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Proteomic investigation and understanding on IgY purification and product development

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ABSTRACT An increasing demand for the development of immunoglobin Y (**IgY**) illustrates the necessity of the component analysis in the process of conduction and quality control. This study investigated the proteomic changes in crude IgY extracts and purified IgY products obtained by sequential polyethylene glycol precipitation (**PEG**) of egg yolks followed by human mycoplasma protein-based affinity chromatography compared with intact egg yolks. After confirming the extraction efficiency and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) analysis, liquid chromatography tandem-mass spectrometry (**LC-MS/MS**) was performed with samples including fresh yolk, IgY extracted product and purified product. A total of 348 proteins were identified, with 36 proteins deleted and 209 newly detected proteins in the purified product compared to the intact egg yolk. The significantly decreased proteins mainly included phosvitin, albumin, and apolipoprotein B whereas the significantly increased proteins were mainly IgY-related proteins. GO analysis showed that the purified IgY product had ATPase activity and purine ribonucleoside triphosphate binding activity, and was mainly involved in purine and nucleic acid metabolism. This study will inevitably fasten the commercial application of IgY antibodies and is of greater significance for promotion, development and approval for new antibody derived drug products.

Key words: immunoglobulin Y (IgY), egg yolk, extraction and purification, label-free proteomic analysis, quality control

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INTRODUCTION

Immunoglobulin Y (**IgY**) is homologous to mammalian IgG and is the main serum immunoglobulin in poultry, amphibians, and reptiles which can be further transferred to egg yolk to provide passive immunity for their embryos and offspring (Zhang et al., 2017). Hen egg yolk contains a high concentration and large quantity of IgY, which can be purified at a much lower cost with high yield and fulfilling the requirements of animal welfare at the same time. Moreover, a large number of studies have shown that IgY has antibacterial and antiviral effects, showing great potential in the passive treatment of epidemic diseases such as the coronavirus disease 2019 (Lee et al., 2021). Therefore, IgY technology involving its generation, extraction and purification has been widely applied for broad biomedical purposes including passive immunotherapy, immunoassays, and as an alternative to antibiotics (Schade et al., 2007). In the field of food research, studies have demonstrated the potential of IgY antibodies not only as food additives to passively prevent and treat human digestive tract diseases (Müller et al., 2015), but also for food preservation, such as IgY antibodies specifically targeting *Listeria monocytogenes* (Sui et al., 2011).

With the deepening of IgY application practice, efficient and standardized extraction and purification of IgY is to meet the needs of this era. The main components of yolk are lipids and proteins, and most of the proteins are insoluble lipoproteins, which are present in the yolk granules (Chang et al., 2018). Therefore, the extraction of IgY primarily needs the removal of the lipoprotein in egg yolk and further separation of IgY from water soluble protein. The remaining egg yolk can be used in food products or can be

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subjected to further fractionation to obtain some other biologically active components (Zhang et al., 2021). Briefly, the extraction and purification of IgY involves 2 stages. The initial step involves separation of the water-soluble fraction containing immunoglobulins from the egg yolk and the latter is the further purification of IgY, even specific IgY, from the rest of the proteins. Different extraction and purification methods have been developed for the improvement of various parameters such as IgY yield, purity, work capacity, time consumption, and convenience (Ren et al., 2016; Bizanov, 2018).

Water dilution (**WD**) and polyethylene glycol (**PEG**) precipitation are the most used IgY extraction methods. The WD method has been recognized as a simple and inexpensive way in obtaining high yields of IgY where as a comparative study found higher purity of IgY obtained by the PEG precipitation method (Renet al., 2016). PEG is a nondenaturing water-soluble polymer having the ability to precipitate protein from aqueous solution. PEG is chemically inactive and does not alter even at high temperatures and high concentrations, does not interact with proteins. In addition to the 2 methods mentioned above, ammonium sulphate precipitation is often used to extract IgY from egg yolk (Ko and Ahn 2007; Leiva, et al., 2023). However, compared with ethanol or ammonium sulphate, the target product precipitated by PEG can be more easily separated by centrifugation (Ramshaw, et al., 1984). Affinity chromatography based on protein A and/or protein G is considered as a common and efficient method for purifying antibodies in both laboratory and industrial scales (Hage, 1999). However, due to the structural difference of the Fc fragment between IgY and IgG, these common affinity chromatography tags do not bind to IgY (Łupicka-Słowik, et al., 2014). To overcome this limitation, many other ligands, such as C2 domain of streptococcal protein G (Alves, et al., 2021) and some specific synthetic ligands (Dong, et al., 2008), were attempted to bind to IgY antibodies for affinity purification. In addition, we had developed human mycoplasma protein (protein M) (Grover, et al., 2014) based affinity chromatography which can effectively purify IgY by removing non-IgY proteins (Jiang, et al., 2016). Therefore, the combination of PEG precipitation and protein M chromatography would be a good choice to obtain IgY in high purity.

Despite in some application scenarios, IgY related products can be developed in a simple and practical way, such as egg yolk powder containing specific IgY for the prevention of neonatal diarrhea (Rosa, et al., 2015), or toothpaste against human caries (Rosaet al., 2015), industrialization of biological products requires standardized production process and stable intermediate and end products, which are essential for the production, transportation, storage and use of protein biologics (Crommelin, et al., 2003). Unlike other small molecule formulations, it should be taken into consideration that the registration of new protein products must clearly define the active ingredient(s), such as whole eggs, yolk alone or purified yolk antibodies (Wang and Chow, 2012; Herdeiro, et al., 2016). Based on proteomic analysis using liquid chromatography tandem-mass spectrometry (**LC-MS/MS**), IgY was identified as one of the most abundant proteins in egg yolk (Mann and Mann, 2008), but the study did not focus on the extraction and purification steps of IgY. Also in this year, another MS-based proteomics analysis took the WD-derived IgY preparation with low lipoprotein content as the research object, and successfully determined that the preparation contained 25 proteins in addition to IgY (Nilsson, et al., 2008). However, this study has not performed proteomic analysis of further purified IgY products. In addition, when compared with 2-dimensional polyacrylamide gel electrophoresis, LS-MS/MS analysis, which does not rely on electrophoretic separation, has greater advantages in identifying low-abundance proteins in complex samples (Bunai and Yamane, 2005).

A recent scientometric study shows that researchers have conducted a large number of studies on IgY, including the understanding of the definition and evolutionary status of IgY, extraction and purification of IgY under laboratory conditions, and the application of IgY antibodies in human and veterinary medicine (Wu, et al., 2022). However, these studies were mainly small-scale attempts under laboratory conditions, and industrialization of IgY products was not sufficiently studied. Our study aimed to reveal the possible difference of IgY extraction and purification process in terms of their protein types and contents, to identify non-IgY components generated during the extraction and purification of IgY antibodies and lay the foundation for further standardized commercial production of IgY products.

MATERIALS AND METHODS

Preparation of Egg Yolk Sample

A total of 6 Leghorn eggs were collected in triplicates (2 eggs per replicate), with the same size and color. The yolks were separated carefully and the yolk membrane was removed. The obtained egg yolk content was used for further experiments, after leaving a small portion as a control (group A).

