

Identification of the Duck Egg White N-Glycoproteome and Insight into the Course of Biological Evolution

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Supporting Information

ABSTRACT: Protein glycosylation is a ubiquitous posttranslational modification that modulates protein properties, thereby influencing bioactivities within a system. Duck egg white (DEW) proteins exhibit diverse biological properties compared with their chicken egg white (CEW) counterparts, which might be related to glycosylation. N-Glycoproteome analysis of DEW was conducted, and a total of 231 N-glycosites from 68 N-glycoproteins were identified. Gene ontology analysis was used to elucidate the biofunctions of DEW N-glycoproteins and compare them with those of CEW, which showed that the differences mostly involved molecular functions and biological processes. The biological functions of DEW N-glycoproteins were illuminated through bioinformatics analysis and comparison with CEW orthologues, which showed different allergenicities and antibacterial abilities. These divergences might be initiated by specific alterations in glycosylation, which can enhance the proteolysis resistance and protein steric hindrance. These results provide new insights for discovering the effects of N-glycosylation on biofunctions during the divergence of homologous proteins.

KEYWORDS: duck egg white, N-glycoproteins, bioinformatic analysis, bioactivities, evolution

INTRODUCTION

Protein glycosylation is a posttranslational modification that is involved in modifying protein properties and bioactivities.^{1,2} N-Glycosylation increases the complexity of egg protein conformations, resulting in diverse functional properties.^{3–5} For example, Quan et al. found that desugarization of duck egg white (DEW) markedly improved its gelling properties and made the gel network more compact and denser.³ In addition, some protein bioactivities can be affected by the binding ability of carbohydrate moieties. For instance, the antivirus activity of egg ovomucin could be increased by the interaction between Mg²⁺ and the carbohydrate moieties of ovomucin.⁴ Furthermore, the glycosylation of certain egg white proteins was also recognized as affecting egg-induced allergies.⁵ Alcántara et al. reported a patient with an allergy to DEW without a chicken egg white (CEW) allergy and suggested that ovalbumin (OVA) might be the responsible protein.⁶ It was speculated that the specific egg allergenicity between Anseriformes and Galliformes may be due to glycoprotein differences.

East Asia is one of the major areas for duck domestication, and it has a 2000 year history of duck farming.⁷ Duck is a kind of omnivorous waterfowl that often nests in high humidity regions conditions. Globally, hen eggs are the most consumed avian eggs, whereas duck eggs are specifically utilized for food processing in East Asia; in particular, they are processed into salted, dried, and preserved eggs.⁸ About 3 to 4 million ducks are raised each year in China, which provides an important

supplement to the poultry industry. Besides the utilization of DEW in the food processing industry, some DEW proteins also show diverse functions compared with their counterparts in CEW.^{9–11} For instance, DEW cystatin is phosphorylated and glycosylated, whereas that of CEW is not.⁹ Gels from DEW exhibited higher cohesiveness and better water binding properties than gels from CEW.¹⁰ In addition to the differences in protein structures and property, the enzymatic activity of lysozyme in DEW is higher than that of its CEW counterpart.¹¹ Geng et al. speculated that the CEW ovostatin lost some conserved N-glycosites after they compared it with the other avian egg white ovostatins; this might be related to the longer domestication process of chickens.¹² In our previous proteomic study, we performed comparisons of specific proteins between duck and chicken egg whites, but discrepancies in their glycosylation levels have not been investigated.¹³

Glycomics has been used to study the protein glycosylation in avian egg whites and its relationship with avian evolution.^{14–16} A large-scale glycans investigation in different evolutionary lineages was made through the advanced glycoblotting-based high-throughput glycomics, which sug-

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gested that the differences in the glycan types and amounts between Anseriformes and Galliformes might be related to their lifestyles and diets.¹⁴ The Gal β 1–4Gal epitope glycans from nine avian species egg whites were investigated. The different expression of Gal β 1–4Gal among the nine species may be due to evolutionary differences, leading to different susceptibilities to specific infectious diseases.¹⁵ Recently, a glycoblotting study on the egg white proteins of four different quail species revealed various N-glycan structures.¹⁶ Besides glycomics, glycoproteomics has also been used for analyzing species differences. The characterization and comparison of whey N-glycoproteins from bovine and human colostrum and mature milk may provide insight into the application of infant formulas.¹⁷ A detailed N-glycosylation analysis of the royal jelly proteins between western and eastern honeybees revealed species-specific modifications and variations in immune functions.¹⁸ A N-glycoproteomic analysis of chicken egg yolk was conducted and provided further understanding of embryo development, egg storage, and processing.¹⁹ Although glycomics analysis has been extensively used to study avian egg whites, studies involving glycoproteomics are scarce. Our former omics analysis of CEW glycoproteins identified 71 N-glycosylation sites on 26 glycoproteins, which gave a basic understanding of CEW glycoproteins for further comparison with other avians.²⁰

Hence, the comparative analysis of egg white N-glycoproteins between chicken and duck is expected to provide insight into the avian evolution.²⁰ Previous studies have investigated the N-glycoproteins in some avian eggs, but little is known on the DEW N-glycoproteome. The DEW protein was digested by trypsin and subsequently deglycosylated by PNGase F in H₂¹⁸O. The glycosylation on asparagine was transferred to aspartic acid with a mass increase of 2.98 Da, which is detectable by LC-MS/MS. The glycoprotein characterization of DEW and the glycoproteome comparison with CEW are important for revealing species diversity in terms of bioactivities. Furthermore, this study will facilitate better understanding of glycoprotein evolution among different avian species.

MATERIALS AND METHODS

Sample Collection. Duck eggs laid within 24 h were collected from the Poultry Research Centre farm of Huazhong Agricultural University (Wuhan, Hubei). To reduce variation, duck eggs were selected from a flock of 40-week-old Peking ducks reared in separate floor pens under standard feeding conditions. A total of nine eggs were randomly collected, and egg whites were manually separated from egg yolks. Three sampling repeats were homogenized, frozen at –80 °C, and stored at this temperature until the following analysis.

Protein Extraction. The frozen DEW (1 mL) lyophilized powder was ground and sonicated (150 W, 30 s each time) in four volumes of lysis buffer (10 mmol/L dithiothreitol, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor); this was followed by centrifugation with isometric Tris-saturated phenol at 5500g at 4 °C for 10 min. Then, the supernatant was mixed with four volumes of ammonium acetate/methanol (0.1 mol/L) and incubated overnight. After that, the remaining precipitate was washed with methanol and acetone. The protein was redissolved in 8 mol/L urea, and then the protein concentration was determined by using a BCA kit according to the manufacturer's instructions.²¹

Trypsin Digestion. The method was based on our previous protocol.²⁰ For digestion, the DEW protein solution was treated with 5 mmol/L dithiothreitol for 30 min at 56 °C and then with 11 mmol/L iodoacetamide for 15 min at room temperature in the dark. The alkylated samples were diluted by adding 100 mmol/L NH₄HCO₃ to

make the urea concentration less than 2 mol/L. Finally, trypsin (Promega) was added, with 1:50 (w/w) trypsin/protein for the first digestion (overnight) and 1:100 (w/w) trypsin/protein for a second 4 h digestion.

HPLC Fractionation. The tryptic peptides were fractionated by high pH reverse-phase HPLC using a Thermo Betasil C18 column (5 μ m particles, 10 mm inner diameter, 250 mm length). The peptides were first separated into 60 fractions with a gradient of 8 to 32% acetonitrile (pH 9.0) over 60 min (Figure S2A). Then, the peptides were combined into four fractions and dried by vacuum centrifugation (Figure S2B).

Glycopeptide Enrichment. To enrich the DEW glycosylation peptides, tryptic peptides were dissolved in the enrichment buffer (40 μ L, 80% acetonitrile and 1% trifluoroacetic acid) and then transferred to the hydrophilic interaction liquid chromatography microcolumn (HILIC, SeQuant) in a centrifuge at 4000g for 15 min. After that, the enrichment buffer was used to wash the hydrophilic microcolumn three times with HILIC.²² After 10% acetonitrile was used to elute the glycopeptides, the eluate was collected and lyophilized. Then, the glycopeptides were redissolved in 50 mmol/L ammonium bicarbonate buffer (50 μ L), 2 μ L of PNGase F (Roche, 11365185001) was added, and the solution was incubated at 37 °C overnight. For LC-MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore) according to the manufacturer's instructions, and the enriched DEW glycopeptides were lyophilized.

