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Proteomic analysis of egg white proteins during the early phase of embryonic development

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ABSTRACT

Avian egg albumen participates in embryonic development by providing essential nutrients as well as antimicrobial protection. Although various biological functions of egg white proteins were suggested during embryogenesis, global changes of these proteins under incubation conditions remained uninvestigated. This study presents a proteomic analysis on the change of egg white proteins during the first week of embryonic development. By using 2-DE, together with MALDI-TOF MS/MS, thirty protein spots representing eight proteins were identified showing significant changes in abundance during incubation. An accelerating degradation of ovalbumin was observed in a wide range of molecular weight. In addition, four protein complexes were predicted according to the detected molecular weight increase. Among these speculated protein complexes, an ovalbumin spot coupled with RNA-binding protein was detected. The absence of these protein complexes before incubation, followed by the constant increase in abundance during incubation indicates conceivable pivotal roles in embryonic development. To better understand the function of the proteins identified in this study, discrepancies of egg white protein changes between fertilized and unfertilized chicken eggs were additionally demonstrated. These findings will provide insight into the embryogenesis process to improve our knowledge of egg white proteins in regulating and supporting early embryonic development.

1. Introduction

The chicken egg white represents a substantial part of essential nutrients for the developing embryo and also protects the embryo against invading bacteria. Albumen proteins form half of the non-incubated egg protein content and participate, to a great extent, in regulating whole-body protein synthesis in chicken embryos during incubation [1]. Egg white is absorbed and utilized for the energetic and nutritional needs of the embryo during the incubation process [2]. Many changes take place during the embryonic development, i.e., egg white pH increased at the first 2 days of incubation and then decreased with incubation time, meanwhile, the lysozyme enzymatic activity decreased rapidly during the first 12 days of incubation [3]. Conformational changes of certain proteins (i.e. ovalbumin) may be of some difference in developing eggs compared to that of unfertilized eggs [4]. According to Moran (2007) [5], embryonic development can be divided into three major phases. The first phase, which includes the first week of incubation, is characterized as establishment of germ by the formation of several egg compartments (including amnion, chorion, allantois, and yolk sac) that support the survival of the growing embryo [6]. During

Abbreviations: PG, Prostaglandin; MW, molecular weight; PCBP3, poly (rC) binding protein 3; OTf, ovotransferrin.

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this period, albumen solids are hardly absorbed by the embryo [2]. Egg white is absorbed and utilized gradually in the second phase (day 8 to day 18 of incubation) to support the rapid embryonic growth. The third phase, including the last three days of incubation, is characterized by the consumption of amnion and accumulation of glycogen reserves for the embryo emergence.

Recently, the role of incubation and egg white proteins in protecting eggs from microbial infection was widely focused [7–10]. However, the mechanism for this inhibition is still unknown. Besides the physicochemical or environmental factors such as viscous property, alkaline pH, and the incubation temperature, certain egg white proteins may also play an important role in the egg white antimicrobial activity. The most widely studied antimicrobial proteins of egg white are lysozyme and ovotransferrin [11]. Lysozyme, also known as N-acetylmuramic-hydrolase, has potent bactericidal activity against gram-positive bacteria [12]. Ovotransferrin is also extensively evidenced as the major egg white protein protecting the embryo from *Salmonella enterica* serovar *Enteritidis* infection [13]. Its antimicrobial activity mainly depends on the action of iron chelation, which leads to the iron deprivation for bacterial growth [14]. Moreover, some protein inhibitors, such as ovoinhibitor, ovomucoid, ovostatin, and cystatin were also suggested to be involved in the antimicrobial defense of developing eggs through *in vitro* experiments [15–18]. In addition, the generation of some antimicrobial proteins (such as Tenp [19]) and peptides (such as defensin-family proteins [20,21]) through the action of proteases [22] may also affect the antimicrobial ability of egg white during incubation. The systemic analysis needs to be performed to reveal the change of egg white proteins in the early stage of incubation, which may serve for the better absorption of albumen content in the latter phases and/or defense the embryo from microbial infections.

In the past few years, proteomics has been applied to the identification of novel proteins in chicken egg white. In the year 2006, Guerin-Dubiard et al. identified 16 proteins in egg white including two novel proteins (Tenp and VMO-1) [19]. Then, the high confidence identification of 78 proteins in egg white was reported using MS-based high throughput proteomic techniques [23]. Later in 2008, the use of two types of peptide ligand libraries in conjunction with LC-ESI-IT-MS/MS allowed the identification of 148 unique egg white protein species including a number of low-abundance proteins [24]. Recently, using a dual pressure linear ion trap Orbitrap instrument (LTQ Orbitrap Velos), Mann et al. identified 158 albumen proteins including 79 found in egg white for the first time [25]. These findings support the further investigation of the comparative proteomic analysis of egg white proteins. To study the change of egg white proteins during storage at the proteomic level, Omana et al. used 2-DE and LC-MS to elucidate how protein changes affect the egg white thinning [26].

As egg white absorption seldom take place during the first week of incubation, albumen proteins may mainly behave as the barrier of defense against microbial infection, and prepare for better absorptions in the latter phases of incubation. To obtain more information on the changes of egg white proteins occurring during the early phase of incubation, samples from fertilized and unfertilized chicken eggs were collected at 2 and 7 days of incubation for 2-DE based proteomic analysis. The objective of this study was to determine the changes at proteomic level to further understand the importance of the early phase on the development of chick embryo.

2. Materials and methods

2.1. *Egg white sampling*

Fertilized and unfertilized chicken eggs (60±0.5 g, average weight) from Lohmann White Single Comb White Leghorn (of the same flock, 40 weeks of age) laid within 24 h were collected from the Poultry Research Centre farm of Huazhong Agricultural University and used in this study. The collected eggs were incubated at 38±0.5 °C and 65% relative humidity in a forced-air incubator for 7 days. Eggs were randomly selected during the sampling, and egg white was carefully obtained every 24 h. Fresh egg (0 day) was considered as control. Three biological replicates were performed during the following experiments for 2-DE analysis to reduce variations.

2.2. *Protein extraction*

Protein extraction was performed basically according to the method reported by Omana et al. [26], with minor modification. Egg white was carefully separated from yolk and gently homogenized with a magnetic stirrer for 30 min to reduce the viscosity. Part of the white homogenate was used for pH and SDS-PAGE analysis. The whites of fertilized and unfertilized chicken eggs after 2 and 7 days of incubation were selected respectively for 2-DE analysis. To prepare the samples for 2-DE analysis, 150 μL of the egg white homogenate was extracted with 1.5 mL of ice-cold acetone containing 10% w/v trichloroacetic acid and 5 mM DTT. The samples were kept at −20 °C overnight, and were centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in a 1.5 mL of ice-cold acetone containing 5 mM DTT. This centrifugation-and-resuspension process was repeated three times. The final pellets were vacuum-dried and resuspended in 200 μL rehydration buffer (Bio-Rad). After adequately vortexing, the samples were centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was collected and the protein content was quantified using a 2-DE Quant Kit (GE Healthcare, Piscataway, NJ, USA).