Extraction of IgY Antibodies Using PEG 6000 Precipitation Method

The extraction of IgY had already been reported (Pauly, et al., 2011) and described briefly as below. The yolk isolated from the egg was mixed with twice the amount of phosphate buffer solution (**PBS**, 0.01M, pH 7.4). PEG 6000 at a final concentration of 3.5% (W/V) was added to the yolk mixture to remove lipids and lipoprotein. After shaking at room temperature (20°C−25° C) for 20 min, the mixture was centrifuged (HC-3018RAnhui USTC Zonkia Scientific instruments, China) at 12,000 × *g* for 20 min at 4°C. Supernatant was collected and mixed with 12% PEG 6000 (W/V). After thorough stirring, the suspension was centrifuged at 12,000 × *g* for 20 min at 4°C. The precipitate was dissolved in 10 mL of PBS and 12% (W/V) PEG 6000 was added further. After thorough mixing, the content was

again centrifuged under above mentioned conditions. Finally, the precipitate was collected and dissolved in 1.2 mL of PBS, transferred into a dialysis bag (10-kDa COMW; Solarbio, Beijing, China) and dialyzed against PBS at 4°C overnight. A portion of the obtained IgY extract was labeled as group B, whereas the rest was further used for purification.

IgY Purification Using Protein M

Protein M (4 mg, prepared in our lab to obtain) was coupled to 4 mL NHS-activated Sepharose 4FF (SUNHUI, Suzhou, China) according to the manufacturer's instructions. The coupled sepharose (4 mL) was packed into a 10 mL gravity column and equilibrated with 5 bed volumes of binding buffer (0.01 M PBS, pH 7.4) at room temperature. Then the dialyzed egg yolk dilution was directly injected into the column and binding buffer was used to remove unbound materials. The bound IgY was eluted with elution buffer (0.1 M glycine solution, pH 3.0) and immediately neutralized with neutralization buffer (1 M Tris-HCl, pH 9.0). The obtained fractions were labeled as Group C and 30 μL was taken for Western-blotting analysis (See Supplementary Figure S1). Appropriate amounts of samples from the 3 groups A, B, and C were analyzed by SDS-PAGE (The samples were loaded onto a gel that consisted of 12% separating gel and 5% stacking gel. The gel was stained using Coomassie brilliant blue). And the rest of the samples were used for digestion and LC-MS/MS analysis.

Protein Digestion

SDT buffer (4% SDS in w/v, 0.1 M Tris-HCl pH 7.6, 0.1 M DTT) was used for sample lysis and protein extraction. The amount of protein was quantified with the BCA Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Protein sample (200 μg) was measured and added into 30 μL SDT buffer (4% SDS, 0.1 M DTT, 0.15 M Tris-HCl pH 8.0) for trypsin digestion by following filter aided sample preparation (**FASP**) method (Wiśniewski et al., 2009). The detergent DTT and other low molecular weight components were removed using UA buffer (8 M Urea, 0.15 M Tris-HCl pH 8.0) by repeated ultrafiltration. Then, iodoacetamide (100 μL, 0.1 M IAA in UA buffer) was added to block the reduced cysteine residues and the samples were incubated for 30 min at room temperature under dark condition. After collection of all the samples in the ultrafiltration tube, UA (100 μL) was added before centrifugation for 15 min (12,000× g, 4°C), followed by triplicate operation of adding NH_4HCO_3 (100 μL, 0.05 M) and centrifugation for 10 min (12,000× g, 4°C). Finally, the protein suspensions were digested with trypsin (4 μg; Merck, Darmstadt, Germany) in NH_4HCO_3 buffer (40 μL, 0.025 M) overnight at 37°C and the resulting peptides were collected as a filtrate. The digested peptides of each sample were desalted on C18 Cartridges (Empore SPE Cartridges C18 in standard density, bed I.D. 7 mm, volume 3 mL; Merck, Darmstadt, Germany), concentrated by vacuum centrifugation and reconstituted in formic acid (40 μL, 0.1%, v/v). The peptide content was estimated by UV light spectral density at 280 nm and quantified based on the frequency of tryptophan and tyrosine in vertebrate proteins.

LC-MS/MS Analysis

Each sample was separated by HPLC liquid phase system EasynLC with nano-liter flow rate. The peptides were loaded onto a reverse phase trap column (100 μm × 2 cm; Thermo Fisher Scientific, Waltham, MA), connected to the C18-reversed phase analytical column (length: 10 cm, inner diameter: 75 μm) in buffer A (0.1% formic acid), and then separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology (Cholewa et al., 2014). After chromatographic separation, the samples were analyzed by Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The mass spectrometer was operated in the form of positive ions, with the scan range of the precursor ion 300 to 1,800 m/z and the related parameters as follow: automatic gain control target: 3e6, the maximum inject time: 10 ms, dynamic exclusion duration: 40.0 s, resolution of survey scans: 70,000 at m/z 200, resolution for HCD spectra: 17,500 at m/z 200, isolation width: 2 m/z, normalized collision energy: 30 eV, and underfelt ratio (specifies the minimum percentage of the target value likely to be reached at maximum fill time) of 0.1%. The peptide recognition mode of the instrument was selected, and 20 fragments was collected after each full scan.

Identification and Quantitation of Proteins

The MS raw data of each sample was combined and searched using the MaxQuant search engine (1.5.3.17) for identification and quantitation analysis (Cox and Mann, 2008; Schwanhäusser et al., 2011). Tandem mass spectra were searched against the National Center for Biotechnology Information (**NCBI**) database. Related parameters and instructions were as follow: cleavage enzyme: trypsin (allowing up to 2 missing cleavages), mass tolerance for precursor ions: 20 ppm in the first search and 6 ppm in the main search, carbamidomethyl on cysteine residues: fixed modification, oxidation on methionine residues: variable modifications, the false discovery rate (**FDR**): ≤ 0.01. The same protein was used for subsequent Perseus analysis if it is identified at least twice in 3 repeated biological tests. Protein abundance was represented by LFQ (label-free quantitative) value (Cox et al., 2014), with A to B ratio > 2 and P value < 0.05 defined as expression increase, ratio < 0.5 and P value < 0.05 as expression decrease.

Perseus (version 1.3.0.4) and Statistical Package for the Social Sciences (**SPSS**) (version 22.0) were applied to perform statistical analysis on the database search files obtained by MaxQuant. Gene Ontology (Ashburner

et al., 2000) (http://geneontology.org/) gene function classification system was used to analyze the function of differentially expressed proteins.

RESULTS

High purity IgY Was Obtained After Sequential PEG Precipitation and Protein M Column Chromatography

The egg yolk sample, IgY extract (PEG precipitation) and purified product (protein M affinity chromatography) were analyzed by gel electrophoresis. Egg yolk samples contained many proteins; most had a molecular weight greater than 66.2 kDa. After PEG precipitation, the total content of protein was reduced, along with a significant reduction in the large molecular weight proteins (>66.2 kDa), and an increase in the small molecular weight proteins (<35 kDa). After purification, total proteins were largely removed, leaving only 2 bands on the gel, representing the light chain (≈27 kDa) and the heavy chain (≈67 kDa) of the IgY antibody, respectively (Figure 1). SDS-PAGE results showed the high homogeneity of replicated samples (Figure 1).

