EuPA OPEN PROTEOMICS 2 (2014) 38–59

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Proteomic analysis of fertilized egg white during early incubation

*Jiapei Wang, Jianping Wu**

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

ARTICLE INFO

Article history:
Received 23 September 2013
Received in revised form
29 October 2013
Accepted 4 November 2013

Keywords:
Fertilized egg whites
2-DE
Proteomics
Incubation
Quantification
Embryo

ABSTRACT

Proteomic analysis of egg white proteins was performed to elucidate their metabolic fates during first days of embryo development using 2-DE coupled with a LC–MS/MS. A total of 91 protein spots were analyzed, representing 37 proteins belonging to 'Gallus gallus', of 19 proteins were detected in egg whites for the first time, such as lipoproteins, vitellogenin and zona pellucida C protein. All ovomucoid spots with one exception were significantly ($P < 0.05$) increased. Marker protein and one flavoprotein spot were significantly increased while hemopexin, serum albumin precursor, Ex-FABP precursor and Galline Ex-FABP were significantly decreased.

1. Introduction

Egg white provides not only many essential nutrients supporting the development of new life but also bioactivities for the protection of embryo against microorganisms [1–5]. With the aid of proteomics, new egg white proteins with bioactivities have been characterized. Desert et al. investigated the proteome-level of hen egg white with 2-DE–LC–MS/MS for the first time, leading to the characterization of a small acidic protein Ch21 which is a developmentally regulated protein in chick embryo skeletal tissues and belongs to the superfamily of lipophilic molecule carrier proteins [6,7]. Guerin-Dubiard et al. identified a total of 16 proteins, of two egg white proteins, Tenp, a protein with strong homology with a bacterial permeability-increasing protein family (BPI), and VMO-1, an outer layer vitelline membrane protein, were found for the first time [8]. Mann detected 78 egg white proteins, out of 54 new proteins, using 1-DE and LC–MS/MS [9]. D'Ambrosio et al. reported a total of 148 proteins from egg whites using 2-DE combined with a protein enrichment (peptide ligand libraries) technology [10]. Recently, Mann et al. identified 158 egg white proteins with a dual pressure linear ion trap Orbitrap instrument (LTQ Orbitrap Velos), out of 79 were found for the first time in egg white [11]. A quiescence-specific protein precursor in egg white was also identified [12]. Currently, more than 200 proteins have been identified and characterized from unfertilized eggs.

We have previously reported how storage would affect unfertilized egg white proteins, and suggested that the

* *Corresponding author at*: Department of Agricultural, Food and Nutritional Science (AFNS), 4-10 Ag/For Center, University of Alberta, Edmonton, Alberta, Canada T6G 2P5. Tel.: +1 780 492 6885; fax: +1 780 492 4265.
E-mail address: jianping.wu@ualberta.ca (J. Wu).

2212-9685
http://dx.doi.org/10.1016/j.euprot.2013.11.001

degradation of ovalbumin/clusterin was, at least partially, responsible for egg white thinning during egg storage [13]. Although egg white proteins are used widely as a versatile food ingredient, by nature, eggs are laid for new life. During incubation, egg white proteins go complex biochemical changes to fulfill the needs of chick embryonic development. Ovotransferrin is responsible for transferring ferric ions from hen oviduct to developing embryo; ovoinhibitor presents inhibitors of serine proteinases such as trypsin, chymotrypsin, subtilisin and porcine elastase; lysozyme functions mainly as an anti-microbial protein during embryo development; flavoprotein could protect embryos against bacterial attack by binding riboflavin to sustain embryo's growth until hatching [14–16]. There is very limited information available to characterize the changes of egg white proteins during incubation. A proteomic analysis of egg white proteins during the first week of embryonic development was recently reported [17] in which eight proteins, presented in 30 protein spots, were identified. Further characterization of the biochemical changes of egg white proteins during incubation therefore is needed to help our understanding on how egg white proteins function in embryonic development. Therefore, the objective of the study was to investigate the change of egg white proteins during early embryonic development.

2. Materials and methods

2.1. *Materials*

Fresh fertilized White Leghorn Eggs (60 ± 0.5 g), laid within 24 h from the Poultry Research Centre farm of the University of Alberta, were used for the study. Eggs were incubated in an incubator (small P20, Jamesway Incubator Company Inc., Saint Paul, MN, USA) with two turnings per day at 37.2 ± 0.5 °C and 53 ± 3% relative humidity with automatic ventilation. Eggs were sampled at 0, 1, 3, 5, 7 and 9th day, and 10 eggs per replicate were performed in triplicate for each time point. Eggs were weighed individually before and after incubation.

2.2. *Extraction of egg white proteins*

Egg white proteins were extracted as described previously with slight modifications [12]. Briefly, eggs were manually broken on the smaller end, the whites were carefully separated from the egg yolk (or the whole embryo part) and homogenized with a magnetic stirrer for 30 min, and then 100 μL of egg white was finely homogenized in 1.5 mL of ice-cold acetone containing 10% (v/v) trichloroacetic acid (TCA, Sigma, Louis, MO, USA) and 0.07% (v/v) dithiothreitol (DTT, Bio-Rad, Hercules, CA, USA). After one hour incubation at −20 °C, the mixture was centrifuged (10,000 × *g*, 15 min, 4 °C), and the resulting pellet was washed three times with 1 mL of ice-cold acetone containing 0.07% (v/v) DTT. The pellet was subsequently air-dried for 20 min in a Speedvac (Hetovac VR-1; Heto Laboratory Equipment A/S, Birkerod, Denmark), and then resolubilized in 150 μL of rehydration buffer (Bio-Rad) containing 1.0% (v/v) tributylphosphine (TBP, Bio-Rad) and incubated overnight at 4 °C. The sample was centrifuged (10,000 × *g*, 15 min, 4 °C) and the supernatant was subsequently transferred into a 1.5 mL centrifuge tube and stored at −20 °C till 2-DE analysis. Protein extractions were performed three times as the technical replicates and 10 fertilized eggs were used for each independent biological replicates. Protein concentrations were determined using a modified Bradford assay with protein assay dye reagent (Bio-Rad) and bovine serum albumin (BSA, Sigma) as the standard [18].

2.3. *2-DE analysis*

IEF (isoelectric focusing) in the linear pH gradient (the first dimension) and nonlinear molecular mass gradient separation (the second dimension) by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were performed as described previously [12]. IPG strips (11 cm, pH 4–7, Bio-Rad) were rehydrated overnight at room temperature with 100 μg of extracted egg white proteins in 125 μL of rehydration sample buffer. IEF was performed on a PROTEAN IEF cell (Bio-Rad) with the following settings: 250 V for 15 min, linear increase to 4000 V over 3 h, focused for 20,000 V h, and held at 500 V. Each focused IPG strip was equilibrated in 1 mL of equilibration buffer-I (6 M urea, 2% (w/v) SDS, 0.37 M Tris–HCl, pH 8.8, 20% (v/v) glycerol and 130 mM DTT) for 10 min twice and then incubated with 1 mL of equilibration buffer-II (6 M urea, 2% SDS, 0.37 M Tris–HCl, pH 8.8, 20% glycerol and 135 mM iodoacetamide (IAA, Bio-Rad) for 10 min twice. The equilibrated strip was then placed on top of 13% (w/v) polyacrylamide gel and the second dimension electrophoresis was performed in a PROTEAN II xi Cell (Bio-Rad) with the Precision Plus Protein Standard (Bio-Rad). After electrophoresis, the gel was stained with a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA).

2-DE images were recorded with a GS-800 calibrated densitometer (Bio-Rad) and the gel image was assembled in a matchset using PDQuest software (version 7.3.1, Bio-Rad). Automated spot detection was performed with PDQuest software, and then the matched spots were verified and adjusted manually. The intensity of each matched spot was analyzed and significantly ($P < 0.05$) altered spots were identified with the Student's *t*-test feature of the software. Moreover, the relative abundance of protein expression between each pair of eggs was compared.

2.4. *Digestion*

All matched protein spots were excised from gels and then were digested, in-gel, with trypsin based on 'Agilent 1100 LC–MS getting started guide'. Protein spots excised from gels were washed for 5 min with HPLC grade water and dehydrated with acetonitrile (ACN) for 30 min. After removing the liquid, samples were dried with speed vacuum equipment at room temperature for 10 min. The dry samples were subsequently destained with the mixture of 100% ACN and 0.1 M of NH_4HCO_3 (with the volume ratio of 1:1), and then dehydrated them again as described above. The proteins from these samples were incubated with 0.1 M of NH_4HCO_3 (containing 10 mM DTT) at 56 °C for 30 min and washed with 100% ACN. Then, these proteins were alkylated at room temperature with 0.1 M of NH_4HCO_3 (containing 55 mM of IAA) in the dark for 20 min. The samples were dehydrated

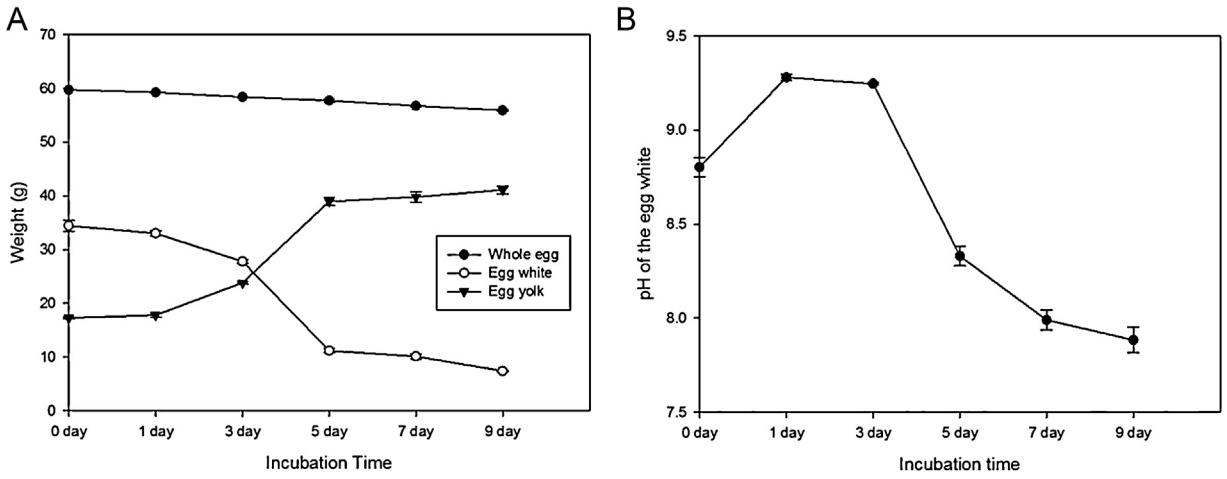


Fig. 1 – (A) The weight of whole shell-egg weight, the fertilized egg white, and the embryo part with incubation time. (B) The change of fertilized egg white pH with incubation time. Ten eggs per biological replicate were performed in triplicate for each time point.

again as described above. Digestion was performed by adding trypsin (0.02 μg/μL) with 40 mM NH_4HCO_3 and 10% of ACN at 37 °C for 16 h. Digestion was terminated by adding formic acid (0.1%, v/v) and stirring for 40 min. After removing the liquid, the hydrolysates (peptides) were extracted with 50% of ACN and 0.1% of formic acid for 30 min twice and then samples were stored at −20 °C till MS analysis.

