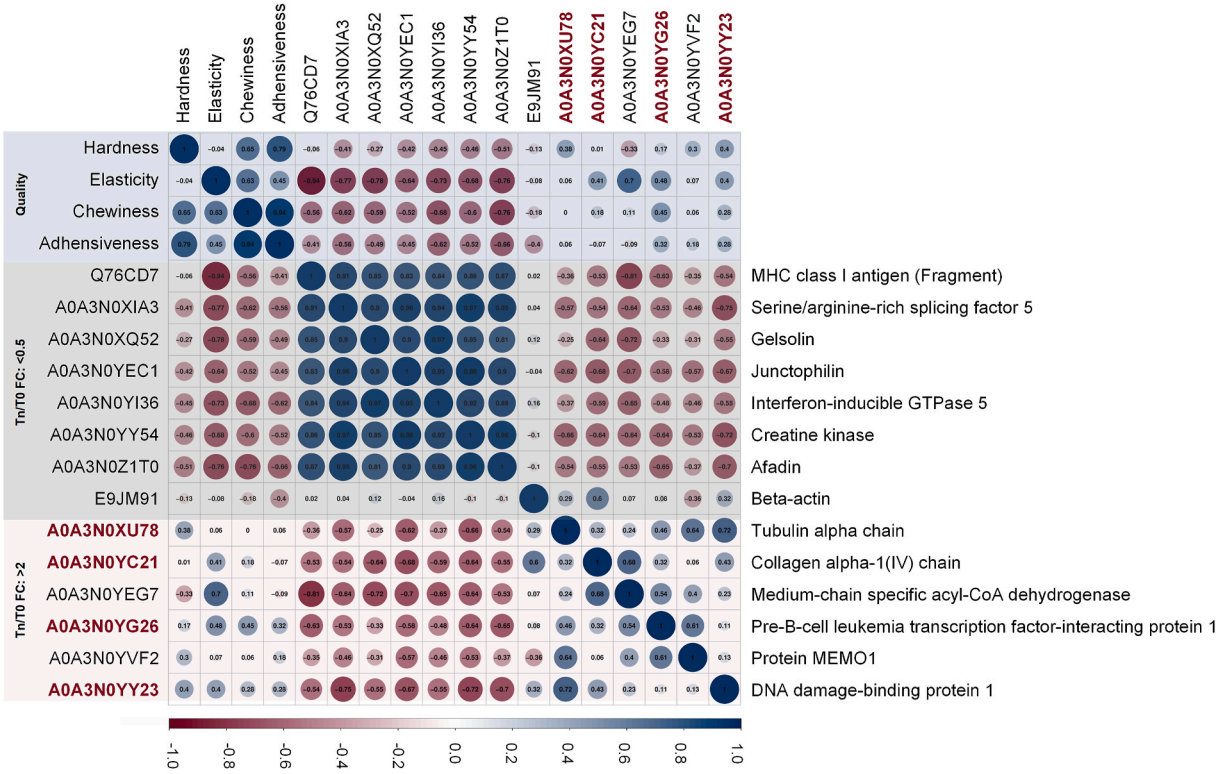

Fig. 8. Correlation analysis of differentially expressed proteins with quality attributes during the thermal processing of CGC. For each differentially expressed protein, a Pearson correlation coefficient was calculated for each quality attribute, including hardness, elasticity, chewiness, and adhesiveness. The correlation coefficient ranges from -1.0 to 1.0, where 1.0 indicates a perfect positive linear relationship, 0 indicates no linear relationship, and -1.0 indicates a perfect negative linear relationship.

and validation, we identified a large number of peptides and proteins, the richness of which highlights the complexity of CGC as a biological tissue. Further analysis revealed that there were 516 proteins common to all sample groups, a finding that revealed that even in unprocessed CGC, a certain number of common proteins existed. These shared proteins may represent the foundational proteome of CGC and offer critical clues for understanding its biological characteristics and processing responses.

