Proteomic analysis of fertilized egg yolk proteins during embryonic development

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ABSTRACT Egg yolk is an important source of nutrients for embryo development. In this study, the egg yolk protein composition at 0, 10, and 18 D of incubation was analyzed by 2-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry. A significant difference in the abundance of 42 protein spots representing 12 proteins were identified ($P < 0.05$). The 2-DE gel image analysis exhibited that the molecular weight (MW) of 29 protein spots was lower than their theoretical value, in which 14 vitellogenin (VTG) fragments were lower than the theoretical value. There were 13 protein spots showed a higher MW including 5 ovotransferrins with MW of 87.2 kDa. The gene ontology enrichment analysis suggested that biological process of the differentially expressed proteins were mainly involved in lipid transport and lipid localization at 10 and 18 D of incubation. The molecular function of the differentially expressed proteins was involved in nutrient reservoir activity, lipid transporter activity, and antigen binding at 10 D of incubation. At 18 D of incubation, the differentially expressed proteins mainly participated in nutrient reservoir activity and substrate-specific transporter activity. The high abundance of VTGs at 10 D of incubation might participate in lipid localization and lipid transportation to facilitate the yolk nutrient transport to embryo. The low expression of ovotransferrins at 10 D of incubation indicated the chondrogenesis of embryo.

Key words: egg yolk protein, embryonic development, proteomic, vitellogenin, ovotransferrin

INTRODUCTION

Egg yolk accounts for about 30% of egg weight and is rich of various fatty acids, vitamins, mineral elements, and proteins, which is an important source of nutrients for embryonic development. The yolk protein requirement of chicken embryo varies with the developmental stage of hatching. Currently, there are some articles exploring the composition of egg yolk proteins. There were 119 proteins identified from chicken egg yolk using 1-D SDS-PAGE, LC-MS/MS, and MS. Among them, serum albumin, the vitellogenin (**VTG**) cleavage products, apovitellenins, Immunoglobulin Y (IgY), and ovalbumin have the highest abundance in egg yolk plasma (Mann and Mann, 2008). Using 3 combinatorial peptide ligand libraries, 255 unique proteins were identified in egg yolk plasma (Farinazzo et al., 2009). Using 2-dimensional gel electrophoresis (**2-DE**) combined with matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (**MALDI-TOF MS/MS**) to study the changes of egg yolk protein during egg storage, Gao et al. (2017) identified 12 protein spots showed significant abundant changes. D'Ambrosio et al. (2008) using 2-DE combined with a protein enrichment (peptide ligand libraries) technology found 148 proteins in egg white. The above studies provide a theoretical basis for further exploration of proteomics for egg yolk. However, still no research on compositional changes of yolk protein during chicken embryonic development.

Chicken embryonic development can be divided into 3 stages: the first stage of embryogenesis (0–7 embryonic D [**ED**]), the second for embryo development (8–14 ED), and the third (15–21 ED) of rapid absorption of nutrients for hatching (Moran, 2007). The first 10 D of chicken embryo development is crucial for early embryogenesis, corresponding to the establishment of the embryonic germ and the formation of chorionic sac and

allantoic cavity. The physicochemical properties of egg yolk changed during incubation, and the yolk protein component plays a key role in embryo development. The third stage includes a transfer of remaining egg white into the amniotic sac and a rapid oral absorption of the resulting mixture of nutrients by the embryo to expand body glycogen reserve (Moran, 2007). Réhault-Godbert et al. (2014) analyzed the difference in protein content of water-soluble fraction between 12 embryonic days chicken egg yolk and the unfertilized egg yolk, in which the alpha-fetoprotein and 2 unidentified proteins (F1NHB8 and F1NMM2) were identified for the first time by LC-MS/MS. Although the protein in the water-soluble component of egg yolk accounts for more than 80% of the total egg yolk protein, the proteomic characteristics of the egg yolk in fertilized egg during incubation are still not fully explained. In addition, considering the characteristic of differentially expressed proteins in egg yolk during incubation, 2-DE combined with MALDI-TOF MS/MS was used to analyze the proteomic differences of total protein in chicken yolk at 0, 10, and 18 ED of incubation. The results will provide new insights for understanding the regulatory mechanism of egg yolk protein for chicken embryo development.

MATERIALS AND METHODS

Egg Samples

A total of 92 fertilized eggs (50 ± 1 g of eggs) produced from Huainan Partridge chicken (40 wk, Anhui, China) within 3 connective days were collected. Eggs were incubated under normal conditions (2 h per turning at $37.8 ± 0.1°C$ and $58 ± 3\%$ relative humidity with automatic ventilation). At 0, 10, and 18 ED, 9 eggs were randomly selected for yolk separation. After eggs were opened, embryos were carefully peeled off for yolk collection.

Protein Extraction from Egg Yolk

The extraction of protein from egg yolk was carried out according to the instructions of Tissue or Cell Total Protein Extraction kit (C510003, Sangon Biotech, Shanghai, China). Ice-cold lysis buffer 1 mL containing 5 μL of phosphatase inhibitor, 1 μL of protease inhibitor, and 10 μL of phenylmethanesulfonyl fluoride was added to 0.2 g of egg yolk and then homogenized at 4°C for 3 min. The homogenized sample was centrifuged at 4°C, $12,000 × g$ for 10 min. After centrifugation, the supernatant was considered as the egg yolk whole protein extract. The desalination of protein extract was performed basically as described by Wang and Wu, (2014). The extracted protein of 100 μL was added to 400 μL of ice-cold acetone and mix well. After overnight incubation at $-20°C$, the mixture was centrifuged ($12,000 × g$, 10 min, 4°C), and the supernatant was discarded. The pellet was subsequently air dried for 15 min in a draft cupboard (FGG1500, Kebei, Wuhan, China)

and then resolubilized by rehydration loading buffer I containing 8 mol urea, 4% CHAPS, 65 mmol dl-dithiothreitol, 0.2% (w/v) Bio-Lyte, and 0.001% bromophenol blue. The sample was homogenized for 10 min to fully dissolve and then centrifuged at $10,000 × g$, 10 min, 4°C. The supernatant was subsequently transferred into a 1.5 mL centrifuge tube and stored at $-20°C$. Protein samples were quantified by Bradford Protein Quantification Kit (20202ES76, Yeasen, Shanghai, China).

Three extracted protein samples from each embryonic day were equally mixed to form 1 biological replicates. Three biological replicates were performed during the following experiments for 2-DE analysis to reduce variations.