There Were 81,173 and 254 Proteins in Groups A, B, and C, Respectively

The 3 samples were divided into different components by HPLC, a total of 2026 peptides and 348 proteins were identified by mass spectrometry. Eighty-one proteins were identified in egg yolk samples and the top 10 proteins with the highest LFQ intensity included phosvitin (F1NFL6, A0A1D5NUW2, A0A3Q2U347), apolipoprotein B, uncharacterized protein (A0A1D5NWD7), Ig-like domain-containing protein (A0A3Q2UAA5, A0A3Q2U8X8), albumin, and ovotransferrin. After PEG precipitation, 18 proteins were removed and 110 proteins were added, resulting in a total of 173 proteins identified in crude IgY extract, with major proteins including, but not limited to, Ig lambda chain C region, Ig-like domain-containing protein, vitellogenin-2, apolipoprotein B, ovotransferrin, and complement component 3. Subsequently, in the purified IgY samples, 80 proteins were removed, 161 proteins were added, and a total of 254 proteins were obtained, with the major proteins of Ig lambda chain C region, Ig-like domain-containing protein, uncharacterized protein (A0A1D5NWD7), actin, cytoplasmic 1, and vitellogenin-2. More importantly, compared with group A, group C ultimately reduced 36 proteins, but increased as many as 198 proteins. In addition, it is worth noting that 41 proteins were present in the entire process of IgY extraction and purification (Figure 2I). The proteins contained in each of the 3 groups A, B, and C was shown in Supplementary Table S1. In addition, the 3 biological replicates of the intragroup samples had almost identical protein contents with 82, 88, and 79 proteins identified in group A and 176, 178,175 in group B and 249, 255, 258 in group C respectively (Figure 2II–IV) which were consistent with the SDS-PAGE results.

Differentially Expressed Proteins Were Found in 3 Comparison Groups (B/A, C/B, and C/A)

Quantitative results for proteins with significant changes in abundance ($P < 0.05$, threshold of fold change >2 or <0.5) were shown in the following 3 comparison groups: IgY extract obtained by PEG precipitation versus egg yolk sample (B/A), IgY refined product

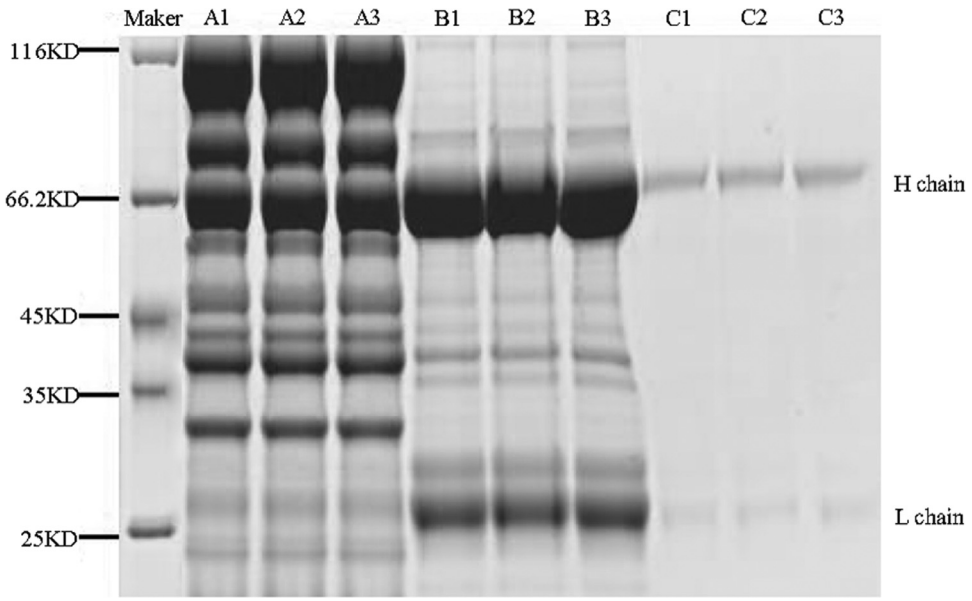

Figure 1. Gel image of egg yolk, IgY extract and IgY purification. Lane 1: Protein marker; Lane 2, 3, 4: A1, A2, A3 Egg yolk sample in triplicates; Lane 5, 6, 7: B1, B2, B3 IgY Crude extract samples in triplicates; Lane 8, 9, 10: IgY Purified product sample in triplicates (H-heavy chain & L- light chain). 10 μL of each sample was loaded onto a gel that consisted of 10% separating gel and 5% stacking gel. The gel was stained using Coomassie brilliant blue.

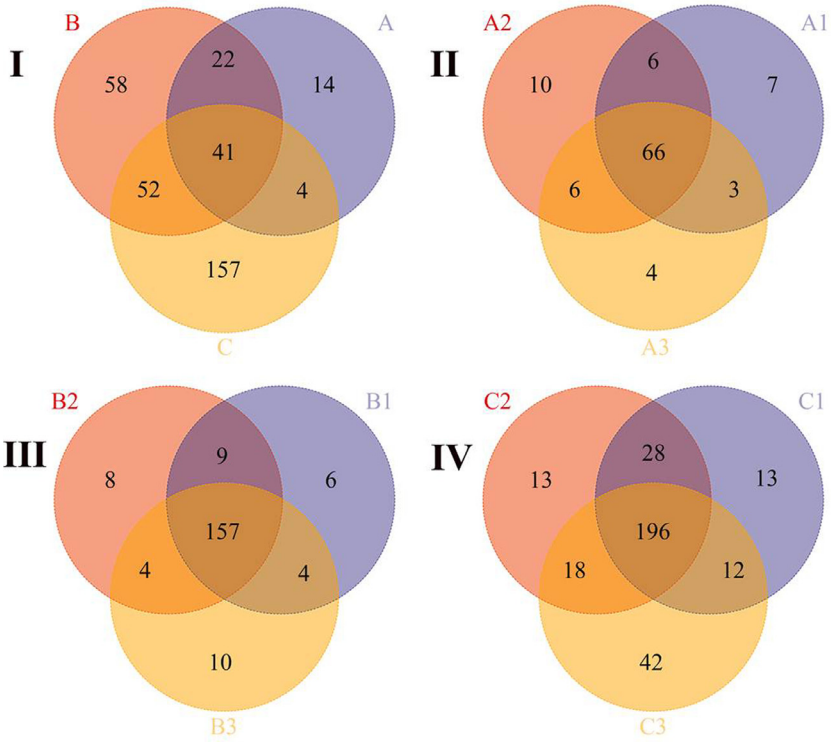

Figure 2. Comparison of protein content among 3 groups of samples (I) and within each sample group (II, III, IV). (A, B, C) egg yolk, IgY crude extract, IgY purification products; overlapping section: number of shared proteins; nonoverlapping sections: number of specific proteins.