2.3. *2-DE analysis*

Proteins were separated by 2-DE analysis using the Ettan IPGphor 3 System (GE Healthcare, USA) for the first dimension isoelectric focusing (IEF) and the Ettan DALTSix System (GE Healthcare) to perform SDS-PAGE in the second dimension. IEF was performed with DryStrip IPG strips of 24 cm (pH 4–7) which were rehydrated overnight at room temperature (20 °C) with 125 μL (100 μg of protein) of the protein sample in the rehydration buffer (Bio-Rad). The Isoeletric focusing was performed at 20 °C, step 1: 300 V for 0.5 h, step 2: 700 V for 0.5 h, step 3: 1500 V for 1.5 h, step 4: 9000 V for 3 h, step 5: 9000 V for 5 h, for a total of 64,000 Vh. After completion of the IEF program, the strips were stored at −20 °C.

Prior to second dimension analysis, the individual strips were equilibrated for two times in order to resolubilise proteins and reduce disulfide bond: 15 min in 10 ml denaturing solution (6 M urea, 30% glycerol, 2% SDS, 0.375 M Tris (pH 8.8) containing 0.1 M DTT, a trace of bromophenol blue) and subsequently for 15 min in 10 ml equilibration buffer containing 250 mM IAA (iodoacetamide). After equilibration, the second dimension electrophoresis was performed on a 12.5% SDS polyacrylamide gel. The gels were run at 2 W per gel for the first 45 min and followed by 17 W per gel for about 4.5 h until the dye front reached the bottom of the gel. The protein spots were visualized via silver staining or Coomassie Brilliant Blue G-250 staining. Subsequently, gel evaluation and data analysis were carried out using the ImageMaster v 7.0 program (GE Healthcare). The differences of 2-DE data were evaluated by a one-way ANOVA and a Tukey's significance test ($p<0.01$) using SPSS 13.0 (SPSS, Chicago, IL, USA).

2.4. *Gel analysis and protein identification*

To analyze the protein patterns, stained gels were scanned and calibrated using a PowerLook 1100 scanner (UMAX), followed by analysis of protein spots via ImageMaster v 7.0 program (GE Healthcare). Only those with significant and reproducible changes ($p<0.01$) were considered to be differentially accumulated proteins in relative abundance at 2 and 7 days of incubation compared to control (0 day). The target protein spots were manually excised from the stained gels and washed and then digested with sequencing-grade trypsin (Promega, Madison, WI, USA). MALDI-TOF spectra were calibrated using trypsin autodigestive peptide signals and matrix ion signals. The protein sample with an equivalent matrix solution (HCCA) was applied to further MALDI-TOF MS/MS analysis which was performed by a fuzzy logic feedback control system (Ultraflex MALDI-TOF-TOF mass spectrometer Bruker, Karlsruhe, Germany) equipped with delayed ion extraction. Peptide masses were searched against the non-redundant sequence database (NCBInr v20110513) to identify the protein using the MASCOT program (http://www.matrixscience.com). Search parameters were set, with tolerance to 100 ppm peptide mass variance, for Carbamidomethyl (C) as fixed modification, and Oxidation (M) as variable modification. For the MASCOT search results, protein scores, when greater than 74, were considered significant ($p<0.05$).

3. Results

The pH of albumen from fertilized eggs increased to 9.41 in the second day of incubation and then deceased gradually to 8.34 at the end of the early embryonic development phase (the 7th day) (Fig. 1). Comparatively, the albumen pH from unfertilized eggs kept increasing within 7 days incubation (Fig. 1). To investigate the egg white protein alterations correlated to these pH changes, egg white from fertilized eggs after 2 days and 7 days of incubation together with that before incubation were collected respectively for 2-DE-based proteomic analysis. Unfertilized egg whites were also collected at the same sampling time using for comparison.

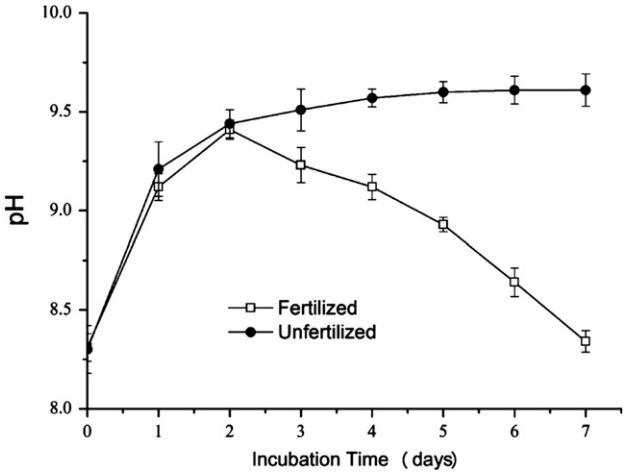

Fig. 1 – pH of egg white collected from fertilized and unfertilized chicken eggs during the first week of incubation at 24-hour intervals.

3.1. *Alterations of protein abundance in fertilized egg white during the incubation*

Comparing the 2-DE gels of fertilized egg white samples during the early phase of incubation (after 2 and 7 days respectively) to that of the control (0 day), 30 protein spots which showed significant ($p<0.01$) change were identified (Table 1) and marked in Fig. 2. The detected sequences via MS/MS of every protein spots were listed in Table 1. Among these altered proteins, 17 protein spots were identified as ovalbumin, which is the most abundant egg white protein (comprising 54% of the total proteins) [27]. Many spots representing ovalbumin, such as spot 6, 9, 10–13, showed a much lower molecular weight than the theoretical value (42.9 kDa), indicating the degraded fragments of ovalbumin (Table 1). Nevertheless, two protein spots (spot 26 and 27), which did not exist in the control (0 day) and increased in abundance during incubation in both fertilized and unfertilized eggs, exhibited higher molecular weight than the theoretical value (Table 1 and Fig. 3).

Except for ovalbumin, 7 other proteins exhibiting alterations in abundance were identified, including clusterin (spot 1, 7, 29 and 30), ovotransferrin (spot 20 and 25), Prostaglandin D2 synthase (PG D2 synthase) (spot 2 and 5), ovoinhibitor (spot 22 and 24), Lysozyme C (spot 23), ovalbumin-related protein Y (spot 21) and uncharacterized protein (spot 28) (Table 1 and Fig. 4). Spot 28 harbored a mixture of two proteins: uncharacterized protein with RNA-binding domain and ovalbumin (Table 1). Fig. 4 depicted observed increases and decreases in protein abundance for these spots. Alterations of these proteins were shown in Fig. 4.

3.2. *Discrepant changes in protein abundance between fertilized and unfertilized egg whites*

To gain insight into the possible different changes in protein abundance between fertilized and unfertilized eggs during early stage of incubation, further comparative analysis of the identified

Table 1 – List of significantly altered fertilized chicken egg white proteins during the first week of incubation.

