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Postfertilization Changes in Nutritional Composition and Protein Conformation of Hen Egg

Xiang Duan,[‡] Mei Li,[‡] Fengfeng Wu,[‡] Na Yang,[‡] Mehdi Nikoo,[‡] Zhengyu Jin,[†,‡] and Xueming Xu*[,†,‡]

[†]State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, China

[‡]School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, China

ABSTRACT: Fertilized hen egg is traditionally considered as a dietary supplement in many Asian countries. This work aimed to obtain information on the effect of fertilization on total nutritional composition of egg contents and protein conformation. Chemical analysis showed that the lipid level in fertilized egg began to decrease after day 9. Fertilized egg before day 9 was higher in essential free amino acids (EFAA) and monounsaturated fatty acids (MUFA) contents but lower in cholesterol and polyunsaturated fatty acids (PUFA) than unfertilized counterparts. Fertilized egg proteins were characterized by an increase in hydrophobicity and a decrease in electrostatic interaction. Circular dichroism analysis showed that β-sheet decreased with increasing incubation time, whereas unordered structure increased. The findings observed in this work provide a crucial basis for understanding nutritional composition and protein conformation of fertilized egg, with the potential of being utilized as an EFAA/MUFA-rich, low-cholesterol dietary supplement.

KEYWORDS: *fertilized egg, chemical composition, dietary supplement, protein conformation*

INTRODUCTION

Hen egg has been recognized as an excellent source of nutrients and foods for centuries.[1] There are two distinct types of hen egg, unfertilized and fertilized eggs. The eggs in the market are mostly those that are unfertilized.[2] In fact, fertilized egg is traditionally considered as a natural dietary supplement in some Asian countries. The *Compendium of Materia Medica*, the most famous Chinese pharmacopoeia, has recorded that fertilized egg contributes to appetite increase and immune function enhancement, especially for pregnant women and the elderly.[3]

There have been some reports describing changes in chemical composition during incubation period, such as developmental relationships between yolk and embryo body composition,[4] changes in amount of carbohydrates in yolk,[5] and utilization of docosahexaenoic acid (DHA) and some amino acids.[6,7] These research studies[4−8] paid more attention to mobilization and utilization of biochemical composition by the chick embryo and to egg white and yolk alterations from the perspective of physiology. However, information regarding the change in total nutritional composition of egg contents from the perspective of food is still limited.

Hen eggs are also used as a vital ingredient in several foods, especially for their exceptional functional properties, which mainly depend on the physicochemical properties of egg proteins.[9] These proteins might be expected to undergo conformational and structural changes during embryonic development. An understanding of these proteins' structural characteristics provides the fundamental basis for investigation of their applications.[10] To our knowledge, this is the first study to examine changes in the conformation and structure of egg proteins during incubation.

The objectives of the present study were to (1) investigate the change in total nutritional composition of egg contents and (2) determine the protein conformation during the incubation period, thereby providing information for application of fertilized egg.

MATERIALS AND METHODS

Materials. Unfertilized and fertilized hen eggs were obtained from a local producer (Zudai Poultry Co., Wuxi, Jiangsu, China). The eggs were incubated at 37.8 °C and 60% relative humidity in a benchtop incubator (Brinsea Products, Banwell, UK). Eggs were turned automatically through an angle of 90° every hour. Fertilized eggs were obtained on days 3, 6, 9, 12, and 15 of the incubation period. On each day, representing the development stage studied, 10 eggs were obtained and the entire contents were homogenized in a DS-1 high-speed tissue triturator (Specimen Model Factory, Shanghai, China). The obtained samples were then lyophilized for analysis. The process was repeated three times for each development stage. All animal protocols in this study adhered to the *Guide for the Care and Use of Laboratory Animals* (Ministry of Science and Technology of China, 2006) and were approved by the animal ethics committee of Jiangnan University. We made all efforts to minimize animal use and suffering in these experiments. Wide molecular weight markers (NEB, P7702) (New England Biolabs Ltd., Beijing, China) were used to identify proteins in SDS-PAGE. The protein and phospholipid (PL) standards for studying the molecular weight (MW) distribution and PL composition were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade (Shanghai Chemical Reagents Co., Shanghai, China).

Analysis of Protein Composition. *Protein Content.* The crude protein content of unfertilized and fertilized egg was determined by AOAC's Kjeldahl method conducted according to the procedure of Tkachuk,[11] and a nitrogen conversion factor of 6.25 was used to quantify the crude protein content.

Protein Extraction. The unfertilized and fertilized samples were defatted by means of washing with at least 20 volumes (w/v) of cold

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acetone and dried overnight at 4 °C. Dried acetone powder was stored at −70 °C. Proteins were extracted from the defatted dried powdered pulp by agitation with PBS overnight at 4 °C. After centrifugation at 2500*g* for 15 min at 4 °C, the supernatant was collected, filtered, and centrifuged at 12000*g* for 3 min. The proteins extracted were dialyzed using a molecular weight cutoff (MWCO) of 200 Da to remove salts. The resulting retentates were lyophilized for further research. The protein concentration was estimated by using the biuret method of Hatefi et al.,[12] and the protein contents of lyophilized samples at different stages were about 91%; there were no significant differences (data not shown) among different samples. All extracts were stored at −70 °C for further research.