Two-Dimensional Electrophoresis

The 2-DE experiments were performed using the PROTEAN i12 IEF cell (Bio-Rad, Hercules, CA) for the first dimension isoelectric focusing (IEF) and the Mini-PROTEANs tetra cell (Bio-Rad) to perform SDS-PAGE in the second dimension as described by Omana et al. (2011). The IPG strips (7 cm, pH 3-10) were position rehydrated for 14 h at 18°C in PROTEAN i12 IEF cell with 125 μL of the protein sample (437.5 μg of protein) in rehydration loading buffer I. The conditions used for IEF were as follows: step 1, 100 V for 0.5 h; step 2, 250 V for 0.5 h; step 3, 500 V for 1 h; step 4, 1,000 V for 1 h; step 5, 2,000 V for 1 h; step 6, 3,000 V for 1 h; step 7, 4,000 V for 2 h; step 8, 4,000 V for 20,000 Vh; for a total of 35,000 Vh. Each focused IPG strip was equilibrated in 2.5 mL of equilibration buffer-I (6 mol urea, 2%SDS, 0.375 mol Tris-HCl, pH 8.8, 20% glycerol and 0.1 mol dl-dithiothreitol) for 15 min each and then incubated with 2.5 mL of equilibration buffer-II (6 mol urea, 2% SDS, 0.375 mol Tris-HCl, pH 8.8, 20% glycerol and 250 mmol iodoacetamide) for 15 min each. Second-dimensional electrophoresis was performed on 10% (w/v) SDS polyacrylamide gel using a Mini-PROTEANs tetra cell (Bio-Rad). A power of 60 V per gel was applied for 30 min, and a power of 120 V per gel was then applied for about 1.3 h until the bromophenol blue dye front reached the bottom of the gel. Proteins were then visualized by Coomassie Brilliant Blue G-250 staining.

Gel Analysis and Differentially Expressed Protein Spots Identification

All gels were scanned and calibrated using a GS-900 calibrated densitometer (Bio-Rad), and then, protein spots were detected by PDQuest software version 8.0.1 (Bio-Rad). The differentially expressed protein spots were selected and excised from the gel and transferred into a 1.5 mL centrifuge tube, subsequently. The protein-containing gel was washed with ddH$_2$O and then decolorized by adding 50 μL of decolorizing solution. After washing the gel with ddH$_2$O again, the protein-containing gel was washed with 100 μL of 25 mmol NH$_4$HCO$_3$ 50% acetonitrile and 100%

acetonitrile until the gel was whitened. A total of 5 µL of 0.01 µg/µL sequencing-grade trypsin (Promega, Madison, WI) was added to each tube to make the gel absorbed thoroughly on ice box until the gel became transparent. Then, 10 µL of 50 mmol NH_4HCO_3 solution containing 10% acetonitrile was added, and the mixture was digested overnight in a 37°C thermostat water bath. After digestion, the supernatant was lyophilized and then re-dissolved in 0.1% trifluoroacetic acid. The dissolved samples were subjected for analysis by 5800 MALDI-TOF-TOF Analyzer (ABSCIEX, Redwood City, CA). Peptide masses were searched against the nonredundant sequence database (NCBInr) through the Mascot program (http://www.matrixscience.com). Search parameters were set with tolerance to 150 ppm peptide mass variance, for carbamidomethyl (C) as fixed modification and oxidation (M) as variable modification (Qiu et al., 2012). With the Mascot search results, protein scores, when greater than 59, were considered significantly believable ($P < 0.05$).

### Bioinformatic Analysis and Statistical Analysis

The multiomics data analysis tool, OmicsBean, was used to analyze the obtained proteomics data (http://www.omicsbean.com:88/), in which distributions in biological function, cellular component, and molecular function were assigned to each protein based on gene ontology (**GO**). The protein expression level of the 2-DE data were subjected for one-way ANOVA by SPSS 20.0 (SPSS, Chicago, IL). For GO analysis, the protein spots at 0 ED was set as control when compared with 10 ED which was also set as control when compared with 18 ED. Student t test was used for comparing the difference between 2 groups. The P-value less than 0.05 was considered as significant difference.