2.5. *LC–MS/MS analysis*

The extracted peptide mixtures were analyzed on an Agilent 1100 Series LC/MSD Trap XCT System (Agilent Technologies, Palo Alto, CA, USA) operated in the unique peptide scan auto-MS/MS mode. Five microliters of peptide mixture was loaded onto a concentration column (Zorbax 300SB-C18, 5 μm, 5 × 0.3 mm) by an autosampler, followed by a separation column (Zorbax 300S B-C_{18}, 5 μm, 150 × 0.3 mm). The column was eluted at a flow rate of 4 μL/min with 3% buffer B for 5 min, gradient increased to 15% buffer B over 3 min, to 45% buffer B over 42 min, to 90% buffer B over 10 min, and decreased to 3% buffer B over 1 min, and maintained at 3% buffer B for 14 min for next injection, in which buffer A is 0.1% TFA in H_2O and buffer B is 0.1% TFA in ACN (v/v). The peptide ion fragmentation was performed with an MS 300–2000 *m/z* scan and the three most intense ions were analyzed. Every MS/MS data was shown with the ChemStation Data Analysis module in a Mascot Generic File (*.mgf) and subsequently analyzed using MS/MS ion search module of Mascot software. The NCBI non-redundant (NCBInr) database and all taxonomic categories were considered during the search: two missing cleavage allowance by trypsin, fixed modification of carbamidomethyl, ±1.2 Da of peptide tolerance, ±0.8 Da of MS/MS tolerance, 1+, 2+ and 3+ of peptide charges, monoisotopic ions with no precursor and ESI-TRAP instrument. Individual ion scores obtained by the Mascot search based on peptide mass fingerprints were calculated as $-10 \times \log(P)$, where a random was presented as the probability (P) of the observed match. All ion scores with a threshold value >66 were considered to indicate identity or extensive similarity ($P < 0.05$) as defined by the Mascot probability analysis.

3. Results and discussion

The weight of whole egg was decreased linearly at a rate of 0.4–0.5 g/day during the first 9 days of incubation (Fig. 1A), which was well known due to the evaporation of egg moisture [19]. An accelerating decrease of the egg white weight was also observed during the first 5 days of incubation, and then leveled off afterwards (Fig. 1A). For the egg yolk (embryo part), the opposite trend occurred compared to the egg white. The weight of egg yolk increased slowly for the first 3 days of incubation, and rapidly from the 3rd to the 5th day and then laid off to the ninth day of incubation (Fig. 1A); this was mainly caused by the transferring of egg white content to embryo [2,20,21].

The pH of egg white increased rapidly from pH 8.8 to pH 9.3 at the first day of incubation, stabilized for two days, and then decreased rapidly from pH 9.2 to pH 8.3 from the 3rd to the 5th day of incubation, and further decreased to pH 7.9 at the ninth day (Fig. 1B). Previous studies showed that the pH of unfertilized egg white increased to around pH 9.5 after 3 day of storage at 4 and 22 °C, but remained stable afterwards [17,22]. The change in pH in fertilized egg whites was caused not only by gas exchange at elevated temperature, but also the metabolic process occurred during the embryonic development.

Changes of egg white proteins during incubation were analyzed using two-dimensional gel electrophoresis (2-DE) and 127 protein spots were subjected to LC–MS/MS. A total of 37 proteins, belonging to 'Gallus gallus', were identified from 91 detected protein spots (Fig. 2). Nineteen were identified in egg white for the first time and their gi numbers are 162952006, 229157, 10120552, 15988035, 157829560, 157831653, 157831883, 157831884, 262367842, 325053940, 345100466, 212881, 52138705, 71896765, 6729806, 14719680, 5738946, 363729530 and 365813051, respectively (Table 1). Significant changes in abundance vary for different protein spots during incubation; some major protein spots such as

Table 1 – The list of proteins identified by 2-DE and LC–MS/MS.

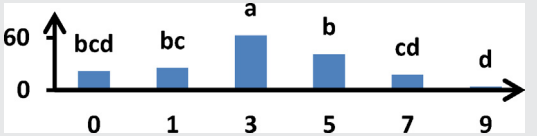
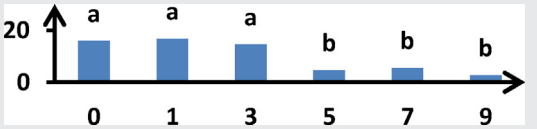
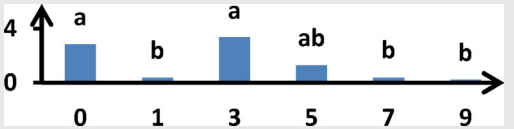
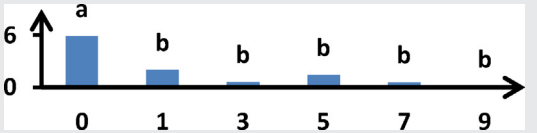
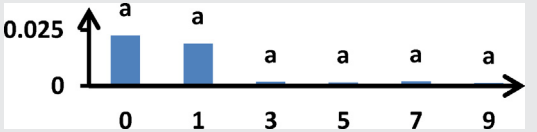
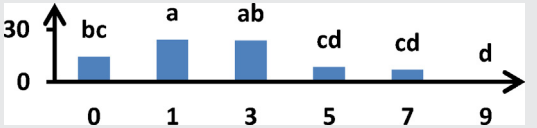
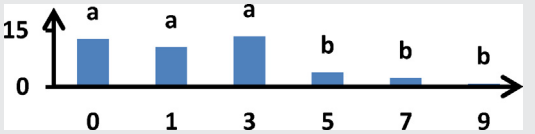
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
73	129293	Ovalbumin	622	52%	11/39	43.2/5.19	58.7/5.44	
69	129293	Ovalbumin	1855	61%	15/118	43.2/5.20	55.4/5.34	
5	129293	Ovalbumin	862	39%	11/50	43.2/5.19	52.2/4.57	
26	129293	Ovalbumin	761	47%	12/44	43.2/5.19	51.6/4.75	
29	129293	Ovalbumin	72	5%	1/4	43.2/5.20	51.3/5.11	
51	129293	Ovalbumin	695	35%	10/52	43.2/5.20	51.3/5.11	
24	129293	Ovalbumin	1443	51%	14/85	43.2/5.20	51.3/4.97	

Table 1 – (*Continued*)

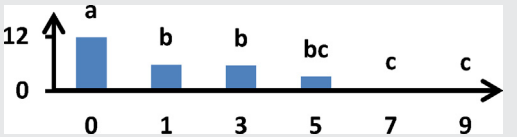
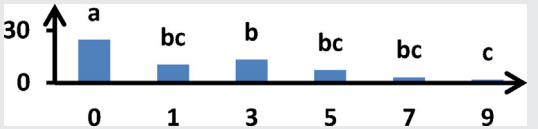
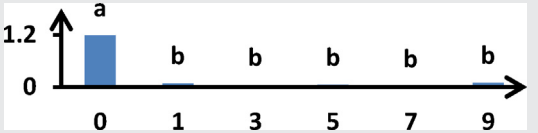
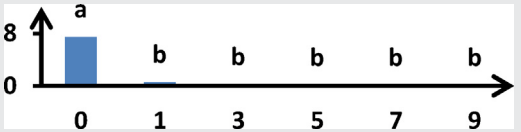
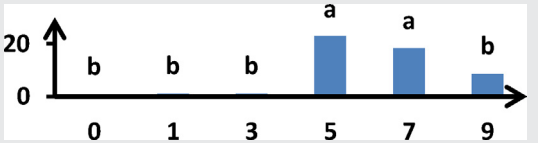
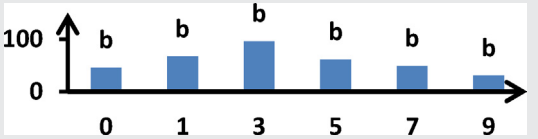
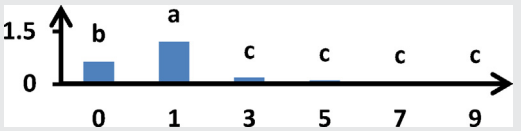
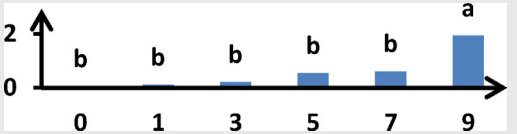
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
25	129293	Ovalbumin	938	43%	10/44	43.2/5.20	51.3/4.86	
52	129293	Ovalbumin	94	92%	2/8	4.34/5.36	50.8/5.03	
72	129293	Ovalbumin	832	47%	13/52	43.2/5.19	50.2/5.55	
71	129293	Ovalbumin	1513	54%	14/99	43.2/5.19	48.0/5.46	
36	129293	Ovalbumin	300	18%	4/14	43.2/5.19	47.0/6.03	
70	129293	Ovalbumin	1339	39%	12/103	43.2/5.20	46.5/5.34	
68	129293	Ovalbumin	483	26%	8/19	43.2/5.19	41.5/5.32	
46	129293	Ovalbumin	97	15%	2/2	43.2/5.19	39.1/5.15	

Table 1 – (*Continued*)