Molecular Weight Distribution. The proteins extracted as indicated above were used to investigate the MW distribution. The MW distribution profiles were estimated according to the method described by Liu et al.[13] A Waters 600 liquid chromatography system (Waters Co., Milford, MA, USA) equipped with a 2487 UV detector was used for this experiment. The UV detector was set at 215 nm. The column used was a TSK gel filtration column, 300 × 7.8 mm (Tosoh Co., Tokyo, Japan), whereas the mobile phase consisting of PBS was delivered at a flow rate of 0.5 mL/min, and 20 μL of sample was injected into the high-performance liquid chromatography (HPLC) system. A MW calibration curve was obtained from the following standards from Sigma: bovine serum albumin (66000 Da), carbonic anhydrase (29000 Da), and aprotinin (6500 Da).

SDS-PAGE. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Laemmli.[14] The proteins extracted were dissolved in a 0.1 M Tris-HCl dissociation buffer containing 4% (w/v) SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.05 mg/mL bromophenol blue (tracker dye), at a concentration of 3 mg of powder/mL of buffer, heated in a boiling water for 5 min, and then allowed to cool at room temperature. Samples were then centrifuged at 10000*g* for 10 min, and 15 μL of the supernatant was subjected to SDS-PAGE, using 5% stacking gel and 10% separating gel. Electrophoretic separation was performed in an SE 600 Ruby (GE Healthcare Bio-Sciences, Piscataway, NJ, USA)n applying a maximum voltage of 500 V. At the end of the run, proteins were stained with 0.1% Coomassie brilliant blue in 12.5% trichloroacetic acidn and Coomassie stain was visualized using a GelDoc-It TS Imaging System (UVP, Upland, CA, USA). The optical densities of bands in the digitized images were determined with the Gel-Pro Analyzer 32 program (Media Cybernetics, Rockville, MD, USA).

Analysis of Free Amino Acid (FAA) Composition. FAA composition was measured according to the method described by Lyndon et al.[15] HPLC separation of the amino acid derivatives was carried out on ODS HYPERSIL (Agilent Technologies, Palo Alto, CA, USA), UV detector set at 338 nm (262 nm for Pro, Hypro). The flow rate was set at 1.0 mL min^{-1}. Stepwise elution was performed, and the results were expressed as milligrams per gram of dry matter.

Analysis of Lipid Composition. *Lipid Extraction.* Lipid from unfertilized and fertilized eggs was extracted according to the method described by Kaewmanee.[16] Sample (10 g) was homogenized with 200 mL of a mixture composed of chloroform, methanol, and distilled water (50:100:50, v/v/v) by an Ultra-Turrax homogenizer (IKA T10 basic, IKA Werke GmbH and Co., Staufen, Germany) for 2 min. The homogenate was then treated with 50 mL of chloroform and homogenized for 1 min. Distilled water (25 mL) was added, and the mixture was homogenized for 30 s at the same speed. The mixture was centrifuged at 3000*g* for 10 min and transferred into a separating funnel. The chloroform phase (bottom phase) was drained off into the Erlenmeyer flask containing anhydrous sodium sulfate (1−2 g) and stirred thoroughly to remove the residual water. Lipid in chloroform was decanted into a round-bottom flask through a filter paper. The chloroform was evaporated at 35 °C, with a rotary evaporator (RE52CS, Yarong Biochemical Instrument Factory, Shanghai, China), and the residual solvent was removed under a stream of nitrogen. The lipid content was measured gravimetrically. Lipid samples were kept in an amber vial under nitrogen at −20 °C until further analysis.

Phospholipid Class. The lipid extracted as indicated above was used to measure the phospholipid class. Lipid classes were separated by high-performance thin layer chromatography (HPTLC) with a Camag TLC Scanner 3 (CAMAG Co., Muttenz, Switzerland) according to the method described by Cejas et al.[17] Lipid sample (15 μL) was spotted on the TLC plates using a microsyringe. The solvent system used was methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9 by vol). Lipid classes were quantified by charring with a copper acetate reagent followed by calibrated scanning densitometry using a Shimadzu CS-9001PC dual-wavelength flying spot scanner (254 nm). PL standards from Sigma were used to identify and quantify PLs.

Cholesterol Content. The cholesterol content was measured according to the method described by Bragagnolo and Rodriguez-Amaya.[18] Lyophilized sample (0.3 ± 0.01 g) of unfertilized and fertilized egg was mixed with 5 mL of 2% KOH in absolute ethanol and heated at 50 °C for 120 min in a water bath. The mixture was then cooled under running water, and 5 mL of distilled water was added. Cholesterol was extracted twice with 10 mL of hexane. An aliquot (3 mL) of the hexane extract was dried under nitrogen, redissolved in 3 mL of acetonitrile/isopropanol (70:30), and injected into the Shimadzu LC-20A HPLC system (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a Spherisorb ODS-2 (5 μm), 4.6 × 150 mm, preceded by a 4.6 × 10 mm Spherisorb ODS-2 (5 μm) guard column. The mobile phase (flow rate = 1 mL min^{-1}) consisted of acetonitrile/isopropanol (70:30 v/v). Each run took 15 min. Absorption spectra were taken at 190−300 nm and the chromatograms at 210 nm. Quantitative analysis was carried out by external standardization method.

Fatty Acid Composition. The lipid extracted as indicated above was used to measure the fatty acid (FA) composition. The FA composition was measured as fatty acid methyl esters (FAME), by a gas chromatograph GC-2010 (Shimadzu Co., Tokyo, Japan) equipped with a flame ionization detector (FID).[16] PEG-20 M (30 m, 0.32 mm i.d.) was used for separation. Nitrogen was used as the carrier gas at a flow rate of 3.0 mL/min. The initial column temperature was set at 120 °C for 3 min, followed by a ramp of 10 °C min^{-1} to 190 °C, and then at 2 °C min^{-1} to reach a final temperature of 220 °C, which was held for 20 min. The detector temperature at the injection port was maintained at 250 °C. Retention times for FAME standards were used to identify chromatographic peaks. Fatty acid content was expressed as grams per 100 g of lipid.