RESULTS

Differential Expression of Protein Abundance During Different Incubation Periods

The 2-DE combined with MALDI-TOF MS/MS was used to compare and analyze the egg yolk protein of chicken embryos at different incubation periods (0, 10, and 18 ED). There were 42 protein spots showed differential expression among different ED ($P < 0.05$, Figure 1). Compared with 0 ED, there were 29 protein spots differentially expressed at 10 ED, in which 16 spots (7, 8, 9, 10, 12, 17, 24, 26, 27, 28, 29, 30, 31, 33, 34, and 35) showed increased abundance, and 13 spots (1, 4, 6, 11, 14, 15, 18, 22, 23, 25, 36, 37, and 40) showed decreased abundance. Compared with 10 ED, there were 37 protein spots differentially expressed at 18 ED, in which 13 spots (13, 15, 16, 19, 31, 32, 33, 36, 37, 38, 39, 40, and 41) showed increased abundance, and 24 spots (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18, 20, 21, 23,
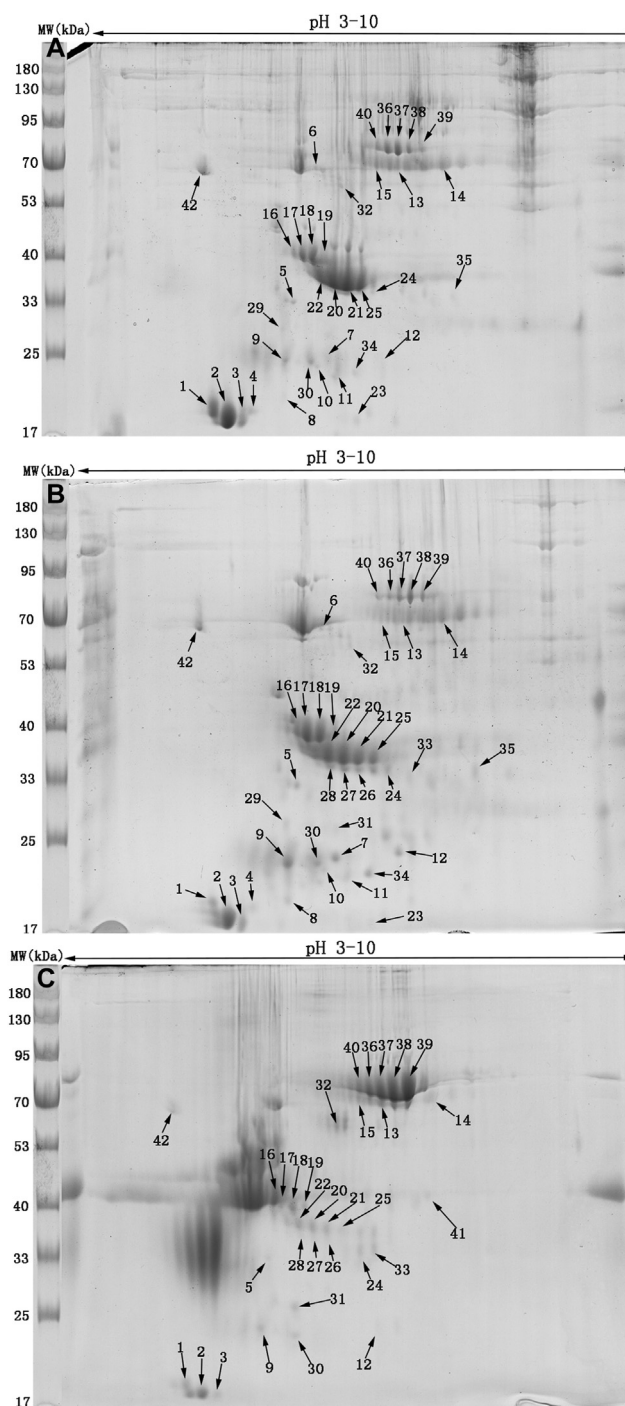
Figure 1. Representative 2-DE gel images of proteins from chicken egg yolk prepared by IEF/SDS-PAGE separation followed by staining with Coomassie Brilliant Blue G-250: (A) egg yolk proteins after 0 D of incubation; (B) egg yolk proteins after 10 D of incubation; (C) egg yolk proteins after 18 D of incubation. Spots that significantly changed in abundance during incubation are indicated by numbers and arrows. The molecular weights (MW) and pI scales are indicated. Each gel is representative of 3 independent replicates. 2-DE, 2-dimensional gel electrophoresis; IEF, isoelectric focusing.

25, 26, 27, 28, 29, 30, 34, and 35) decreased in abundance.

The molecular weight (MW) of the 14 protein spots representing the VTG fragment (including 4 VTG-I precursors, 9 VTG-II, and 1 VTG-III) was extremely lower than its theoretical molecular value. Especially, the

Table 1. List of significantly altered fertilized chicken egg yolk proteins during incubation.

Spot[1]	Accession[2]	Protein name	Score[3]	% Sequence coverage	MW (kDa/pI) Thero.	MW (kDa/pI) Obs.
1	268370086	Vitelline membrane outer layer protein 1 [Gallus gallus]	496	33	21.5/5.21	19.8/5.29
2	268370086	Vitelline membrane outer layer protein 1 [Gallus gallus]	501	33	21.5/5.21	18.3/5.47
3	268370086	Vitelline membrane outer layer protein 1 [Gallus gallus]	445	33	21.5/5.21	17.7/5.64
4	766944282	Serum albumin precursor [Gallus gallus]	250	6	71.9/5.51	19.1/5.76
5	766944282	Serum albumin precursor [Gallus gallus]	62	2	71.9/5.51	32.5/6.31
6	766944282	Serum albumin precursor [Gallus gallus]	183	6	71.9/5.51	78.7/6.58
7	766944282	Serum albumin precursor [Gallus gallus]	176	4	71.9/5.51	23.6/6.77
8	266634462	Immunoglobulin lambda light chain precursor [Gallus gallus]	65	11	23.2/5.66	19.8/6.21
9	266634462	Immunoglobulin lambda light chain precursor [Gallus gallus]	169	11	23.2/5.66	23.4/6.21
10	266634462	Immunoglobulin lambda light chain precursor [Gallus gallus]	130	11	23.2/5.66	22.5/6.63
11	266634462	Immunoglobulin lambda light chain precursor [Gallus gallus]	107	11	23.2/5.66	21.8/6.92
12	266634462	Immunoglobulin lambda light chain precursor [Gallus gallus]	267	24	23.2/5.66	23.9/7.59
13	614458442	Immunoglobulin Y heavy chain constant region [Gallus gallus]	376	18	43.5/6.11	78.6/7.43
14	614458442	Immunoglobulin Y heavy chain constant region [Gallus gallus]	376	18	43.5/6.11	78.6/8.10
15	614458442	Immunoglobulin Y heavy chain constant region [Gallus gallus]	270	14	43.5/6.11	78.6/7.23
16	52138705	Vitellogenin-I precursor [Gallus gallus]	459	4	212.6/9.16	39.7/6.31
17	52138705	Vitellogenin-I precursor [Gallus gallus]	601	4	212.6/9.16	39.7/6.44
18	52138705	Vitellogenin-I precursor [Gallus gallus]	618	4	212.6/9.16	39.7/6.60
19	52138705	Vitellogenin-I precursor [Gallus gallus]	127	1	212.6/9.16	39.3/6.77
20	212881	Vitellogenin-II [Gallus gallus]	571	4	206.9/9.22	36.1/6.89
21	212881	Vitellogenin-II [Gallus gallus]	511	3	206.9/9.22	36.1/7.06
22	212881	Vitellogenin-II [Gallus gallus]	667	5	206.9/9.22	36.1/6.69
23	212881	Vitellogenin-II [Gallus gallus]	159	2	206.9/9.22	17.9/7.23
24	212881	Vitellogenin-II [Gallus gallus]	293	2	206.9/9.22	34.7/7.43
25	212881	Vitellogenin-II [Gallus gallus]	579	4	206.9/9.22	36.1/7.26
26	212881	Vitellogenin-II [Gallus gallus]	356	3	206.9/9.22	34.7/7.06
27	212881	Vitellogenin-II [Gallus gallus]	361	3	206.9/9.22	34.7/6.89
28	212881	Vitellogenin-II [Gallus gallus]	264	1	206.9/9.22	34.7/6.69
29	971408444	Vitellogenin-III [Gallus gallus]	200	1	193.3/8.93	27.4/6.21
30	1708509	Ovoinhibitor precursor [Gallus gallus]	130	7	54.4/6.16	23.6/6.52
31	1708509	Ovoinhibitor precursor [Gallus gallus]	94	4	54.4/6.16	27.2/6.63
32	1708509	Ovoinhibitor precursor [Gallus gallus]	217	10	54.4/6.16	65.1/6.97
33	1708509	Ovoinhibitor precursor [Gallus gallus]	366	13	54.4/6.16	34.9/7.71
34	113206052	Apolipoprotein B precursor [Gallus gallus]	465	2	52.5/8.51	22.1/7.21
35	113206052	Apolipoprotein B precursor [Gallus gallus]	142	1	52.5/8.51	34.2/8.52
36	71274075	Ovotransferrin BB type [Gallus gallus]	962	22	79.6/6.85	87.2/7.43
37	71274075	Ovotransferrin BB type [Gallus gallus]	1,148	23	79.6/6.85	87.2/7.59
38	71274075	Ovotransferrin BB type [Gallus gallus]	1,222	23	79.6/6.85	87.2/7.71
39	71274075	Ovotransferrin BB type [Gallus gallus]	484	14	79.6/6.85	87.2/7.81
40	71274075	Ovotransferrin BB type [Gallus gallus]	857	20	79.6/6.85	87.2/7.30
41	129293	Ovalbumin [Gallus gallus]	682	27	43.2/5.19	39.3/7.97
42	46395491	PIT54 protein precusor [Gallus gallus]	569	25	52.7/4.61	71.9/5.04

