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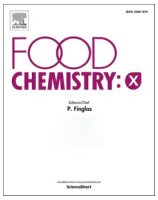

N-glycoproteomic profiling reveals structural and functional alterations in yellow primary preserved egg white under saline-alkali treatment

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ABSTRACT

The posttranslational *N*-glycosylation of food proteins is important to their structure and function. However, the *N*-glycoproteomics of yellow preserved egg white were rarely reported. This study explored the changes of *N*-glycoproteome in yellow preserved eggs white after salt and alkali treatment. A total of 213 *N*-glycosites were identified on 102 glycoproteins, revealing prevalent glycosylation motifs and multiple *N*-glycosites within proteins. Salt and alkali treatment significantly altered the glycosylation patterns, impacting major proteins differently. GO analysis indicated the roles of differentially expressed glycoproteins in responding to stimuli and biological regulation. KEGG analysis emphasized the importance of salivary secretion pathway in enzyme secretion and peptide generation. Protein domain analysis highlighted the downregulation of Serpin. Protein-protein interaction networks revealed Apolipoprotein B as central players. This study provides essential structural information on the glycosylation modifications of egg white proteins, contributing to our understanding of the mechanisms behind the functional properties of preserved eggs.

1. Introduction

Preserved eggs are a unique traditional pickled food in China, where poultry eggs are pickled to create preserved eggs, extending their shelf life and imparting a distinct flavor. During the curing process, the action of strong alkali leads to the degradation of proteins into various small-molecule active peptides, imbuing the eggs with multifunctional activities such as anti-inflammatory, antioxidant, and anticancer properties (Mao et al., 2018; Ding et al., 2019; Liang et al., 2020). Therefore, they enjoy significant popularity among consumers. Preserved eggs can be categorized based on the type of poultry eggs used, including duck, chicken, quail, and other poultry eggs. Additionally, they can be classified by their color into traditional eggs (with brown egg white and dark green yolk) and yellow eggs (with light yellow egg white and orange yolk) (Gao et al., 2021; Xue et al., 2022; Wang et al., 2021). Initially, the production process of preserved eggs involved wrapping fresh poultry eggs in a mixture of mud, plant ash, soda ash, and quicklime for 4–6 weeks. However, this method was intricate and contained lead, posing harm to humans and constraining the advancement of preserved egg production. Subsequently, an alkaline solution containing sodium hydroxide, sodium chloride, and water was employed as a replacement for soda ash and quicklime. Immersion was adopted instead of the traditional "mud coating" method, simplifying the process and enhancing safety.

Nonetheless, there remains a scarcity of research on yellow preserved eggs processed through the immersion method. Consequently, the quality attributes, functions, and mechanisms of such eggs are not well understood. Primary eggs with higher protein ratios, eggshell strength, and thickness were chosen as the raw material for producing superior quality yellow preserved eggs in our previous study (Shi et al., 2023). We employed quantitative proteomics to screen for proteins associated with food quality and made preliminary inferences about their potential functional mechanisms. Proteomics examines protein types, structures, functions, and interactions within biological systems (Lee et al., 2021). Quantitative proteomics might not capture the potential protein functional changes caused by glycosylation modifications. These modifications could lead to alterations in protein structure or interactions that might not be reflected in conventional proteomic

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analyses. *N*-Glycoproteomics, a subset of proteomics, focuses on the study of glycosylation modifications on proteins. Glycosylation, a prevalent and complex post-translational modification, regulates protein functions in various ways (Zhu et al., 2017). The alterations in functional properties observed in food proteins during processing are frequently linked to the presence of *N*-glycosylated proteins. Sun et al. (2010) found that glycosylation modification can markedly heighten the gel properties of egg white proteins. The results of Wu et al. (2022) also showed that egg white proteins formed a denser gel after glycosylation reaction. Wang et al. (2022) found that ovalbumin, as the predominant *n*-glycosylated protein in proteins, plays an important role in the early stages of protein thermogel formation due to the heterogeneity of the molecular structure resulting from highly *N*-glycosylated modifications. In addition, it has been shown that *N*-glycosylation modifications can effectively increase protein solubility by introducing hydrophilic sugar chains and improve protein emulsification properties due to the spatial site-blocking effect of sugar chains (Zhang et al., 2019). At the same time, different glycosylation sites may lead to different positions and lengths of the glycosyl chains, thus causing changes in the three-dimensional structure of the protein. It may also affect the interactions between the protein and other molecules. For example, binding to cell surface receptors or other proteins may vary as a result of differences in glycosylation, which in turn may affect signalling or cell-to-cell interactions. Therefore, studying the number and relative abundance of *N*-glycosites of food proteins during processing through quantitative *N*-glycosylation proteomics can help researchers reveal the molecular mechanisms underlying changes in the functional properties of food proteins (Tian et al., 2023).

In the process of making preserved eggs, egg white form a gel during salt and alkaline treatment, resulting in the distinctive texture of preserved eggs. Previous studies have focused on their flavour, gel properties and some functional components (Zhao et al., 2013; Zhao et al., 2014; Zhao et al., 2016; Zhang et al., 2018). However, in-depth studies on the *N*-glycosylation modification of preserved eggs white and the associated functional mechanisms have not been reported. Therefore, we investigated the differential glycoproteins and corresponding glycosylation sites of primary eggs white and yellow preserved eggs white using reversed-phase high performance liquid chromatography (RP-HPLC) combined with tandem mass tagging (TMT) labeling technology in the study. This study aims to investigate changes in *N*-glycosylation site expression and biological functions during the transformation of primary eggs into yellow preserved eggs through strong alkali and high salt treatment. The anticipated outcomes will provide a comprehensive theoretical foundation and application support for understanding the functional activity mechanisms of yellow preserved eggs. Simultaneously, to guide our subsequent research on the functional activity and molecular mechanisms of dermatogelin gels, we are examining the impact of glycosylation site changes on the spatial conformation of the corresponding proteins. This exploration will establish the groundwork for the subsequent study of complete *N*-glycopeptide modification genomics.