Analysis of Protein Conformation. *Free Sulfhydryl (SH) Content.* The SH content was measured according to the method of Li et al.[19] Ellman's reagent was prepared by dissolving 40 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 10 mL of Tris−Gly buffer (10.4 g of Tris, 6.9 g of Gly, and 1.2 g of disodium ethylenediaminetetraacetic acid (EDTA) in 1000 mL of deionized water, pH 8.0). Unfertilized and fertilized egg proteins were dissolved with the Tris−Gly−SDS buffer (45 mL Tris−Gly buffer containing 5 mL of 25% SDS aqueous solution) to a final concentration of 10 mg/mL. Four milliliter samples were mixed with 0.04 mL of DTNB (4 mg/mL) solution. Subsequently, the mixture was shaken and incubated at room temperature for 10 min. The supernatant was centrifuged (10000*g*, 20 min), and the absorbance of supernatant was measured at 412 nm on a UV−vis spectrophotometer (UV1100, Beijing Ruili Instrument Co., Beijing, China). A standard curve using reduced glutathione was used, and the results were expressed as micromoles of SH per gram of protein.

Surface Hydrophobicity (H_o). Surface hydrophobicity (H_o) of proteins was measured according to the method of Alizadeh-Pasdar et al.,[20] using 1-anilinonaphthalene-8-sulfonic acid (ANS) as the fluorescence probe. Protein dispersions were diluted (0.005−0.2% w/v) in 10 mM phosphate buffer (pH 7.0). Then, aliquots (20 mL) of ANS (8.0 mM in the same buffer) were added to 4 mL of sample. Fluorescence intensity was measured with a Hitachi F4500 fluorescence spectrometer (Tokyo, Japan) at wavelengths of 390 nm (excitation) and 470 nm (emission). The initial slope of fluorescence intensity versus protein concentration plot was used as an index of H_o.

Zeta-Potential. Zeta-potential of proteins was measured using a Zetasizer 2000 (Malvern Instruments, Southborough, UK). The unfertilized and fertilized egg proteins were diluted with PBS, with a

Table 1. Protein and Lipid Amounts (Grams per Egg) in Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

	unfertilized egg	day 3	day 6	day 9	day 12	day 15
protein	6.56 ± 0.36a	6.31 ± 0.44a	6.50 ± 0.32a	6.76 ± 0.48a	6.49 ± 0.30a	6.28 ± 0.28a
lipid	5.61 ± 0.51a	5.21 ± 0.41ab	5.41 ± 0.32ab	4.91 ± 0.45ab	4.81 ± 0.21b	4.71 ± 0.51b

[a]Different letters (a, b) in the same row indicate significant differences ($P < 0.05$).

Table 2. Molecular Weight Distribution (Percent) of Proteins in Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

sample	unfertilized egg	day 3	day 6	day 9	day 12	day 15
>30000 Da	80.26 ± 3.24ab	77.15 ± 3.63bc	73.28 ± 2.97c	70.75 ± 2.87d	74.24 ± 3.12cd	84.39 ± 2.56ad
5000−30000 Da	14.38 ± 1.25c	16.17 ± 0.87bc	17.38 ± 1.54b	20.78 ± 1.38a	17.84 ± 1.09b	8.65 ± 1.14d
<5000 Da	5.36 ± 0.27d	6.68 ± 0.41c	9.34 ± 0.54a	8.46 ± 0.59ab	7.92 ± 1.04b	6.97 ± 0.63bc

[a]Different letters (a−d) in the same row indicate significant differences ($P < 0.05$).

concentration around 2 mg/mL. The samples were injected into the apparatus, and the averages of three measurements were reported as zeta-potential.

Secondary Structures. The secondary structures of proteins were determined using circular dichroism (CD) spectra. The CD spectra was scanned at the far-UV range (250−190 nm) with a spectropolarimeter (MOS-450/AF-CD, Bio-Logic, France), using a quartz cuvette with a 1 mm optical path length at room temperature. The CD spectra of unfertilized and fertilized egg proteins were measured using distilled water as a blank solution, with a scan speed of 30 nm/min and a bandwidth of 1.0 nm. The CD spectra could be used to estimate the relative content of protein secondary structures. The secondary structures of proteins were analyzed by the algorithm SELCON3. Four secondary structures were calculated: α-helices, β-sheets, β-turns, and unordered.

Dielectric Constant. The dielectric constants (ε') of unfertilized and fertilized egg proteins were measured using a Precision Impedance Analyzer (65120B, Wayne Kerr Electronics, London, UK) within the range from 10 kHz to 15 MHz.

Statistical Analysis. The experiments were run in triplicate. The results are expressed as the mean ± standard deviation and were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze data, and Duncan's multiple-range tests were conducted to determine differences between the means. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Changes in Protein and Lipid Contents. As shown in Table 1, there was no significant difference in protein content between unfertilized and fertilized eggs. The lipid level in fertilized egg was lower than that of unfertilized egg, and a significant decrease in lipid content was observed after day 9 of incubation. Brian et al.[21] reported that glucose and amino acids formed the main energy source at early stages of development, and then the energy metabolism was dominated by utilization of lipid. This observation may contribute to the decrease in lipid level after day 9 of incubation.