LC-MS/MS Analysis. The separation and analysis of the glycopeptides were carried out by UPLC-ESI-MS/MS with an ESAY-nLC 1000 UPLC system (Thermo Fisher Scientific). The tryptic glycopeptides were dissolved in 0.1% formic acid and 2% acetonitrile (solvent A). The gradient of solvent B (0.1% formic acid in 98% acetonitrile) was increased from 5 to 20% over 26 min, from 20 to 32% over 8 min, and then from 32 to 80% over 3 min, and the concentration was held at 80% for another 3 min. The constant flow rate was all at 700 nL/min. Then, the peptides were subjected to NSI source followed by tandem mass spectrometry in an Orbitrap Fusion. The electrospray voltage applied was 2.2 kV. The first-order *m/z* scan range was 350 to 1550, and intact peptides were detected at a resolution of 60 000 in the Orbitrap. Then, the peptides were detected in the Orbitrap at a resolution of 15 000. A data-dependent procedure was used that alternated between one MS scan followed by 20 MS/MS scans with 15 s of dynamic exclusion. Automatic gain control was set at 5×10^4 .

Database Search. The secondary mass spectrometry data was retrieved using Maxquant search engine (v1.5.2.8) and compared against the NCBI databases (organism: *Anas platyrhynchos*). As the cleavage enzyme, trypsin was allowed up to two missing cleavages. The first search and the main search of mass tolerances of the precursor ions were set at 20 and 5 ppm, respectively. The subsequent mass tolerance was set to 0.02 Da for the fragment ions. Carbamidomethyl on Cys was specified as a fixed modification, and oxidation on Met and deamidation with ¹⁸O on Asn were specified as variable modifications. For identification, the false discovery of the peptide and site level rate was specified as 1%.

Bioinformatics Analysis. Gene ontology (GO) analysis of the identified DEW glycoproteins was derived using the database for annotation. The pathways were identified according to the Kyoto Encyclopedia of Genes and Genomes database. All identified glycoprotein name identifiers were searched against the STRING database version 10.5 for protein–protein interactions. The protein–protein interaction network of the glycoproteins was also established with the use of Cytoscape software. The functional description of ovotransferrin (OTF) was annotated against the database of InterProScan (<http://www.ebi.ac.uk/interpro/>). The locations of the N-glycosylation sites of OTF were visualized and presented by the Illustrator for Biological Sequences 1.0 program.²³ The data of the CEW N-glycoproteins from our previous study were used for bioinformatics analysis and comparison with the data for DEW.²⁰

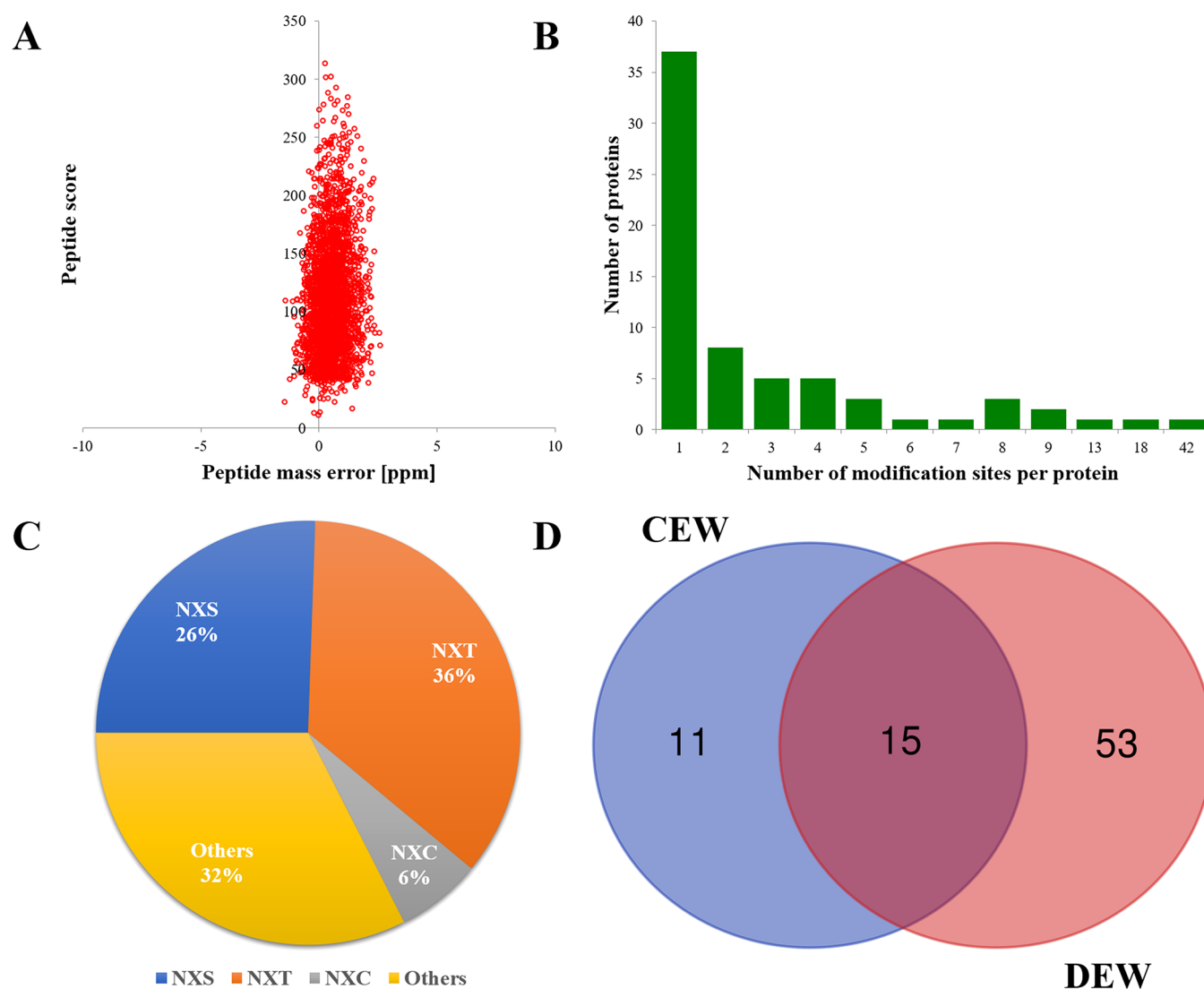


Figure 1. Characteristics of the identified duck N-glycoproteins. (A) Mass error distribution of the identified DEW N-glycopeptides. (B) Distribution of single and multiple DEW N-glycoproteins. (C) Distribution of recognized sequence motifs in DEW N-glycoproteins. (D) Venn diagrams of identified N-glycoproteins between duck and chicken egg whites. The list of CEW N-glycoproteins was from ref 20.

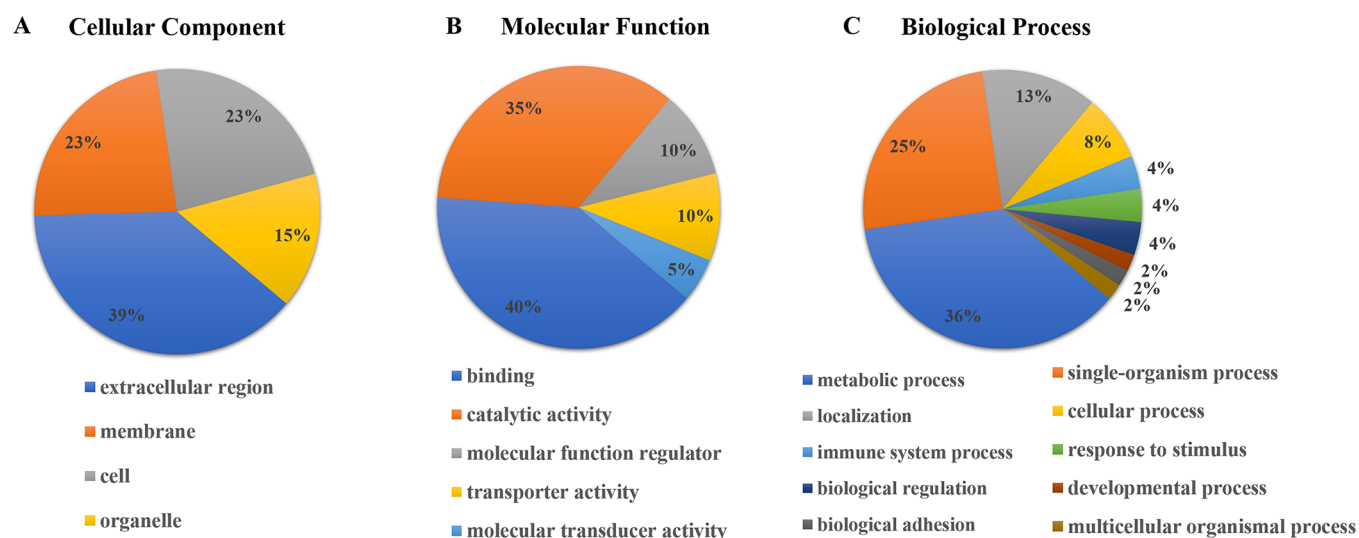


Figure 2. Gene ontology (GO) analysis (level 2) of the N-glycoproteins in DEW.

