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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

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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).

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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