Egg proteins are nutritionally complete proteins with an unsurpassable balance of amino acids. According to the World Health Organization, egg protein has the highest true digestibility among major food proteins.[2] Our result showed that there was no significant difference in protein amount between unfertilized and fertilized eggs. In addition, the egg yolk lipid content has usually been labeled as a troublesome dietary factor. Our result exhibited that the lipid level in fertilized egg after day 9 was lower than that of unfertilized egg. This nutritional property might make fertilized egg suitable for people with special dietary requirements.

Protein Pattern. Results of MW distribution are shown in Table 2. The pattern of MW distribution of proteins was assessed at >30000, 5000−30000, and <5000 Da. The percentage of fraction higher than 30000 Da decreased gradually up to day 9 and then began to increase. The increase after day 9 indicated that the chemical synthesis of new protein molecules occurred during incubation. Correspondingly, the percentage of MW lower than 5000 reached a maximum on day 6 and then decreased gradually in later stages. The decrease in fraction below 5000 Da, mainly including peptides and amino acids, was in accordance with the speculation that peptides and amino acids might participate in protein synthesis during embryogenesis.[22]

The SDS-PAGE analysis of protein pattern for fertilized egg is shown in Figure 1. SDS-PAGE was processed using a 5% stacking gel and a 10% separating gel. During the incubation period, the amount of yolk-derived proteins (apovitellins, apovitellenins, and livetins) decreased. Conversely, optical density results indicated that white-derived protein (ovotransferrin, ovoglobulin, and ovalbumin) amounts displayed little fluctuation (data not shown).

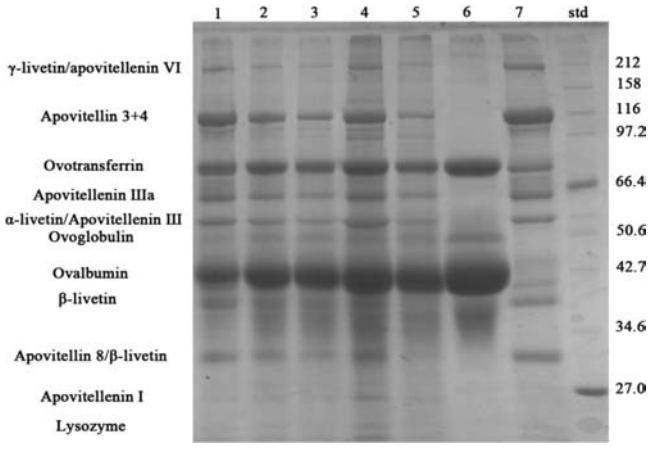

Figure 1. SDS-PAGE analysis of egg proteins on 5% stacking gel and 10% separating gel. Lanes: 1−5 for fertilized egg proteins on days 3, 6, 9, 12, and 15, respectively; 6, for unfertilized egg white proteins; 7, for unfertilized egg yolk proteins; std, for MW standard.

Table 3. Free Amino Acid Composition (Milligrams per Gram of Dry Weight) of Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