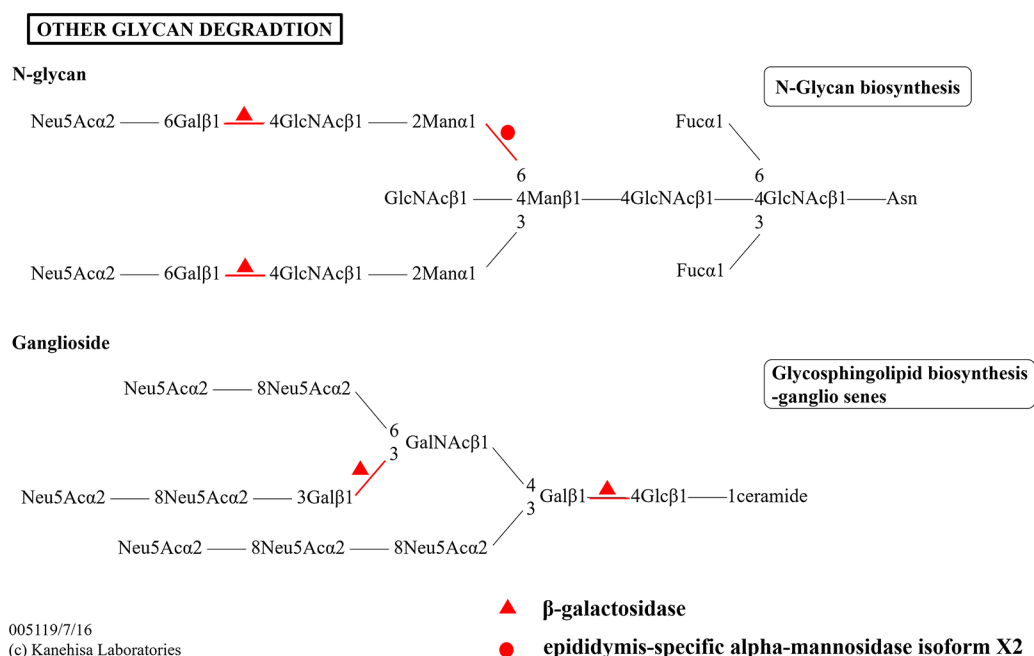


Figure 3. Duck egg white glycoproteins involved in the glycan degradation KEGG pathway. Red lines denote the severable glycosyl bonds. Copyright Kanehisa Laboratories.²⁶ Reprinted with permission.

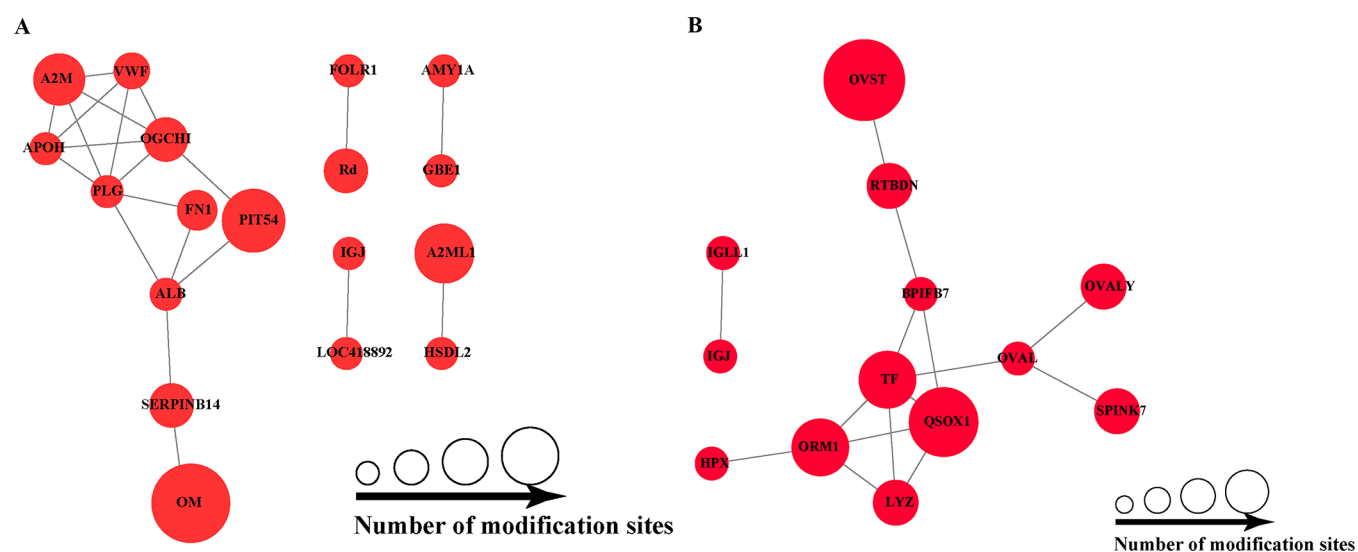


Figure 4. Protein–protein interaction network analysis of the N-glycoproteins in (A) duck egg white and (B) chicken egg white. The list of CEW N-glycoproteins was from ref 20. Disconnected nodes are hidden.

RESULTS AND DISCUSSION

Mapping of the N-Glycosites in DEW Proteins. To study the N-glycoproteins in the DEW samples, the tryptic DEW peptides were treated with PNGase F to remove the N-glycans for subsequent LC-MS/MS analysis. On the basis of the shift in protein molecular mass, a total of 68 N-glycoproteins carrying 231 N-glycosites were identified (Table S1). All the peptide ions had mass tolerances of less than 5 ppm, indicating high precision (Figure 1A). The distribution of DEW N-glycosites is shown in Figure 1B. Of these N-glycoproteins, 37 glycoproteins from DEW contained a single glycosite, which was more than that of CEW (12 glycoproteins).²⁰ The number of glycosites per DEW protein varied from 1 to 42. The highest number of N-glycosites (42 sites) was detected in mucin-5B (Table S1), whereas only 15

N-glycosites were identified in CEW mucin-5B.²⁰ Most of the N-glycosites identified in DEW matched the N-X-[T/S] (X ≠ proline, P) motif, and the proportion of the N-X-T motif (36%) was higher than that of N-X-S (26%, Figure 1C). In addition to the conserved N-X-[T/S] motif, N-X-C was shown to be present at a high frequency in this study, and the frequency of the less detected motifs was higher in DEW (32%) than in CEW (9%).²⁰

Bioinformatic Analysis of DEW N-Glycoproteins. The detected N-glycoproteins of DEW were analyzed via GO analysis. As shown in Figure 2, all mapped N-glycoproteins were classified in accordance with their biological processes, cellular components, and molecular functions at level 2. Multiple N-glycoproteins coevolved with the rise of extracellular processes, potentially signifying significant roles in

organism development, embryo growth, and organ formation.²⁴ In the present study, the distribution of mapped glycoproteins was mainly clustered in the extracellular region (Figure 2A), which was in accordance with the coevolution theory. In terms of molecular function (Figure 2B), the largest proportion of identified N-glycoproteins in DEW were involved in binding (GO:0005488), followed by catalytic activity (GO:0003824), which was performed by a variety of enzymes. In this study, various glycosylated hydrolases, especially glycosylases, were identified in DEW (Table S1). Moreover, the β -galactosidase in DEW were involved in some pathways, as shown in Figure 3. It was shown that there was a greater variety of hydrolases found in DEW than in CEW, suggesting certain unique functions for waterfowl.²⁵ It was reported that the N-linked glycans were required for the enzyme activity of β -glucosidase,²⁷ which was related to the carbohydrate metabolic process. Therefore, the glycans of DEW glycosidases might play an important role in stabilizing their enzymatic activities to release sugars for meeting the need for energetic fuel during embryo development.

The N-glycoproteins of CEW were identified in our previous study.²⁰ Here, comparative GO analysis between DEW and CEW was performed. The distribution of cellular components in DEW and CEW N-glycoproteins was similar (Figures 2A and S1A). A higher percentage of CEW N-glycoproteins (58%), compared with DEW N-glycoproteins (40%), were clustered into the molecular function of binding, whereas more DEW N-glycoproteins (35%) were enriched in catalytic activity compared with CEW N-glycoproteins (11%, Figures 2B and S1B). Major N-glycoproteins in DEW were associated with the metabolic process and single-organism process, whereas CEW glycoproteins were mainly distributed in biological regulation (Figures 2C and S1C). A larger number of metabolic process related glycoproteins were found in DEW (36%) than in CEW (8%). It was reported that the enzymatic efficiency could be modulated by the glycoprotein N-glycans,²⁷ and the alteration of enzymatic efficiency might be related to the higher energy demand for duck.