purified by protein M versus IgY extract (C/B) and IgY pure product versus egg yolk sample (C/A). The original data of all the differentially expressed proteins were shown in Supplementary Table S2. A total of 38 proteins were significantly upregulated and 10 were significantly downregulated in B/A group, 18 proteins were significantly upregulated and 34 were significantly downregulated in C/B group, and 14 proteins were significantly upregulated and 10 proteins were significantly downregulated in C/A group. In B/A group, protein with the most upregulated expression was peptidase S1 domain containing protein (increased by 242 times), followed by Ig-like domain containing protein, which accounts for half of the upregulated proteins. The most downregulated proteins were mainly vitellogenin-1, apovitellenin-1, epididymal secretory protein E1, ovalbumin (Fragment), and phosvitin. In C/B group, the top 10 upregulated proteins were 14-3-3 protein gamma, 14-3-3 protein zeta, heat shock protein HSP 90-alpha, actin, polyubiquitin (fragment), heat shock cognate protein HSP 90-beta, synaptotagmin-1, Ig-like domain-containing protein (A0A3Q2TX27, A0A3Q2TYH6) and heat shock cognate 71 kDa protein. On the other hand, many proteins were downregulated, including but not limited to alpha-2-macroglobulin-like 4 protein, vitamin K-dependent protein S and complement factor H. In C/A group, among the 14 upregulated proteins, 11 proteins were all Ig-like domain-containing protein and the other 3 proteins were peptidase S1 domain containing protein, Ig lambda chain C region and complement C8 alpha chain. The most downregulated proteins were mainly phosvitin (A0A3Q2U347, A0A1D5NUW2), albumin and apolipoprotein B (Figure 3).

Differentially Expressed Proteins in 3 Comparison Groups Exhibited Different Functions

GO enrichment analysis showed that the differentially expressed proteins of the 3 comparison groups were enriched in different functional items. In B/A group, the top 3 biological processes were complement activation, protein activation cascade and humoral immune response; the top 3 molecular functions were lipid transporter activity, peptidase inhibitor activity and endopeptidase inhibitor activity; the top 3 cellular components were extracellular region, extracellular space, and extracellular region part (Figure 4I). In C/B group, differentially expressed proteins were enriched in 5 biological processes (purine-containing compound metabolic process, nucleobase-containing small molecule metabolic process, purine nucleotide metabolic process, nucleoside phosphate metabolic process, and nucleotide metabolic process), 2 molecular functions (tubulin binding and cytoskeletal protein binding), and 3 cellular components (cytoplasm, neuron part, and cytoplasmic part) (Figure 4II). Similarly, in C/A group, the differentially expressed proteins also showed different enrichment results from the above 2 comparison groups. Significant changes have taken place in 3 important biological processes (purine-containing compound metabolic process, nucleobase-containing small molecule metabolic process and purine nucleotide metabolic process), 3 molecular functions (ATPase activity, small molecule binding and purine ribonucleoside triphosphate binding), and 3 cellular components (cytoplasm, intracellular and intracellular part, Figure 4III).

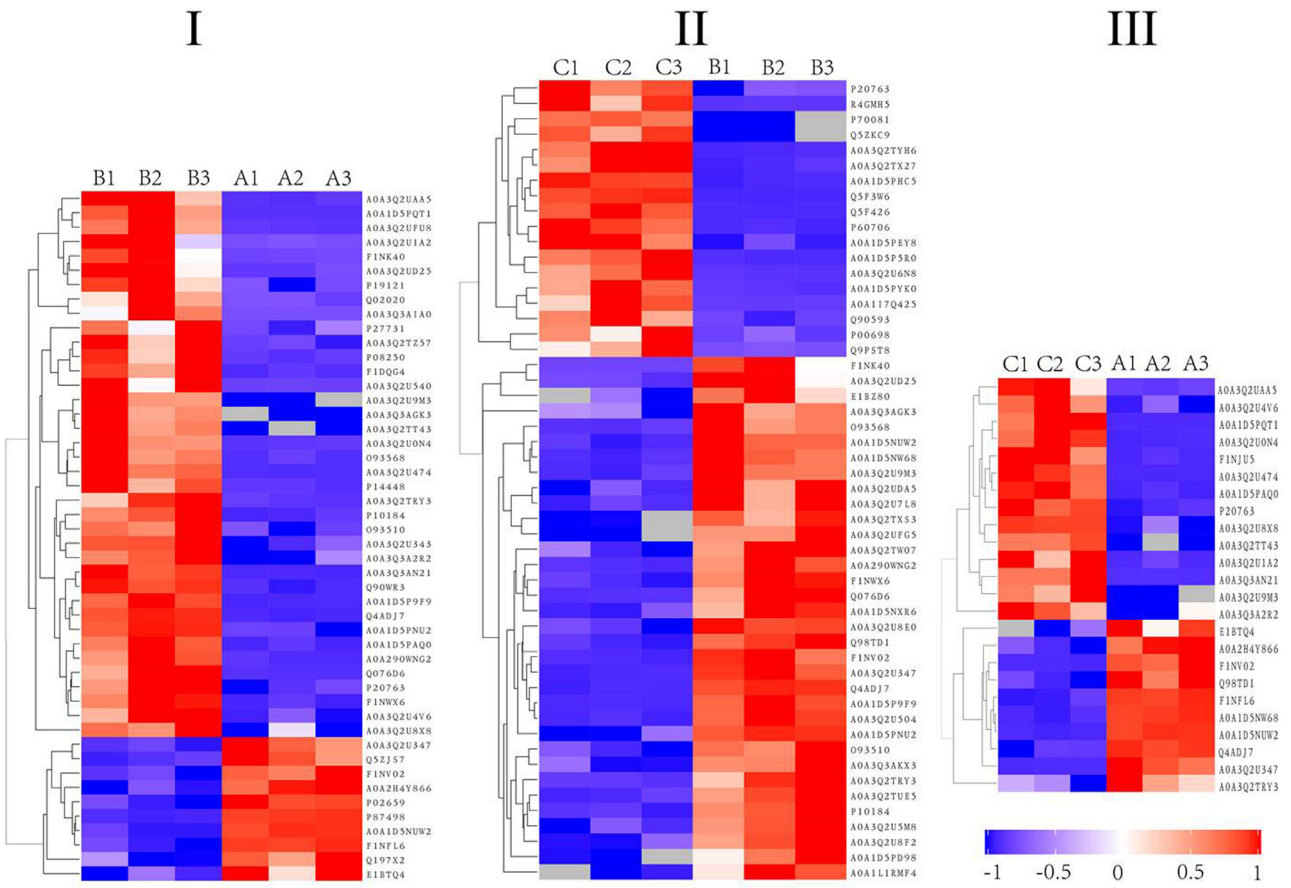

Figure 3. Differentially expressed proteins identified in groups A, B, and C using cluster analysis. I, II, III: the cluster analysis of differentially expressed proteins in B/A group, C/B group and C/A group respectively. A1, A2, A3; B1, B2, B3; C1, C2, C3: 3 biological replicates of groups A, B, and C respectively; red and blue: significantly upregulated and downregulated proteins respectively; gray: no quantitative information.