FAA	unfertilized egg	day 3	day 6	day 9	day 12	day 15
nonessential						
Asp	1.24 ± 0.21ab	1.30 ± 0.17a	1.16 ± 0.14ab	1.12 ± 0.09ab	(9.27 ± 1.32) × 10^{-1}b	(9.88 ± 2.11) × 10^{-1}b
Glu	1.32 ± 0.11b	1.93 ± 0.08a	(9.88 ± 1.19) × 10^{-1}c	1.90 ± 0.19a	1.02 ± 0.10c	1.11 ± 0.25bc
Ser	(2.03 ± 0.38) × 10^{-2}b	(6.10 ± 0.43) × 10^{-2}a	(2.81 ± 0.32) × 10^{-3}c	(1.68 ± 0.26) × 10^{-2}b	(4.04 ± 0.32) × 10^{-4}e	(1.03 ± 0.13) × 10^{-3}d
Gly	(7.21 ± 0.53) × 10^{-1}c	(8.15 ± 0.67) × 10^{-1}ab	(7.82 ± 0.41) × 10^{-1}bc	(7.16 ± 0.38) × 10^{-1}c	(7.46 ± 0.44) × 10^{-1}bc	(8.96 ± 0.28) × 10^{-1}a
Ala	(4.42 ± 0.41) × 10^{-1}ab	(3.87 ± 0.29) × 10^{-1}bc	(4.36 ± 0.26) × 10^{-1}ab	(4.97 ± 0.53) × 10^{-1}a	(2.65 ± 0.24) × 10^{-1}d	(3.56 ± 0.31) × 10^{-1}c
Cys-s	(1.58 ± 0.11) × 10^{-2}d	(2.97 ± 0.32) × 10^{-2}b	(2.26 ± 0.38) × 10^{-2}c	(3.95 ± 0.19) × 10^{-2}a	(6.90 ± 0.21) × 10^{-3}e	(7.86 ± 0.54) × 10^{-3}e
Pro	1.96 ± 0.22a	1.87 ± 0.32a	2.04 ± 0.19a	1.89 ± 0.32a	1.72 ± 0.18a	1.79 ± 0.32a
∑NEFAAs	5.72 ± 0.63abc	6.40 ± 0.67a	5.43 ± 0.41abc	6.18 ± 0.69ab	4.78 ± 0.48c	5.14 ± 0.84bc
essential						
His	(1.10 ± 0.21) × 10^{-1}a	(1.37 ± 0.24) × 10^{-1}a	(1.05 ± 0.11) × 10^{-1}a	(1.10 ± 0.19) × 10^{-1}a	(9.31 ± 0.56) × 10^{-2}a	(1.33 ± 0.11) × 10^{-1}a
Thr	(5.21 ± 0.22) × 10^{-1}cd	(5.72 ± 0.27) × 10^{-1}c	(6.73 ± 0.33) × 10^{-1}b	(7.89 ± 0.41) × 10^{-1}a	(7.32 ± 0.82) × 10^{-1}ab	(4.81 ± 0.21) × 10^{-1}d
Arg	(6.49 ± 0.25) × 10^{-1}c	(7.60 ± 0.53) × 10^{-1}ab	(7.36 ± 0.61) × 10^{-1}bc	(8.61 ± 0.25) × 10^{-1}a	(6.32 ± 0.27) × 10^{-1}c	(8.63 ± 1.02) × 10^{-1}a
Tyr	(5.41 ± 0.34) × 10^{-1}c	(5.65 ± 0.43) × 10^{-1}bc	(6.35 ± 0.86) × 10^{-1}ab	(6.60 ± 0.21) × 10^{-1}a	(4.11 ± 0.19) × 10^{-1}d	(2.34 ± 0.22) × 10^{-1}e
Val	(5.47 ± 0.32) × 10^{-1}c	(5.81 ± 0.18) × 10^{-1}bc	(6.12 ± 0.43) × 10^{-1}b	(6.81 ± 0.39) × 10^{-1}a	(1.51 ± 0.15) × 10^{-1}d	(7.70 ± 0.24) × 10^{-2}e
Met	(1.39 ± 0.15) × 10^{-1}c	(2.27 ± 0.13) × 10^{-1}a	(2.38 ± 0.21) × 10^{-1}a	(1.71 ± 0.11) × 10^{-1}b	(1.48 ± 0.17) × 10^{-2}d	(3.28 ± 0.29) × 10^{-2}d
Phe	(3.96 ± 0.21) × 10^{-1}ab	(4.39 ± 0.30) × 10^{-1}a	(4.14 ± 0.31) × 10^{-1}ab	(3.78 ± 0.13) × 10^{-1}b	(1.62 ± 0.28) × 10^{-1}c	(1.50 ± 0.14) × 10^{-1}c
Ile	(3.83 ± 0.16) × 10^{-1}a	(4.05 ± 0.21) × 10^{-1}a	(4.16 ± 0.35) × 10^{-1}a	(4.28 ± 0.33) × 10^{-1}a	(2.60 ± 0.25) × 10^{-1}b	(1.93 ± 0.17) × 10^{-1}c
Leu	(6.17 ± 0.25) × 10^{-1}b	(7.04 ± 0.34) × 10^{-1}a	(6.70 ± 0.41) × 10^{-1}ab	(6.06 ± 0.56) × 10^{-1}b	(2.80 ± 0.07) × 10^{-1}c	(2.68 ± 0.21) × 10^{-1}c
Lys	(5.61 ± 0.28) × 10^{-1}b	(6.98 ± 0.87) × 10^{-1}a	(6.17 ± 0.66) × 10^{-1}ab	(5.81 ± 0.41) × 10^{-1}b	(4.28 ± 0.34) × 10^{-1}c	(6.50 ± 0.54) × 10^{-1}ab
∑EFAAs	4.46 ± 0.21b	5.29 ± 0.35a	5.12 ± 0.43a	5.27 ± 0.30a	3.16 ± 0.24c	3.05 ± 0.23c
TFAA content	10.18 ± 0.84a	11.69 ± 1.02a	10.55 ± 0.84a	11.45 ± 0.99a	7.94 ± 0.72b	8.19 ± 1.07b

[a]Different letters (a–d) in the same row indicate significant differences ($P < 0.05$).

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Table 4. Phospholipid Composition (Grams per Egg) of Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

phospholipid	unfertilized egg	day 3	day 6	day 9	day 12	day 15
PC	1.17 ± 0.10a	1.07 ± 0.09a	1.12 ± 0.07a	1.02 ± 0.09a	1.03 ± 0.05a	1.01 ± 0.08a
PE	0.37 ± 0.03a	0.36 ± 0.03a	0.38 ± 0.02a	0.35 ± 0.03a	0.30 ± 0.02b	0.23 ± 0.02c
PI	0.11 ± 0.01ab	0.12 ± 0.01a	0.09 ± 0.01c	0.09 ± 0.01c	0.10 ± 0.01bc	0.10 ± 0.01bc
LPE	0.04 ± 0.00d	0.05 ± 0.00c	0.04 ± 0.00d	0.05 ± 0.00c	0.08 ± 0.00b	0.09 ± 0.01a
SM	0.02 ± 0.00c	0.03 ± 0.0b0	0.02 ± 0.00c	0.02 ± 0.00c	0.03 ± 0.00b	0.04 ± 0.00a
total PLs	1.71 ± 0.15a	1.63 ± 0.13a	1.66 ± 0.11a	1.53 ± 0.14a	1.56 ± 0.08a	1.47 ± 0.16a

[a]Different letters (a–d) in the same row indicate significant differences ($P < 0.05$).