2. Material and methods

2.1. Sample preparation

Fresh primary eggs without break, crack and odor were obtained from the Zhong cheng layer breeding cooperative located in Guizhou Province. The yellow preserved eggs were prepared according to the method reported in our previous study (Shi et al., 2023), and the production process was as follows: the primary eggs were soaked in an alkaline solution (5.5 % NaOH + 3 % NaCl) for one week, then washed and placed in a freshness cabinet at 15 °C for one week to obtain high quality yellow preserved primary eggs. After peeling the shell, the egg white and yolk were separated. The egg white of the primary eggs was labeled as PEW and served as the control group, while the egg white of the yellow preserved primary eggs was labeled as YPPEW and served as the experimental group.

2.2. Protein extraction

Five milligrams of mashed PEW and YPPEW samples were weighed separately, then added 4 times the volume of lysis buffer (1 % SDS, 1 % protease inhibitor), and ultrasonically lysed. The samples were centrifuged at 4 °C, 12,000 g for 10 min to remove cellular debris. The supernatant was then transferred, and the protein concentration of the samples was determined using the bicinchoninic acid (BCA) protein assay kit (P0010; Beyotime, Shanghai).

2.3. Trypsin digestion and TMT labelling

An equal amount of each sample protein was taken for enzymatic lysis and the appropriate amount of standard protein Glutathione S-transferase (GST) and Maltose Binding Protein (MBP) was added. The volume was adjusted to consistency with the lysis solution. One volume of pre-cooled acetone was added, vortexed and mixed well, followed by the addition of four times the volume of pre-cooled acetone. The mixture was then precipitated at −20 °C for 2 h. After centrifugation at 4,500 g for 5 min, the supernatant was discarded, and the precipitate was washed twice with pre-cooled acetone. After drying the precipitate, a final concentration of 200 mM Triethylamonium bicarbonate (TEAB) was added, and the precipitate was ultrasonicated to break up the precipitate. Trypsin was added at a ratio of 1:50 (protease: protein, m/m), and the enzyme was digested overnight. Dithiothreitol (DTT) was added to a final concentration of 5 mM and reduced at 56 °C for 30 min. Then, iodoacetamide (IAA) was added to a final concentration of 11 mM and incubated at room temperature away from light for 15 min. The peptides were solubilised in 0.5 M TEAB and labeled according to the instructions of the TMT kit (Thermo Fisher Scientific, Waltham, MA, USA). The procedure was as follows: the labeling reagent was thawed and dissolved in acetonitrile, mixed with the peptides and incubated at room temperature for 2 h. The labeled peptides were mixed and desalted with Strata X C18 (Phenomenex) and freeze-dried in vacuum.

2.4. Modification enrichment

Peptides were dissolved in 200 μL of enrichment buffer solution (80 % acetonitrile/5% trifluoroacetic acid), and the supernatant was transferred to a hydrophilic (HILIC) microcolumn and centrifuged at 1000 g for about 15 min to complete the enrichment. The hydrophilic microcolumn was then washed three times with enrichment buffer. The glycopeptides were eluted with 0.1 % trifluoroacetic acid, 50 mM ammonium bicarbonate solution and 50 % acetonitrile, respectively, and the combined eluents were collected and freeze-dried under vacuum. After evacuation, the glycopeptides were redissolved in 50 μL of 50 mM ammonium bicarbonate buffer dissolved in heavy oxygen water and 2 μL of PNGase F glycosidase was added and digested at 37 °C overnight. Finally, salt was removed by using Strata X C18 (Phenomenex, Inc., Torrance, CA, USA) instructions and vacuum freeze-dried for liquid-mass spectrometry analysis.

2.5. RP-HPLC fractionation and LC-MS/MS analysis

The peptides were loaded onto a Agilent 300 Extend C18 column (5 μm, 4.6 × 250 mm, Agilent, Santa Clara, CA, USA) to the fractionation by RP-HPLC. The RP-HPLC gradient was as follows: 8–32 % acetonitrile (pH 9.0) over 60 min into 60 collected tubes. Then, the peptides were combined into 4 fractions, and the combined fractions were vacuum freeze-dried for subsequent operations. The peptides were dissolved in solvent A and then separated using the EASY-nLC 1000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) with a flow rate of 500 nL/min. Solvent A consisted of 0.1 % formic acid in 2 % acetonitrile, and