The protein–protein interaction network was determined for the interactions of N-glycoproteins in DEW (Figure 4A) and CEW (Figure 4B). The edges and nodes represent the interactions of glycoproteins. The DEW glycoprotein interaction network contain 21 predicted interactions involving 18 N-glycoproteins. Among these interacting glycoproteins, some bear enzymatic activities, including A2M, PLG, OM, and A2ML1. SPINK7 in CEW and SERPINB14 in DEW are both ovalbumin, but they have one and four N-glycosites, respectively. The proteins predicted to interact with DEW and CEW ovalbumin are also different. Whether and how diverse glycosylation affects the interactions among these glycoproteins require further study.

Characterization of the N-Glycoproteome in DEW. In the comparison of the DEW and CEW N-glycoproteomes (Figure 1D), 15 N-glycoproteins were shown to be conserved in these two species. On the basis of the GO analysis, these conserved N-glycoproteins were involved in protein binding, protein localization, and enzyme activity regulation. There were 53 unique glycoproteins identified in DEW, such as legumain, carboxypeptidase D precursor, and deleted-in-malignant-brain-tumors-1-protein-like (DMBT1PL), which might be involved in species-specific properties or bioactivities. For instance, the glycosylation of immature legumain was important for its localization and processing into its active

form.²⁸ DMBT1PL, as a specific glycoprotein in DEW, was presented with nine SRCR domains, in which the glycans were identified as affecting innate immunity on the basis of the ligand binding properties.²⁹ The glycosylation domains adjacent to the SRCR domains harbor an essential role in binding viruses.³⁰ Therefore, the carbohydrate moieties on DMBT1PL might enhance the DEW antibacterial activity.

Antibacterial-Related N-Glycoproteins in DEW. Considering the duck eggshell from commercial hatcheries is mainly contaminated with Gram-positive cocci, it is probable that the cavity-nesting environment may lead to more potent egg white antimicrobial activities to defend against attacks by Gram-positive bacteria.^{31,32} Lysozyme is a major antimicrobial protein in DEW that could inhibit most Gram-positive bacteria by hydrolyzing the peptidoglycan cell wall.³³ In addition, some proteins like OTF and riboflavin-binding protein (RFBP) inhibit the bacteria by competitively binding elements required by the bacteria. For example, RFBP can eliminate free riboflavin in egg white, and the riboflavin binding activity may be decreased by deglycosylation.³⁴

In this study, nine N-glycosites were identified in DEW OTF, but only three glycosites were found in CEW.²⁰ A comparative study suggested that the varying OTF N-glycoforms between chicken and pheasant might influence biofunctions by changing the primary structure of OTF.³⁵ According to the high sequence identity between the DEW and CEW OTFs, the higher amount of N-glycosites in DEW OTF might be the main reason for its variation in antimicrobial activity. Ghanbari et al. reported that the glycans on the C-lobe of OTF might affect the layout of the iron binding site residue and subsequently affect the transferrin function.³⁶ As shown in Figure 5, most of the N-glycosites were concentrated on the

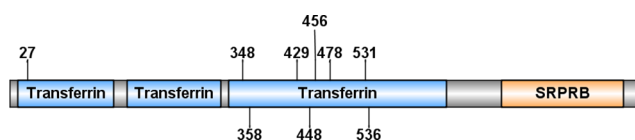


Figure 5. Locations of the identified N-glycosites on DEW ovotransferrin.

transferrin domain (PF00405), which contained active sites showing ferric iron binding activity. Consequently, it could be suggested that these concentrated N-glycans may influence the dynamics of OTF structures and subsequently affect the antibacterial activity.

In DEW lysozyme, a total of four N-glycans were detected. All of the N-glycosites were localized at the noncanonical motifs (NXC, NXR, NXD, and NXE), which were similar to those of CEW lysozyme.³⁷ It was suggested that nesting under humid conditions might enhance bacterial contamination, and thus waterfowl might evolve higher antimicrobial activity.^{31,32} Additionally, the conjugation of polysaccharide and lysozyme could strengthen the protein surface activity, allowing it to attack bacteria, which contain hydrophobic material on their envelopes, whereas the native lysozyme without polysaccharide fails to lyse Gram-negative bacteria.³⁸ Thus, the different antibacterial spectra or activity between CEW and DEW may rely on the lysozyme carbohydrate moieties; however, this requires further study.

In this study, 42 N-glycosites were detected in DEW Mucin-5B, which is a subunit of ovomucin.³⁹ Mucin-6, another subunit of ovomucin, was identified with only one N-glycosite. It was

reported that the glycopeptides derived from CEW ovomucin could prevent bacterial adherence to porcine erythrocytes.⁴⁰ Other studies demonstrated that N-glycans also play a vital role in protein dimerization and polymerization, which prevent egg white thinning during storage.^{41,42} Therefore, the relative higher viscosity in DEW may be caused by the abundant N-glycosites of duck mucin-5B, which can also enhance the antibacterial activity by limiting bacterial movement.

Allergy-Related N-Glycoproteins in DEW. Among the 13 glycosites identified in DEW ovomucoid (OM), 5 of them (N7, N51, N73, N156, and N173), with motif N-X-[S/T] (X ≠ proline, P), were canonical sites, whereas 8 were noncanonical sites (N11, N37, N45, N76, N102, N110, N164, and N167). There is a controversy about whether the carbohydrate moieties are the active fragments of antigenicity.^{43–45} Zhang et al. reported that the OM glycans had an inhibitory effects on the protein binding properties to IgG and IgE,⁴⁴ and another study found that N-linked glycans, especially the mannose chain on OM domain III, were related to the suppression of allergic response.⁴⁵ Moreover, OM domain III was more related to allergenic effects than domains I and II.⁴⁴ Many N-glycosites were identified in the third domain of DEW OM, which might reduce the allergenicity. On the other hand, it was reported that the carbohydrate moieties were responsible for increased resistance to proteolysis and resulted in its allergenic potency.⁴⁶ It has been reported that two specific IgE recognition epitopes exist in CEW OM (DCSRFPNATDKE and FNPVCGTDGVTYDN), and the N-glycosites were both identified in our former work.^{20,47} Here, similar peptides (DCSRFPNTTNEE and FSPVCGTDGVTYDN) were glycosylated in DEW, which might be related to its allergenicity. Therefore, it may be speculated that the glycans on DEW OM can not only prevent IgE-recognition epitopes from being degraded but also affect the protein steric hindrance. The discrepancy in the glycosylation of OM between duck and chicken egg whites may contribute to their specific allergenicities.^{6,20}

Two independent N-glycosites and a pair of adjacent glycosites were identified in duck RFBP (Table S1). It was reported that the secondary and tertiary structures of RFBP could be affected by its carbohydrate moieties.⁴⁸ Moreover, a study on the effects of the adjacent serine O-glycosylation showed that the clustered carbohydrates shifted the conformational equilibrium as well as the target binding sites.⁴⁹ Moreover, the carbohydrate moieties, especially sialic acid, were essential for protein transport and biological functions.³⁴ Thus, it could be postulated that the adjacent glycosites on duck RFBP might affect IgE binding activity by changing the protein structure.

Lysozyme is also recognized as being associated with allergies. The specific glycosylation of hen egg lysozyme could result in steric hindrance on the protein interfacial region and, subsequently, reduce the antigenicity dramatically.⁵⁰ Moreover, OVA, as one of the most studied allergenic proteins, was identified with four N-glycosites in DEW. It has been demonstrated that the deglycosylation of OVA N-terminal glycan decreases the allergenicity and antigenicity.⁵ It was reported that glycation by a Maillard reaction could influence the allergenicity of ovalbumin, which could contribute to the alteration of the resistance to digestive enzymes.⁵¹ Thus, the difference in OVA carbohydrate moieties between DEW and CEW might also contribute to the

alteration of species-specific allergenicity, which demands further investigation.

To conclude, the N-glycoproteome of DEW was investigated and compared with that of CEW via bioinformatics analysis in this study. In total, 231 N-glycosites on 68 glycoproteins were identified in DEW. According to the restriction of trypsin digestion strategy, however, some N-glycosites might still be undetected, which needs further analysis with a combination of different enzymes. Meanwhile, the simultaneous existence of O-glycans might also interfere with the identification of N-glycosite information. GO functional annotations of the N-glycoproteins in DEW and CEW revealed their variations in biological functions. The comparison of glycosites between CEW and DEW N-glycoproteins elicited differences in their allergenicities as well as their antibacterial activities. The divergence of these glycoprotein homologues was suggested as having evolved from adaption to various environmental stresses, such as the need to defend against infections. Meanwhile, the N-glycosite variations between DEW and CEW proteins may be related to the poultry domestication process, providing new insight into the evolutionary history of poultry.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b03059.