Yolk-derived proteins were likely transformed into tissues or organs and provided essential nutrients and energy to embryos. This was in agreement with a previous study in which yolk residue decreased rapidly after day 4 of incubation.[23] Previous research reported that biological functions of egg white proteins are the prevention of microorganisms' penetration into the yolk and supply of nutrients to the embryo during the late stages of development.[24] This is agreement with the SDS-PAGE result that the major white-derived proteins (ovotransferrin, ovoglobulin, and ovalbumin) displayed little fluctuation before day 15. Ovalbumin is the most dominant white protein. Despite the intensive investigations undertaken on ovalbumin, its biological function remains largely unknown.[24] Ovotransferrin plays an important role in bacteriostatic activity for its high affinity for metal iron.[24] The biological function of ovoglobulins has not been clearly elucidated, but they appear to be important in the foaming capacity of egg white.[24] In addition, there are other white-derived proteins not detected in the SDS-PAGE for their low concentrations, including ovomucoid, ovomucin, lysozyme, ovoinhibitor, avidin, and others. These proteins possessed unique functional properties, such as antimicrobial, enzymatic, inhibitory, cell growth stimulatory, metal binding, vitamin binding, and immunological activities.[2] Thus, distinct from yolk-derived proteins, white-derived proteins play an essential role in maintaining a stable environment for embryonic development.

In recent years, a number of novel egg proteins have been identified using proteomic techniques, including separation techniques such as 2-D electrophoresis and chromatography, mass spectrometry applications, and search in protein databases.[25] These proteomic techniques may also be broadly utilized for studying biological functions of egg proteins. This is a meaningful area of research that needs further efforts.

Free Amino Acid Composition. FAA composition of unfertilized and fertilized eggs is shown in Table 3. The amount of essential free amino acids (EFAA) increased before day 9 and then began to decrease. Similarly, total free amino acids (TFA) content displayed sequential fluctuation before day 9 and then began to decrease, being mainly due to a decrease in essential amino acids. This was in accordance with the change tendency of protein fraction below 5000 Da (Table 2).

With an unsurpassable balance of nutritional amino acids, hen egg is useful as a supplement to those foods with low essential amino acid concentration.[2] Our work showed that the total essential free amino acids content in fertilized egg before day 9 was higher than that of unfertilized egg. This meant that fertilized egg has the potential of being utilized as an amino acid-rich dietary supplement.

Phospholipid Composition. Phospholipid composition in unfertilized and fertilized eggs is shown in Table 4. There was no significant difference in total PL contents between unfertilized and fertilized eggs. Phosphatidylcholine (PC), the most dominant class of phospholipid for both unfertilized and fertilized eggs, showed no change in content between unfertilized and fertilized eggs. The phosphatidylethanolamine (PE) amount remained stable up to day 12 and then decreased at day 15. Conversely, the phosphatidylinositol (PI) amount decreased on day 6 and then remained stable up to day 15. Lysophosphatidylethanolamine (LPE) and sphingomyelin (SM) displayed fluctuation before day 9 and then significantly increased during incubation up to day 15.

PLs are natural biosurfactants as well as important materials for building cells and tissues. Polyunsaturated fatty acids are especially concentrated in the PL fraction.[2] Egg yolk PC is a significant source of choline, which is an important nutrient in brain development, liver function, and cancer prevention.[2] PUFAs, that is, arachidonic acid (ARA) and DHA, are found in egg yolk PC but not in soy PC.[2] PE is the second most abundant PL in egg yolk. The greatest proportions of ARA and DHA were found in the PE fraction.[21] Recently, because of health consciousness and a dieting boom, many people are reducing fat in their diets. This leads to low intake of PLs and choline, which is not a healthy trend.[2] As there were no significant differences in amounts of total PLs and PC, fertilized eggs with lower lipid level could be utilized as a novel dietary supplement to enhance PLs and choline.

Cholesterol Content. As shown in Figure 2, the cholesterol level kept decreasing with prolonged incubation time. After 15 days of incubation, the cholesterol content decreased from 0.33 to 0.21 g/egg. Besides, the data indicated that the rate of decline was maximal between days 9 and 12.

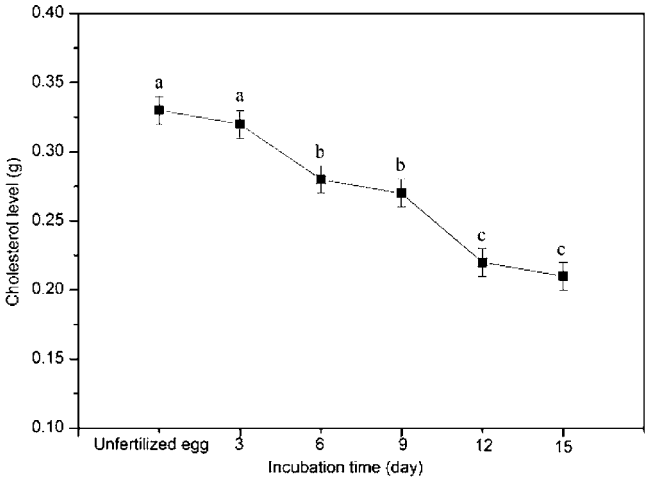

Figure 2. Cholesterol level (g/egg) in unfertilized egg and fertilized egg at different developmental stages. Different letters (a–c) on the bars indicate significant differences ($P < 0.05$).