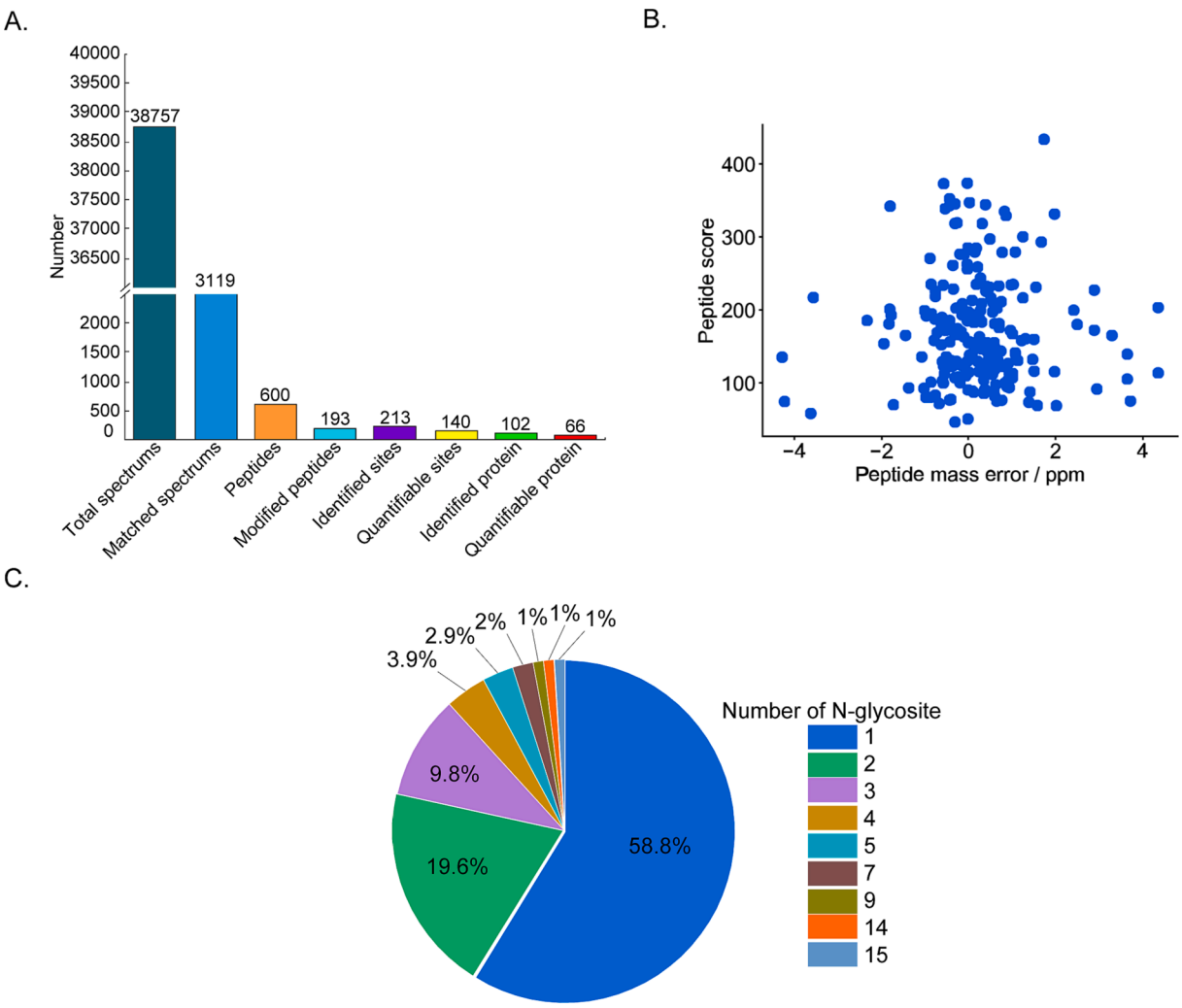

Fig. 1. Characteristics of the identified *N*-glycopeptides. A. Number of spectrograms; B. Mass error distribution of identified *N*-glycopeptides; C. Percentage of *N*-glycopeptides possessing different glycosylation sites.

solvent B consisted of 0.1 % formic acid in 90 % acetonitrile. The gradient was created by increasing solvent B from 8 to 22 % over 20 min, increasing to 35 % B over 13 min, increasing to 80 % B over 4 min, and then retaining 80 % B for 3 min. The separated peptides were subjected to NSI source and then were identified by Q Exactive™ Plus (Thermo Fisher Scientific, Waltham, MA, USA). The electrospray voltage was 2.2 kV. The primary mass spectrometry scanning range was set at 400–1500 *m/z* with a scanning resolution of 70000, while the secondary mass spectrometry scanning range was set at a fixed starting point of 100 *m/z*, and the resolution of the secondary scanning range was set at 17500. The data acquisition mode used a data-dependent scanning (DDA) procedure, whereby the top 20 peptide parent ions with the highest signal intensity were selected to enter the higher-energy collisional dissociation (HCD) in sequence, and the collision energy was 30 %. To improve the effective utilisation of the mass spectrometry, the automatic gain control (AGC) was set to 3E6, the signal threshold was 6.3E4 ions/s, the maximum injection time was 50 ms, and the dynamic exclusion time for tandem mass spectrometry scanning was set to 30 s to avoid repeated scanning of the parent ions.

2.6. Statistical analysis

Mass spectrometry data from the raw data were searched using Maxquant (v1.6.15.0) software.The mass error tolerance for primary parent ions was set to 20 ppm and 4.5 ppm for First search and Main search, respectively, and 20 ppm for secondary fragment ions.The cysteine alkylation (C) was set as fixed modification, and variable modifications as oxidation of methionine, acetylation of the protein *N*-terminus, and deamidation (NQ). The quantification method was set to TMT-6plex and the false discovery rate (FDR) of protein identification was no more than 1 %. IBM SPSS 25.0 (SPSS Inc., Chicago, ill, U.S.A.) was used for analysis of variance and Duncan test, and $P < 0.05$ was statistically significant. The model of sequences constituted with amino acids in specific positions of modify-21-mers (10 amino acids upstream and downstream of the site) in all protein sequences was analyzed using Soft MoMo (motif-x algorithm). Subsequently, Gene Ontology (GO) analysis (http://www.uniprot.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (https://www.genome.jp/kegg/), protein domain (PfamScan, https://www.ebi.ac.uk/Tools/pfa/pfamscan) and protein–protein interaction network (PPI, STRING software, version 10.5) were performed for the differentially expressed *N*-glycoproteins.

3. Results and discussion

3.1. Identification of N-glycoproteins and mapping of N-glycosites

A total of 38,757 spectrograms were obtained by quantitative *N*-glycoproteomics technique with TMT-labeling, among which 3119 were matched (Fig. 1A). N- glycosylation was observed in 193 of the 600 peptides. We identified 213 *N*-glycosites on 102 glycoproteins, of which

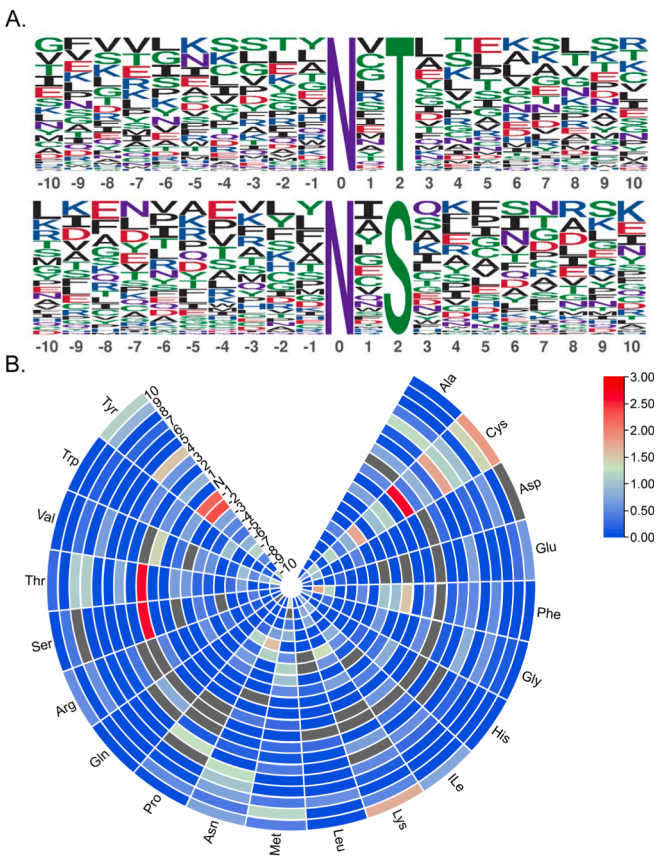

Fig. 2. *N*-glycosylation modification site motif.