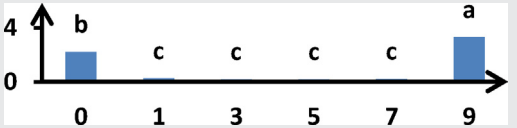
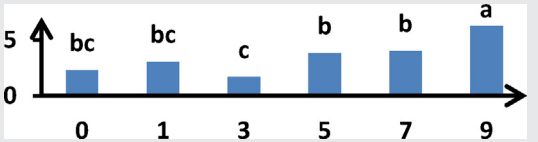
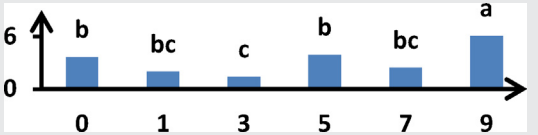
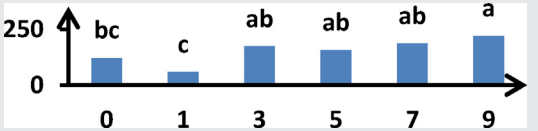
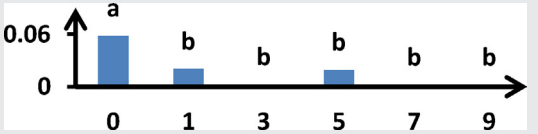
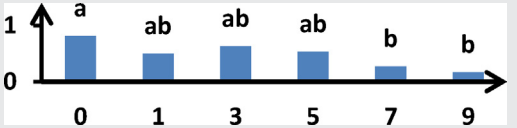
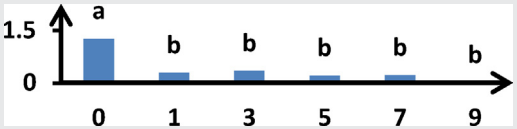
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
66	129293	Ovalbumin	165	12%	2/5	43.2/5.19	38.7/5.44	
49	129293	Ovalbumin	396	37%	8/20	43.2/5.19	37.3/5.23	
67	129293	Ovalbumin	556	39%	9/30	43.2/5.20	36.1/5.44	
3	129293	Ovalbumin	68	5%	1/2	43.2/5.19	35.9/6.55	
17	129293	Ovalbumin	79	10%	2/4	43.2/5.19	33.1/4.85	
64	129293	Ovalbumin	609	48%	10/39	43.2/5.20	30.4/5.31	
43	129293	Ovalbumin	94	9%	2/2	43.2/5.19	30.0/5.19	
84	129293	Ovalbumin	83	17%	3/6	43.2/5.19	29.0/5.67	

Table 1 – (*Continued*)

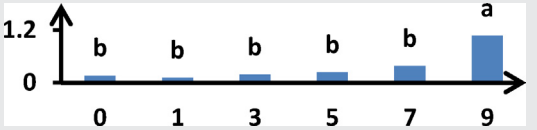
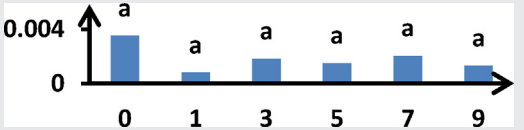
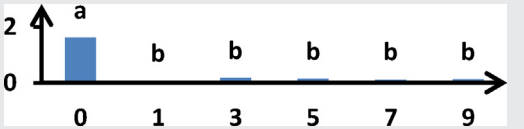
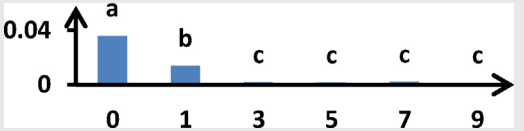
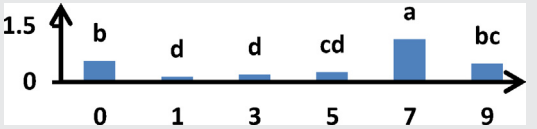
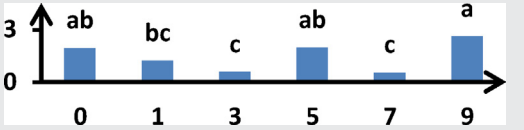
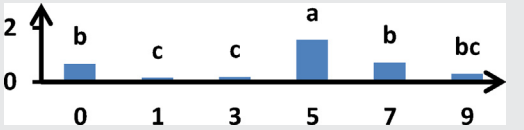
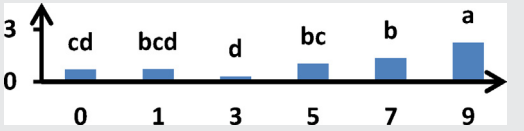
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
63	129293	Ovalbumin	182	14%	3/7	43.2/5.19	28.5/5.44	
65	129293	Ovalbumin	150	19%	3/7	43.2/5.19	27.5/5.28	
62	129293	Ovalbumin	356	23%	6/12	43.2/5.19	25.1/5.45	
31	129293	Ovalbumin	93	22%	3/4	43.2/5.19	22.5/5.14	
30	129293	Ovalbumin	99	10%	2/5	43.2/5.19	22.1/5.21	
61	129293	Ovalbumin	259	15%	4/9	43.2/5.19	21.8/5.37	
32	129293	Ovalbumin	129	11%	3/5	43.2/5.19	20.7/5.14	
60	129293	Ovalbumin	252	25%	6/16	43.2/5.19	19.9/5.51	

Table 1 – (*Continued*)

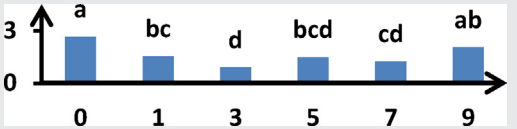
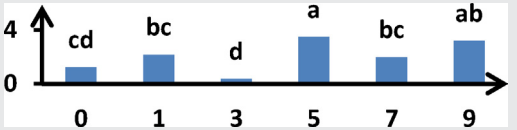
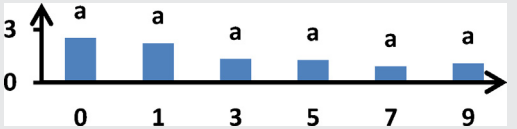
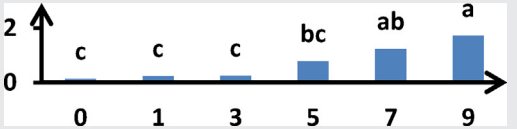
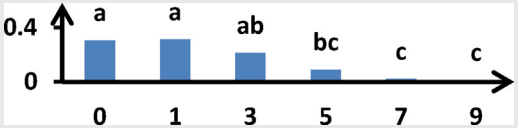
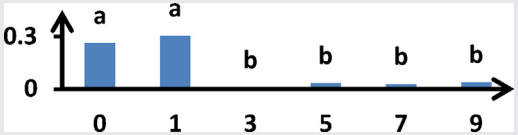
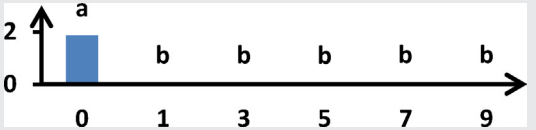
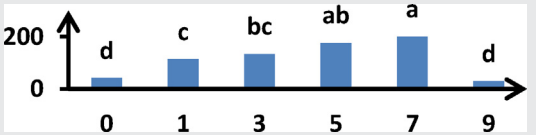
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
56	129293	Ovalbumin	262	22%	3/14	43.2/5.20	18.0/5.39	
57	129293	Ovalbumin	225	21%	4/12	43.2/5.20	16.3/5.39	
81	129293	Ovalbumin	182	18%	5/11	43.2/5.19	16.2/5.67	
59	129293	Ovalbumin	326	23%	6/19	43.2/5.20	15.2/5.28	
55	71897377	Ovalbumin-related protein Y	64	9%	2/4	44.0/5.20	24.3/5.35	
58	71897377	Ovalbumin-related protein Y	67	3%	1/2	44.0/5.20	20.6/6.39	
21	448824824	PREDICTED: ovalbumin-related protein Y	144	17%	5/10	45.0/5.98	51.2/6.07	
1	14719680	Ovotransferrin	1816	74%	19/239	37.2/6.73	74.8/6.66	

Table 1 – (*Continued*)

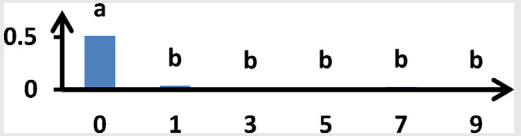
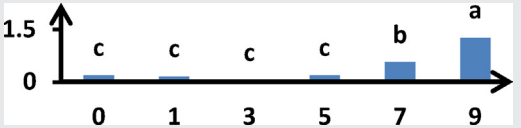
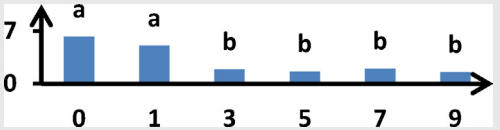
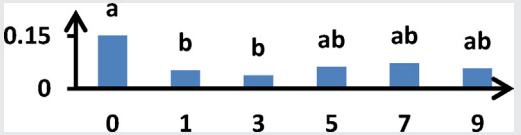
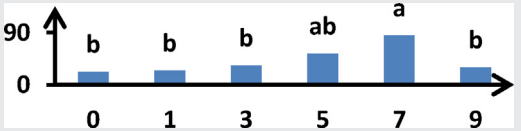
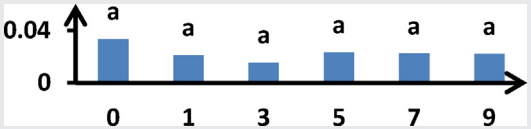
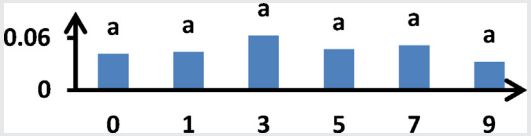
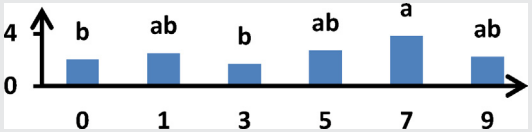
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
28	83754919	Ovotransferrin	1408	50%	24/126	77.5/6.70	48.7/6.27	
40	6729806	Ovotransferrin	79	7%	1/3	36.9/6.72	29.9/6.14	
82	1351295	Ovotransferrin	70	7%	2/3	79.6/6.85	21.2/6.30	
75	71274079	Ovotransferrin BC type	2334	48%	23/302	79.6/7.08	79.59/7.08	
11	71274079	Ovotransferrin BC type	561	23%	10/45	79.6/7.08	67.9/6.26	
33	71274079	Ovotransferrin BC type	461	34%	15/38	79.6/7.08	51.5/6.34	
27	71274079	Ovotransferrin BC type	201	7%	3/4	79.6/7.08	49.9/6.17	
50	71274079	Ovotransferrin BC type	101	7%	2/5	79.6/7.08	36.6/6.33	

Table 1 – (*Continued*)