[1]Spot ID represents the protein spot number on the 2-DE gel image.
[2]Accession numbers of matched proteins according to the NCBInr database.
[3]MASCOT score. The Mascot threshold score for all of these identified proteins is 59.

VTG-III (spot 29) decreased from the theoretical value of 193.3 kDa to 27.4 kDa (Table 1, Figure 1). Compared with 10 ED, the abundances of 10 protein spots (17, 18, 20, 21, 23, 25, 26, 27, 28, and 29) were significantly decreased, whereas the abundance of spots 16 and 19 was significantly increased at 18 ED (Figure 2 and Figure 3); The MW of 3 spots representing the IgY heavy chain constant was 78.6 kDa, higher than its theoretical value of 43.5 kDa (Table 1, Figure 1). The abundances of 3 protein spots (11, 14, and 15) decreased significantly after 10 ED. Compared with 10 ED, there were 5 protein spots (8, 9, 10, 11, and 12) with a significant decrease in abundance at 18 ED, especially 3 protein spots (8, 10, and 11) were not detected at 18 ED (Figure 2).

The MW of proteins (spots 1-3 representing vitelline membrane outer layer protein 1 and spots 4, 5, and 7 representing serum albumin precursor) were lower than their theoretical value (Table 1, Figure 1). The abundance of spots 1, 4, and 6 were decreased in 10 ED and spots 2 to 7 decreased in abundance at 18 ED compared with 10 ED (Figure 2); The MW of the 3 ovoinhibitor
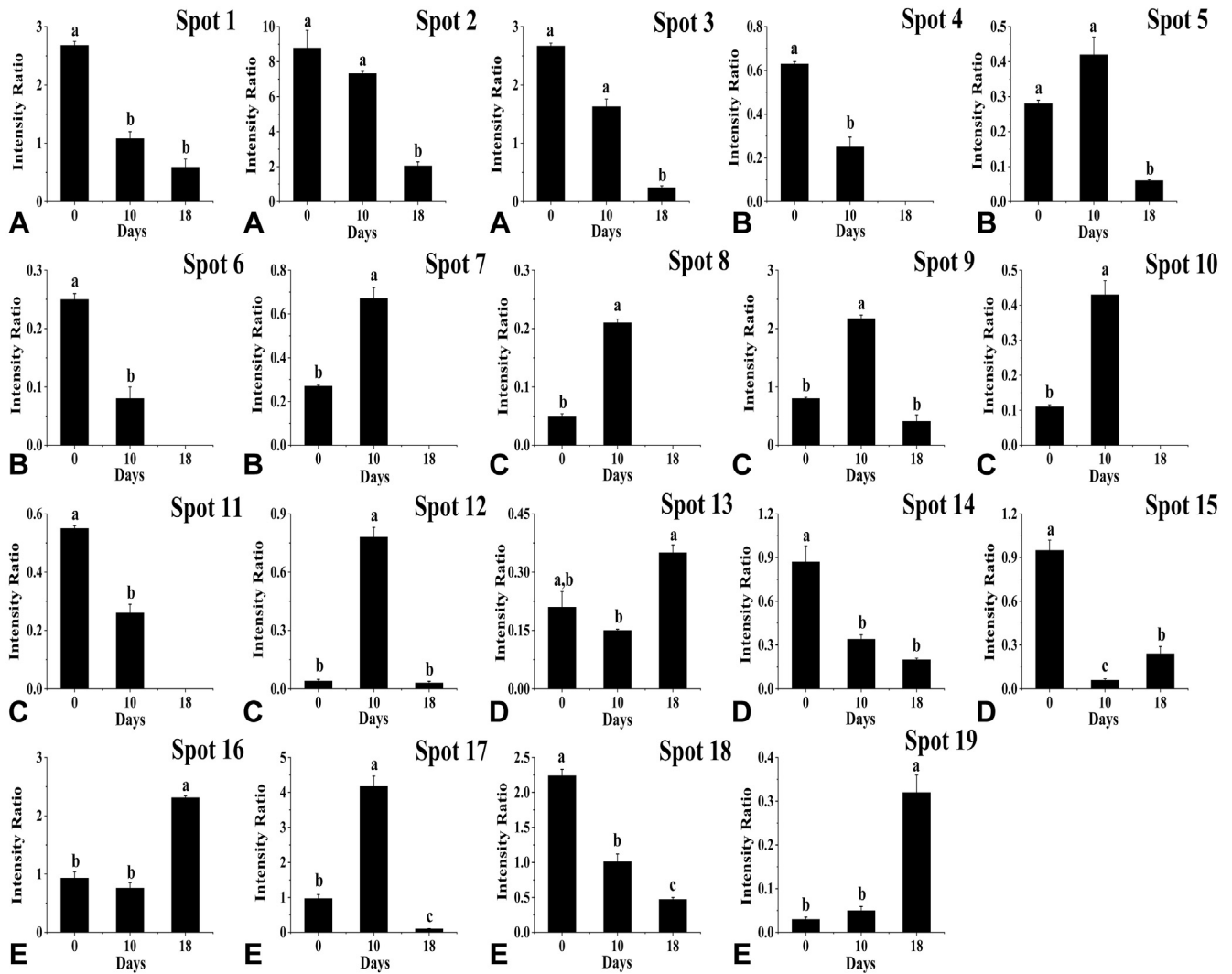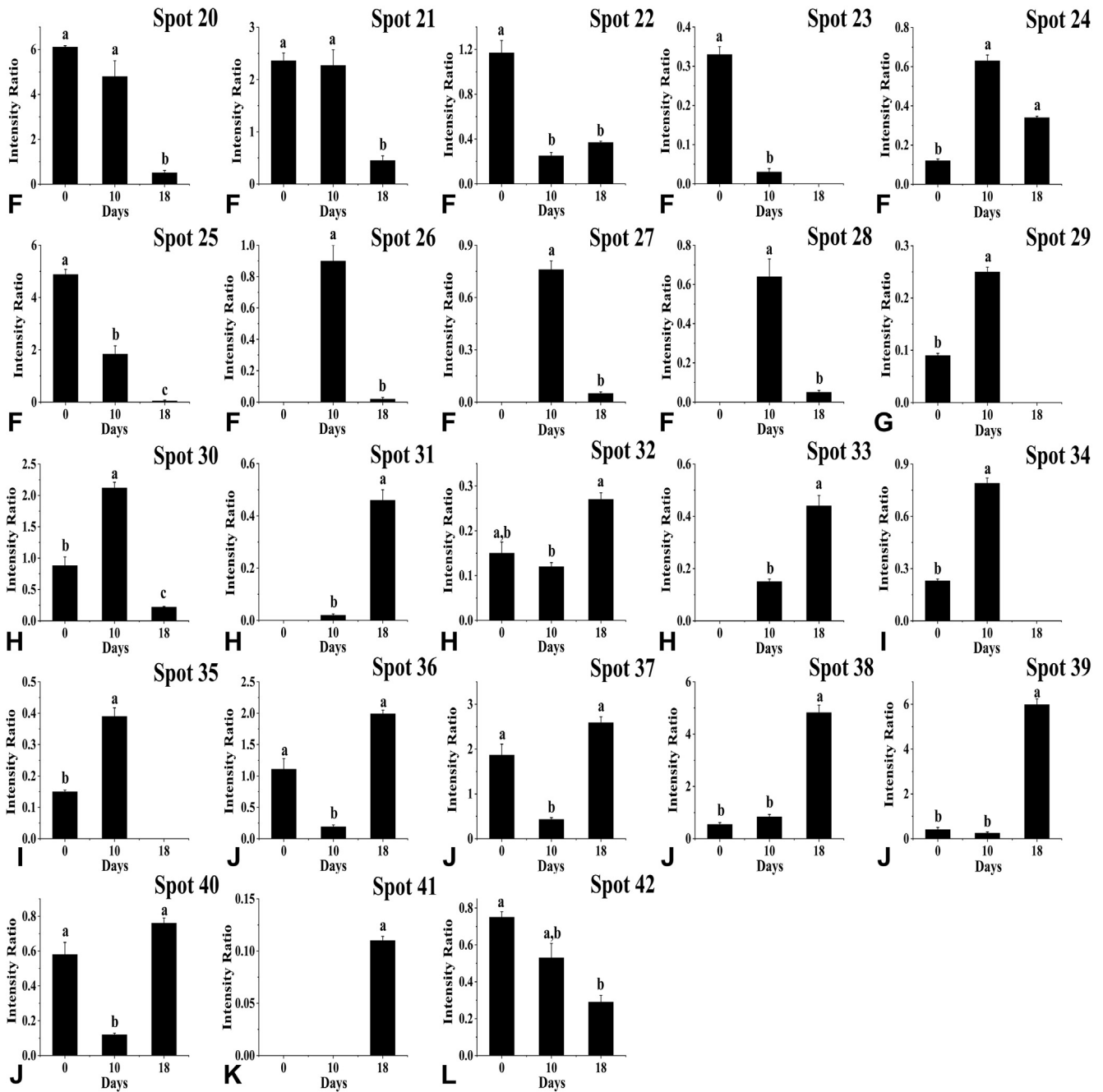
Figure 2. Changes in the intensity ratio of egg yolk proteins after 0, 10 and 18 D of incubation. (A) vitelline membrane outer layer protein 1; (B) serum albumin precursor; (C) immunoglobulin lambda light chain precursor; (D) immunoglobulin Y heavy chain constant; (E) vitellogenin-I precursor. Column profiles of these proteins are shown as bar charts. Only spots that showed significant ($P < 0.05$) difference in intensity were given. The data represent the mean intensity values ($\pm$SD) from 3 replicates at each incubation period. The bars with no common letter (a–c) indicate significant difference ($P < 0.05$).