Spot [a]	Accession [b]	Protein name	Score [c]	C [d]	Experimental pI/Mr (kDa)	Theoretical pI/Mr(kDa)	Sequence [e]
Serpin family							
3	129293	Ovalbumin [*Gallus gallus*]	134	17	4.54/41	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
4	129293	Ovalbumin [*Gallus gallus*]	375	32	4.53/44	5.19/42.9	R.DILNQITKPNDVYSFSLASR.L R.LYAEER.Y K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
6	129293	Ovalbumin [*Gallus gallus*]	261	14	5.08/25	5.19/42.9	R.DILNQITKPNDVYSFSLASR.L R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V
8	129293	Ovalbumin [*Gallus gallus*]	252	23	4.86/39	5.19/42.9	R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E K.HIATNAVLFFGR.C
9	129293	Ovalbumin [*Gallus gallus*]	302	21	4.80/17	5.19/42.9	R.DILNQITKPNDVYSFSLASR.L R.LYAEER.Y K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E R.VTEQESKPVQMMYQIGLFR.V
10	129293	Ovalbumin [*Gallus gallus*]	227	18	4.57/24	5.19/42.9	K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V
11	129293	Ovalbumin [*Gallus gallus*]	134	18	4.80/25	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
12	129293	Ovalbumin [*Gallus gallus*]	132	14	4.89/26	5.19/42.9	R.LYAEER.Y K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V
13	129293	Ovalbumin [*Gallus gallus*]	325	34	4.59/28	5.19/42.9	R.DILNQITKPNDVYSFSLASR.L R.LYAEER.Y R.LYAEERYPILPEYLQCVK.E R.YPILPEYLQCVK.E K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E K.HIATNAVLFFGR.C
14	129293	Ovalbumin [*Gallus gallus*]	149	19	4.45/33	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E R.ADHPFLFCIK.H
15	129293	Ovalbumin [*Gallus gallus*]	187	22	4.42/40	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E R.ADHPFLFCIK.H K.HIATNAVLFFGR.C
16	129293	Ovalbumin [*Gallus gallus*]	127	13	4.64/39	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.ISQAVHAAHAEINEAGR.E
17	129293	Ovalbumin [*Gallus gallus*]	257	25	4.78/45	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E K.HIATNAVLFFGR.C
18	129293	Ovalbumin [*Gallus gallus*]	347	33	4.39/47	5.19/42.9	R.LYAEERYPILPEYLQCVK.E R.YPILPEYLQCVK.E K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E R.ELINSWVESQTNGIIR.N K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E K.HIATNAVLFFGR.C
19	129293	Ovalbumin [*Gallus gallus*]	134	17	4.46/45	5.19/42.9	R.LYAEER.Y R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
26	129293	Ovalbumin [*Gallus gallus*]	358	35	4.63/82	5.19/42.9	R.DILNQITKPNDVYSFSLASR.L R.LYAEERYPILPEYLQCVK.E

Table 1 (*continued*)

Spot [a]	Accession [b]	Protein name	Score [c]	C [d]	Experimental pI/Mr (kDa)	Theoretical pI/Mr(kDa)	Sequence [e]
							R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V R.VTEQESKPVQMMYQIGLFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E R.ADHPFLFCIK.H K.HIATNAVLFFGR.C
27	129293	Ovalbumin [*Gallus gallus*]	326	27	4.50/82	5.19/42.9	R.LYAEER.Y K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
28	129293	Ovalbumin [*Gallus gallus*]	267	36	4.39/82	5.19/42.9	K.ELYRGGLEPINFQTAADQAR.E R.GGLEPINFQTAADQAR.E K.AFKDEDTQAMPFR.V K.LTEWTSSNVMEER.K K.ISQAVHAAHAEINEAGR.E
	118086587	Uncharacterized protein [*Gallus gallus*]	133	13	4.39/82	9.30/34.5	R.INISEGNCPER.I R.ESTGAQVQVAGDMLPNSTER.A K.IANPVEGSTDR.Q
21	129296	Ovalbumin-related protein Y [*Gallus gallus*]	184	18	4.50/59	5.20/43.8	K.TFSVLPEYLSCAR.K R.KFYTGGVEEVNFK.T K.IAFNTEDTR.E K.TINFDKLR.E K.HSLELEEFR.A R.ADHPFLFFIR.Y R.YNPTNAILFFGR.Y
Clusterin family							
1	45382467	Clusterin [*Gallus gallus*]	177	18	6.54/33	5.47/51.3	K.EHQAMLHTLEETKR.R K.ICHSGSGLVGR.Q R.IDALLDR.E R.RFEDLEER.F R.TPPFGGFR.E R.EAFVPPVQR.V R.NSAGCLRMR.D R.FMEIVAEQALQHYK.Q
7	45382467	Clusterin [*Gallus gallus*]	144	13	6.20/34	5.47/51.3	K.EHQAMLHTLEETKR.R K.ICHSGSGLVGR.Q R.RFEDLEER.F R.TPPFGGFR.E R.EAFVPPVQR.V R.NSAGCLRMR.D
29	45382467	Clusterin [*Gallus gallus*]	171	16	6.05/33	5.47/51.3	K.EHQAMLHTLEETKR.R K.ICHSGSGLVGR.Q R.RFEDLEER.F R.TPPFGGFR.E R.EAFVPPVQR.V R.LVPPRR.R R.FMEIVAEQALQHYK.Q
30	45382467	Clusterin [*Gallus gallus*]	144	13	5.79/34	5.47/51.3	K.EHQAMLHTLEETKR.R K.ICHSGSGLVGR.Q R.RFEDLEER.F R.TPPFGGFR.E R.EAFVPPVQR.V R.NSAGCLRMR.D
Lipocalin family							
2	45383612	PG D2 synthase, brain [*Gallus gallus*]	321	32	5.35/21	6.30/20.8	R.NSLYIR.T R.FSYTNPR.W R.WGSNHDIR.V R.VVETNYDEYALVATQISK.S K.STGSSNMVLLYSR.T R.TKEVAPQR.L
5	45383612	PG D2 synthase, brain [*Gallus gallus*]	321	32	5.84/21	6.30/20.8	R.NSLYIR.T R.FSYTNPR.W R.WGSNHDIR.V R.VVETNYDEYALVATQISK.S K.STGSSNMVLLYSR.T R.TKEVAPQR.L
Transferrin family							
20	1351295	Ovotransferrin [*Gallus gallus*]	348	21	6.08/56	6.85/77.8	K.SVIRWCTISSPEEK.K R.DLTQQER.I K.ATYLDCIK.A K.KGTEFTVNDLQGK.T K.TSCHTGLGR.S K.FFSASCVPGATIEQK.L K.HTTVNENAPDQKDEYELLCLDGSR.Q R.VAAHAVVAR.D K.SDFHLFGPPGK.K R.KDQLTPSPR.E K.DQLTPSPR.E K.TDERPASYFAVAVAR.K R.RANVMDYR.E
25	1351295	Ovotransferrin [*Gallus gallus*]	300	18	6.93/81	6.85/77.8	R.DLTQQER.I K.HTTVNENAPDQKDEYELLCLDGSR.Q K.TCNWAR.V K.SDFHLFGPPGK.K K.TDERPASYFAVAVAR.K R.TGTCNFDEYFSEGCAPGSPPNSR.L K.YFGYTGALR.C K.NLQMDDFELLCTDGR.R R.ECNLAEVPTHAVVVRPEK.A
Protease inhibitors kazal family							
22	1708509	Ovoinhibitor [*Gallus gallus*]	229	22	4.50/59	6.16/51.9	K.DGTSWVACPR.N R.EHGANVEKEYDGECRPK.H K.EYDGECRPK.H R.TLVACPR.I R.QEIPEIDCDQYPTR.K R.CREEVPELDCSK.Y R.CEEDITKEHCR.E K.VSPICTMEYVPHCGSDGVTYSNR.C R.CFFCNAYVQSNR.T