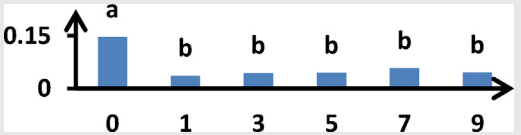
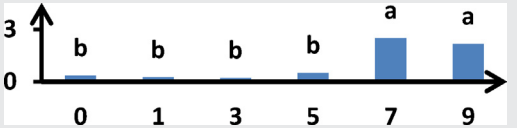
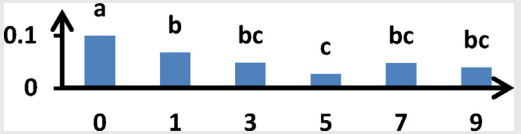
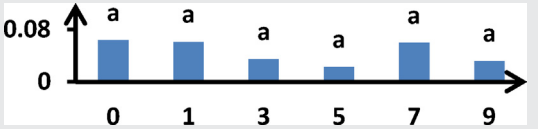
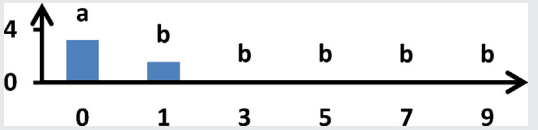
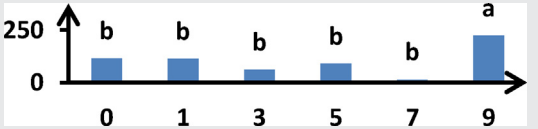
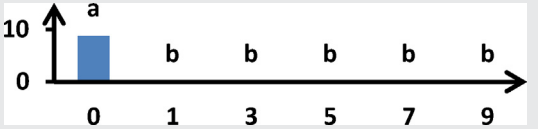
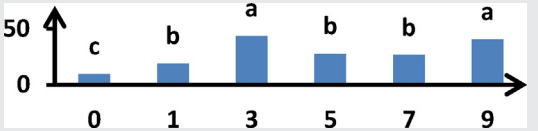
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
86	71274079	Ovotransferrin BC type	120	3%	1/2	79.6/7.08	36.0/5.75	
44	71274079	Ovotransferrin BC type	217	11%	3/10	79.6/7.08	32.5/6.41	
88	71274079	Ovotransferrin BC type	64	3%	1/2	79.6/7.08	29.9/5.96	
41	71274079	Ovotransferrin BC type	165	7%	2/8	79.6/7.08	27.8/6.31	
15	71895337	Ovoinhibitor precursor	565	23%	11/31	54.4/6.16	68.4/6.38	
9	71895337	Ovoinhibitor precursor	717	41%	16/56	54.4/6.16	66.6/6.49	
8	71895337	Ovoinhibitor precursor	167	17%	9/22	54.4/6.16	62.8/6.18	
23	223464	Ovomucoid	215	43%	4/13	21.2/4.78	41.7/4.80	

Table 1 – (*Continued*)

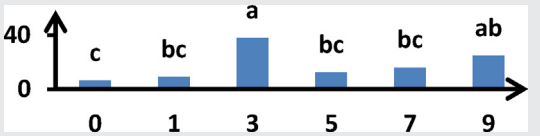
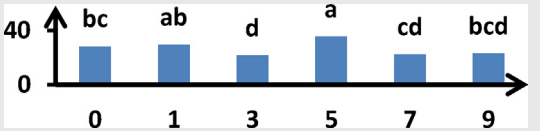
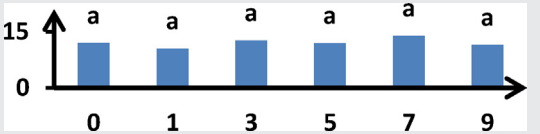
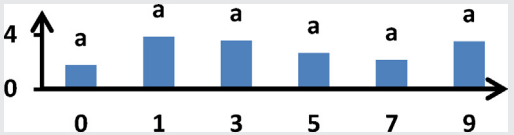
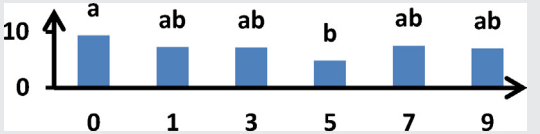
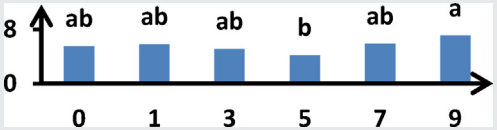
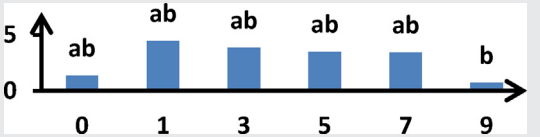
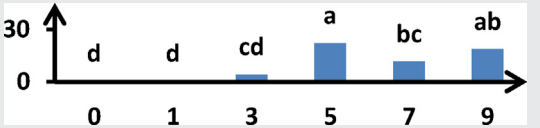
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
20	223464	Ovomucoid	187	37%	4/9	21.2/4.78	41.0/4.65	
14	223464	Ovomucoid	181	43%	4/9	21.2/4.78	37.8/4.81	
12	223464	Ovomucoid	226	49%	6/17	21.2/4.78	36.5/4.66	
10	223464	Ovomucoid	83	31%	3/6	21.2/4.78	36.5/4.42	
16	223464	Ovomucoid	192	49%	6/18	21.2/4.78	36.1/4.93	
19	223464	Ovomucoid	121	43%	4/8	21.2/4.78	32.6/4.59	
13	223464	Ovomucoid	150	43%	4/9	21.2/4.78	28.6/4.85	
53	223464	Ovomucoid	154	40%	4/8	21.2/4.78	10.2/5.53	

Table 1 – (*Continued*)

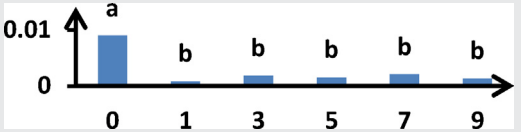
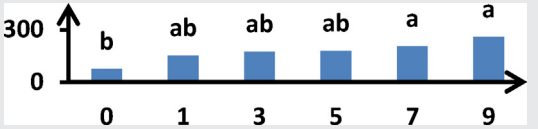
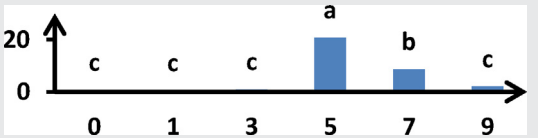
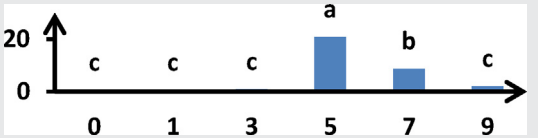
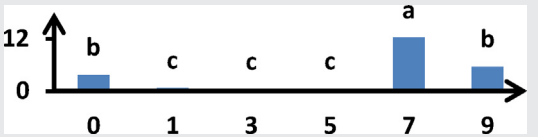
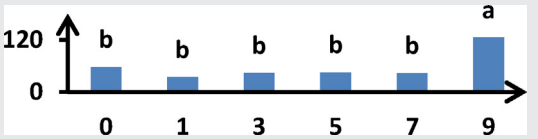
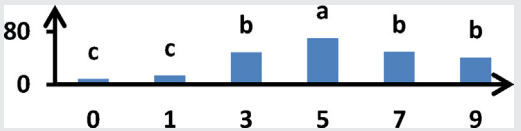
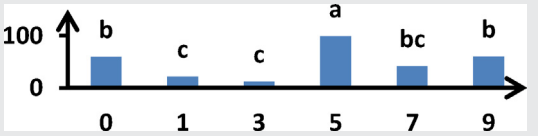
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
18	124757	Ovomucoid	168	53%	5/10	23.7/4.75	28.8/4.77	
35	162952006	Ovomucoid precursor	279	53%	5/12	23.5/4.75	32.4/4.93	
37	15988035	Lysozyme	173	45%	4/7	14.8/9.32	43.3/6.29	
37	157831884	Lysozyme	155	27%	3/5	14.7/9.46	43.3/6.29	
4	157831883	Lysozyme	86	12%	1/2	14.7/9.46	12.4/6.56	
6	229157	Lysozyme	328	85%	10/44	14.8/9.18	12.2/7.00	
90	229157	Lysozyme	340	72%	7/34	14.8/9.18	11.4/6.50	
7	157829560	Lysozyme	221	61%	4/43	14.7/8.49	11.6/6.65	

Table 1 – (*Continued*)

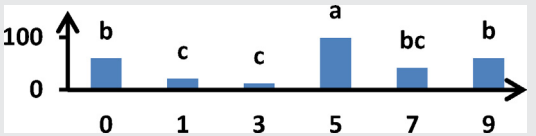
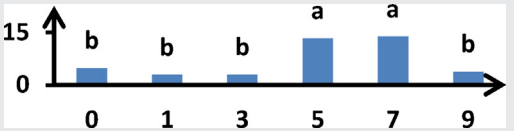
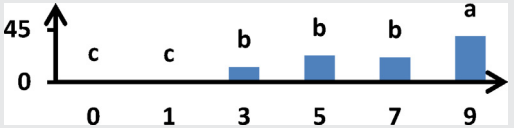
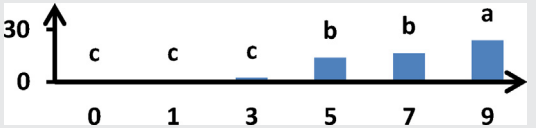
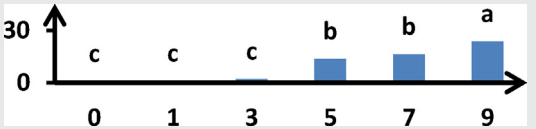
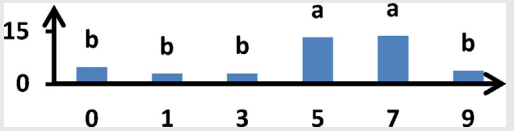
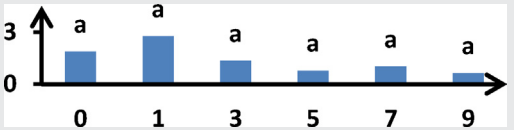
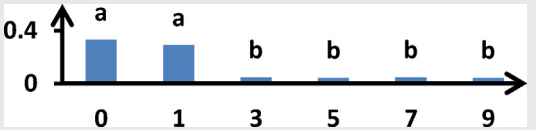
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
7	262367842	Lysozyme	242	73%	7/77	14.8/9.18	11.6/6.65	
85	126608	Lysozyme	109	34%	3/4	16.7/9.37	11.5/6.36	
77	10120552	Lysozyme	121	21%	2/3	14.8/9.32	10.8/5.97	
78	157831653	Lysozyme	111	21%	2/3	14.8/9.18	10.8/5.84	
78	325053940	Lysozyme	89	21%	2/4	14.8/9.46	10.8/5.84	
85	345100466	Lysozyme	107	38%	3/4	14.8/9.46	11.5/6.36	
38	45382467	Clusterin precursor	93	13%	4/8	51.9/5.48	37.4/5.05	
54	45382467	Clusterin precursor	86	3%	2/2	51.9/5.48	36.1/6.13	

Table 1 – (*Continued*)