Table S1. Details of DEW N-glycopeptides resulting from a database search of LC-MS/MS data (XLSX)

Figure S1. Gene Ontology analysis (level 2) of the N-glycoproteins in CEW (the list of CEW N-glycoproteins was from ref 20) (PDF)

Figure S2. (A) HPLC separation chromatogram data of trypsin digested protein mixtures and (B) illustration of the 60 components separated into 4 fractions (PDF)

Figure S3. Chromatograms from the LC-MS/MS analysis of trypsin digested DEW glycoproteins (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DEW, duck egg white; CEW, chicken egg white; HILIC, hydrophilic interaction liquid chromatography; GO, gene ontology; DMBT1PL, deleted in malignant brain tumors 1;

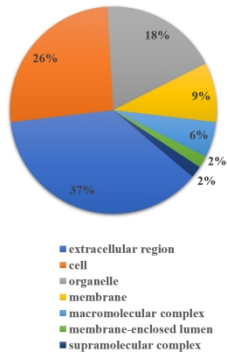
OTF, ovotransferrin; RFBP, riboflavin-binding protein; OM, ovomucoid; OVA, ovalbumin

REFERENCES

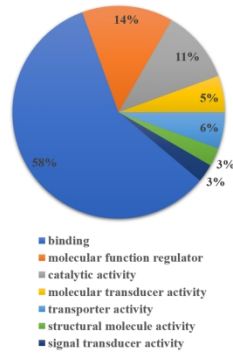
- (1) Wormald, M. R.; Dwek, R. A. Glycoproteins: glycan presentation and protein-fold stability. *Structure* **1999**, *7*, R155–R160.
- (2) Hart, G. W.; Copeland, R. J. Glycomics hits the big time. *Cell* **2010**, *143*, 672–676.
- (3) Quan, T. H.; Benjakul, S. Quality, protease inhibitor and gelling property of duck egg albumen as affected by storage conditions. *J. Food Sci. Technol.* **2018**, *55*, 513–522.
- (4) Shan, Y.; Xu, Q.; Ma, M. Mg²⁺ binding affects the structure and activity of ovomucin. *Int. J. Biol. Macromol.* **2014**, *70*, 230–235.
- (5) Park, H. Y.; Yoon, T. J.; Kim, H. H.; Han, Y. S.; Choi, H. D. Changes in the antigenicity and allergenicity of ovalbumin in chicken egg white by N-acetylglucosaminidase. *Food Chem.* **2017**, *217*, 342–345.
- (6) Alcántara, V. M.; Palacios, C. L.; Anaya, A. S.; Bustamante, O. L.; Jimeno, N. L. Duck egg allergy in an adult patient without allergy to chicken egg. *J. Investig. Allergol. Clin. Immunol.* **2019**, *29*, 245–246.
- (7) Clutton-Brock, J. A history of domesticated animals. *J. Anim Ecol.* **1964**, *33*, 215.
- (8) Quan, T. H.; Benjakul, S. Duck egg albumen: physicochemical and functional properties as affected by storage and processing. *J. Food Sci. Technol.* **2019**, *56*, 1104–1115.
- (9) Warwas, M.; Gburek, J.; Osada, J.; Golab, K. Purification and characterization of cystatin from duck egg white. *Acta Biochim. Pol.* **1995**, *42*, 351–356.
- (10) Pikul, J. Characteristics of duck eggs and the quality of duck eggs products. *Arch. Geflügelk.* **1998**, *62*, 72–82.
- (11) Naknukool, S.; Hayakawa, S.; Uno, T.; Ogawa, M. CHAPTER 18-Antimicrobial activity of duck egg lysozyme against almonella enteritidis. *Global Issues in Food Science & Technology* **2009**, 293–307.
- (12) Geng, F.; Huang, X.; Majumder, K.; Zhu, Z.; Cai, Z.; Ma, M. Mass spectrometry and two-dimensional electrophoresis to characterize the glycosylation of hen egg white ovomacroglobulin. *J. Agric. Food Chem.* **2015**, *63*, 8209–8215.
- (13) Hu, S.; Qiu, N.; Liu, Y.; Zhao, H.; Gao, D.; Song, R.; Ma, M. Identification and comparative proteomic study of quail and duck egg white protein using 2-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry analysis. *Poult. Sci.* **2016**, *95*, 1137–1144.
- (14) Hirose, K.; Amano, M.; Hashimoto, R.; Lee, Y. C.; Nishimura, S.-I. Insight into glycan diversity and evolutionary lineage based on comparative Avio-N-glycomics and sialic acid analysis of 88 egg whites of Galloanserae. *Biochemistry* **2011**, *50*, 4757–4774.
- (15) Suzuki, N.; Nawa, D.; Su, T.-H.; Lin, C.-W.; Khoo, K.-H.; Yamamoto, K. Distribution of the Gal β 1–4Gal epitope among birds: species-specific loss of the glycan structure in chicken and its relatives. *PLoS One* **2013**, *8*, No. e59291.
- (16) Sanes, J. T.; Hinou, H.; Lee, Y. C.; Nishimura, S. I. Glycoblotting of Egg White Reveals Diverse N-glycan Expression in Quail Species. *J. Agric. Food Chem.* **2019**, *67*, 531–540.
- (17) Cao, X.; Yang, M.; Yang, N.; Liang, X.; Tao, D.; Liu, B.; Wu, J.; Yue, X. Characterization and comparison of whey N-glycoproteomes from human and bovine colostrum and mature milk. *Food Chem.* **2019**, *276*, 266–273.
- (18) Feng, M.; Fang, Y.; Han, B.; Xu, X.; Fan, P.; Hao, Y.; Qi, Y.; Hu, H.; Huo, X.; Meng, L.; Wu, B.; Li, J. In-Depth N-Glycosylation Reveals Species-Specific Modifications and Functions of the Royal Jelly Protein from Western (*Apis mellifera*) and Eastern Honeybees (*Apis cerana*). *J. Proteome Res.* **2015**, *14*, 5327–5240.
- (19) Geng, F.; Xie, Y.; Wang, J.; Majumder, K.; Qiu, N.; Ma, M. N-Glycoproteomic Analysis of Chicken Egg Yolk. *J. Agric. Food Chem.* **2018**, *66*, 11510–11516.
- (20) Geng, F.; Wang, J.; Liu, D.; Jin, Y.; Ma, M. Identification of N-Glycosites in Chicken Egg White Proteins Using an Omics Strategy. *J. Agric. Food Chem.* **2017**, *65*, 5357–5364.
- (21) Wisniewski, J.; Zougman, A.; Nagaraj, N.; Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6*, 359–362.
- (22) Zacharias, L. G.; Hartmann, A. K.; Song, E.; Zhao, J.; Zhu, R.; Mirzaei, P.; Mechref, Y. HILIC and ERLIC Enrichment of Glycopeptides Derived from Breast and Brain Cancer Cells. *J. Proteome Res.* **2016**, *15*, 3624–3634.
- (23) Liu, W.; Xie, Y.; Ma, J.; Luo, X.; Nie, P.; Zuo, Z.; Lahrmann, U.; Zhao, Q.; Zheng, Y.; Zhao, Y.; Xue, Y.; Ren, J. IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* **2015**, *31*, 3359–3361.
- (24) Zielinska, D.; Gnadt, F.; Schropp, K.; Wiśniewski, J.; Mann, M. Mapping N-Glycosylation Sites across Seven Evolutionarily Distant Species Reveals a Divergent Substrate Proteome Despite a Common Core Machinery. *Mol. Cell* **2012**, *46*, 542–548.
- (25) Sun, C.; Liu, J.; Li, W.; Xu, G.; Yang, N. Divergent Proteome Patterns of Egg Albumen from Domestic Chicken, Duck, Goose, Turkey, Quail and Pigeon. *Proteomics* **2017**, *17*, 1700145.
- (26) Kanehisa, M.; Sato, Y.; Furumichi, M.; Morishima, K.; Tanabe, M. New approach for understanding genome variations in KEGG. *Nucleic Acids Res.* **2019**, *47*, D590–D595.
- (27) Boudabbous, M.; Ben Hmad, I.; Saibi, W.; Mssawra, M.; Belghith, H.; Gargouri, A. Trans-glycosylation capacity of a highly glycosylated multispecific β -glucosidase from *Fusarium solani*. *Bioprocess Biosyst Eng.* **2017**, *40*, 559–571.
- (28) Haugen, M. H.; Boye, K.; Nesland, J. M.; Pettersen, S. J.; Egeland, E. V.; Tamhane, T.; Brix, K.; Maeldandsmo, G. M.; Flatmark, K. High expression of the cysteine proteinase legumain in colorectal cancer-implications for therapeutic targeting. *Eur. J. Cancer* **2015**, *51*, 9–17.
- (29) Ligtenberg, A. J. M.; Veerman, E. C. I.; Nieuw Amerongen, A. V.; Mollenhauer, J. Salivary agglutinin/glycoprotein-340/DMBT1: a single molecule with variable composition and with different functions in infection, inflammation and cancer. *Biol. Chem.* **2007**, *388*, 1275–1289.
- (30) Ligtenberg, A. J.; Karlsson, N. G.; Veerman, E. C. Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. *Int. J. Mol. Sci.* **2010**, *11*, 5212–5233.
- (31) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Antimicrobial activity of the Anseriform outer eggshell and cuticle. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2008**, *149*, 640–649.
- (32) Wellman-Labadie, O.; Picman, J.; Hincke, M. T. Comparative antibacterial activity of avian egg white protein extracts. *Br. Poult. Sci.* **2008**, *49*, 125–132.
- (33) Nakimbugwe, D.; Masschalck, B.; Atanassova, M.; Zewdie-Bosünner, A.; Michiels, C. W. Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. *Int. J. Food Microbiol.* **2006**, *108*, 355–363.
- (34) Miller, M. S.; Buss, E. G.; Claggett, C. O. The role of oligosaccharide in transport of egg yolk riboflavin-binding protein to the egg. *Biochim. Biophys. Acta, Gen. Subj.* **1981**, *677*, 225–233.
- (35) Jiang, K.; Wang, C.; Sun, Y.; Liu, Y.; Zhang, Y.; Huang, L.; Wang, Z. Comparison of Chicken and Pheasant Ovotransferrin N-Glycoforms via Electrospray Ionization Mass Spectrometry and Liquid Chromatography Coupled with Mass Spectrometry. *J. Agric. Food Chem.* **2014**, *62*, 7245–7254.
- (36) Ghanbari, Z.; Housaindokht, M. R.; Bozorgmehr, M. R.; Izadyar, M. The effect of glycosylation on the transferrin structure: A molecular dynamic simulation analysis. *J. Theor. Biol.* **2016**, *404*, 73–81.
- (37) Asperger, A.; Marx, K.; Albers, C.; Molin, L.; Pinato, O. Low Abundant N-linked Glycosylation in Hen Egg White Lysozyme Is Localized at Nonconsensus Sites. *J. Proteome Res.* **2015**, *14*, 2633–2641.
- (38) Amiri, S.; Ramezani, R.; Aminlari, M. Antibacterial Activity of Dextran-Conjugated Lysozyme against *Escherichia coli* and *Staphylococcus aureus* in Cheese Curd. *J. Food Prot.* **2008**, *71*, 411–415.