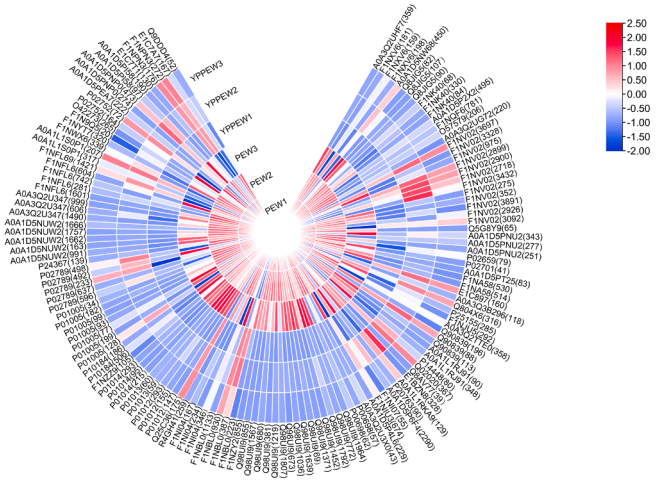

Fig. 3. Expression pattern of quantifiable *N*-glycosites.

140 *N*-glycosites assigned to 66 glycoproteins were quantifiable. There were 180 peptides identified with a score above 100, accounting for 84.51 %. Most of them scored between 100 and 200, accounting for 56.34 %. All the peptide ions had mass tolerances of less than 5 ppm, meaning that the mass accuracy of the MS data fit the requirements (Fig. 1B). More than half of the proteins contained a single *N*-glycosite and 19.6 % contained 2 *N*-glycosites. Proteins contained multiple *N*-glycosites accounted for 21.57 % (Fig. 1C). The differences in the glycosylation levels of proteins, suggesting the possibility of multiple biological functions or roles under salt-alkali treatment, and provides a basis for a deeper understanding of protein structure and function. The conservative *N*-glycosylation pattern is *N*-X-[S/T], where N stands for Asn, S for Ser, T for Thr, and X for any amino acid except proline (Zhou et al., 2021). To understand the properties of the *N*-glycosylation complexes identified in YPPEW samples, we used the Motif-X algorithmic tool to analyse the position-specific frequencies of amino acid residues. As shown in Fig. 2.A, threonine (T) and serine (S) were significantly overrepresented at the + 2 position, suggesting that most of the identified egg white *N*-glycosites were localized at the canonical sequence. More specifically, based on the unique peptide, the *N*-glycosites that matched with *N*-X-T (accounting for 62.43 %) occurred more frequently than those that matched with *N*-X-S (37.57 %). This indicated that glycoproteins that contain the *N*-X-T sequence have a higher abundance. The motif with the highest frequency (from the 10th site upstream to the 10th site downstream) was MFFCNKCFYYNCS/TCYCNTTCC.

3.2. *Expression of N-glycosites*

After salt and alkali treatment, more glycosylation sites were found to be significantly downregulated, suggesting that most glycoproteins had a deglycosylation process, which might reduced their solubility and contributed to the gelation of YPPEW samples (Fig. 3) (He et al., 2022; Yu et al., 2020). Surprisingly, some of the major proteins shown in previous proteomic studies, such as DNA topoisomerase I, zona pellucida sperm-binding protein, and P-type structural domain proteins, showed less change in glycosylation levels, even though they were more significantly altered at the proteomic level (Shi et al., 2022). Of the 213 identified *N*-glycosites, 8 proteins had five or more *N*-glycosites, namely Mucin-5B (Q98UI9), Apolipoprotein B (F1NV02), Ovomucoid (P01005), Phosvitin VTG1 (A0A1D5NUW2), Phosvitin VTG2 (F1NFL6), Ovotransferrin (P02789), Ovalbumin (P01012) and Ovalbumin-related protein Y (P01014). Mucin-5B was the most heavily *N*-glycosylated protein in YPPEW with 15 *N*-glycosites, followed by Apolipoprotein B, Ovomucoid, Phosvitin VTG1 and Phosvitin VTG2 with 14, 9, 7 and 7 *N*-glycosites, respectively. It is noteworthy that Apolipoprotein B, Phosvitin VTG1, and Phosvitin VTG2 were frequently detected in the egg yolk. This occurrence may be attributed to the denaturation of proteins in the egg yolk under the influence of salt and alkali, causing the connections between proteins to loosen. Consequently, proteins become more easily released from the egg yolk and migrate into the proteins of the preserved egg. It was reported that Apolipoprotein B was upregulated during the storage of hot spring egg white (Chen, et al., 2023). The migration process of these proteins from the egg white to the egg yolk and its potential impact on the unique texture, flavor, and potential functional activities of preserved egg proteins has not been reported and requires further investigation.

Consistent with the results reported in previous studies, Mucin-5B was the protein with the most *N*-glycosites in egg white (Geng et al., 2017; Xiao et al., 2020). The *N*-glycosites of Mucin-5B were typical, including N69, N381, N673, N680, N772, N855, N1036, N1219, N1371, N1452, N1567, N1639, N1792, N1807, and N1964, indicating that high-frequency *N*-glycosylation tended to obey the canonical *N*-X-[S/T] rule. Except for N1792, which had no differential change, the others were significantly downregulated in YPPEW ($P < 0.05$). The highly glycosylated ovalbumin in proteins was believed to be the main reason for the high protein viscosity, providing more skeletal structure for the gel formation process (Shan et al., 2020). Mucin-5B underwent a deglycosylation process induced by salt and alkali, which affected the folding and function of mucin-5B, thereby altering the hardness and elasticity of the gel texture (Xiao et al., 2020).