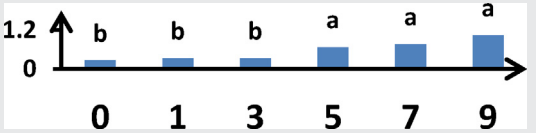
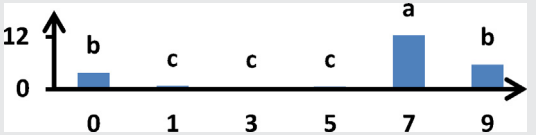
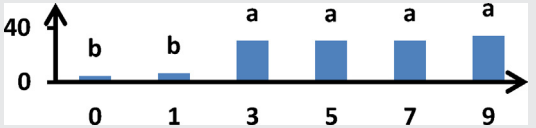
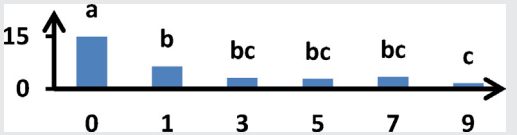
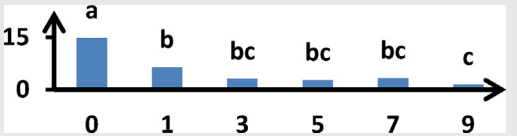
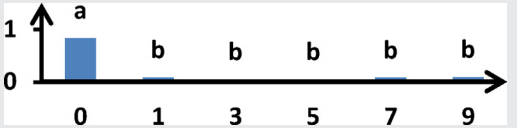
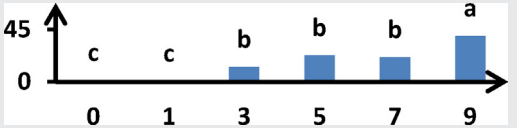
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
83	45382467	Clusterin precursor	111	6%	3/5	51.9/5.48	36.1/5.90	
42	45382467	Clusterin precursor	96	5%	2/6	51.9/5.48	34.1/5.12	
4	45383131	Hep21 protein precursor	82	15%	1/2	12.6/6.55	12.4/6.56	
22	45383093	Ovoglycoprotein precursor	165	15%	3/6	22.5/5.11	39.6/4.94	
79	365813051	Extracellular fatty acid-binding protein	312	40%	5/12	18.3/5.94	19.8/5.74	
79	45382221	Extracellular fatty acid-binding protein precursor	279	37%	5/14	20.3/5.56	19.8/5.74	
80	45383612	Prostaglandin D2 synthase, brain precursor	213	29%	4/12	21.0/6.30	19.4/5.87	
77	211503	Marker protein	115	21%	3/4	20.2/5.37	10.8/5.97	

Table 1 – (*Continued*)

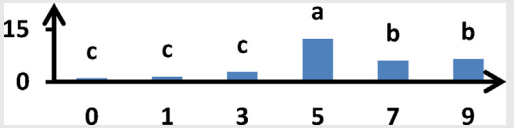
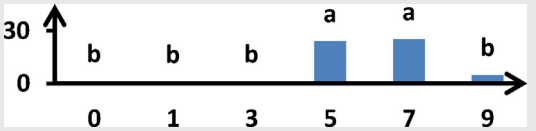
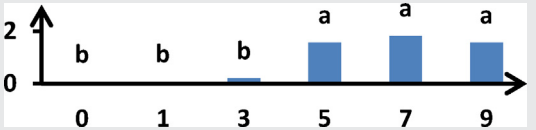
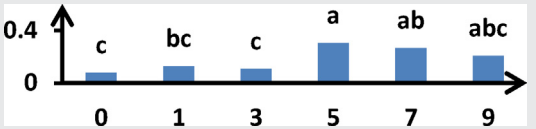
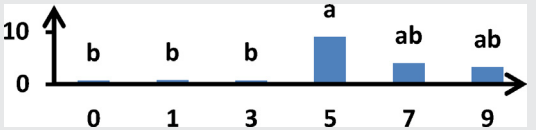
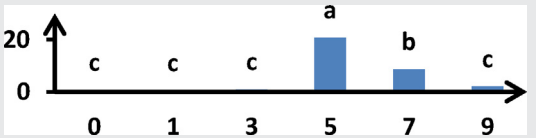
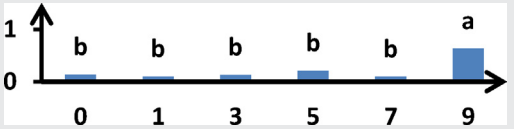
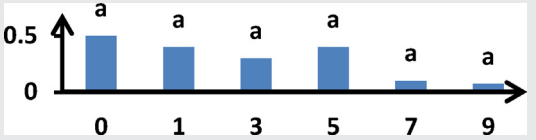
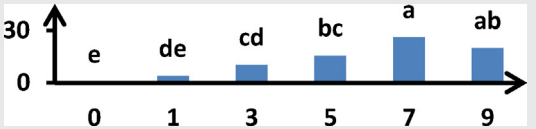
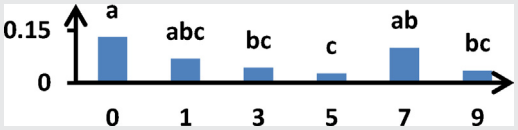
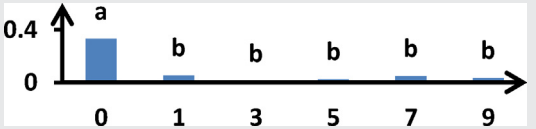
No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
2	212881	Vitellogenin	386	5%	8/24	20.7/9.22	43.9/6.71	
39	212881	Vitellogenin	132	2%	4/6	206.9/9.22	43.1/6.53	
34	212881	Vitellogenin	169	3%	4/13	20.7/9.22	42.0/6.07	
87	212881	Vitellogenin	264	4%	7/13	20.7/9.22	27.9/5.85	
89	52138705	Vitellogenin-1	160	1%	4/9	21.3/9.16	47.3/5.83	
37	71896765	Vitellogenin-2 precursor	150	2%	4/8	206.7/9.23	43.3/6.29	
91	5738946	Zona pellucida C protein	75	3%	1/2	48.6/5.72	42.0/5.75	
48	352173	Protein, riboflavin binding	151	31%	5/10	26.1/4.95	41.8/5.24	

Table 1 – (*Continued*)

No.[a]	gi[b]	Name	S[c]	C[d]	M[e]	Thr. Mr/pI	Obs. Mr/pI	Relative abundance[f]
47	352173	Protein, riboflavin binding	155	10%	2/3	26.1/4.95	36.3/5.16	
74	363729530	PREDICTED: hemopexin	297	38%	8/17	43.5/5.36	70.0/5.48	
45	45383974	Serum albumin precursor	220	24%	10/25	71.9/5.51	67.5/5.70	
76	45383974	Serum albumin precursor	181	32%	19/37	71.9/5.51	67.5/5.60	

[a] Protein sport number corresponding in Fig. 2.
[b] Protein accession number in NCBI database.
[c] Scores of Mascot and the threshold of score over 64.
[d] Sequence coverage (%).
[e] Number of the sequence-unique peptides/the number of total Marched peptides.
[f] Each column denotes the relative abundance of protein spot automatically calculated by 2-DE image analysis software based on their area and intensity; the number 0, 1, 3, 5, 7 and 9 in the X-axis denotes the incubation time, before incubation, 1 day, 3 days, 5 days, 7 days and 9 days; dissimilar letters on the top of columns denote significant difference ($P < 0.05$).

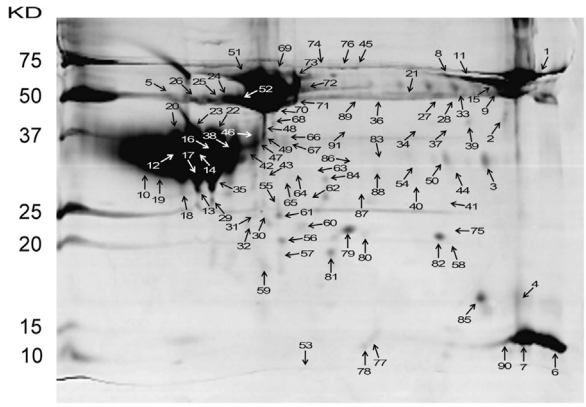


Fig. 2 – A representative 2-DE gel image of fertilized egg white proteins without incubation treatment (0 day incubation). An amount of 100 μg of total protein was loaded on the strip (11 cm, pH 4–7) and performed on SDS-PAGE following the staining with a Colloidal Blue Staining Kit.

all three vitellogenin protein spots (spots 34, 39 and 89), one ovalbumin spot (spot 36), one ovotransferrin spot (spot 50), one clusterin spot (spot 54), and one protein complex of lysozyme and vitellogenin-2 (spot 37) showed a rapid increase from 5 to 7 days of incubation and a slight reduction in abundance afterwards (within frame area of Fig. 3, Table 1). Changes of egg white proteins during incubations are presented based on protein family. The quantification of each protein spot was investigated by comparison of 'relative abundance' among different incubation day. One hundred micrograms of extracted egg white proteins was loaded in each 2-D gel. The increase or decrease of each protein means the proportion of this protein increase or decrease against the whole weight of egg white protein. Seven protein spots, spot 4, 7, 37, 77, 78, 79 and 85, were identified as protein complex: spot 4 (Lysozyme, gi 157831883; Hep21 protein precursor, gi 45383131), spot 7 (lysozyme, gi 157829560 and 262367842), spot 37 (lysozyme, gi 157831884 and 15988035; vitellogenin-2 precursor, gi 71896765), spot 77 (lysozyme, gi 10120552; marker protein, gi 211503), spot 78 (lysozyme, gi 157831653 and 325053940), spot 79 (extracellular fatty acid-binding protein precursor, gi 45382221 and 45382221) and spot 85 (lysozyme, gi 126608 and 345100466).