- (39) Robinson, D. S.; Monsey, J. B. Studies on the composition of egg white ovomucin. *Biochem. J.* **1971**, *121*, 537–547.
- (40) Sun, X.; Gänzle, M.; Wu, J. Identification and Characterization of Glycopeptides from Egg Protein Ovomucin with Anti-Agglutinating Activity against Porcine K88 Enterotoxigenic *Escherichia coli* Strains. *J. Agric. Food Chem.* **2017**, *65*, 777–783.
- (41) Bell, S. L.; Xu, G.; Khatri, I. A.; Wang, R.; Rahman, S.; Forstner, J. F. N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2. *Biochem. J.* **2003**, *373*, 893–900.
- (42) Kato, A.; Ogato, S.; Matsudomi, N.; Kobayashi, K. Comparative study of aggregated and disaggregated ovomucin during egg white thinning. *J. Agric. Food Chem.* **1981**, *29*, 821–823.
- (43) Besler, M.; Steinhart, H.; Paschke, A. Allergenicity of hen's egg-white proteins: IgE binding of native and deglycosylated ovomucoid. *Food Agric. Immunol.* **1997**, *9*, 277–288.
- (44) Zhang, J. W.; Mine, Y. Characterization of IgE and IgG epitopes on ovomucoid using egg-white-allergic patients' sera. *Biochem. Biophys. Res. Commun.* **1998**, *253*, 124–127.
- (45) Rupa, P.; Nakamura, S.; Mine, Y. Genetically glycosylated ovomucoid third domain can modulate Immunoglobulin E antibody production and cytokine response in BALB/c mice. *Clin. Exp. Allergy* **2007**, *37*, 918–928.
- (46) Benede, S.; Lopez-Fandino, R.; Reche, M.; Molina, E.; Lopez-Exposito, I. Influence of the carbohydrate moieties on the immunoreactivity and digestibility of the egg allergen ovomucoid. *PLoS One* **2013**, *8*, No. e80810.
- (47) Martinez-Botas, J.; Cerecedo, I.; Zamora, J.; Vlaicu, C.; Dieguez, M. C.; Gomez-Coronado, D.; de Dios, V.; Terrados, S.; de la Hoz, B. Mapping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *Int. Arch. Allergy Immunol.* **2013**, *161*, 11–20.
- (48) Rohrer, J. S.; White, H. B. Separation and characterization of the two Asn-linked glycosylation sites of chicken serum riboflavin-binding protein. Glycosylation differences despite similarity of primary structure. *Biochem. J.* **1992**, *285*, 275–280.
- (49) Schuman, J.; Qiu, D.; Koganty, R. R.; Longenecker, B. M.; Campbell, A. P. Glycosylations versus conformational preferences of cancer associated mucin core. *Glycoconjugate J.* **2000**, *17*, 835–848.
- (50) Usui, M.; Shimizu, T.; Goto, Y.; Saito, A.; Kato, A. Effective reduction of antigenicity of hen egg lysozyme by site-specific glycosylation. *FEBS Lett.* **2004**, *557*, 169–173.
- (51) Jiménez-Saiz, R.; Belloque, J.; Molina, E.; López-Fandiño, R. Human immunoglobulin E (IgE) binding to heated and glycated ovalbumin and ovomucoid before and after in vitro digestion. *J. Agric. Food Chem.* **2011**, *59*, 10044–10051.

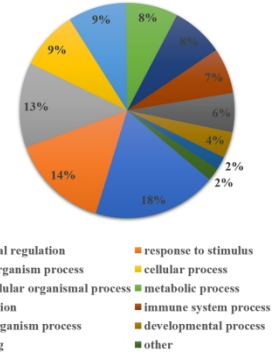
A Cellular Component



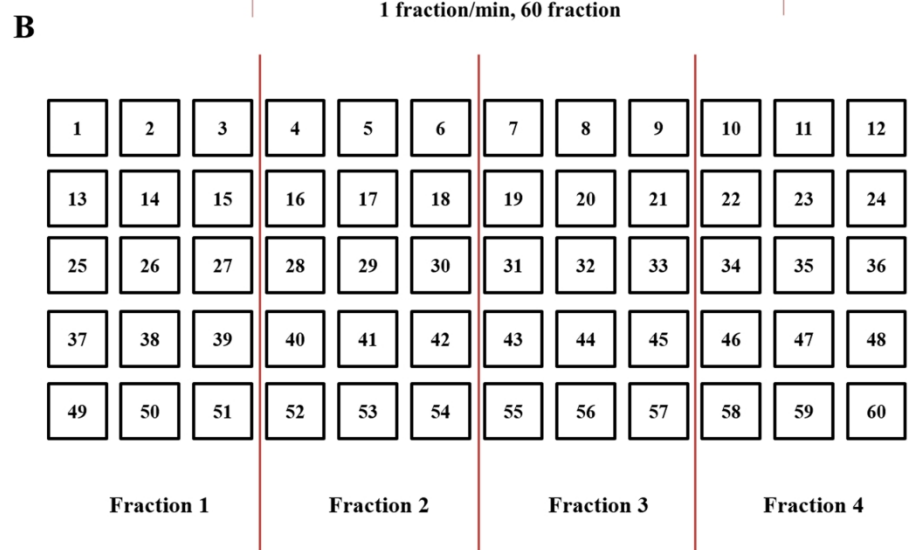
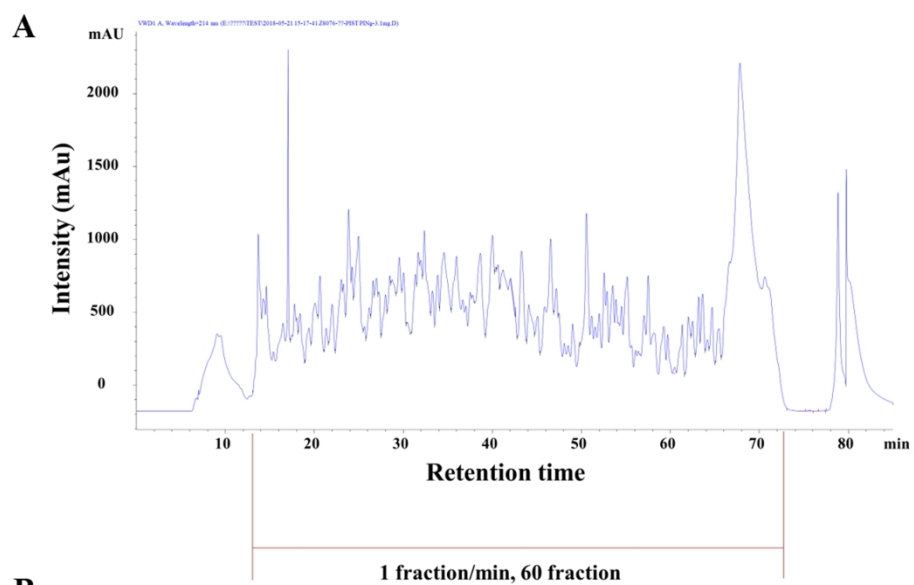
B Molecular Function