DISCUSSION

To meet the diversified application needs, increased IgY products have been developed and distributed in the markets, in the form of new drugs, food, or feed additives in R&D pipeline, including clinical studies for anti-SARS-CoV-2 virus (Kreuzberger et al., 2021; Vieira-Pires et al., 2021). However, the development of IgY technology still faces some technical barriers to be solved. There is a lack of uniform standards and mature methods for the preparation, purification, and use of egg yolk antibodies (Kovacs-Nolan and Mine 2012; da Silva et al., 2021). In many current applications, egg yolk antibodies are often used in the form of crude products against specific pathogens, which do not necessarily require high purity of IgY and thereby further IgY purification procedure may not apply. However, to maximize the use of IgY in human and veterinary diagnostic and therapeutic fields and to achieve large scale commercialization of IgY, there is a need to clarify what substances are contained in IgY products with its safety aspects for humans and animals.

Compared to other protein quantification methods, such as array-based, gel-based, or tag-based methods, label-free mass spectrometric protein quantitation is being widely used in differentially expressed proteomics studies owning to its high accuracy, reproducibility and cost performance (Lai et al., 2013). In this study, bottom-up label-free LC-MS/MS was performed with samples including fresh yolk, IgY extracted product and purified product. In contrast to the original egg yolk samples, the proteins in the 2 different IgY preparations obtained were significantly changed in terms of both total protein amount and protein type.

SDS-PAGE showed that the total protein content was gradually reduced after PEG treatment and protein M chromatography. In the final purified product, IgY was obtained, which was confirmed by Western-blotting analysis (Supplementary Figure S1). A subsequent cross-sectional comparison of the proteomes for these 3 comparison groups showed a significant decrease in total protein quantification as extraction and purification proceeded (Figure 5, blue part compared to purple and orange part), which was consistent with the SDS-PAGE observation. This reduction was mainly due to a large decrease in the levels of apolipoprotein B, phosvitin, albumin, ovotransferrin, and complement component, which were highly abundant in egg yolk (Mann et al., 2008) and were necessarily removed in large amounts after stepwise extraction and purification (Figure 6I). Simultaneously, a significant rise in the contents of Ig-like domain-containing protein and Ig lambda chain C region was observed (Figure 6II). Ig-like domains were similar in amino acid sequence and structure to the Ig domains of immunoglobulins (Edelman, 1987), constitute immunoglobulins superfamily that is extensively

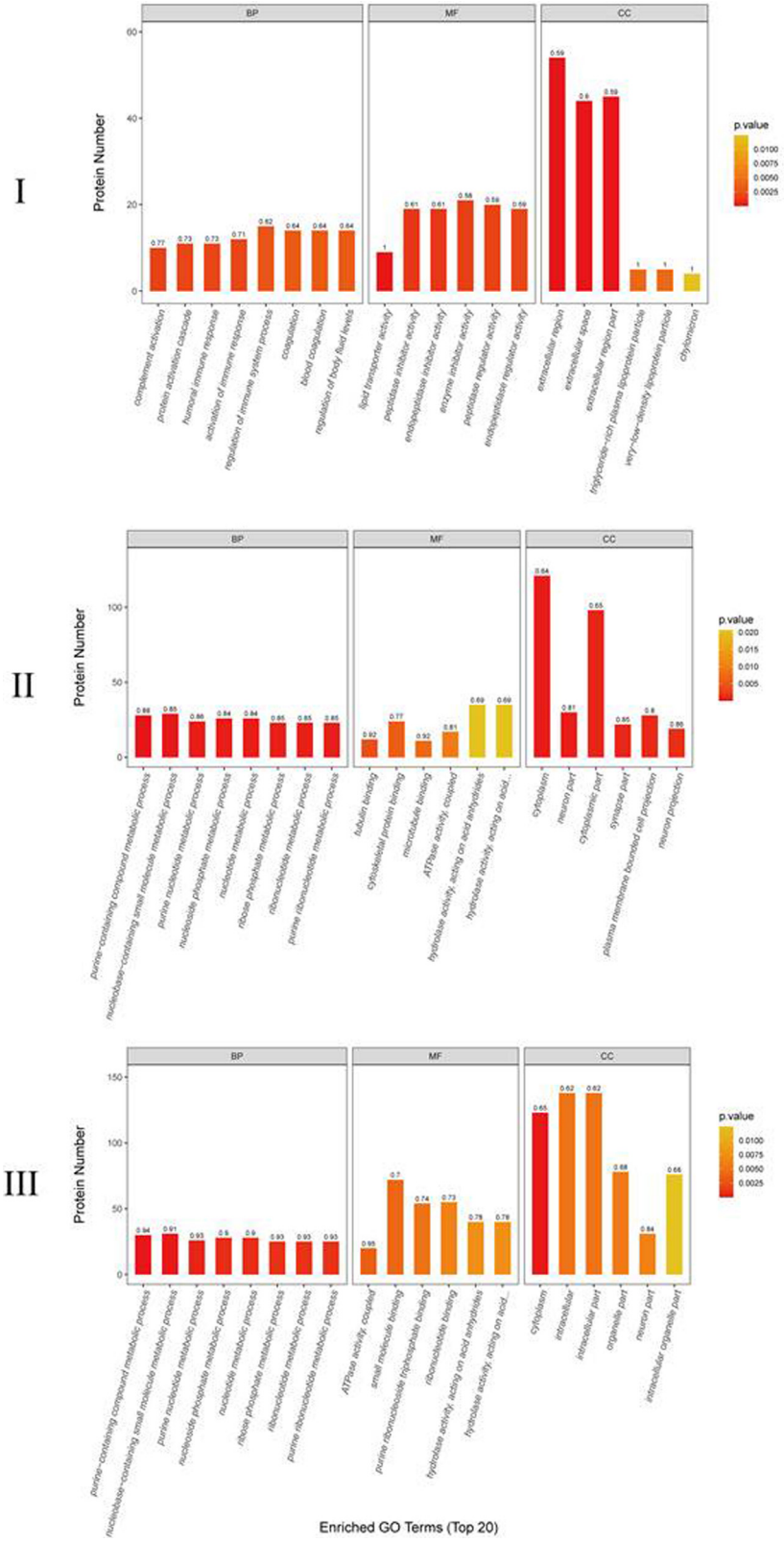

Figure 4. GO enrichment results of differentially expressed proteins. I, II, III: the enrichment results of differentially expressed proteins in B/A, C/B, and C/A comparison groups respectively; abscissa: GO term; ordinate: the number of differentially expressed proteins corresponding to the GO term; BP: biological process; MF: molecular function; CC: cellular component; the colors of the bubble: the significance of the enriched GO terms; the color gradient from yellow to red: the increasing significant difference; numbers on bars: enrichment factor (≤1) of each pathway (numbers of differential proteins/numbers of characterized proteins).

involved in immune responses and intercellular receptor-ligand binding processes (Halaby and Mornon, 1998). These results indicate a combined procedure of PEG precipitation and protein M-based affinity chromatography is feasible for the purification of IgY antibodies from a proteomics point of view.