When heat treatment was applied to CGC at different temperatures, we noted that as the temperature increased, the diversity of protein expression also increased, with this being particularly pronounced at 90 °C. These changes may be related to protein denaturation (Li et al., 2022b) or aggregation (Kunnath et al., 2022). Conversely, under mild conditions at 20 °C, we found the least amount of change in protein expression, suggesting that at this low temperature, the structure and function of proteins remain relatively stable, with no significant structural alterations (Soyano and Mushirobira, 2018). Intermediate temperature treatments revealed protein expression differences that lie between the two extremes, suggesting that within this temperature range, the stability and functionality of proteins are only minimally affected. This might be due to partial denaturation of proteins, which have not reached the complete denaturation caused by high temperatures; proteins might partially retain their function or be able to refold and recover function after heating (Rosa et al., 2017). To further analyse the impact of temperature on the CGC proteome, we employed Mfuzz analysis and hierarchical clustering algorithms to categorize the protein expression patterns. Mfuzz analysis revealed ten different expression patterns, among which two exhibited increasing expression trends with increasing temperature, suggesting that these proteins may actively respond to heat stress or maintain cellular function at relatively high expression levels under high temperature. The other two patterns exhibited decreasing expression trends with increasing temperature, indicating that the associated proteins may be prone to denaturation or degradation at high temperatures, leading to reduced expression levels. Four patterns showed peak expression at specific temperatures, such as 20 °C, 40 °C, 50 °C, and 70 °C, which may reflect the impact of heat stress levels corresponding to these temperatures on protein expression, causing denaturation or loss of function. The expression of the remaining two patterns was irregular, with 47 and 77 types of associated proteins, respectively, which may indicate that the response of these proteins to temperature changes does not follow a single trend but is subject to the combined influence of multiple factors. The results of the hierarchical clustering analysis further confirmed that during the heat treatment process, proteins were mainly distributed into three different clusters, reflecting the aggregation trends of proteins at low, intermediate, and high temperatures.

In studies exploring the influence of temperature changes on protein expression, conducting GO and KEGG pathway enrichment analysis on the differential proteins between adjacent temperature-treated groups is a commonly used and effective method. This analytical technique helps us to understand the biological characteristics of proteins whose expression changes under different temperature conditions. For down-regulated proteins, when the treatment temperature increased from 80 °C to 90 °C, enrichment was most significant across all GO categories. This reveals the severe impact of high-temperature treatment on protein expression, particularly at the critical temperature of 90 °C, close to the limit of an organism tolerance, where proteins generally become inactivated or denatured. On the other hand, when the temperature increases from 20 °C to 40 °C, the enrichment of upregulated proteins in GO categories is most pronounced, possibly reflecting the organism adaptive response to the initial changes in temperature. KEGG pathway analysis further revealed key metabolic pathways involved during heat treatment, including those of amino acid metabolism, glutathione metabolism, nucleotide metabolism, carbohydrate metabolism, and

lipid metabolism. Amino acid metabolism suggests that temperature changes may affect the synthesis and degradation of amino acids in muscle tissue. Given that amino acids are the building blocks of proteins and play an essential role in the formation of flavour, such impacts could alter the nutritional value and flavour characteristics of meat (Zheng et al., 2022; Kasumyan, 2016). Changes in lipid metabolism pathways are equally important, as they may change the texture and nutritional qualities of meat, thereby affecting the quality of the final product and consumer acceptance (Yang et al., 2019).

To further explore the impact of heat processing on CGC quality, we conducted a detailed analysis of its composition and characteristics postheat treatment. The results showed that CGC contains relatively high levels of glutamic acid, aspartic acid, lysine, leucine, and arginine. Glutamic acid and aspartic acid are especially crucial, as they comprise the key amino acids responsible for the savory taste perceived by humans—the “umami" flavour—significantly enhancing the overall flavour of food and imparting a more tantalizing taste to fish (Hu et al., 2022). Moreover, lysine and arginine play crucial roles in protein crosslinking during the processing process. This crosslinking not only shapes the structure of the meat but also enhances its elasticity, thereby affecting the texture of the final product (Nash et al., 2021). The analysis also indicates that the content of ash and crude fat remains stable at high temperatures, suggesting that the mineral and lipid components in CGC possess high thermal stability or that their changes are not apparent within the current detection range. Normally, heat treatment is accompanied by moisture loss, so a reduction in water content is expected. When systematically evaluating the quality characteristics of CGC, we observed significant fluctuations in amino acid and protein content at the critical temperatures of 50 °C and 70 °C. Notably, at 70 °C, parameters such as hardness, adhensiveness, chewiness and elasticity of CGC peaked, making this phenomenon particularly striking. This indicates that at this temperature, the protein network in the fish reached its optimum state of firmness and elasticity, possibly due to protein denaturation and reorganization to form a stable gel network (Wu et al., 2019). This gel structure is vital to the texture of meat products, ensuring the appropriate elasticity and continuity during chewing, thereby significantly enhancing the texture of CGC (Chen et al., 2020; Oyinloye et al., 2023). However, if the temperature continues to rise, it may cause excessive coagulation, leading to a loss of the ideal texture. These findings have practical implications for optimizing CGC processing techniques. By precisely controlling the heating temperature, particularly maintaining it within the optimal range of 50 °C to 70 °C, the meat quality characteristics of CGC can be maximally improved and optimized. Future research could delve further into the specific impacts of heat treatment on the functionality of CGC proteins and how these changes affect the sensory experiences of consumers, as well as exploring strategies to adjust processing conditions to mitigate any adverse effects.