Of the 14 *N*-glycosites of Apolipoprotein B, N2899 and N2900 were significantly upregulated, whereas N275, N352, N3328, and N3891 were significantly downregulated ($P < 0.05$), and the other sites were not significantly changed. As with Mucin-5B, most of the sites matched the typical *N*-X-S/N-X-T motif, suggesting a high degree of evolutionary conservation in YPPEW *N*-glycoproteins. Apolipoprotein B had been reported as the predominant glycoprotein in egg yolk (Geng et al.,

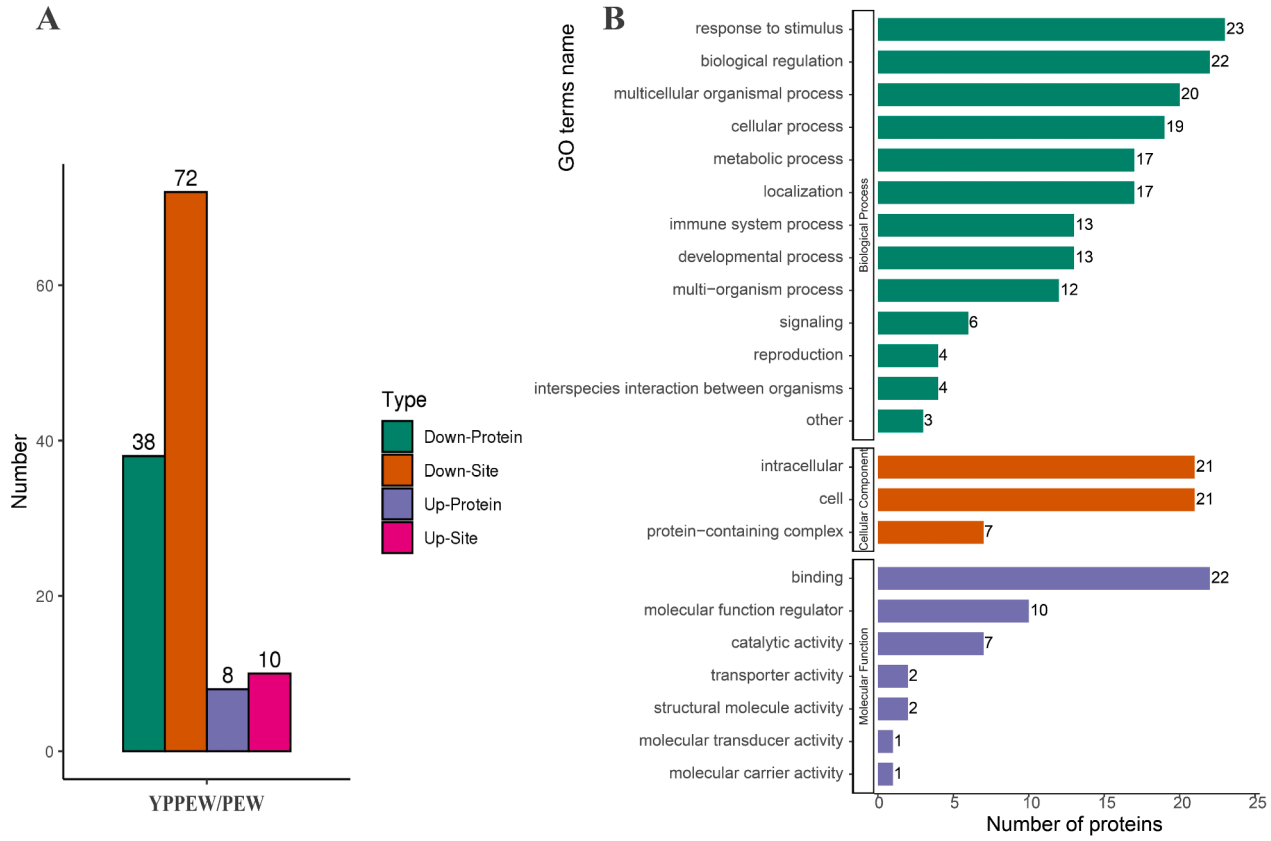

Fig. 4. A. Differential modification site/protein statistics; B. Distribution of differentially expressed proteins in GO secondary annotation statistics.

2018). The large number of glycosylation sites of Apolipoprotein B might be related to the migration of protein. As the precursor protein of egg yolk low-density lipoprotein, Apolipoprotein B has the primary function of binding and transporting lipids. Covalently modified *N*-glycans on apolipoprotein B could play a directional role in the complexation of peptide chains and lipids, and extended *N*-glycans contributed to receptor recognition during transmembrane transport (Ma, 2021). It is clear that *N*-glycosylation modifications are important for the function of apolipoprotein B, further studies on the glycosylation modification are beneficial to our understanding of the mechanisms by which the YPPEW functional effects such as anti-inflammation.

Ovomucoid is the major glycoprotein in egg white, and has been detected at high abundance in the eggshell matrix glycoproteomic analysis (Geng et al., 2017; Yang et al., 2020). Ovomucoid possessed nine *N*-glycosites (N34, N63, N77, N93, N99, N128, N182, N190 and N199), five of which were significantly downregulated, namely N34, N93, N99, N182 and N199. In addition, phosvitin VTG1 had four *N*-glycosites significantly downregulated (N163, N991, N1662, and N1757), while only N1601 was significantly downregulated in phosvitin VTG2. Phosvitins are known to possess a variety of functional activities, including metal chelating, antioxidant, emulsifying, antibacterial and cytotoxic activities (Lee et al., 2017). However, glycosylation modifications on their functional activities have been less reported, especially the variability of modifications at different sites, which needs to be further explored. In addition, we noted opposite changes at different glycosylation sites of a protein, and the differences between these different glycosylation sites may in turn affect the structure of the glycoprotein, leading to some important retention or transformation of the protein's function, which deserves to be verified by further studies.