C Biological Process



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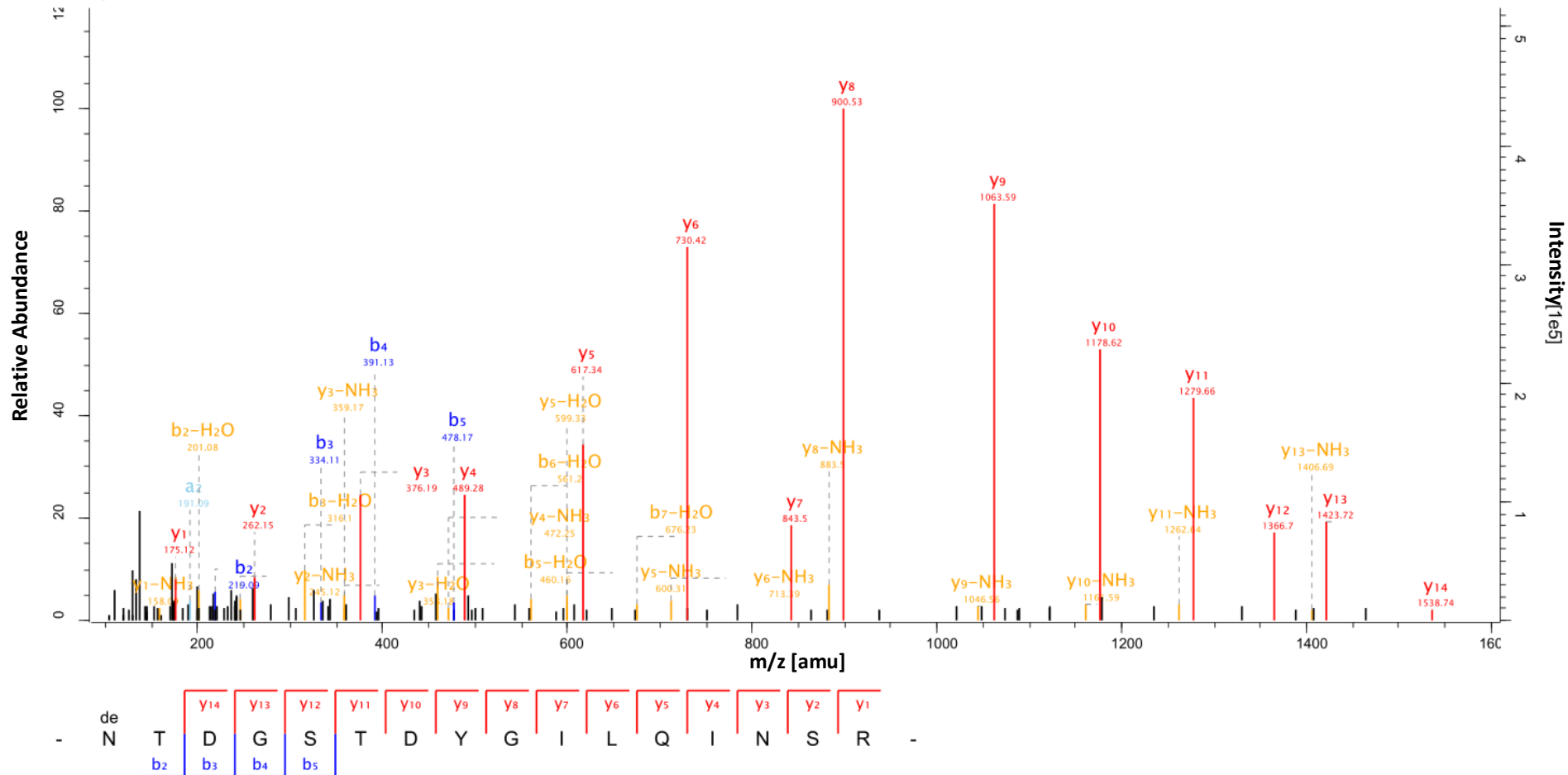
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Lysozyme C

N⁶⁴

N*TDGSTDYGILQINSR

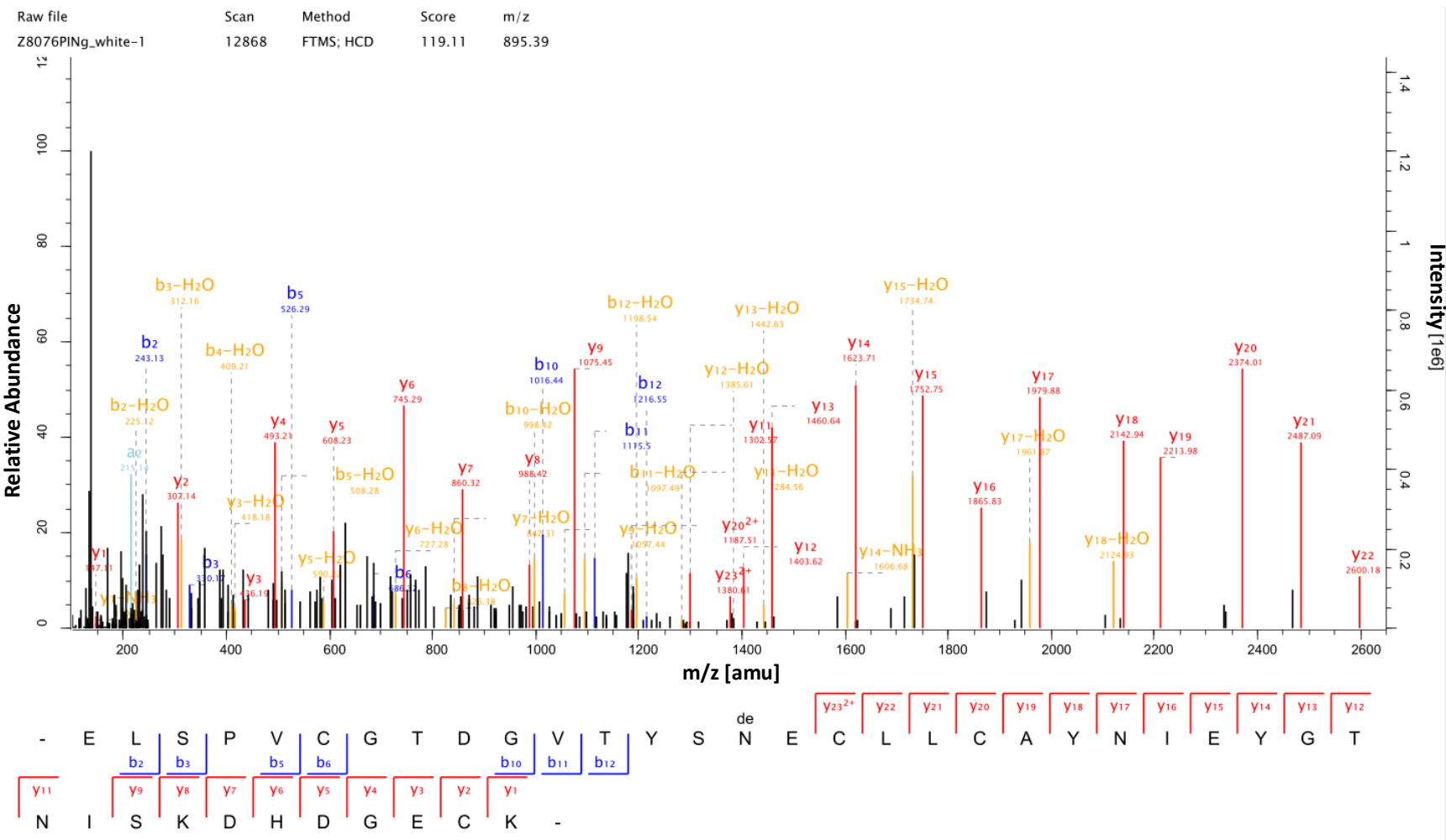
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Ovomucoid