3.1. *Serpin family*

Ovalbumin is a glycoprotein with a molecular mass of 45 kDa [23]. In this study, 33 protein spots were identified as Serpin family proteins: ovalbumin (gi: 129293), ovalbumin-related protein X (gi: 448824824) and ovalbumin-related protein Y (gi: 71897377); out of 22 protein spots showed lower apparent molecular weight than the theoretical one, which was mainly due to the degradation of ovalbumin [17]. Omana et al. reported that the degradation of ovalbumin in infertile egg during storage may be due to the combined effect of proteolysis and increase in pH [13], while the degradation of ovalbumin in fertile egg during incubation might be caused mainly by a broad specificity of aminopeptidase and glutamyl aminopeptidase in egg white [17,24,25] at decreased pH during incubation (Fig. 1B). In addition, Whisstock et al. concluded that ovalbumin could just be a storage protein [26]. Sugimoto et al. suggested that egg white ovalbumin migrates into the developing embryo by changing its form to less ordered structures fitted to transportation. This result suggests that ovalbumin may have a dynamic function in developing organic cells [20]. In this study, 11 ovalbumin protein spots showed higher molecular weight than the theoretical one, probably due to the interaction with other proteins. During the first 9 days of incubation, 10 ovalbumin protein spots were increased significantly ($P < 0.05$) while 16 ovalbumin protein spots were significantly ($P < 0.05$) decreased in abundance (Table 1). In comparison, Qiu et al. identified only 17 ovalbumin spots and reported 14 ovalbumin protein spots increased while 3 ovalbumin protein spots decreased during the first 7 days of incubation [17]. Since ovalbumin consists of 54% (w/w) of egg white proteins [23], change of these major ovalbumin spots, such as spot 3, 17 and 69 in Fig. 2 (not identified by Qiu et al.),

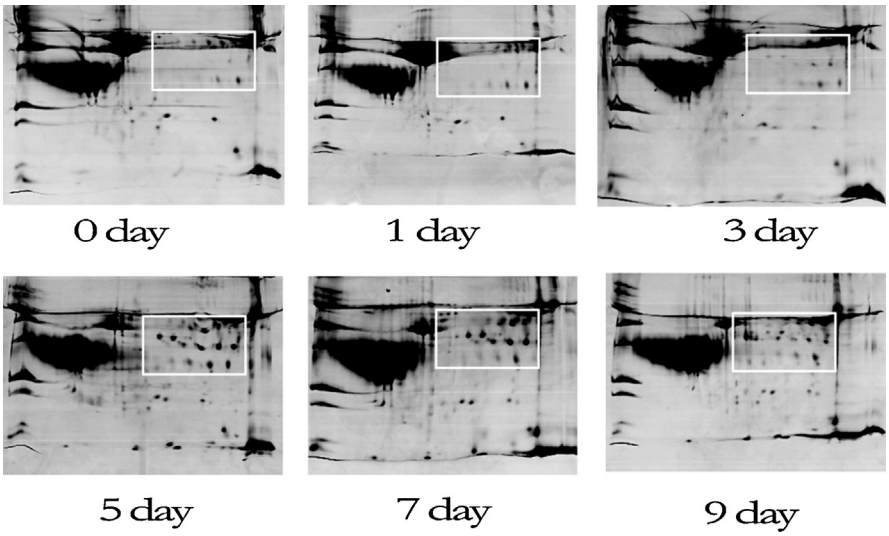


Fig. 3 – Comparison of 2-DE gel image of fertilized egg white proteins at different incubation days: 0, 1, 3, 5, 7 and 9 days.

probably play more important role than those faint ovalbumin spots.

Members of the ovalbumin family belong to serine proteinase inhibitors (serpins show strong inhibition against serine or cysteine proteases, with the exception of ovalbumin [27,28]. Ovalbumin-related proteins X and Y have been reported previously [8,12]. The prediction of its sequence (ovalbumin gene X gene) was confirmed by Guerin-Dubiard et al. [29]. A rapid increase of protein spot of ovalbumin-related protein Y from fertilized egg whites was observed at 7 days of incubation [17]. In this study, spot 21 was identified as ovalbumin-related protein X with gi number of 448824824, while 55 and 58 were identified as ovalbumin-related protein Y with gi number of 71897377 (Table 1). In contrast, our study showed that both of them were significantly ($P < 0.05$) decreased in abundance during the first 9 days of incubation. Ovalbumin-related protein Y was reported to have 21 potential phosphorylation sites and 4 potential N-glycosylations sites (NetPhos and NetNGlyc procedures at http://www.cbs.dtu.dk); further study is needed to understand whether these changes are related to embryo development.

3.2. *Transferrin family*

Ovotransferrin (conalbumin), a member of the transferrin family, is responsible for the transfer of ferric ions from hen oviduct to the embryo [30]. Keung and Azari suggested that there are two active sites for iron-transferring activity, FeNF and FeCF (NH_2- and COOH-terminal domains of iron-ovotransferrin) [31]. Giansanti et al. reported the antiviral activity of ovotransferrin toward chicken embryo fibroblast infection by Marek's disease virus (MDV) [32]. In this study, a total of 5 proteins, presenting in 13 protein spots, were identified as ovotransferrin (gi: 1351295, 6729806, 1471968 and 83754919) and ovotransferrin BC type (gi: 71274079), of two proteins (gi: 6729806 and 1471968) were detected in egg whites for the first time (Table 1). Ovotransferrin is the second most protein in egg white (12%) [23]. Qiu et al. identified two ovotransferrin spots [17]. Five major ovotransferrin spots 11, 27, 33, 50 and 82 were detected in this study, showing the change of ovotransferrin in abundance during embryo development.

Increase in abundance of one ovotransferrin spot was reported in infertile eggs during 10 days of storage at 22 °C [13]. In fertilized eggs, significant increase of ovotransferrin was also determined by Qiu et al., who reported increase of two ovotransferrin spots during early incubation stages (0–5 days) [17,33]. In this study, five protein spots (spot 28, 75, 82, 86 and 88) were significantly ($P < 0.05$) decreased at the first 3 days of incubation while two protein spots (spot 40 and 44) were significantly ($P < 0.05$) increased from 5 to 7 days of incubation (Table 1). Gentili et al. found ovotransferrin and its receptor played an important role during chondrogenesis and endochondral bone formation in developing chick embryo [34]. The increasing in abundance of two ovotransferrin spots in our study agreed with the result of Genrili et al.'s result. They found that ovotransferrin was first observed in the 7 day cartilaginous rudiment, and at the later stage the high level of ovotransferrin receptors was observed in the 13 day old tibia in the diaphysis collar of stacked-osteoprogenitor cells, the layer of derived osteoblasts, and the primordial of the menisci [34].

3.3. *Protease inhibitors Kazal family*

Ovoinhibitor, containing seven domains in a single polypeptide chain, is a member of protease inhibitor Kazal family with inhibitory activity against serine proteinase, such as trypsin, chymotrypsin, subtilisin and elastase [8,14]. It also exhibits antimicrobial activities against *Bacillus thuringiensis* to protect chick embryo [35]. Ovoinhibitor spots were detected from fertilized egg whites during incubation and also from unfertilized egg whites during storage [13,17]. In our study, three protein spots (gi: 71895337) were identified as ovoinhibitor precursor in egg whites; spots 8 and 15 were significantly ($P < 0.05$) decreased at the first day of incubation while spot 9 was significantly ($P < 0.05$) increased from the 7th to 9th day of incubation (Table 1). Baintner and Feher found that trypsin inhibitor content of albumen gradually passed into the amniotic cavity and was taken up by chick embryo during embryo development, which was supposed that between 11 and 18 days of embryonic development the trypsin inhibitor passes from the gut to the yolk sac through the vitellointestinal duct; the thin yolk contained only traces of trypsin inhibitor; and the allantoic fluid was entirely free from it [36].

Ovomucoid, one of the major egg white proteins (11%), is a member of protease inhibitor Kazal family [37], presenting inhibition effect on trypsin and chymotrypsin as well as locusta trypsin and chymotrypsin-like enzyme isolated from the African migratory locust *Locusta migratoria* [38]. Ovomucoid is also the prominent albumen protein that could be detected in blood during embryo development and after hatching [39]. Information on change of ovomucoid during incubation has not been reported in Qiu et al.'s study [17]. In this study, a total of 3 proteins, presenting in 11 protein spots, were identified as ovomucoid (gi: 124757 and 223464) and ovomucoid precursor (gi: 162952006), which was detected in egg whites for the first time (Table 1). Protein spot 18 was significantly ($P < 0.05$) decreased at the first day of incubation while four protein spots (spot 20, 23, 35 and 53) were significantly ($P < 0.05$) increased during the 9th day of incubation. It is noteworthy that all three major ovomucoid spots (20, 23 and 35) increased, indicating that ovomucoid was not used during early stage of incubation (9 days). Their apparent molecular weights are all higher than theoretical one, due to the formation of complexes with other proteins [22]. This might also contribute to increased protein abundance observed.

3.4. *Family 22 of glycosyl hydrolases*

Lysozyme, representing in hen egg white by lysozyme C, is a well-studied antimicrobial protein (3.4% of total egg white proteins) [37,40]. In this study, 11 proteins were identified as lysozyme, of 10 proteins, with the gi numbers of 229157, 10120552, 15988035, 157829560, 157831653, 157831883, 157831884, 262367842, 325053940, 345100466, were detected in egg whites for the first time (Table 1). Lysozyme protein spots were usually identified as protein complexes, such as spot 7 containing 2 proteins (gi: 157829560 and 262367842), spot 37 containing 2 proteins (gi: 15988035 and 157831884), spot 78 containing 2 proteins (gi: 325053940 and 157831653), spot 85 containing 2 proteins (gi: 126608 and 345100466), due mainly to the interactions between lysozyme and other avian proteins

[12,17]. Lysozyme has the pI value of 9.4; however, the pI value of other spots such as 6, 7 and 90, is around 6.5–7.0, which is mainly due to the limitation of the gel detecting-ability (pI 4–7). The biological function of lysozyme in fowl eggs is unclear, but it probably serves as a defense mechanism before the embryo reaches the ability to produce immunoglobulins [41]. Three spots 37, 85 and 90 were significantly ($P < 0.05$) increased during the first 5 days of incubation and then were significantly ($P < 0.05$) decreased from the 5th to 9th day of incubation, spots 6, 77 and 78 were also significantly ($P < 0.05$) increased during the 9 days of incubation, especially the spot 6 is one of the major lysozyme spots (spot 6, 7 and 85) in this study. Qiu et al. detected only one protein spot as lysozyme C with a MW of 66 kDa after 2 days of incubation, followed by slight decrease after 2 days of incubation [17]. Fang et al. reported that the concentration of lysozyme remained stable during early incubation stage [33]. However, the activity of lysozyme from fertilized eggs was reported to rapidly decrease during the first 12 incubation days due to its physical unavailability, resultant from interaction with conalbumin [42].

3.5. *Lipocalin family*

Glycoprotein in chicken egg whites has been known for a long time; however, its functional and biological properties are still not fully understood [43,44]. Eight glycoprotein spots were detected by Guerin-Dubiard et al. [8]. Qiu et al. did not detect this protein in fertile egg. In this study, one protein spot (spot 22) was identified as glycoprotein precursor with gi number of 45383093; its abundance was significantly ($P < 0.05$) increased from 1 to 3 days of incubation, and then keep stable until 9th day (Table 1).