The observed positive correlations between upregulated proteins and the textural properties of CGC upon thermal processing provide valuable insights into the molecular underpinnings of meat quality. The strong association of the tubulin alpha chain (A0A3N0XU78) with CGC hardness may reflect its role in maintaining cytoskeletal integrity, which could influence the firmness of meat postcooking (Chen et al., 2019). Similarly, the correlation of the collagen alpha-1 (IV) chain (A0A3N0YC21) with elasticity and chewiness aligns with the known function of collagen in providing structural support and flexibility to tissues (Wang et al., 2018). The broad positive correlation between pre-B-cell leukemia transcription factor-interacting protein 1 (A0A3N0YG26) and DNA damage-binding protein 1 (A0A3N0YY23) and all the assessed textural parameters suggested that these proteins may be involved in complex pathways that confer multiple textural characteristics to CGC (Zhao et al., 2020; Hu et al., 2015). Their upregulation could be indicative of cellular responses to heat stress, potentially leading to changes in meat texture. The identification of these proteins as potential biomarkers for CGC quality after thermal processing is particularly intriguing as it opens up the possibility of developing predictive models for meat quality based on protein expression profiles. Moreover, these findings could pave the way for targeted interventions in meat processing to optimize textural properties, enhancing consumer acceptability. Further investigations are warranted to elucidate the exact mechanisms by which these proteins influence the textural properties of CGC. Such studies could involve functional assays to confirm the roles of these proteins and to explore their interactions with other molecular constituents during thermal processing. Additionally, the practical application of these biomarkers in industrial settings needs to be explored, including their stability and reliability across different meat types and processing conditions.

In summary, this study utilized advanced 4D label-free proteomics technology combined with in-depth data mining methods to comprehensively reveal the impact of heat treatment on the CGC proteome composition. We identified protein expression heterogeneity at different treatment temperatures and elucidated the extensive diversity of their biological functions. These research findings not only provide molecular-level insights into the quality management and processing of CGC but also have profound implications for research in aquatic product science, nutrition, and food processing. By conducting a comprehensive evaluation of CGC quality characteristics at different heating temperatures, this study identified 70 °C as the critical temperature point where CGC meat quality is optimal. These findings provide important scientific evidence for understanding how temperature affects the CGC proteome composition and fish quality characteristics, with practical application value for improving heat processing techniques to optimize CGC quality. Furthermore, factors such as batch variability in CGC, varying heating conditions, differences in proteomic detection methods, and complex data processing may limit the generalizability and robustness of the results. Future studies should expand to encompass a broader array of species, diverse thermal processing conditions, a variety of analytical approaches, and comprehensive performance evaluations. Such investigations will further elucidate the protein changes in CGC and other fish species, thereby providing a more holistic framework to aid in controlling the food processing workflow and optimizing the sensory qualities and nutritional value of the final product. This multifaceted approach will not only contribute to a deeper understanding of thermal-induced proteomic alterations but also pave the way for the development of improved food processing techniques that can enhance consumer satisfaction and health benefits.

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CRediT authorship contribution statement

Wan-ling Lin: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Funding acquisition. **Hui Huang:** Conceptualization, Formal analysis, Investigation, Resources. **Ya-qun Liu:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Han-xu Liu:** Methodology, Formal analysis, Investigation, Data curation. **Ya Wei:** Validation, Resources. **Yong-qiang Zhao:** Validation, Resources. **Yue-qi Wang:** Investigation, Data curation. **Yan-yan Wu:** Validation, Resources. **Sheng-jun Chen:** Resources, Data curation. **Lai-hao Li:** Investigation, Validation.

Declaration of competing interest

All authors declare that No conflict of interest exists.

Data availability

Data will be made available on request.

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