N¹³⁹

ELSPVCGTDGVTYSN*ECLLCAYNIEYGTNISKDHDGECK

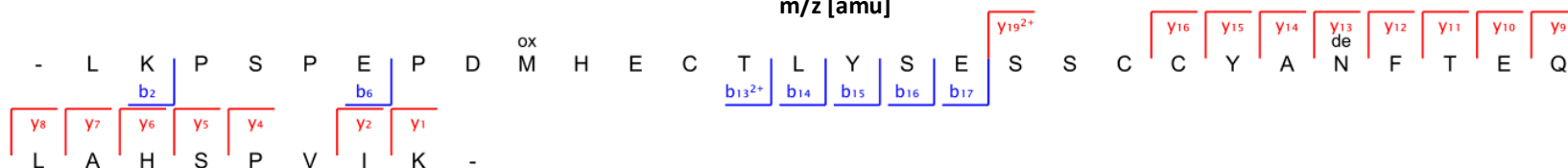
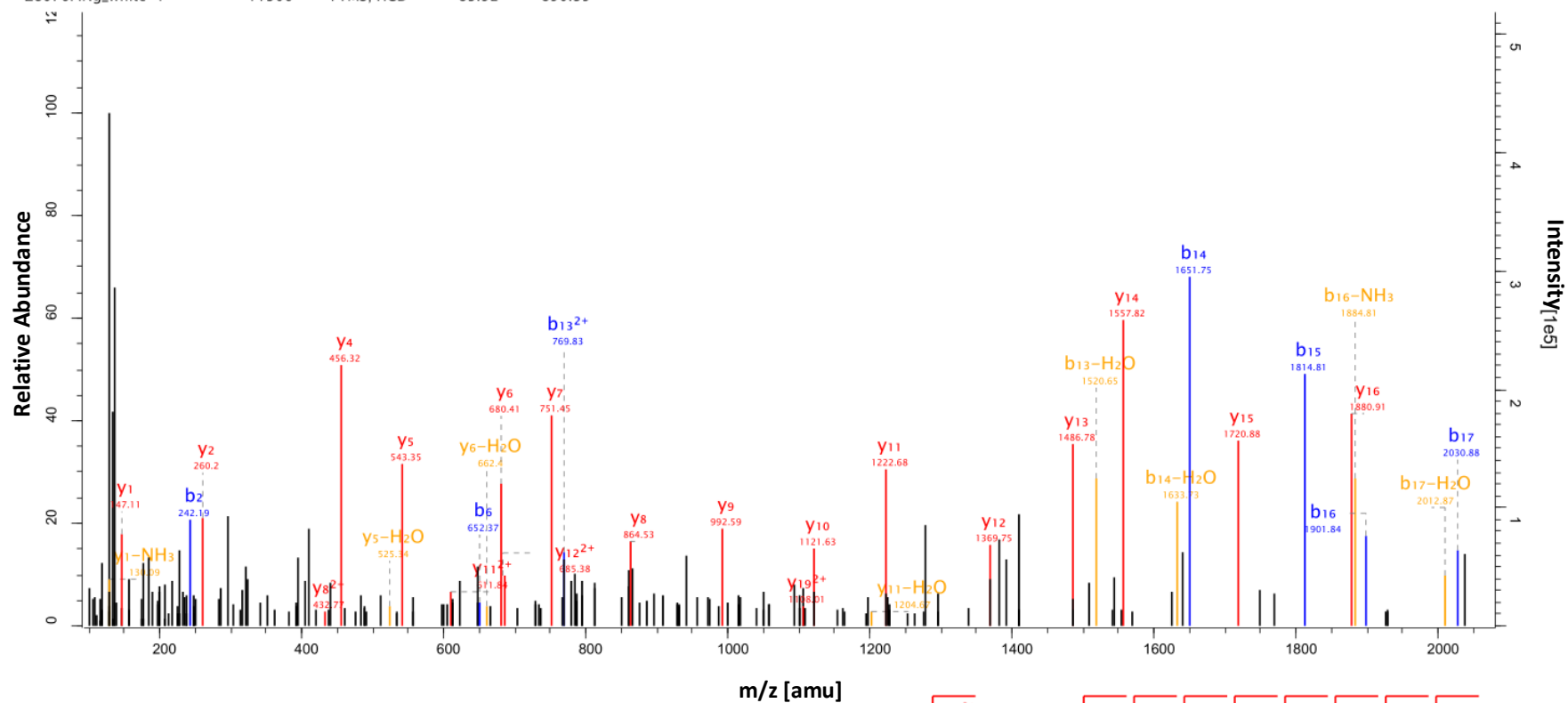


Riboflavin-binding protein

N⁵³

LKPSPEPDMHECTLYSESSCCYAN*FTEQLAHSPVIK

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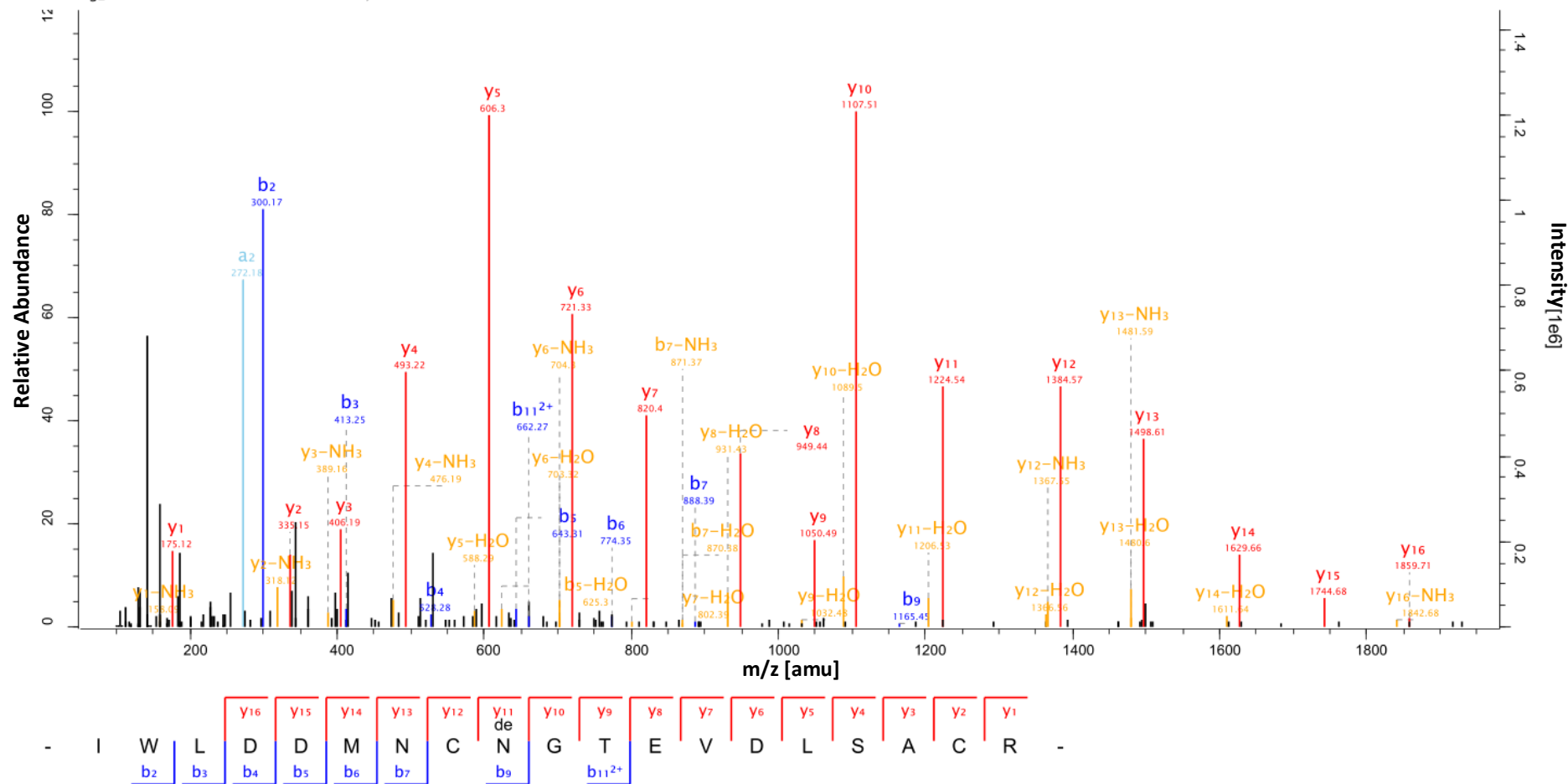


Deleted in malignant brain tumors 1 protein-like

N¹¹⁵

IWLDDMNCN*GTEVDLSACR

Raw file Scan Method Score m/z
Z8076PINg_white-1 13335 FTMS; HCD 193.27 1136.48



Ovotransferrin

N³⁹⁵

WSVVSNGEVECTVADN*TK

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m/z: 999.96