(*continued on next page*)

Table 1 (*continued*)

Spot [a]	Accession [b]	Protein name	Score [c]	C [d]	Experimental pI/Mr (kDa)	Theoretical pI/Mr(kDa)	Sequence [e]
24	1708509	Ovoinhibitor [*Gallus gallus*]	328	25	6.40/71	6.16/51.9	K.DGTSWVACPR.N R.EHGANVEKEYDGECRPK.H K.EYDGECRPK.H R.TLVACPR.I R.ILSPVCGTDGFTYDNECGICAHNAEQR.T R.QEIPEIDCDQYPTRK.T R.CREEVPELDCSK.Y K.VSPICTMEYVPHCGSDGVTYSNR.C
Lysozyme family							
23	126608	Lysozyme C [*Gallus gallus*]	227	50	5.93/66	9.36/16.2	R.HGLDNYR.G K.FESNFNTQATNR.N R.NTDGSTDYGILQINSR.W K.MVSDGNGMNAWVAWR.N

[a] Spot ID represents the protein spot number on the 2-DE gel image.
[b] Accession numbers of matched proteins according to the NCBInr database.
[c] MASCOT score. The Mascot threshold score for all these identified proteins is 74.
[d] Percent of sequence coverage.
[e] The sequence of matched peptides.

30 proteins with the corresponding spots from the unfertilized egg white 2-DE gels (as the reference) were performed. Concerning the 17 ovalbumin spots, abundance changes were similar between fertilized and unfertilized egg whites and no statistically significant difference ($p<0.05$) were observed except for spot 6 (Fig. 3). Different changing patterns in intensities were observed of three clusterin spots (Fig. 4 A), two ovoinhibitor spots (Fig. 4 D), one Lysozyme spot (Fig. 4 E) and on spot representing ovalbumin-related protein Y (Fig. 4 F). Significant differences ($p<0.05$) in protein abundance were detected among these spots comparing between fertilized and unfertilized egg whites.

4. Discussion

Changes of egg white contents during the embryonic development were of wide interest in the past decades in order to reveal the functions of egg white proteins to embryogenesis [1–5,28]. The variations of certain proteins have been demonstrated to possibly affect the anti-microbial ability or the protein degradation and uptake during embryogenesis [4,29,30]. To improve our understanding of these variations, a systematical characterization at proteomic level of albumen protein changes during incubation is needed. Previous

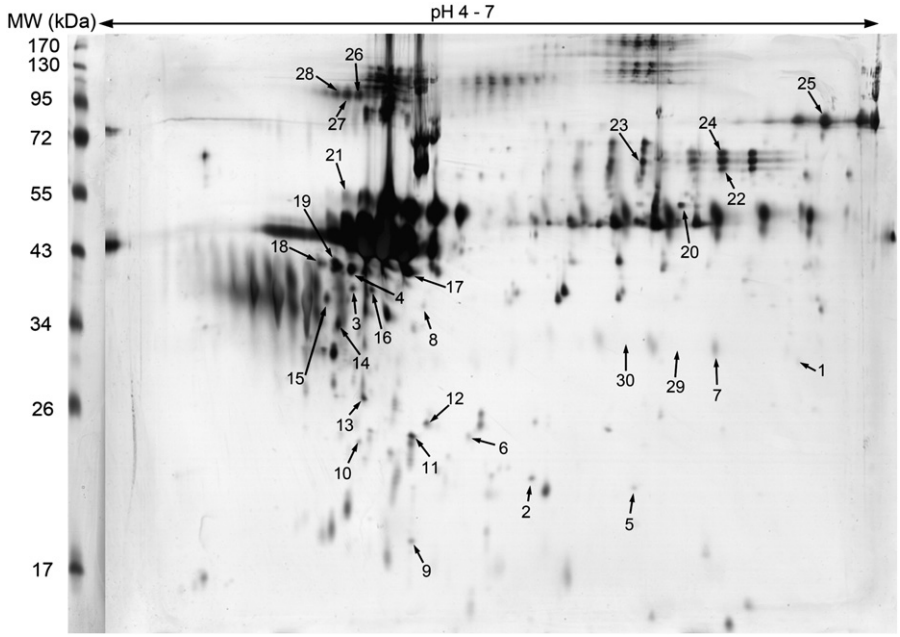

Fig. 2 – Representative 2-DE gel image of the proteins from fertilized chicken egg white prepared by IEF/SDS-PAGE separation and subsequently silver stained. Spots that significantly ($p<0.01$) changed in abundance during incubation process are indicated by numbers and arrows. The image shown represents the three independent 2-DE gel replicates of the fertilized chicken egg white at the end of early incubation (7 day).