We also observed that as extraction and purification proceeded, increasing kinds of proteins emerged (81,

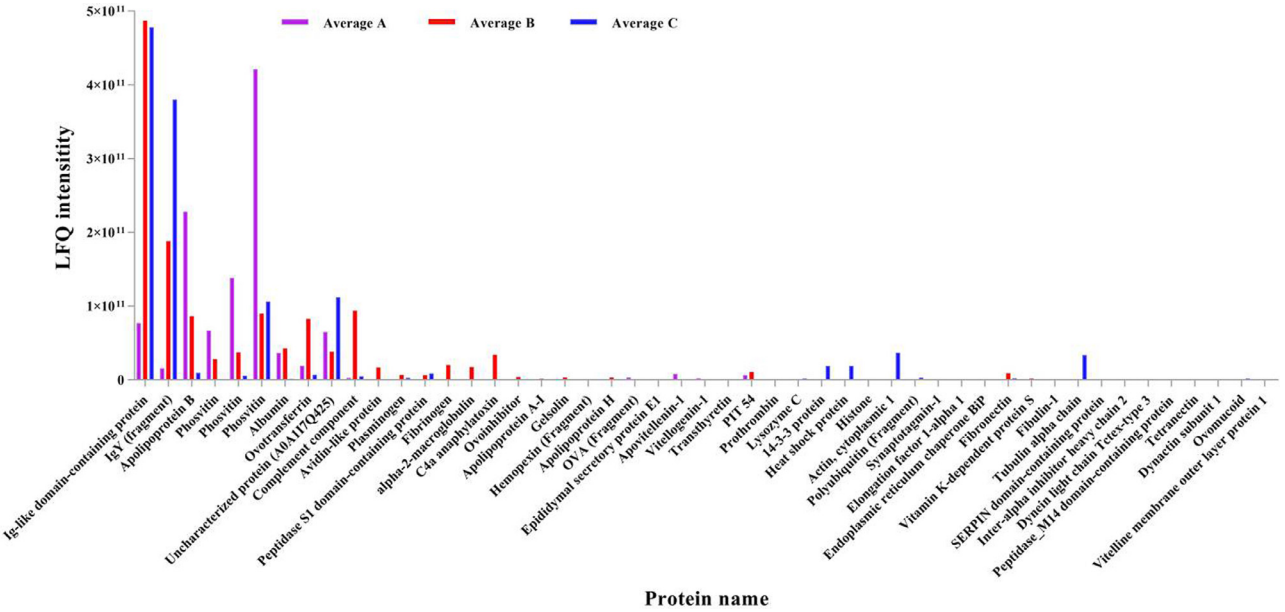

Figure 5. Changes in protein content in the original egg yolk group (A), IgY crude extract (B), and IgY purification products (C). Average (A, B, C), the average of LFQ intensity of the 3 replicate groups in groups A, B, and C, respectively.

173, and 254 proteins in egg yolk, IgY crude extract and IgY purified product, respectively). Clearly, the analysis of the protein composition of crude and purified IgY extracts, especially the emerging proteins, is important for the safety assessment and quality control of both IgY preparations. A comprehensive investigation identified 119 proteins in hen egg yolk, mainly including VTG-derived proteins (i.e., phosvitin, apovitellenin-1), proteases and protease inhibitors, vitamin- and cofactor-binding proteins, serum proteins, and some proteins from egg white (Mann and Mann, 2008). In our study, 81 proteins were identified from egg yolks, with major proteins such as albumin and yolk glycoproteins, which is in agreement with previous study. The presence of these identified proteins, which are naturally present in hen's eggs and are therefore considered nontoxic and generally safe, may also be a challenge to the safety of IgY preparations as they may still cause allergic reactions in those who consume them due to individual differences (Dona and Suphioglu, 2020). Quantitative analysis showed that 173 proteins were identified after PEG precipitation. PEG precipitates the proteins and subsequently removes a large amount of lipids from the yolk by centrifugation. As shown in Figure 7I, antibody-associated proteins (both Ig-like domain-containing protein and Ig lambda chain C region) had the highest abundance in crude IgY extract and were much higher than all types of lipoproteins. In contrast, some of the lipoproteins in IgY preparations obtained by the WD method were in higher abundant than the IgY products we needed (Nilssonet al, 2008), indicating that PEG precipitation is more favorable than aqueous extraction method for removing lipoproteins. PEG could be used at room temperature without any risk of protein denaturation and the PEG added could be removed in subsequent dialysis (De Meulenaer and Huyghebaert, 2001), which seems to contradict the large increase in protein types in the crude extracts. A plausible explanation is the detrimental effect of high protein abundance on the detection of low protein abundance. For example, the detection of biomarkers in human plasma often requires the removal of high-abundance proteins, also known as protein depletion, because a few proteins dominate the plasma proteome profile, which masks the signal of less abundant proteins (Polaskova et al., 2010). Similarly, some lipoproteins are the main components of egg yolk proteins, and these are removed in large quantities as extraction and purification proceeds, exposing many less abundant proteins. We also note the addition of certain proteins in the form of fragments in both extraction and purification products, such as fibrinogen beta chain (Q02020) and VH1 protein (A2N883). It is interesting to note that that some physical stimuli, such as sound waves and shock waves can have physical and chemical effects on the structure and aggregation of molecules, which can further affect the properties of food ingredients (Arzeni et al., 2012). Therefore, another important reason for the detection of increasing proteins could be that some proteins are broken down into multiple smaller proteins and peptides during the separation and purification process due to some physical or chemical stimulations, such as sound waves and shock waves, on the structure and aggregation of molecules.

After IgY purification, antibody-associated proteins were clearly the predominant components, followed by uncharacterized proteins (A0A1D5NWD7) and a small number of lipoproteins (e.g., Vitellogenin-2; Figure 7II). Quantitative studies showed that 254 proteins were identified in purified IgY products, significantly increased as compared to 81 proteins in the original egg yolk. Specifically, 14 proteins were upregulated and 10 proteins were downregulated in expression after purification as compared to original yolk, and 198 proteins were newly detected. Obviously, the appearance of those