precursor spots (30, 31, and 33) was lower than their theoretical value of 54.4 kDa (Table 1). The abundances of spots (30, 31, and 33) increased significantly at 10 ED, especially spots 31 and 33 were not detected at 0 ED. Compared with 10 ED, the abundance of ovoinhibitor spots were significantly increased at 18 ED (except for spot 30, Figure 3); The MW of the 5 ovotransferrin BB type spots (36, 37, 38, 39, and 40) was 87.2 kDa, which is higher than the theoretical value of 79.6 kDa (Table 1). The abundance of the 3 spots (36, 37, and 40) was significantly decreased at 10 ED. Compared with 10 ED, the 5 ovotransferrin spots increased significantly at 18 ED (Figure 3); The abundance of apolipoprotein B (**ApoB**) precursor spots (34 and 35) were increased at 10 ED and were not detected at 18 ED. The MW of ApoB precursor was lower than its theoretical value (Table 1, Figure 3). The abundance of PIT54 precursor (spot 42) was significantly decreased at 18 ED

and showed a higher MW than its theoretical value (Table 1, Figure 3); Ovalbumin was only detected at 18 ED with a lower MW (Table 1, Figure 1).

GO Enrichment for Differentially Expressed Proteins

The identified differentially expressed proteins were classified into 12 categories. The GO enrichment analysis was performed on biological processes, cellular components, and molecular functions. As compared with 0 ED, there were 86 annotations in biological process, 11 annotations in cellular components, and 20 annotations in molecular function enriched in 10 ED ($P < 0.05$). As compared with 10 ED, there were 116 annotations in biological process, 20 annotations in cellular components, and 25 annotations in molecular function enriched in 18 ED ($P < 0.05$).