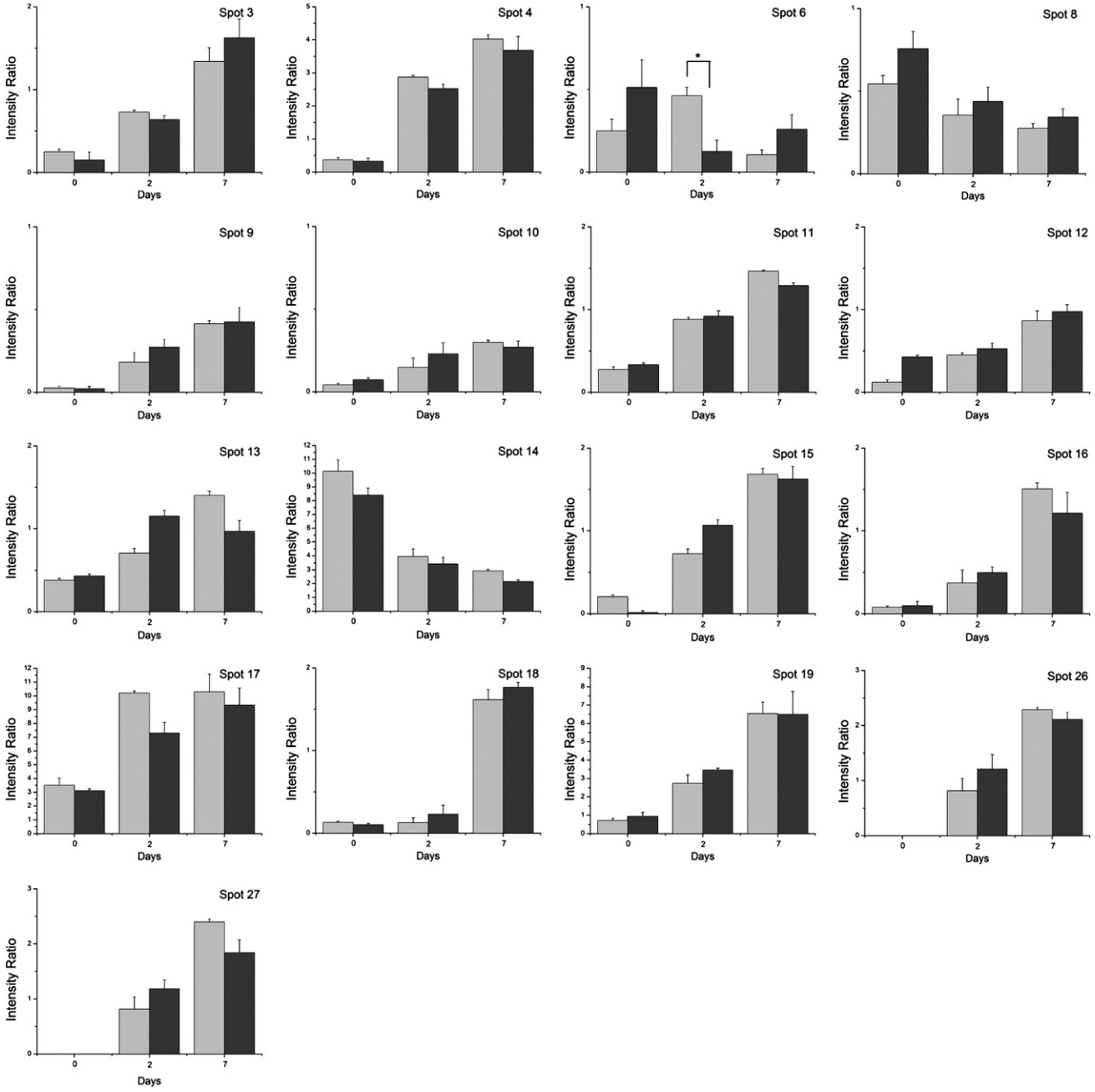

Fig. 3 – Changes in the intensity ratio of egg white ovalbumin during the first week of incubation. Column profiles of ovalbumin abundance from fertilized (light gray) and unfertilized (dark gray) chicken eggs were shown as bar charts. Only spots with significant ($p<0.01$) difference were given and error bars were shown. The asterisks indicate the statistically significant difference ($p<0.05$) between fertilized and unfertilized eggs.

proteomic studies mainly focused on the identification of total proteins of hen egg, and the novel minor proteins in particular [19,23,25,31–33]. Recently, Omana et al. firstly performed a comparative proteomic analysis by the means of 2-DE and LC-MS to describe the albumen protein changes during storage at ambient temperature of 22 °C [26]. It was found that most alterations in protein abundance were occurred during the initial 10 and 20 days of storage period.

Fig. 1 indicated the albumen pH decrease from the second day of incubation, which was in accordance with former reports [3]. As egg yolk is slightly acidic and a hydrogen ion concentration gradient exists between albumen and yolk, such pH decrease in egg white may play an important role in early embryonic development [34]. Corresponding to this pH change, alterations at proteomic level still need to be revealed. In the present study, albumen protein changes of fertilized chicken eggs during the early phase of incubation were investigated by proteomic tools for the first time. Over 100 protein spots were determined through 2-DE analysis, among which 36 protein spots were observed with significant ($p<0.01$) change in intensities and 30 spots were further identified by MALDI-TOF MS/MS analysis.