Prostaglandin (PG) D_2 synthase has been observed in many organs that may play important roles in reproduction, regulation of sleep and pain responses, and multiple aspects of allergy and inflammation through its receptor systems [45]. In this study, protein spot 80 was identified as prostaglandin D2 synthase, brain precursor, with gi number of 45383612. This protein started to decrease significantly ($P < 0.05$) from the 1st day of incubation, and then keep stable until 9th day (Table 1). Two PG D2 synthase spots from fertilized egg white were identified by Qiu et al.; one decreased in abundance during 7 days of incubation while another increased at first two days and then decreased from 2 to 7 days of incubation [17]. PG D2 synthase was significant decreased in unfertilized egg whites during 30 days of storage at room temperature [13].

Extracellular fatty acid binding protein (Ex-FABP) is a 21 kDa lipocalin specifically binding fatty acids, expressed during chicken embryo development in hypertrophic cartilage, muscle fibers and blood granulocytes [46]. Desert et al. and Guerin-Dubiard et al. detected a form of Ch21 in unfertilized egg whites by 2-DE [6,8]. In our study, three proteins were identified as extracellular fatty acid-binding protein precursor (gi: 45382221), marker protein (also called Ch21 protein with gi number of 211503) and extracellular fatty acid-binding protein (gi: 365813051). Galline Ex-FABP was detected in egg whites for the first time in this study; this protein was reported to function as an antibacterial siderocalin and a lysophosphatidic acid sensor through dual ligand specificities [47]. Marker protein (spot 77) significantly ($P < 0.05$) increased in abundance during the 9 days of incubation. Protein spot 79 was identified as a protein complex of extracellular fatty acid-binding protein precursor and extracellular fatty acid-binding protein and its abundance significantly ($P < 0.05$) decreased during the 9 day incubation (Table 1). This protein was not reported by Qiu et al. [17].

In this study, four protein spots were identified as clusterin precursor with the gi number of 45382467; spot 54 was significantly ($P < 0.05$) decreased from 1 to 3 days of incubation while spot 83 was significant ($P < 0.05$) increased in abundance from 3 to 5 days of incubation. In comparison, Qiu et al. [17] also reported that four protein spots of clusterin from fertilized egg whites were decreased in abundance during 7 days of incubation [17]. Omana et al. reported that clusterin of unfertilized eggs underwent degradation but its abundance increased during storage, which might be partially responsible for egg white thinning during storage [13]. Clusterin is a widely expressed secreted glycoprotein in numerous biological fluids and mainly acts as an extracellular chaperone to inhibit stress-induced aggregation and precipitation during embryo development [48,49]. Base on our result about the changes of clusterin, lysozyme and ovotransferrin, the changes of clusterin might be considered as a response of the changes of lysozme and ovotransferrin, especially all of them present significant changes in different extent during the first 5 days of incubation.

Hep21 protein is a member of the uPAR/CD59/LY-6/snake neurotoxin superfamily identified by Nau et al. [50]. In this study, spot 4 was identified as Hep21 protein precursor with gi number of 45383131. Spot 4 was first decreased at the first day of incubation, increased from 5 to 7 days of incubation, and finally decreased from 7 to 9 days of incubation (Table 1). Qiu et al. did not detect this protein in their study [17]. The uPAR protein is an important mediator in the cellular process of cancer development and invasion, angiogenesis, and metastasis; the Ly-6 type antigens are involved in T-lymphocyte activation; the CD59 proteins are the most potent inhibitors of complement-mediated lysis [50]. However, it is hard to conclude a role of Hep21 because of its various characters in structure and function with above proteins in this the uPAR/CD59/LY-6/snake neurotoxin superfamily [50].

3.6. *Folate receptor family*

Egg-white riboflavin binding protein (flavoprotein), constituting approximately 0.8% of the total egg white proteins, was first isolated and characterized by Li-Chan and Nakai [37] and Hamazume et al. [51]. It is a phosphoglycoprotein, comprising of 219 amino acid residues, found in egg white as well as in egg yolk and serum of laying hens [37]. In this study, two protein spots (spot 47 and 48) were identified as riboflavin binding protein with gi number of 352173. Spot 48 did not show change ($P > 0.05$) while spot 47 was significantly increased ($P < 0.05$) in abundance during the 9 days of incubation (Table 1). Qiu et al. did not detect flavoprotein in fertile egg white [17]. Flavoprotein is one of the most acidic proteins in egg white with an isoelectric point close to 4.0, and its biological function is to store and bind riboflavin (vitamin B2), an essential vitamin, to sustain embryo's growth until hatching [15]. It was reported that embryos died of riboflavin deficiency on or near 13 days

of incubation and the embryos could be rescued by injecting riboflavin or riboflavin-5′ phosphate into the eggs [52], which suggests the requirement of riboflavin for embryos development will occur before 13 days. The increase of riboflavin binding protein during first 9 days of incubation suggested that more riboflavin might be stored by riboflavin binding protein until for further use. Egg-white riboflavin binding protein was reported to vary over a 10-fold range, which may reflect the possibility that it also serves as a scavenger of riboflavin and thus protect embryos against bacterial attack [16,52].

3.7. *Serum albumin precursor*

Two protein spots, spots 45 and 76, were identified as serum albumin precursor with the same gi number of 45383974. Our previous work reported there is no significant ($P < 0.05$) difference of serum albumin precursor in abundance among six commercial unfertilized eggs [12]. In this study, both spots were significantly ($P < 0.05$) decreased in abundance at the first day of incubation, and then keep stable until the 9th day (Table 1). Qiu et al. did not detect serum albumin precursor in fertile egg white [17]. Chicken serum albumin genes and apolipoprotein II genes specify the most abundant mRNA species present in livers of hens [53]; however, little information related to the biological functions of serum albumin precursor was reported.

3.8. *Lipoprotein of yolk*

Vitellogenin is the major yolk precursor protein characterized in the livers of oviparous vertebrates [54]. As the precursor of the lipoprotein and phosphoprotein, vitellogenin makes up most of the protein content of yolk [54–56], in which HDLs (high-density lipoproteins) are the result of the proteolytic cleavage of a precursor, vitellogenin [57]. It is also reported that vitellin membrane is gradually weakened during storage or incubation at high temperature [58]. However, even at the 0 day without incubation, vitellogenin was still detected in egg white sample, which suggests it should not come from the degradation of vitelline membrane during the embryo development. In addition, characterization data showed that the score values were pretty high, which indicated it was impossibly from yolk as the contamination with trace amount during sampling. In this study, the absolute abundance of vitellogenin spots (calculated based on the spot area and density as described in Section 2.3, *date not shown*) is much higher than detecting-limitation, which excluded the possibility of samples contamination by egg yolk while sampling. Therefore, in this study, vitellogenin proteins were detected in egg whites for the first time. Three proteins were identified as vitellogenin (gi: 212881; spots 2, 34, 39 and 87), vitellogenin-1 (gi: 52138705; spot 89) and vitellogenin-2 (gi: 71896765; spot 37). All vitellogenin protein spots significantly ($P < 0.05$) increased in abundance from 3 to 5 days of incubation (Table 1). Qiu et al. did not detect these vitellogenin proteins in fertile egg white [17]. As the derivatives of vitellogenin, HDLs, a mading up of 75–80% proteins and 20–25% lipids in native egg yolk, are complexed to phosvitins to form the granular structure through phosphocalcic bridges [57]. Further investigation was required to understand the biological functions of vitellogenin proteins in chicken egg white.

3.9. *Protein from other tissue*

Chicken hemopexin was previously found in chicken serum and cerebrospinal fluid [59,60]. Using peptide ligand libraries coupled with 2-DE technique, a protein, 'similar to hemopexin', was identified [10]. In this study, hemopexin protein was detected in spot 74 with the gi number of 363729530 in egg white for the first time. Qiu et al. did not detect hemopexin in fertile egg white [17]. Its abundance was significantly ($P < 0.05$) decreased during 9 days of incubation (Table 1). Hemopexin is a serum glycoprotein that binds heme, and transports it to the liver for iron release and free hemopexin to return to the circulation [61].

The zona pellucida protein, secreted by both the oocyte and the follicular cells, is a membrane glycoprotein surrounding the plasma membrane of an oocyte [62]. The gene sequence of zona pellucida C protein was firstly reported by Kono and Matsuda [63]. In this study, protein spot 91 was identified as zona pellucida C protein with the gi number of 5738946. Zona pellucida C protein was detected in egg whites for the first time. Qiu et al. did not detect this protein in fertile egg white [17]. Its abundance was significantly ($P < 0.05$) increased from 7 to 9 days of incubation (Table 1). Chicken zona pellucid is composed of three proteins. Its function is mainly involved in the prevention of polyspermy and the protection of the developing embryo prior to implantation [62]. Conner et al. recently suggested its potential defects of zona genes and their proteins associated with defined pathology [62].

4. Conclusion

Qiu et al. conducted a proteomic analysis of fertile egg white proteins during the first week of embryonic development [17]; however, only eight egg white proteins in egg white were identified. In comparison, 37 proteins were identified in the current study, out of 19 were identified for the first time both in fresh and incubated fertilized egg whites. Four lipoproteins vitellogenin, vitellogenin-1, vitellogenin-2 and zona pellucida C protein, reported previously only in egg yolk and chicken oocyte were also identified in egg white for the first time. Therefore our study provided greater spectrum of metabolic fate of egg white proteins during embryo development, such as all ovomucoid protein spots with one exception were significantly ($P < 0.05$) increased, the abundance of vitellogenin protein, zona pellucid C protein, glycoprotein, marker protein and one flavoprotein spot (spot 47) were significantly ($P < 0.05$) increased while that of hemopexin, serum albumin precursor, extracellular fatty acid-binding protein precursor and Galline Ex-FABP were significantly ($P < 0.05$) decreased. Understanding the metabolic fates of egg proteins during incubation will facilitate unlock their biological roles during embryo development.

Acknowledgements

This work was supported by grants from Natural Sciences and Engineering Research Council of Canada (NSERC), Alberta

Livestock and Meat Agency (ALMA) and Alberta Egg Producers (AEP) to J. Wu.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2013.11.001.