Figure 3. Changes in the intensity ratio of egg yolk proteins after 0, 10 and 18 D of incubation (continue with figure 2). (F) vitellogenin-II; (G) vitellogenin-III; (H) ovoinhibitor precursor; (I) apolipoprotein B precursor; (J) ovotransferrin BB type; (K) ovalbumin; (L) PIT54 protein precursor. Column profiles of these proteins are shown as bar charts. Only spots that showed significant ($P < 0.05$) difference in intensity were given. The data represent the mean intensity values ($\pm$SD) from 3 replicates at each incubation period. The bars with no common letter (a-c) indicate significant difference ($P < 0.05$).

GO Annotation of Differentially Expressed Proteins in Biological Process

At 10 ED, 9 differentially expressed proteins were involved in lipid transport (GO: 0006869), lipid localization (GO: 0010876), humoral immune response mediated by circulating Ig (GO: 0002455), complement activation (GO: 0006956), protein activation cascade (GO: 0072376), Ig-mediated immune response (GO: 0016064), and B cell–mediated immunity (GO: 0019724) in

biological process (Figure 4, Supplementary Table 1). Among the 9 terms, VTG-I precursor, VTG-II, VTG-III, and ApoB precursor were involved in lipid transport and lipid localization. Ig lambda light chain and IgY heavy chain proteins were significantly enriched in immune-related biological processes (Supplementary Table 1). At 18 ED, 10 differentially expressed proteins were involved in lipid transport (GO: 0006869), response to mineralocorticoid (GO:0051385), response to corticosterone (GO:0051412), lipid localization (GO: 0010876),
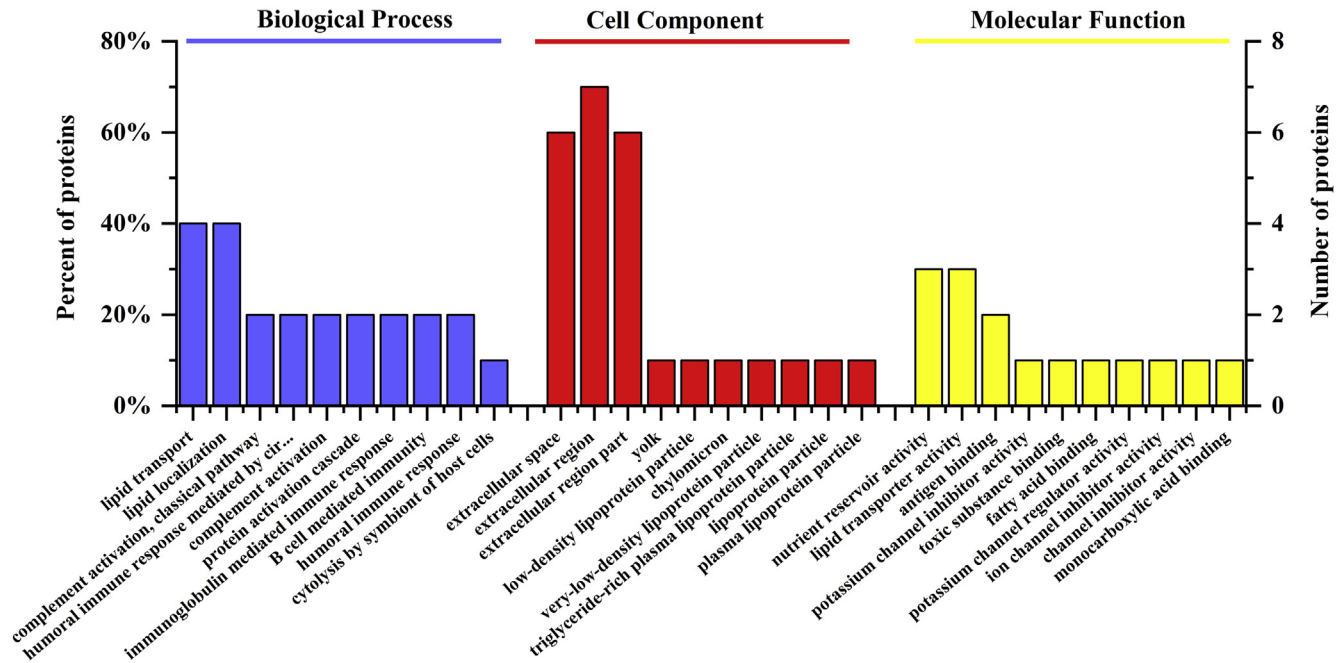
Figure 4. GO annotation of differentially expressed proteins in 3 categories: biological process, cellular component, and molecular function after 10 D of incubation compared with the control (0 D). Only the top 10 annotations for *P*-values are listed. GO, gene ontology.

response to glucocorticoid (GO: 0051384), response to corticosteroid (GO: 0031960), response to ketone (GO:1901654), amino acid homeostasis (GO:0080144), triglyceride mobilization (GO:0006642), and transport (GO:0006810, Figure 5, Supplementary Table 2). Ovoinhibitor precursor and ovalbumin were mainly involved in various hormonal responses, whereas VTG-I precursor, VTG-II, VTG-III, and ApoB precursor were involved in lipid transport and lipid localization (Supplementary Table 2).