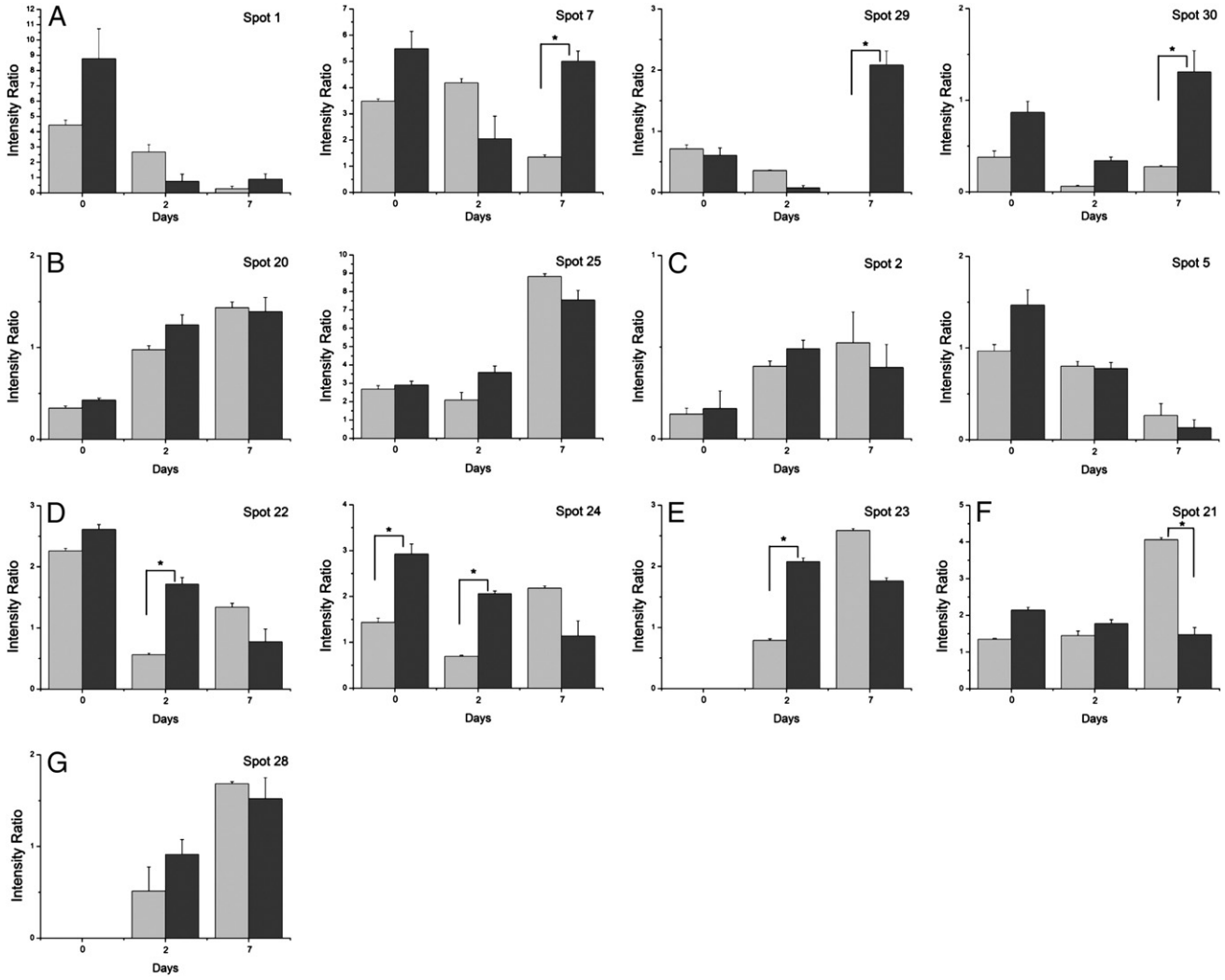

Fig. 4 – Changes in the intensity ratio of egg white proteins during the first week of incubation. (A) Clusterin; (B) Ovotransferrin; (C) Prostaglandin D2 synthase, brain; (D) Ovoinhibitor; (E) Lysozyme C; (F) Ovalbumin-related protein Y; (G) Uncharacterized protein and Ovalbumin mixture. Column profiles of these proteins from fertilized (light gray) and unfertilized (dark gray) chicken eggs were shown as bar charts. Only spots with significant ($p < 0.01$) difference were given and error bars were shown. The asterisks indicate the statistically significant difference ($p < 0.05$) between fertilized and unfertilized eggs.

These identified proteins were further clustered to six families: serpin (ovalbumin and ovalbumin-related protein Y), clusterin (clusterin), transferrin (ovotransferrin), lipocalin (PG D2 synthase), kazal-type protease inhibitors (ovoinhibitor) and lys (Lysozyme C). Of these 30 identified protein spots, 17 proteins were characterized as ovalbumin. As the predominant egg white protein, ovalbumin is a member of the serine proteinase inhibitor (serpin) family but was considered unable to inhibit proteinases because of its inability to form irreversible complexes with proteinases [35]. Previous studies have proved that ovalbumin undergoes conformational changes to a heat stable form while migrating into the embryonic organs from egg white [36]. Omana et al. reported that 6 ovalbumin protein spots were detected with significant change in abundance during storage of 10 or 20 days [26]. These spots were scattered in a wide range of pH and molecular weight (MW), indicating degradations and modifications of ovalbumin during the storage. In this study, 17 ovalbumin protein spots were displayed in a relatively narrower pH range but a wider MW range (Fig. 2 and Supplementary Fig. S1). Most of the ovalbumin proteins, which showed a lower MW than the theoretical value increased consistently in abundance during the first week of incubation both in the fertilized and unfertilized samples (Fig. 3). This may indicate an accelerating degradation of ovalbumin under the incubation conditions. Two ovalbumin spots (spot 8 and 14) were found deceased in abundance during the incubation. This was especially to the intensity change of spot 14 that decreased obviously after 2 days of incubation compared to the slightly decrease in abundance of spot 8. Spot 14 already existed before the incubation (0 day) and may be further degraded to peptides of lower MW or be absorbed to egg yolk. Even though the albumen absorption does not take place in great amount in the first week of incubation, proteins with the immunological behavior of ovalbumin and

ovotransferrin (OTf, formerly conalbumin) have been found in embryonic fluids at 5 to 8 days of incubation [2]. Although the pH value which affects the conversion of ovalbumin to S-ovalbumin showed obviously different tendency between fertilized and unfertilized egg whites (Fig. 1), the changes in protein abundance were mostly compatible between these two kinds of samples (Fig. 3). The rare exceptions were only represented for spot 6 and 13, where the abundance changed differently in unfertilized samples compared to that from fertilized samples (Fig. 3).

Most ovalbumin spots already existed at 0 day, indicating the launch of ovalbumin degradation before the incubation process. Nevertheless, three adjacent protein spots (spot 26, 27 and 28) that also characterized as ovalbumin did not exist before incubation (0 day) and began to emerge after 2 days of incubation. It is peculiar to find that these 3 protein spots shared the same MW (82 kDa), which is about twice of the ovalbumin theoretical MW (Table 1, Fig. 2 and Supplementary Fig. S1) and supposed as ovalbumin dimer [19]. Spot 28 was identified as a complex of ovalbumin and an uncharacterized protein similar to poly (rC) binding protein 3 (PCBP3). PCBP3 is a widely conserved protein family containing the RNA-binding domains and plays roles in mRNA stabilization, translational activation, and translational silencing. Hence, the possible interaction of ovalbumin and PCBP3 may play a key role in pre-mRNA processing as well as important pathways regulating embryonic development. Spot 26 and 27 may also consist of ovalbumin dimer or protein complex, however, the proteins possibly interact with ovalbumin (or fragment) were not identified. The conversion of less α-helices and more β-sheet in ovalbumin secondary structure was found under incubating conditions to form inhibitory ovalbumin (I-ovalbumin), which showed proteinase inhibitory property [35]. This heat-induced altered form of ovalbumin exhibits hydrophobic exposure and intermolecular interaction [37] which was speculated to enhance its binding ability to form protein complex. Therefore, such interactions between ovalbumin and other egg proteins may effectively stimulated under incubation conditions. In addition, these putative polymers or protein complex still existed after urea, SDS, and DTT treatments, which indicated the interaction due to covalent bond and did not involve the hydrophobic interactions or disulfide bonds [19]. Ovalbumin-related protein Y is also a member of serpin family with low abundance in egg white. There were five ovalbumin-related protein Y spots identified in previous studies [19] but were not selected by variance analysis of protein abundance [26]. The identified ovalbumin-related protein Y (spot 21) in this study was firstly detected and showed a lower pI value and higher MW (Fig. 2) than the theoretical ones indicating a possible phosphorylation or glycosylation of the native protein [38]. It is interesting to note the abruptly increase of spot 21 in protein abundance at 7 days incubation for fertilized samples (Fig. 4F). As the protease inhibition activity of ovalbumin-related protein Y was still unverified, the biological function of its enhancing at the end of the early embryonic development phase remained difficult to define.

Another protein (spot 23) which also showed much higher MW than the theoretical value was identified as lysozyme C (Table 1, Fig. 2 and Supplementary Fig. S1). Similarly to that of spot 26–28, spot 23 did not exist before incubation (0 day) and appeared after 2 days of incubation (Fig. 4E). Rapid decreasing of lysozyme activity has been found in albumen of fertile incubated eggs [3]. The interactions between lysozyme and other egg white proteins such as ovomucin have also been detected under sterile conditions and were suggested as one of the major cause leading to egg white thinning [39]. Whether lysozyme complex actually existed and enhanced during the incubation as depicted for spot 23 in this study and how this hypothetic protein complex affects the embryonic development as well as albumen antibacterial abilities still needs further studies.