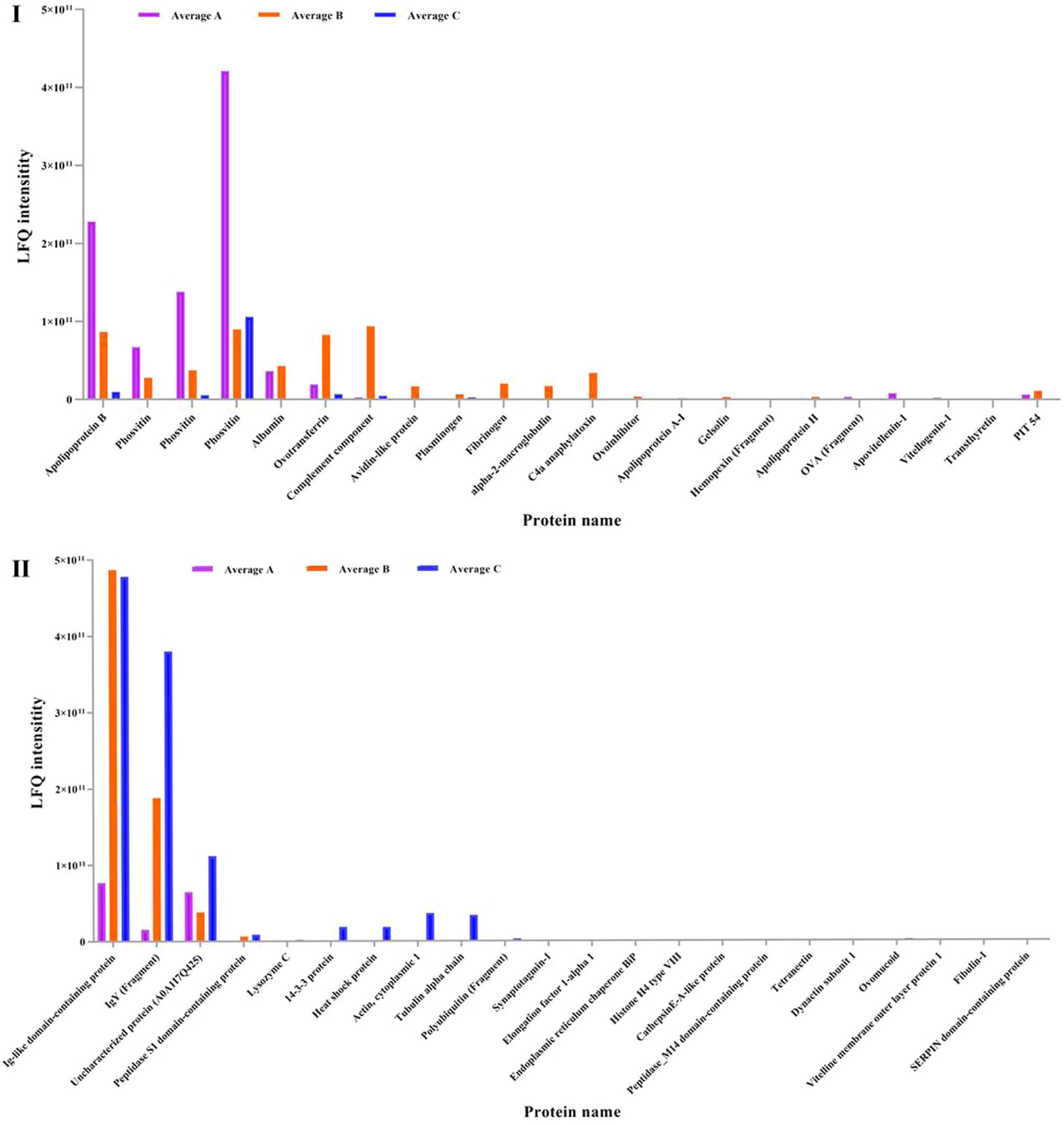

Figure 6. Profiles of proteins with decreased (I) and increased (II) expression during extraction and purification. Average (A, B, C), the average of LFQ intensity of the 3 replicate groups in groups A, B, and C, respectively.

newly detected proteins cannot be simply attributed to the depletion of high abundance proteins and the physical damage caused by the manipulation process, which led to an increase in protein types. There is no doubt that protein M is the key to complete the purification process. In this study, it was obtained by cloning the pM gene sequence from human Mycoplasma genome and expressing it in *Escherichia coli*. Protein M is a transmembrane protein of *Mycoplasma reproductiveis*, and an important finding is that homologs of this protein are present on a variety of *mycoplasma,* such as *Mycoplasma pneumonia, Mycoplasma iowae,* and *Mycoplasma fowleri* from avian species (Groveret al, 2014; Ishfaq et al., 2020), as analyzed by proteomic and mass spectrometric studies. Therefore, it is a crucial concern to determine whether the addition of protein M could introduce the exogenous proteins in the final products. We compared the purified product (group C) with extracted crude product (group B) and found that after protein M purification, as many as 152 new proteins appeared, with sodium/potassium-transporting ATPase subunit alpha-3, tubulin beta chain and ATP synthase subunit alpha being the 3 most abundant proteins (with the highest LFQ intensity values). Tubulin is a highly conserved $\alpha\beta$-dimeric protein and an essential component of microtubules. The dynamic changes of α and β subunits is associated with guanosine triphosphate hydrolysis (Binarová and Tuszynski, 2019). Na,