GO Annotation of Differentially Expressed Proteins in Cellular Component

At 10 and 18 ED, 7 and 8 differentially expressed proteins, respectively, were mainly involved in extracellular space (GO: 0005615), extracellular region (GO: 0005576), and extracellular region part (GO: 0044421) among the cell components (Figure 4, Figure 5, Supplementary Table 3 and Supplementary Table 4).
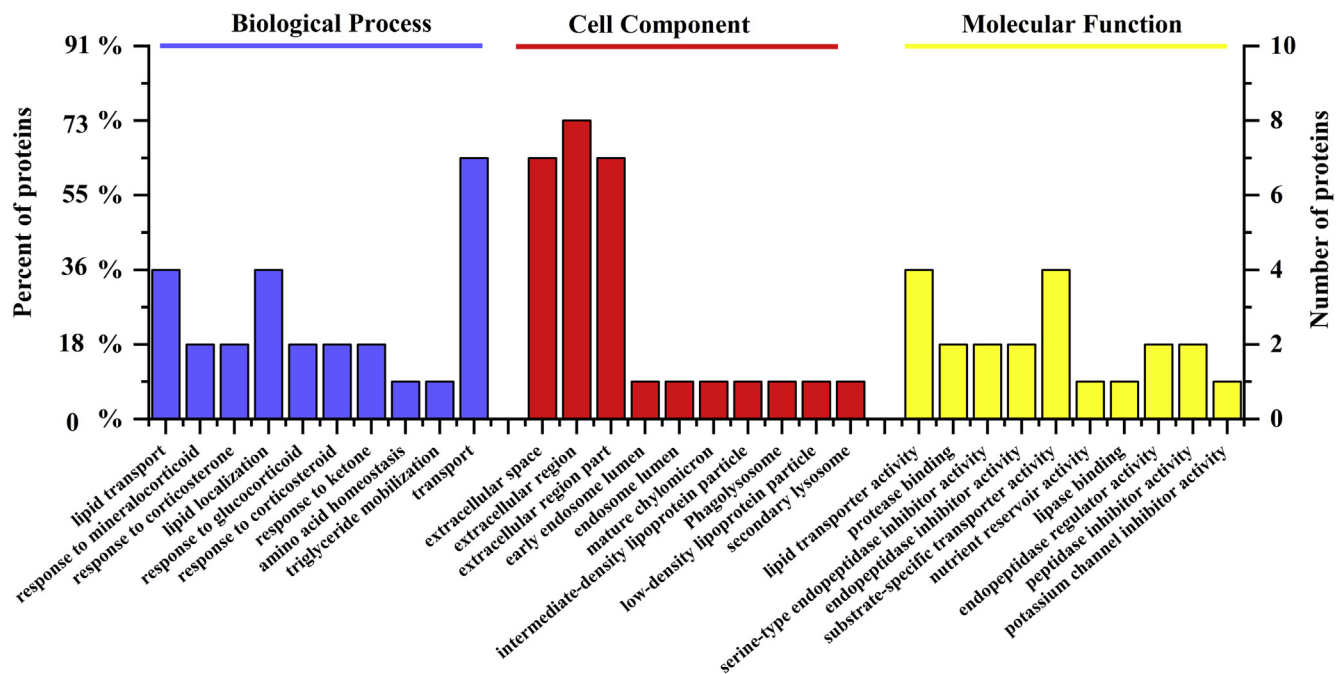

Figure 5. GO annotation of differentially expressed proteins in 3 categories: biological process, cellular component, and molecular function after 18 D of incubation compared with the control (10 D). Only the top 10 annotations for *P*-values are listed. GO, gene ontology.

GO Annotation of Differentially Expressed Proteins in Molecular Function

At 10 ED, VTG-I precursor, VTG-II, and VTG-III were involved in nutrient reservoir activity (GO:0045735) and lipid transporter activity (GO:0005319). Ig lambda light chain and IgY heavy chain proteins were mainly for antigen binding (GO:0003823) function. Serum albumin precursor was characterized as toxic substance binding (GO: 0015643), fatty acid binding (GO: 0005504), and monocarboxylic acid binding (GO: 0033293). Ovoinhibitor precursor was expressed as potassium channel inhibitor activity (GO: 0019870), potassium channel regulator activity (GO: 0015459), ion channel inhibitor activity (GO: 0008200), and channel inhibitor activity (GO: 0016248, Figure 4, Supplementary Table 5). At 18 ED, ovoinhibitor precursor and ovalbumin exhibited protease activity (GO: 0002020), serine-type endopeptidase inhibitor activity (GO: 0004867), endopeptidase inhibitor activity (GO: 0004866), endopeptidase regulator activity (GO: 0061135), and peptidase inhibitor activity (GO: 0030414). VTG-I precursor, VTG-II, VTG-III, and ApoB precursor were involved in lipid transporter activity (GO: 0005319) and substrate-specific transporter activity (GO:0022892, Figure 5, Supplementary Table 6).

DISCUSSION

The incubation period of chicken embryos is about 21 D, and the 10th D is in the middle of the hatching period. During this period, the chicken embryo is under vigorously metabolism, and the egg yolk is relatively thin with high content of water (Peebles et al., 1999). After 18 ED, the yolk sac is condensed, and the content became viscous which is ready for absorption into the abdominal cavity. Therefore, egg yolk hatched for 0, 10 and 18 D were used in this experiment.

Differentially Expressed Proteins for Embryo Nutrient Providing

Vitellogenin is one of egg yolk protein precursor with 3 forms of VTG I, VTG II, and VTG III, which was synthesized by liver in response to estrogenic stimulation. Vitellogenin is mainly responsible for lipids, phosphorus, and metals transport into egg yolk or as nutrient to maintain embryonic development (Wang et al., 1983). The lower MW of 14 VTGs detected in this study might because of its degradation by endogenous aspartic protease and matrix metalloproteinase in egg yolk (Réhault-Godbert et al., 2014). The degradation products of VTG could provide important nutrients for chicken embryo development. Wang and Wu, (2014) first detected VTG in the egg white and the abundance of these protein spots showed a significant increase during incubation. However, the abundance of VTGs decreased significantly at 18 D of incubation in this study. At 18 ED, the chicken embryo was at the end of hatching, and all tissues and organs have been fully developed, and the lung instead of allantoic respiration promotes body with increased metabolism and thus require more nutrient from VTGs.

Serum albumin is partially heat-labile allergen that cause respiratory and food allergy (Quirce et al., 2001). There are few reports on the biological functions of serum albumin in egg yolk. Wang and Wu, (2014) identified the abundance of 2 serum albumins in egg white decreased during incubation. Réhault-Godbert et al. (2014) suggested that serum albumin could provide nutrients for the developing embryo. The results of the decreased abundance or even not identified at 18 ED of serum albumin precursors in this study might be because of its decomposition for embryo utilization, which was in accordance with the above mentioned studies.

Apolipoprotein B is one component of very low density lipoprotein (**VLDL**) particles which are produced by the liver in response to estrogen in birds (Kirchgessner et al., 1987). In accordance with Réhault-Godbert et al. (2014), the significantly increased abundance of ApoB after 10 D of incubation suggested the degradation of LDL as lipid supplementation during embryonic development. Yolk lipids were completely absorbed into yolk sac and then hydrolyzed and re-esterified to form VLDL particles for embryo development (Powell et al., 2004). Therefore, the abundance of ApoB precursors was significantly decreased at 18 ED. The 2 ApoB precursors not detected at 18 D of hatching might be associated with the formation of VLDL particles.