Clusterin is a widely expressed secretory glycoprotein detected in several chicken tissues including magnum, egg shell and egg white [19]. It belongs to the chaperone protein family and shows interaction and stabilization with unfolded or partly folded proteins, which can prevent their aggregation and precipitation. Strong chaperone-like activity of clusterin coupled with egg white ovotransferrin and lysozyme have been suggested during incubation of the developing avian embryo [40]. It is also suggested that chicken clusterin could serve as a marker for follicular atresia and resorption, and may play an important role for embryonic development based on its ability to bind several key proteins [41]. In former 2-DE analysis, three clusterin isoforms (pIs 6.10, 6.30 and 6.60) were revealed with apparent MW 33 kDa and were suggested to be derived from glycosylation of two clusterin monomers (α and β) [19]. In the present study, four clusterin spots which are compatible with those reported [19] were identified with distinct changes during incubation (Table 1, Fig. 2 and Supplementary Fig. S1). All clusterin protein spots from fertilized samples were demonstrated decreasing in abundance in the 7-day period and remained in a trace amount after 7 days of incubation. On the contrary, 3 spots (spot 7, 29 and 30) of unfertilized samples showed relatively much higher intensities than that of fertilized samples (Fig. 4A). This is especially to spot 29 and spot 30 from unfertilized samples, which showed their highest intensity value at 7 days. The intensity increase of these two spots from unfertilized samples in accordance with the reported corresponding changes during egg storage [26], whereas, the decrease of fertilized cluterin spots abundance was not anticipated. Thus, the stabilization of certain egg white proteins interacted by clusterin may stay at a relatively lower level in fertilized eggs than that in unfertilized eggs at the end of the early embryonic development phase, which may serve for the better absorption by embryo in the following development phases.

Ovotransferrin is another abundant protein of egg white responsible for the transfer of ferric ions to developing embryo. OTf was reported to be first observed in the first cartilaginous rudiment during chick embryo tibia development [42]. Recent advances in studies revealed the redox-dependent auto-cleavage of OTf which may be an important regulatory point in the embryonic cascade [43]. OTf also shows antimicrobial activity against a wide range of bacteria (including Gram-positive and Gram-negative) [44]. Five OTf spots were identified in the 2-DE analysis by Guerin-Duviard et al. [19] and one of them were found increased significantly in abundance at 10 days of storage [26]. Through the 2-DE gel analysis, five

formerly discovered OTf spots were clearly differentiated in this study and one of them (spot 25) showed a considerable increase in abundance at 7 days of incubation (Fig. 4B). This increase may be related with the transition of embryonic development phases. Another identified OTf spot (spot 20) showed a relatively lower MW and pI value. Whether the abundance enhancement of spot 20 arose by the OTf self-cleavage together with further modifications and what is the function of such alterations to embryonic development are yet to be unraveled.

Even though an OTf fragment and a wide range of ovalbumin fragments were detected during the incubation which indicating degradations, we could not exclude that some of these components might also be solubilised from the eggshell, or the vitelline membrane or from the egg yolk through the vitelline membrane. As the appearance of protein spots with lower molecular weights than expected is usually correlated with the activity of specific proteinases (activated during incubation by temperature, pH etc.) and their regulation by antiproteases [45], some of the egg white proteinases [23,24] might also be responsible for the degradation products detected in this study. For most of the identified spots, the alterations were consistent between fertilized and unfertilized eggs, which implied these changes may most affected by high temperature rather than the presence of an embryo. Also, the similar variation in abundance observed among most spots in both fertilized and unfertilized eggs indicated that these changes were pH-independent. In contrast, some proteins such as clusterin, ovoinhibitor, lysozyme and Ovalbumin-related protein Y which behaved differently between fertilized and unfertilized eggs, might be pH-dependant (Fig. 4).

Based on 2-DE method and without the elimination of high-abundance proteins, only eight egg white proteins were observed altered significantly in this study, which contained only a small part of the total egg white proteins [19,24,25]. In order to give a detailed view of the alteration of most egg white proteins, the scale of pI 4–7 was select, which missed some well-known protein spots (such as lysozyme C and VMO-1) [19]. The alteration of these proteins as well as the variation of low-abundance egg white proteins during the early embryonic development still needs to be further studied. A lot of ovalbumin spots were detected in a wide MW range in this study. More detailed studies are necessary for the description of the modifications (glycosylation, phosphorylation), protein-protein interactions and probable specific cleavage sites of these ovalbumin spots.

5. Conclusion

In conclusion, a 2-DE based proteomic analysis was firstly performed to investigate the albumen protein alterations of fertilized chicken eggs during the first week of incubation using unfertilized chicken eggs for comparison. Thirty protein spots representing eight proteins were detected with significant changes in abundance during this period. Notably, four spots that emerged after incubation showed much higher molecular weight than the theoretic value indicating a protein complex formed through interactions. Of particular interest is the identified ovalbumin spot containing an RNA-binding protein which may play an important biological role in embryo development. This suggests that ovalbumin may provide the embryo more important biological functions than only nutrient. In addition, a wide range of degradation of ovalbumin was observed before the incubation process. Furthermore, by comparison with the unfertilized egg white samples, some discrepancies in the protein abundance were revealed particularly to clusterin, ovalbumin-related protein Y and ovoinhibitor spots. Further investigations will be needed to demonstrate the relation of these observed discrepancies and embryonic development.

These findings not only give a fundamental understanding of egg white protein alterations during the early embryonic development phase, but also provide the basis for predicting the egg white protein functions in embryogenesis. Forthcoming studies focused on the protein-protein interactions will facilitate a better understanding of such functions.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.12.037.

Conflict of interest statement

The authors have declared no conflict of interest.

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REFERENCES

[1] Muramatsu T, Hiramoto K, Koshi N, Okumura J, Miyoshi S, Mitsumoto T. Importance of albumen content in whole-body protein synthesis of the chicken embryo during incubation. Br Poult Sci 1990;31:101–6.
[2] Carinci P, Manzoli-Guidotti L. Albumen absorption during chick embryogenesis. J Embryol Exp Morphol 1968;20:107–18.
[3] Cunningham FE. Changes in egg white during incubation of the fertile egg. Poult Sci 1974;53:1561–5.
[4] Sugimoto Y, Sanuki S, Ohsako S, Higashimoto Y, Kondo M, Kurawaki J, et al. Ovalbumin in developing chicken eggs migrates from egg white to embryonic organs while changing its conformation and thermal stability. J Biol Chem 1999;274:11030–7.
[5] Moran Jr ET. Nutrition of the developing embryo and hatchling. Poult Sci 2007;86:1043–9.
[6] Ferket PR, De Oliveira JE, Uni Z. Important metabolic pathways in poultry embryos prior to hatch. Worlds Poultry Sci J 2008;64:488–99.
[7] Shawkey MD, Firestone MK, Brodie EL, Beissinger SR. Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. PLoS One 2009;4.
[8] Baron F, Alabdeh M, Lechevalier V, Nau F, Gautier M, Cochet MF, et al. Role of incubation conditions and protein fraction on the antimicrobial activity of egg white against *Salmonella enteritidis* and *Escherichia coli*. J Food Prot 2011;74:24–31.