Table 5. Fatty Acid Profile (Percent of Total Fatty Acid) of Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

fatty acid	unfertilized egg	day 3	day 6	day 9	day 12	day 15
C14:0	0.38 ± 0.04ab	0.34 ± 0.02b	0.41 ± 0.03a	0.35 ± 0.04ab	0.38 ± 0.03ab	0.39 ± 0.02ab
C16:0	26.44 ± 0.44a	26.47 ± 0.60a	27.40 ± 0.11a	26.14 ± 0.41a	26.59 ± 0.31a	27.15 ± 0.36a
C16:1	3.71 ± 0.23c	4.65 ± 0.26a	4.29 ± 0.11ab	4.34 ± 0.26ab	3.99 ± 0.14bc	4.12 ± 0.18b
C18:0	8.86 ± 0.16a	6.68 ± 0.25c	7.56 ± 0.18b	6.61 ± 0.27c	6.44 ± 0.30c	6.54 ± 0.15c
C18:1	43.72 ± 1.22c	50.25 ± 1.33a	47.53 ± 1.63b	49.20 ± 1.12ab	50.24 ± 2.04a	49.43 ± 0.12ab
C18:2	12.69 ± 0.45a	9.72 ± 0.56d	10.83 ± 0.23bc	11.50 ± 0.37b	10.28 ± 0.55cd	10.27 ± 0.65cd
C18:3	0.32 ± 0.04a	0.27 ± 0.03a	0.34 ± 0.07a	0.31 ± 0.03a	0.27 ± 0.02a	0.27 ± 0.01a
C20:4(ARA)	1.43 ± 0.03a	0.56 ± 0.02c	0.62 ± 0.01b	0.52 ± 0.02c	0.63 ± 0.03b	0.63 ± 0.04b
C22:6(DHA)	0.44 ± 0.02a	0.14 ± 0.01d	0.19 ± 0.02c	0.16 ± 0.03cd	0.26 ± 0.01b	0.27 ± 0.02b
SAFA	35.68 ± 0.61a	33.49 ± 0.87bc	35.37 ± 0.47a	32.75 ± 0.52c	33.41 ± 0.64bc	34.08 ± 0.53b
MUFA	47.43 ± 1.69c	54.90 ± 1.59a	51.82 ± 1.74b	53.54 ± 1.38ab	54.23 ± 2.18a	53.55 ± 0.3ab
PUFA	14.88 ± 0.48a	10.69 ± 0.62c	11.98 ± 0.33b	12.49 ± 0.45b	11.44 ± 0.61bc	11.44 ± 0.74bc

[a]Different letters (a–d) in the same row indicate significant differences ($P < 0.05$).

It is well-known that egg existed as a major dietary source of cholesterol in Western diets.[26] Cholesterol content has traditionally been labeled as a troublesome dietary factor.[2] Although many nutritional and physiological trials have shown that cholesterol intake has only a small effect on blood cholesterol compared with dietary saturated fats intake,[27] the effect of dietary cholesterol on human health is still controversial. The latest research[28] indicates that dietary cholesterol was one of the strongest risk factors of the development of nonalcoholic fatty liver disease. Therefore, many reduced-cholesterol egg products have been developed for market requirement.[29] Our work suggested that fertilized egg exhibited lower cholesterol level than unfertilized egg. According to this perspective, fertilized egg is more suitable for people with hypercholesterolemia.

Fatty Acid Composition. The fatty acid composition of unfertilized and fertilized eggs is presented in Table 5. Monounsaturated fatty acids (MUFA) were the most dominant class of fatty acids for both unfertilized and fertilized eggs, followed by saturated fatty acids (SAFAs) and polyunsaturated fatty acids (PUFAs), respectively. The relative amount of MUFAs in fertilized egg was higher than that of unfertilized egg, whereas the percentage of PUFAs was lower than that of unfertilized egg. Throughout the incubation period, the proportions of ARA and DHA dramatically decreased by day 3 and then began to slightly increase during incubation up to day 15.

ARA and DHA play important roles in neural and retina development.[30] Hamburger and Hamilton[31] indicated that neurals, brains, and eyes started to develop at very early stages of embryogenesis. This could explain the result that the ARA and DHA amounts significantly decreased before day 3. In addition, it has been estimated that half of the total amount of fatty acids underwent β-oxidation to provide the energy needed for development.[32] However, it was emphasized that lipid utilization does not simply consist of bulk transfer from yolk followed by nonspecific β-oxidation of fatty acids. Maldjian et al.[33] reported that most of the C_{16} and C_{18} fatty acids transferred during the incubation period were oxidized for energy purposes. In contrast, the C_{20-22} PUFAs appeared to be relatively resistant to β-oxidation, possibly due to their preferential incorporation into membrane phospholipids.[6] This fact explained our result that proportions of ARA and DHA increased relatively after day 9 of incubation.

In addition, as high-MUFA diets have benefits for cardiovascular disease risk factors, a high-MUFA, low-cholesterol diet has attracted much attention recently.[34] On the basis of our research, the potential utilization of fertilized egg in some special diets for cardiovascular patients would merit further studies.

Change of Protein Conformation. During the incubation period, proteins would decompose and then cross-link with each other to form more intricate macromolecular systems.[2] To make clear the change of protein conformation during the incubation period, free sulfhydryl (SH) content, surface hydrophobicity (H_o) and zeta-potential of proteins were investigated.