3.3. GO analysis of significantly changed glycoproteins

The significantly changed proteins were selected using the threshold of fold change of > 1.3 or < 0.83 ($p < 0.05$) comparing YPPEW to PEW. Consequently, 8 proteins were significantly upregulated, while 38 proteins were significantly downregulated in YPPEW (Fig. 4A). GO analysis was performed on proteins that were significantly different in YPPEW in response to salt and alkali treatments. GO annotation was divided into 3 major categories: Biological Process, Cellular Component and Molecular Function, which explain the biological roles of proteins from different perspectives. We counted the distribution of differentially expressed glycoproteins in GO secondary annotations. In terms of biological process, differential proteins were mainly involved in response to stimulus, biological regulation and multicellular organismal process (Fig. 4B). Proteins upregulated in YPPEW were mainly enriched in the multicellular organismal process, biological regulation and metabolic process, while the downregulated proteins were mainly involved in response to stimulus, biological regulation and multicellular organismal process. The results of cellular component analysis showed that the differential proteins were mainly concentrated in intracellular and cell, especially the downregulated proteins. In addition, the molecular functions of the upregulated proteins were mainly binding and structural molecule activity, while the downregulated ones were binding and molecular function regulator. The upregulated proteins were mainly Mucin-6 Fragment, PIT 54 (predicted), Dickkopf-related protein 3, Apolipoprotein B, Olfactomedin-like protein 3, etc. Among them, Mucin-6 Fragment mainly exerted binding and structural molecule activity in intracellular, and the modification sites were N930 and N387. Apolipoprotein B existed as a protein-containing complex in addition to the intracellular, and its molecular functions were binding and transporter activity, with modification sites N2899 and N2900. The downregulated proteins mainly included Mucin-5B, Ovalbumin-related protein, Apolipoprotein D, Lysozyme C, Inter-alpha-trypsin inhibitor heavy chain 3 (predicted), Avidin, Ovotransferrin, etc., most of which were present in intracellular and cell. In addition to binding and transporter activity, they also equipped catalytic activity, involving pathways such as response to

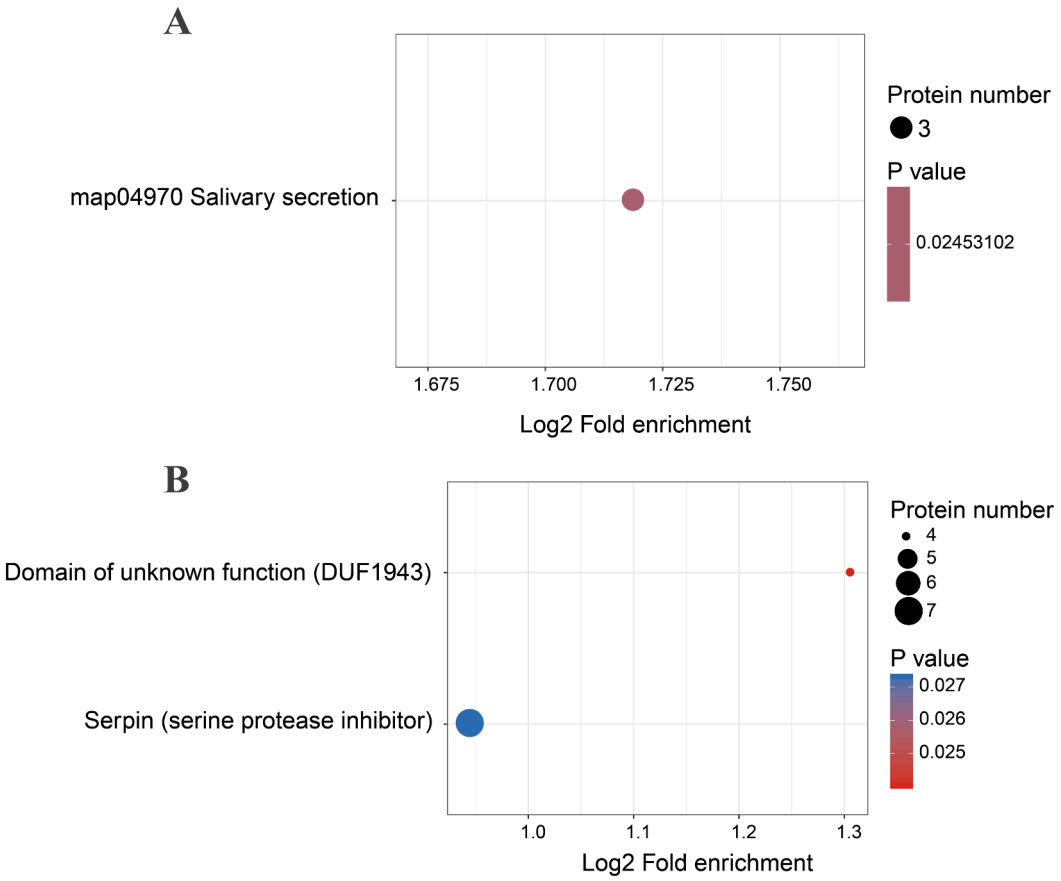

Fig. 5. Functional enrichment analysis of differentially expressed glycoproteins. A. KEGG pathway; B. Protein domain.

stimulus, biological regulation, multicellular organismal process, cellular process, localisation, metabolic process, immune system process, developmental process, multi-organismal process, etc.