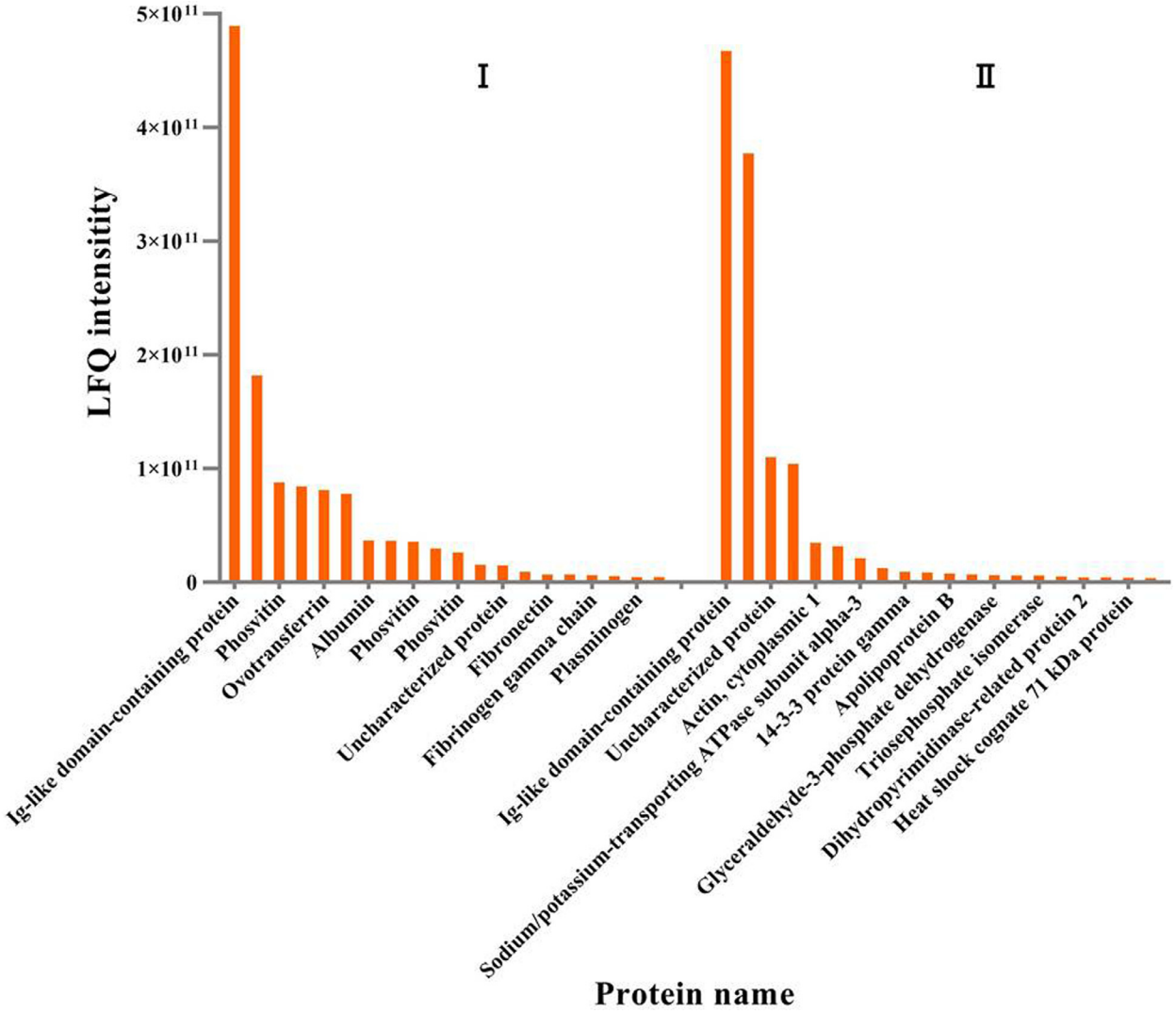

Figure 7. Protein contents in IgY crude extract (I) and purification products (II).

K-ATPase, also known as Na^+ pump, is a transmembrane protein that belongs to P-type ATPase family, and provides ATP-dependent driving force for all sodium-coupled transcellular solute movements (Kaplan, 2002). Furthermore, Na-K-ATPase can interact with cytoskeleton through spectrin, and these proteins are involved in biofilm function (Aperia, 2001), similar to protein M. It is noteworthy that, compared to group B, the expression of 14-3-3 protein was 600 times increased in group C, followed by heat shock protein HSP 90-alpha, actin, cytoplasmic 1 and polyubiquitin (fragment), with 67, 47, and 40-fold increase, respectively; in contrast, the expression of antibody-related proteins increased only up to 23 times. 14-3-3 proteins are a small family of acidic proteins that regulate various intracellular signaling pathways through adapter, scaffold, and chaperone functions (Fu et al., 2000). The expression of 14-3-3 proteins increased steeply at purification stage and this increase may be associated with the addition of protein M. In addition, a protein with accessions A0A1I7Q425 also caught our attention due to its high abundance and a significant increase in content throughout the extraction and purification processes (Figure 6II). According to UniProt database, A0A1I7Q425 is a chicken-derived, uncharacterized protein that may have GTPase activity and may be involved in protein synthesis processes (https://www.uniprot.org/uniprot/A0A1I7Q425), despite this protein is not well studied and its function remains unclear. Functional analysis showed that most differently expressed proteins have ATPase activity and purine ribonucleoside triphosphate binding activity, and are mainly involved in purine and nucleic acid metabolism. Such a situation was also noted in a recent study (Belén González Viacava et al. 2022). They combined enzymatic digestion with ion exchange chromatography to obtain high purity Fab' fragments of IgY, but mass spectrometry identification showed that proteins other than IgY were still present, such as ovotransferrin. It is worth noting that, in addition to proteins such as ovotransferrin and vitellogenin-2 which was demonstrated to cause allergic reactions (De Silva et al., 2016), other molecules were also identified as concurrent with the protein M-purified IgY in our study. And these molecules warrant thorough consideration and investigation to determine whether they may adversely affect the host.

The unique advantages of IgY antibodies has led to the establishment of several companies, mainly focusing on immunoassay and oral therapy applications (Vieira-Pires et al., 2021). However, different application scenarios impose distinct quality requirements on IgY products. Compared to oral/nasal administration, the parenteral use of IgY, especially through injection, necessitates stringent formulation design and quality control due to bypassing the host's natural defense system and barriers (Gulati and Gupta, 2011). The registration of IgY products is subject to certain limitations and challenges. These limitations can arise from regulatory requirements, scientific considerations, and practical aspects. Here are some key points to consider. Firstly, IgY may be contaminated by egg-borne pathogens during the preparation process, such as avian adenovirus (Kang et al., 2017) and leukemia virus (Qu et al., 2016), as well as the introduction of some unknown components. The registration of IgY products as therapeutic agents or diagnostic tools may require adherence to specific regulatory frameworks, such as those set by health authorities like the U.S. Food and Drug Administration (**FDA**) or the European Medicines Agency (**EMA**). These agencies typically require extensive preclinical and clinical data demonstrating safety, efficacy, and quality control measures for product approval. In addition, the availability of standardized guidelines for IgY products might be limited compared to other antibodies, such as IgG. The lack of specific guidelines tailored to IgY may pose challenges in terms of product characterization, validation, and quality control. Besides, While IgY products have been widely used as dietary supplements or alternative treatments in certain regions, their clinical data for specific indications or therapeutic applications are limited. Gathering robust clinical evidence demonstrating their efficacy, safety, and superiority over existing treatments is essential for regulatory approval and acceptance in mainstream medicine. Intellectual property concerns may also arise when registering IgY products, especially if there are existing patents covering similar antibody products or therapeutic targets. Navigating intellectual property landscape and potential conflicts can be challenging and may require licensing agreements or patent considerations. Moreover, Scaling up the production of IgY antibodies can present practical challenges compared to other antibody formats. Issues related to consistency, yield, purification, and quality control need to be addressed to ensure reproducibility, batch-to-batch consistency, and manufacturing scalability. Furthermore, the acceptance of IgY products in the medical and scientific communities can vary. Some areas may have well-established practices and acceptance of IgY-based therapies or diagnostics, whereas in others, it may be less recognized or require additional evidence and education to gain acceptance. To overcome these limitations, collaborative efforts between researchers, industry stakeholders, and regulatory authorities are crucial. Establishing robust scientific evidence, adhering to regulatory requirements, and addressing manufacturing challenges are key steps toward successful registration and wider acceptance of IgY products. We believe our study is an important proof-of-concept, which raises the consideration for IgY practitioners in establishing the standards for quality control and product development and registrations, which has not received attention before.

CONCLUSIONS

A total of 348 proteins were identified using label-free LC-MS/MS proteomics analysis, with 41 shared proteins, 36 fewer and 209 more proteins in IgY purified product as compared to fresh yolk. Using PEG precipitation method, a large amount of lipoprotein was removed from egg yolk and an antibody-lipoprotein complex with IgY antibody as the main body and a small amount of lipoprotein was obtained. After protein M-based purification, high purity IgY was obtained with increased expression of some nonantibody proteins, such as 14-3-3 protein and uncharacterized protein (A0A1I7Q425), which poses new challenges for the assessment of IgY preparations. This study is a necessary reference for the considerations of proteins identification, safety assessment, and process repeatability in further standardization, commercialization, and approval of IgY products.

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Notes: No animal or human subject was used in this study and only fresh eggs were collected directly from local farmer market in Hanzhong, China.

Data Availability: The mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the iProX partner repository with the dataset identifier PXD032865. All other data supporting this study are available from the corresponding author on reasonable request.

Author Contributions: XZ developed the research question. RW conducted the laboratory experiments and analyzed the data. XZ and RW wrote the first draft of the manuscript. XZ, RW and BC critically supervised the manuscript before submission. All authors contributed to the article and approved the submitted version.

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.102843.

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