REFERENCES

[1] Carinci P, Manzoli-Guidotti L. Albumen absorption during chick embryogenesis. J Embryol Exp Morphol 1968;20:107–18.
[2] 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.
[3] Cook Sr MI, Toranzos B, Arendt GAWJ. Incubation reduces microbial growth on eggs shells and the opportunity for trans-shell infection. Ecol Lett 2005;8:532–7.
[4] Shawkey MD, Firestone MK, Brodie EL, Beissinger SR. Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. PLos ONE 2009, http://dx.doi.org/10.1371/journal.pone.0004522.
[5] Alabdeh M, Lechevalier V, Nau F, Gautier M, Cochet MF, Jan S, 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.
[6] Desert C, Guerin-Dubiard C, Nau F, Jan G, Val F, Mallard J. Comparison of different electrophoretic separations of hen egg white proteins. J Agric Food Chem 2001;49:4553–61.
[7] Cancedda FD, Dozin B, Rossi F, Molina F, Cancedda R, Negri A, et al. The Ch21 protein, developmentally regulated in chick embryo, belongs to the superfamily of lipophilic molecule carrier proteins. J Biol Chem 1990;265:19060–4.
[8] 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.
[9] Mann K. The chicken egg white proteome. Proteomics 2007;7:3558–68.
[10] 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 Protemone Res 2008;7:3461–74.
[11] Mann K, Mann M. In-depth analysis of the chicken egg white proteome using an LTQ Orbitrap Velos. Proteome Sci 2011, http://dx.doi.org/10.1186/1477-5956-9-7.
[12] Wang J, Liang Y, Omana DA, Kav NNV, Wu J. Proteomic analysis of egg white proteins from different egg varieties. J Agri Food Chem 2012;60:272–82.
[13] Omana DA, Liang Y, Kav NNV, Wu J. Proteomic analysis of egg white proteins during storage. Proteomics 2011;11:144–53.
[14] Oubre CO, D'Hondt E, Moore RW, Hargis BM, Berghman LR. The chicken pituitary expresses an ovoinhibitor-like protein in subpopulations of some, but not all, hormone-producing cell types. Omestic Anim Endocrinol 2003;25:389–97.
[15] Amoresano A, Brancaccio A, Andolfo A, Perduca M, Monaco HL, Marino G. The carbohydrates of the isoforms of three avian riboflavin-binding proteins. Eur J Biochem 1999;263:849–58.
[16] Huopalahti R, Lopez-Fandino R, Anton M, Schade R. Bioactive egg compounds. Subpart Ib: Egg white compounds. Chapter 10: Riboflavin-binding protein (flavoprotein). Berlin, Heidelberg: Springer-Verlag; 2007. p. 69–74.
[17] Qiu N, Ma M, Cai Z, Jin Y, Huang X, Huang Q, et al. Proteomic analysis of egg white proteins during the early phase of embryonic development. J Proteomics 2012, http://dx.doi.org/10.1016/j.jprot020110120137.
[18] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1967;72:248–54.
[19] Ohi A, Inoue N, Furuta H, Sugawara M, Ohta Y. Development of a method to control the water evaporation of hatching eggs during incubation. Poult Sci 2010;89:551–7.
[20] 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.
[21] Moran Jr ET. Nutrition of the developing embryo and hatching. Poult Sci 2007;86:1043–9.
[22] Wang J, Omana DA, Wu J. Effect of shell egg storage on ovomucin extraction. Food Bioprocess Technol 2011, http://dx.doi.org/10.1007/s11947-011-0558-y.
[23] Li-Chan ECY, Powrie WD, Nakai S. The chemistry of eggs and egg products. In: Stadelman WJ, Cotterill OJ, editors. Egg science and technology. 4th ed. Binghamton, New York: Haworth Press; 1995.
[24] Sharma M, Luthra-Guptasarma M. Degradation of proteins upon storage at near-neutral pH: indications of a proteolytic/gelatinolytic activity associated with aggregates. Biochim Biophys Acta 2009;1790:1282–94.
[25] Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. Factors affecting short-term and long-term stabilities of proteins. Adv Drug Deliv Rev 2001;46:307–26.
[26] Whisstock J, Skinner R, Lesk AM. An atlas of serpin conformations. Trends Biochem Sci 1998;23:63–7.
[27] Worrall DM, Blacque OE, Barnes RC. The expanding superfamily of serpins: searching for the real targets. Biochem Soc Trans 1999;27:746–50.
[28] Hirose J, Doi Y, Kitabatake N, Narita H. Ovalbumin-related gene Y protein bears carbohydrate chains of the ovomucoid type. Biosci Biotechnol Biochem 2006;70:144–51.
[29] Guerin-Dubiard C, Pasco M, Hietanen A, Quiros del Bosque A, Nau F, Croguennec T. Hen egg white fraction by ion-exchange chromatography. J Chromatogr A 2005;1090:58–67.
[30] Richards MP, Huang TL. Metalloprotein analysis by capillary isoelectric focusing. J Chromatogr B 1997;690:43–54.
[31] Keung WM, Azari P. Structure and function of ovotransferrin. II. Iron-transferring activity of iron-binding fragments of ovotransferrin with chicken embryo red cells. J Biol Chem 1982;257:1184–8.
[32] Giansanti F, Rossi P, Massucci MT, Botti D, Antonini G, Valenti P, et al. Antiviral activity of ovotransferrin discloses an evolutionary strategy for the defensive activities of lactoferrin. Biochem Cell Biol 2002;80:125–30.
[33] Fang J, Ma M, Jin Y, Qiu N, Ren G, Huang X, et al. Changes in the antimicrobial potential of egg albumen during the early stages of incubation and its impact on the growth and virulence response of *Salmonella enteritidis*. Ital J Anim Sci 2012;11:92–7.
[34] 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.
[35] Bourin M, Gautron J, Berges M, Attucci S, Le Blay G, Labas V, et al. Antimicrobial potential of egg yolk ovoinhibitor, a multidomain Kazal-like inhibitor of chicken egg. J Agric Food Chem 2011;59:12368–74.
[36] Baintner KJr, Feher G. Fate of egg white trypsin inhibitor and start of proteolysis in developing chick embryo and newly hatched chick. Dev Biol 1974;36:272–8.

[37] Li-Chan E, Nakai S. Biochemical basis for the properties of egg white. Crit Rev Poult Biol 1989;2:21–58.
[38] Huopalahti R, Lopez-Fandino R, Anton M, Schade R. Bioactive egg compounds. Subpart Ib: Egg white compounds. Chapter 13: Antiproteases. Berlin, Heidelberg: Springer-Verlag; 2007. p. 85–92.
[39] ETM. Jr. Nutrition of the developing embryo and hatching. Poult Sci 2007;86:1043–9.
[40] Proctor VA, Cunningham FE. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. CRC Crit Rev Food Sci Nutr 1988;26:359–95.
[41] Huopalahti R, Lopez-Fandino R, Anton M, Schade R. Bioactive egg compounds. Subpart Ib: Egg white compounds. Chapter 6: Lysozyme. Berlin, Heidelberg: Springer-Verlag; 2007. p. 33–42.
[42] Cunningham FE. Changes in egg white during incubation of the fertile egg. Poult Sci 1974;53:1561–5.
[43] Ketterer B. A glycoprotein component of hen egg white. Life Sci 1962;5:163–5.
[44] Ketterer B. Ovoglycoprotein, a protein of hen's-egg white. Biochem J 1965;96:372–6.
[45] Saito S, Tsuda H, Michimata T. Prostaglandin D2 reproduction. Am J Reprod Immunol 2002;47:295–302.
[46] Descalzi-Cancedda F, Dozin B, Zerega B, Cermelli S, Cancedda R. Ex-FABP: a fatty acid binding lipocalin developmentally regulated in chicken endochondral bone formation and myogenesis. Biochim Biophys Acta 2000;1482:127–35.
[47] Correnti C, Clifton MC, Abergel RJ, Allred B, Hoette TM, Ruiz M, et al. Galline Ex-FABP is an antibacterial siderocalin and a lysophosphatidic acid sensor functioning through dual ligand specificities. Structure 2011;19:1796–806.
[48] Mahon MG, Lindstedt KA, Hermann M, Nimpf J, Schneider WJ. Multiple involvement of clusterin in chicken ovarian follicle development. J Biol Chem 1999;274:4036–44.
[49] Poona S, Treweek TM, Wilsonb MR, Easterbrook-Smithc SB, Carvera JA. Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. FEBS Lett 2002;513:259–66.
[50] Nau F, Guerin-Dubiard C, Desert C, Gautron J, Bouton S, Gribonval J, et al. Cloning and characterization of HEP21, a new member of the uPAR/Ly6 protein superfamily predominantly expressed in hen egg white. Poult Sci 2003;82:242–50.
[51] Hamazume Y, Mega T, Ikenaka T. Characterization of hen egg white- and yolk-riboflavin binding proteins and amino acid sequence of egg white-riboflavin binding protein. J Biochem 1984;95:1633–44.
[52] White III HB, Merrill Jr AH. Riboflavin-binding proteins. Ann Rev Nutr 1988;8:279–99.
[53] Hache RJG, Wiskocil R, Vasa M, Roy RN, Lau PCK, Deeley RG. The 5′ noncoding and flanking regions of the avian very low density apolipoprotein II and serum albumin genes Homologies with the egg white protein genes. J Biol Chem 1983;258(7):4556–64.
[54] Nardelli D, van het Schip FD, Gerber-Huber S, Haefliger JA, Gruber M, Ab G, et al. Comparison of the organization and fine structure of a chicken and a *Xenopus laevis* vitellogenin gene. J Biol Chem 1987;262:15377–85.
[55] van het Schip FD, Samallo J, Broos J, Ophuis J, Mojet M, Gruber M, et al. Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. J Mol Biol 1987;196:245–60.
[56] Clark RC. The primary structure of avian phosvitins. Contributions through the Edman degradation of methylmercaptovitins prepared from the constituent phosphoproteins. Int J Biochem 1985;17:983–8.
[57] Huopalahti R, Lopez-Fandino R, Anton M, Schade R. Bioactive egg compounds. Subpart Ia: Egg yolk compounds. Chapter 3: High-density lipoproteins (HDL) or lipovitellin fraction. Berlin, Heidelberg: Springer-Verlag; 2007. p. 13–6.
[58] Reijrink I. Storage of hatching eggs: effects of storage and early uncubation conditions on egg characteristics, embryonic development, hatchability, and chick quality. Thesis from Wageningen University.
[59] Huang SY, Lin JH, Chen YH, Chuang CK, Chiu YF, Chen MY, et al. Analysis of chicken serum proteome and differential protein expression during development in single-comb White Leghorn hens. Proteomics 2006;6:2217–24.
[60] Parada C, Gato A, Aparicio M, Bueno D. Proteome analysis of chick embryonic cerebrospinal fluid. Proteomics 2006;6:312–20.
[61] Altruda F, Tolosano E, Hemopexin:. structure, function, and regulation. DNA Cell Biol 2002;21:297–306.
[62] Conner SJ, Lefievre L, Hughes DC, Barratt CL. Cracking the egg: increased complexity in the zona pellucida. Hum Reprod 2005;20:52–1148.
[63] Kono Y, Matsuda T. Gallus gallus zona pellucida C protein gene; 1999. Published Only in NCBI Database http://www.ncbi.nlm.nih.gov/protein/5738946