Enrichment analysis was further performed on the GO classification of differentially expressed glycoproteins to explore whether there was a significant trend of enrichment in certain functional types (Table S1). The results of the pathway analysis showed that the upregulated pathways contained the immune response-regulating signaling pathway, regulation of alcohol biosynthetic process, regulation of steroid metabolic process, regulation of alcohol biosynthetic process, regulation of steroid metabolic process, toll-like receptor signaling pathway, pattern recognition receptor signaling pathway, regulation of innate immune response, positive regulation of defence response, protein-containing complex assembly, regulation of lipid biosynthetic process, activation of immune response, sterol homeostasis, sterol transport, organic hydroxy ompound transport, lipid homeostasis. The related proteins were Mucin-6 Fragment, Fibrinogen beta chain Fragment, Apolipoprotein B and Dickkopf-related protein 3. The upregulation of immune response-regulating signaling pathways suggests an intriguing aspect of preserved duck eggs. Enhanced immune responses might contribute to improved safety and shelf life, as preserved duck eggs face various environmental challenges during storage. The regulation of alcohol biosynthetic and steroid metabolic processes may affect the composition of preserved duck egg proteins. These alterations could influence the taste and texture of the final product, potentially making it more palatable to consumers. The positive regulation of the innate immune response is particularly interesting. It implies that preserved duck eggs may possess inherent protective mechanisms. This could be advantageous for preventing spoilage and bacterial contamination during prolonged storage. Protein-containing complex assembly suggests changes in the protein matrix of preserved duck eggs. This could impact the overall texture and structural properties of the product, potentially leading to enhanced quality. The regulation of lipid biosynthetic processes, sterol homeostasis, and transport has implications for the lipid profile of preserved duck eggs. Altered lipid composition could affect flavor, stability, and nutritional attributes. The activation of immune responses is intriguing in the context of preserved duck eggs. It suggests that these products may offer unique health benefits, potentially acting as functional foods with immune-boosting properties.

The downregulated pathways were defence response to bacterium, defence response to other organism, lipid transport, response to bacterium, negative regulation of defence response, regulation of lipid metabolic process, sterol homeostasis, response to corticosteroid, organic hydroxy compound transport, sterol The related proteins are Ovalbumin, Ovotransferrin, Lysozyme C, Apolipoprotein H, Mucin-5B, Apolipoprotein D, Alpha-1-acid glycoprotein, Proteinin, and Glycoprotein. glycoprotein, Protein TENP. The downregulation of antibacterial and antimicrobial pathways may indicate greater stability in the preservation period of preserved duck eggs. Although the ability to resist external microorganisms may decrease, it could also reduce autoimmune responses, contributing to product stability. More interestingly, the presence of both upregulation and downregulation within the “Regulation of Lipid Metabolic Process” pathway reflects the intricacies of lipid metabolism in YPPEW. The coordinated action of upregulated and downregulated glycoproteins ensures a balanced lipid composition in YPPEW. Excessive lipid metabolism can lead to off-flavors, while excessive lipid synthesis can result in a greasy or overly fatty taste. In addition, the balance of lipid synthesis and metabolism helps maintain the desired texture in YPPEW. Too much lipid metabolism can lead to a dry or crumbly texture, while excessive lipid accumulation can result in a slimy or oily texture. Reduced responses to hormones might help lower fluctuations in hormone levels within preserved duck eggs, increasing

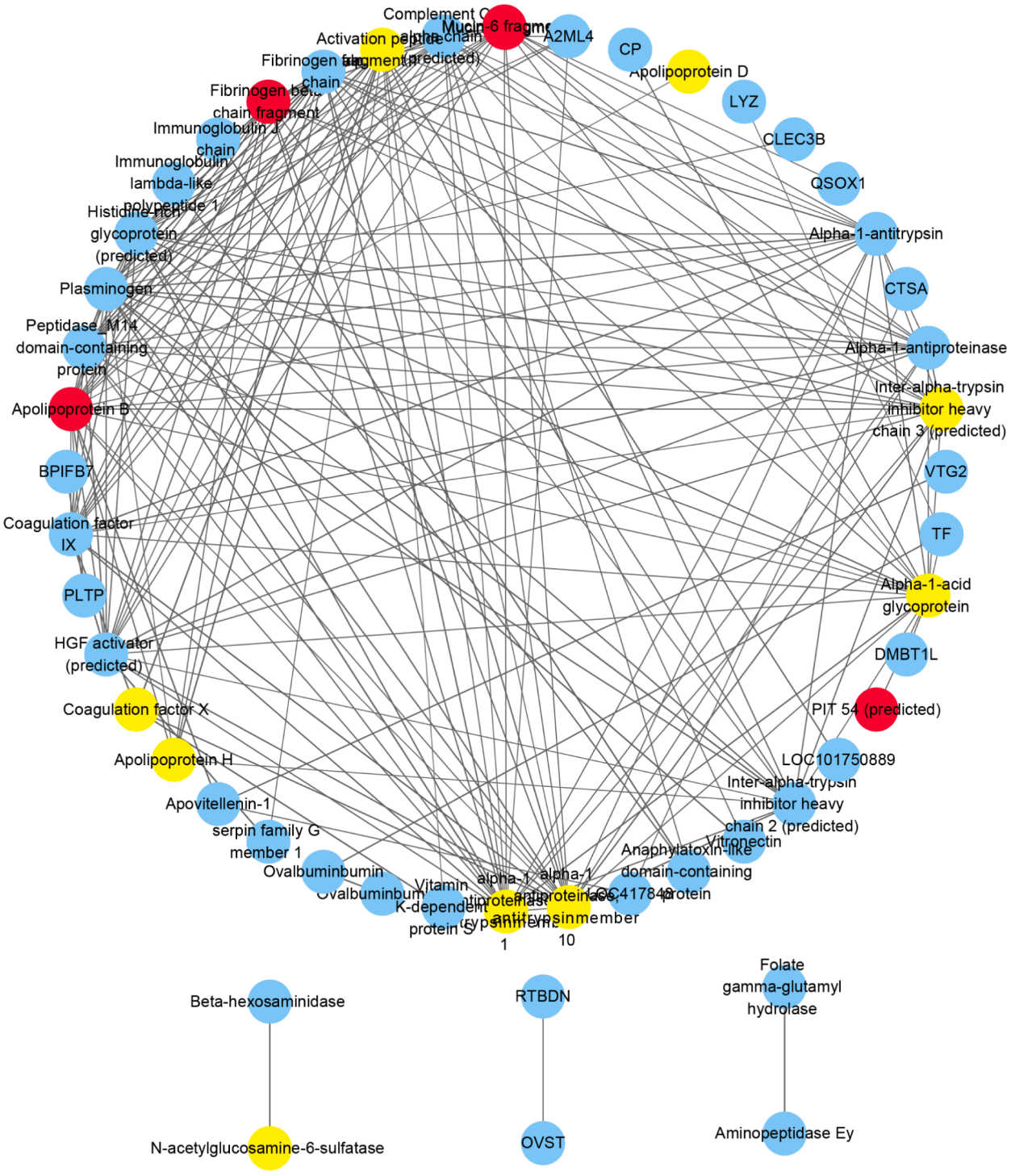

Fig. 6. The interaction between *N*-glycosylated modified proteins. The red circles represent upregulated *N*-glycoproteins, the yellow circles represent downregulated *N*-glycoproteins, and the width of the line represents the strength of the correlation. The blue circles represent *N*-glycoproteins in egg white of PEW and YPPEW samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