[9] D'Alba L, Oborn A, Shawkey MD. Experimental evidence that keeping eggs dry is a mechanism for the antimicrobial effects of avian incubation. Naturwissenschaften 2010;97:1089–95.
[10] Cook MI, Sr B, Toranzos GA, Arendt WJ. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. Ecol Lett 2005;8:532–7.
[11] Hincke MT, Wellman-Labadie O, Picman J. Avian antimicrobial proteins: structure, distribution and activity. Worlds Poultry Sci J 2007;63:421–38.
[12] Hughey VL, Johnson EA. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. Appl Environ Microbiol 1987;53:2165–70.
[13] Baron F, Gautier M, Brule G. Rapid growth of *Salmonella enteritidis* in egg white reconstituted from industrial egg white powder. J Food Prot 1999;62:585–91.
[14] Garibaldi JA. Role of microbial iron transport compounds in bacterial spoilage of eggs. Appl Microbiol 1970;20:558–60.
[15] Miyagawa S, Nishino N, Kamata R, Okamura R, Maeda H. Effects of protease inhibitors on growth of *Serratia marcescens* and *Pseudomonas aeruginosa*. Microb Pathog 1991;11:137–41.
[16] Miyake M, Utsuno E, Noda M. Binding of avian ovomucoid to shiga-like toxin type 1 and its utilization for receptor analog affinity chromatography. Anal Biochem 2000;281:202–8.
[17] Molla A, Matsumura Y, Yamamoto T, Okamura R, Maeda H. Pathogenic capacity of proteases from *Serratia marcescens* and *Pseudomonas aeruginosa* and their suppression by chicken egg white ovomacroglobulin. Infect Immun 1987;55:2509–17.
[18] Wesierska E, Saleh Y, Trziszka T, Kopec W, Siewinski M, Korzekwa K. Antimicrobial activity of chicken egg white cystatin. World J Microbiol Biotechnol 2005;21:59–64.
[19] Guerin-Dubiard C, Pasco M, Molle D, Desert C, Croguennec T, Nau F. Proteomic analysis of hen egg white. J Agric Food Chem 2006;54:3901–10.
[20] Herve-Grepinet V, Rehault-Godbert S, Labas V, Magallon T, Derache C, Lavergne M, et al. Purification and characterization of avian beta-defensin 11, an antimicrobial peptide of the hen egg. Antimicrob Agents Chemother 2010;54:4401–8.
[21] Gong DQ, Wilson PW, Bain MM, McDade K, Kalina J, Herve-Grepinet V, et al. Gallin; an antimicrobial peptide member of a new avian defensin family, the ovodefensins, has been subject to recent gene duplication. BMC Immunol 2010;11.
[22] Mine Y, Ma F, Lauriau S. Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. J Agric Food Chem 2004;52:1088–94.
[23] Mann K. The chicken egg white proteome. Proteomics 2007;7: 3558–68.
[24] D'Ambrosio C, Arena S, Scaloni A, Guerrier L, Boschetti E, Mendieta ME, et al. Exploring the chicken egg white proteome with combinatorial peptide ligand libraries. J Proteome Res 2008;7:3461–74.
[25] K. Mann, M. Mann. In-depth analysis of the chicken egg white proteome using an LTQ Orbitrap Velos. Proteome Sci;9:7.
[26] D.A. Omana, Y. Liang, N.N. Kav, J. Wu. Proteomic analysis of egg white proteins during storage. Proteomics;11:144–153.
[27] Stevens L. Egg white proteins. Comp Biochem Physiol B 1991;100:1–9.
[28] Peebles ED, Gardner CW, Brake J, Benton CE, Bruzual JJ, Gerard PD. Albumen height and yolk and embryo compositions in broiler hatching eggs during incubation. Poult Sci 2000;79: 1373–7.
[29] Golab K, Gburek J, Gawel A, Warwas M. Changes in chicken egg white cystatin concentration and isoforms during embryogenesis. Br Poult Sci 2001;42:394–8.
[30] Jacquinot PM, Leger D, Wieruszeski JM, Coddeville B, Montreuil J, Spik G. Change in glycosylation of chicken transferrin glycans biosynthesized during embryogenesis and primary culture of embryo hepatocytes. Glycobiology 1994;4:617–24.
[31] Farinazzo A, Restuccia U, Bachi A, Guerrier L, Fortis F, Boschetti E, et al. Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. J Chromatogr A 2009;1216:1241–52.
[32] Mann K, Mann M. The chicken egg yolk plasma and granule proteomes. Proteomics 2008;8:178–91.
[33] Mann K, Macek B, Olsen JV. Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 2006;6:3801–10.
[34] Benton Jr CE, Brake J. The effect of broiler breeder flock age and length of egg storage on egg albumen during early incubation. Poult Sci 1996;75:1069–75.
[35] Mellet P, Michels B, Bieth JG. Heat-induced conversion of ovalbumin into a proteinase inhibitor. J Biol Chem 1996;271: 30311–4.
[36] Sugimoto Y, Sanuki S, Ohsako S, Higashimoto Y, Kondo M, Kurawaki J, et al. Ovalbumin in developing chicken eggs migrates from egg white to embryonic organs while changing its conformation and thermal stability. J Biol Chem 1999;274: 11030–7.
[37] Hu HY, Du HN. Alpha-to-beta structural transformation of ovalbumin: heat and pH effects. J Protein Chem 2000;19:177–83.
[38] Nau F, Pasco M, Desert C, Molle D, Croguennec T, Guerin-Dubiard C. Identification and characterization of ovalbumin gene Y in hen egg white. J Agric Food Chem 2005;53:2158–63.
[39] Robinson DS, Monsey JB. Changes in the composition of ovomucin during liquefaction of thick egg white. J Sci Food Agric 1972;23:29–38.
[40] Mann K, Gautron J, Nys Y, McKee MD, Bajari T, Schneider WJ, et al. Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white. Matrix Biol 2003;22:397–407.
[41] Mahon MG, Lindstedt KA, Hermann M, Nimpf J, Schneider WJ. Multiple involvement of clusterin in chicken ovarian follicle development. Binding to two oocyte-specific members of the low density lipoprotein receptor gene family. J Biol Chem 1999;274:4036–44.
[42] Gentili C, Doliana R, Bet P, Campanile G, Colombatti A, Cancedda FD, et al. Ovotransferrin and ovotransferrin receptor expression during chondrogenesis and endochondral bone formation in developing chick embryo. J Cell Biol 1994;124:579–88.
[43] Ibrahim HR, Haraguchi T, Aoki T. Ovotransferrin is a redox-dependent autoprocessing protein incorporating four consensus self-cleaving motifs flanking the two kringles. Biochim Biophys Acta-Gen Subj 2006;1760:347–55.
[44] Valenti P, Visca P, Antonini G, Orsi N, Antonini E. The effect of saturation with Zn^{2+} and other metal ions on the antibacterial activity of ovotransferrin. Med Microbiol Immunol 1987;176:123–30.
[45] Rehault-Godbert S, Gautron JL, Labas V, Belghazi M, Nys Y. Identification and characterization of the precursor of chicken matrix metalloprotease 2 (pro-MMP-2) in hen egg. J Agric Food Chem 2008;56:6294–303.