Ovalbumin is one of the most abundant proteins in egg yolk plasma (Mann and Mann, 2008). Ovalbumin transports from egg white to yolk during long-time storage (Gao et al., 2017). Only 1 protein spot was identified as ovalbumin with molecular weight of 39.3 kDa at 18 ED in this study. The slightly lower molecular mass indicated its undergoing degradation or transportation during long-term incubation. The physiological role of ovalbumin during embryo development was less reported, and its function needs further investigation.

Ovotransferrin, the major protein constituent of egg white, plays an important role in biological iron transport and storage processes, and its spontaneous autocleavage into peptidic fragments offered its alternative biological properties, such as antibacterial and antioxidant activities (Chaipayang et al., 2017). Ovotransferrin was first observed in cartilaginous rudiment at 7 ED and then expressed in high level at the diaphysis collar of stacked-osteoprogenitor cells, the derived osteoblasts, and the menisci primordial cells at 13 ED (Gentili et al., 1994). Ovotransferrin might be transported from the egg yolk to the developing embryo to support the needs of early embryonic cartilage formation, which explains its decreased abundance at 10 ED. Ovotransferrin exhibits antibacterial activity against a variety of bacteria, including gram-positive and gram-negative bacteria (Valenti et al., 1987). The

increased abundance of ovotransferrin at 18 ED might be because of its antibacterial activity at the end of hatching.

Differentially Expressed Proteins Functionally Protect the Developing Embryo

Immunoglobulin Y from bird was synthesized and secreted by mature B cells and then entered the ovary by blood transportation and accumulated in the yolk. Similar to mammalian IgG, IgY also consists of 2 heavy chains and 2 light chains (Chalghoumi et al., 2009; Dias da Silva and Tambourgi, 2010). The disulfide bond between the heavy and light chains might be broken by urea, SDS, and DTT treatments during the experiment; thus, 8 protein spots were identified. During incubation, Ig gradually entered the embryo to provide passive immune protection for chicken embryos and resulted in a decreased abundance in this experiment. The MW of the protein spots representing the IgY heavy chain was 78.6 kDa, indicated a phosphorylation or glycosylation occurred during incubation.

Vitelline membrane outer layer protein 1 (**VMO-1**), a basic protein composed of 163 amino acids, plays essential roles in separating the yolk from the egg white and preventing yolk infection of bacteria by forming a barrier of fibrous layers in avian eggs (Kido et al., 1995; Lim and Song, 2015). Guérin-Dubiard et al. (2006) first identified VMO-1 protein in egg white. In this study, the MW of 3 VMO-1 protein spots (1–3) were lower than their theoretical values, and their abundance decreased significantly at 18 ED indicated that the protein degraded for nutrient absorption. VMO-I could inhibit the wheat germ agglutinin elicited hemagglutination and has a glycan synthetic activity similar to the transferase activity of lysozyme (Kido et al., 1995). However, the physiological function of VMO-1 in chicken embryo development is not very clear.

Ovoinhibitor is a member of the Kazal family of protease inhibitors identified from egg white, which inhibits the activity of trypsin, chymotrypsin, subtilisin, elastase etc., serine proteases (Oubre et al., 2003). Ovoinhibitor protein spots were detected in unfertilized egg white and egg yolk during storage and also in fertilized egg white during incubation (Qiu et al., 2012; Wang and Wu, 2014; Gao et al., 2017). In this study, the MW of the identified ovoinhibitors was lower than their theoretical value suggested that the proteins degraded to keep the activity of serine protease which degrade macromolecular nutrients to support the growth and development of chicken embryo. The increased abundances of ovoinhibitor suggested its transfer from egg white to yolk especially at 10 ED in which the water content in yolk was in highest. At the end of incubation, remaining yolk is gradually inhaled into chicken embryo to keep its early nutritional needs. The increased abundance of ovoinhibitor at 18 ED might

responsible for keeping the integrity of nutrient material from yolk.

The PIT54 is a soluble member of the so-called scavenger receptor cysteine-rich proteins family, which is the major hemoglobin-binding protein in chicken plasma (Georgieva, 2010). The molecular weight of PIT54 protein is 69 kDa (Wicher and Fries, 2006). In this study, The PIT54 precursor identified with higher MW might undergo modification to form PIT54 protein and exert its biological activity. PIT54 functionally converged with haptoglobin to protect nitric oxide signaling by sequestering cell-free Hb in large protein complexes, blocking cardiovascular complications (Schaer et al., 2016).

GO Enrichment Analysis of Differentially Expressed Proteins

Immunoglobulin lambda light chain and IgY heavy chain proteins are involved in biological processes such as humoral immune response mediated by circulating Ig, B cell-mediated immunity and Ig-mediated immune response at 10 ED. During embryo development, Ig lambda light chain and IgY heavy chain proteins provide an effective humoral immunity against the commonest avian pathogens. These antibodies are first adhered in the yolk sac from days 7 to 18 of incubation (Tian and Zhang, 2012). Adipose tissue is the main source of energy during chicken embryo development. On the sixth and eighth D of chicken embryo development, the yolk sac endoderm epithelial cells were mainly composed by yolk particles, lipid droplets of variable sizes, and yolk subdroplets. At 11 ED, the volume of yolk particles and masses of electron-dense fine granules from yolk subdroplets decreased, whereas the number of lipid inclusions and myelin sheaths increased (Kanai et al., 1996). Vitellogenin is a protein that has lipid transport and storage functions. Therefore, VTG-I precursor, VTG-II, VTG-III, and ApoB precursor might play key role in the transport of lipids from the yolk to the yolk sac endoderm at 10 and 18 ED.

At 18 ED, ovoinhibitors and ovalbumin were involved in response to mineralocorticoid, corticosterone, glucocorticoid, corticosteroid, and ketone physiological reaction, while glucocorticoid and mineralocorticoid are involved in the control of a variety of physiological processes, including reproduction, metabolism, salt balance, inflammation, and immunity (Barnes and Adcock, 2003; Fu and Vallon, 2014; Deviche et al., 2017). Therefore, the high abundance of ovalbumin and ovoinhibitors might have significant physiological implications for the embryo development before hatching.

CONCLUSION

The high abundance of VTGs at 10 ED might participate in lipid localization and lipid transportation to facilitate the yolk nutrient transport to embryo. The low expression of ovotransferrins at 10 ED indicated

the chondrogenesis of embryo. The high expression of ovoinhibitor at 18 ED was to inhibit the decomposition of yolk nutrients.

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SUPPLEMENTARY DATA

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