product stability. This may have a positive impact on the flavor and shelf life of YPPEW. The downregulation of the transport pathways for organic hydroxy compounds may suggest greater stability in antioxidants, which could extend the shelf life and flavor longevity of YPPEW.

3.4. KEGG pathway and protein domain analysis

As shown in Fig. 5A, KEGG annotation results showed that the significantly changed glycoproteins in the YPPEW samples were associated with the Salivary secretion pathway, and the up-regulation of this pathway can increase the amount of enzyme secretion, which is conducive to the enzymatic digestion of proteins to produce

polypeptides with different molecular sizes, net charges and solubilities. Current studies have demonstrated that peptides have better anti-inflammatory effects, while egg-derived peptides have potential anti-inflammatory effects, and peptides are more likely to cross the intestinal lumen into the mucosa to interact with target biomolecules, such as membrane receptors, and exhibit better biological activities, such as antioxidant and anti-inflammatory activities (Zhao et al., 2017). The glycoproteins involved are EW135 (predicted), PIT 54 (predicted) and Lysozyme C,where Lysozyme C is a protein hydrolase with antimicrobial properties (Porcheri C & Mitsiadis, 2019).

A protein domain is a conserved part of a given protein sequence and structure that can evolve, function and exist independently of the rest of the protein chain. Each domain forms a compact three-dimensional structure and often can be independently stable and folded. Many proteins consist of several structural domains. One domain may appear in a variety of differentially expressed proteins. Annotation of protein domain of differential glycoproteins based on the Pfam database showed that the domains of differential glycoproteins in YPPEW samples were mainly involved in the downregulation of Serpin (serine protease inhibitor) (Fig. 5B). Serine proteases have many important functions, especially in digestion, coagulation and complement system (Blisnick et al., 2017). Apparently, down-regulation of Serpin favours the functional activity of serine proteases in YPPEW.

3.5. Protein-Protein interaction network of glycoproteins

The interactions of 102 identified *N*-glycoproteins were analysed by STRING, and a highly connected protein–protein interaction network was obtained after extracting protein interactions according to confidence score > 0.7 (high confidence). The network contained 51 identified *N*-glycoproteins and 182 interactions, with 4 significantly upregulated *N*-glycoproteins and 9 downregulated *N*-glycoproteins (Fig. 6). A total of 16 *N*-glycoproteins had more than 10 interactions in the interaction network. Apolipoprotein B interacts with 20 proteins and has the most interactions among the differential proteins. Apolipoprotein B, classified as a lipid-binding protein from the GO enrichment analysis, showed significant changes in *N*-glycosylation levels, including upregulation of N2899, N2900 and downregulation of N275, N352, N3328 and N3891. These results suggested that Apolipoprotein B was an important protein for further investigation of the molecular mechanism of functional activity in YPPEW samples. The results of proteomic and glycoproteomic studies of egg deterioration in high temperature storage by Zhu et al. (2019) also showed that Apolipoprotein B was an important characterisation protein. It is clear that Apolipoprotein B has an important role in regulating the molecular function and structural properties of eggs.

In addition, the Clade A (Alpha-1 Antiproteinase, Antitrypsin, member 10), which contained 16 interactions in the interaction network, was also a candidate for further study. A number of differentially altered *N*-glycoproteins were highly interacted, including Fibrinogen chain, Activation peptide fragment, HGF activator (predicted), Histidine-rich glycoprotein (predicted) and Immunoglobulin. According to the GO annotations in this study, the *N*-glycoproteins in the network that underwent more interactions and had stronger interactions were mainly related to binding, catalytic activity and transporter activity. Clearly, significant changes in the glycosylation levels of individual glycoproteins might have an impact on their neighbouring glycoproteins, leading to changes in molecular function, which need further investigation.

4. Conclusion

This study extensively characterized the glycosylation features of yellow primary preserved egg white proteins under salt and alkali treatment using *N*-glycoproteomics. We identified 213 *N*-glycosites, some of which exhibited significant changes after saline-alkali treatment. These alterations might reflect structural and functional modifications of proteins, providing valuable insights into the properties of preserved egg white proteins. Further functional exploration through GO and KEGG analysis highlighted shifts in biological processes and molecular pathways. Notably, the significant alteration in the salivary secretion pathway suggested enhanced enzyme secretion, favoring the generation of peptides with potential anti-inflammatory properties-a significant aspect of egg white functionality. Additionally, protein domain analysis underscored the downregulation of serpin, potentially enhancing proteinase activity. The protein–protein interaction network revealed the centrality of Apolipoprotein B, suggesting its pivotal roles in the protein interaction network. In conclusion, this study not only advances our understanding of perserved egg white *N*-glycoproteomics but also offers vital clues regarding the impact of saline-alkali treatment on the structural and functional aspects of preserved egg white proteins. These findings hold promise for enhancing food preservation and developing bioactive food components.

CRediT authorship contribution statement

Qi Qi: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Denghui Shi:** Software, Methodology, Investigation, Data curation, Conceptualization. **Wei Su:** Writing – review & editing, Project administration, Conceptualization. **Yingchun Mu:** Supervision, Data curation.

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

No data was used for the research described in the article.

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Ethical approval.

This study does not involve any human or animal testing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101244.

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