Figure 3 shows the free SH content of proteins of unfertilized egg and fertilized egg. The free SH content of unfertilized

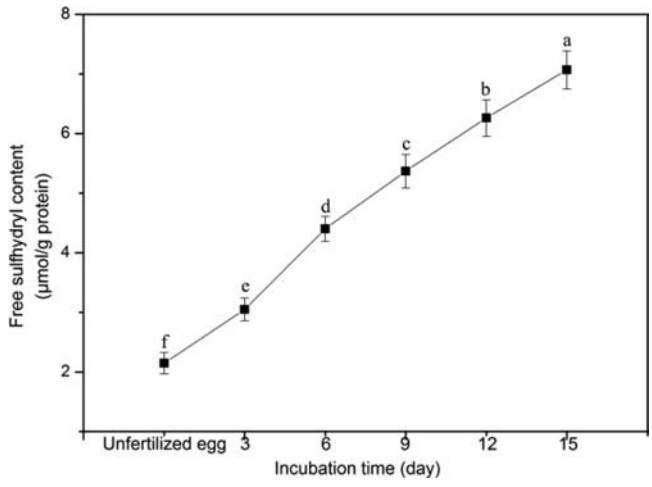

Figure 3. Free sulfhydryl contents of proteins of unfertilized egg and fertilized egg at different developmental stages. Different letters (a–d) on the bars indicate significant differences ($P < 0.05$).

sample was 2.15 μmol/g protein, after 15 days of incubation, and the free SH content increased to 7.07 μmol/g protein. This suggested that the embryogenesis process may induce dissociation and reassociation/cross-linking of protein molecules, accompanying disruption of SS bonds within the protein molecules and release of free SH.

Surface hydrophobicity (H_o) of proteins is one of the structural characteristics to evaluate the change in protein

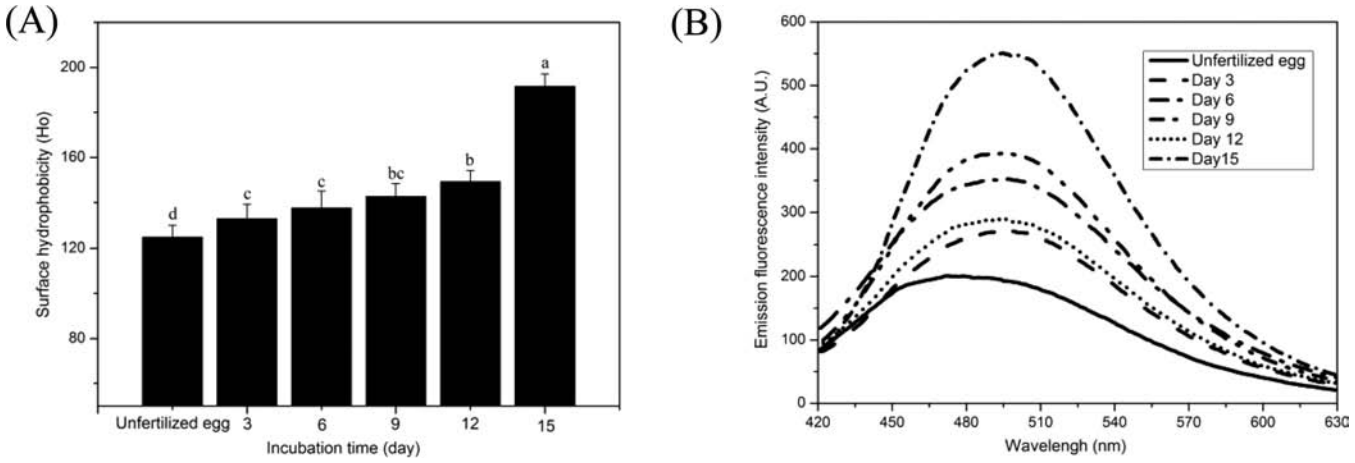

Figure 4. Surface hydrophobicity (H_o) (A) and emission fluorescence spectra of proteins (B) in unfertilized egg and fertilized egg at different developmental stages. Different letters (a–d) above columns indicate significant differences ($P < 0.05$).

conformation. [19] Figure 4A shows that H_o values of fertilized egg proteins were all higher than that of unfertilized egg proteins, and the H_o increased gradually with increasing incubation time. This was in agreement with the change tendency of free SH content, indicating that the changes of molecule conformation caused hydrophobic groups buried inside the molecules to be exposed. Furthermore, the increase of H_o may lead to an increase in the formation of aggregates with high molecular weight.[19] Thus, the increase in the higher MW (>30000 Da) fraction after day 9 (Table 2) may partly be due to aggregation of protein molecules via hydrophobic interactions.

Figure 4B illustrates the emission fluorescence spectra of ANS at unfertilized and fertilized egg proteins. The data showed that unfertilized egg proteins exhibited the lowest level of relative fluorescence intensity over 420–630 nm. During embryonic development, the relative fluorescence intensity peak increased generally with increasing incubation time, with the exception of day 12. The change tendency revealed that the hydrophobic regions of proteins increased with increasing incubation time, which was in accordance with the tendency of surface hydrophobicity.

It is well-known that electrostatic force plays a key role in driving protein to aggregation, and the determination of surface charge will give some important information to explain the molecules' aggregation/precipitation pattern.[35] The zeta-potential of unfertilized and fertilized egg proteins is shown in Figure 5. The zeta-potential of fertilized egg proteins was always lower than that of unfertilized egg and exhibited a decrease with increasing incubation time. The negative charges on egg proteins might be due to the −SH and hydrophobic amino acid side chains exposed.[36] Previous research reported that negative charges may be neutralized by ions released from proteins, and then the hydrophobic interactions took place between neutralized protein molecules.[14] This was in agreement with the result of decrease in zeta-potential and increase in surface hydrophobicity.

To make clear the change in secondary structure of egg proteins during incubation period, circular dichroism (CD) analysis was applied. As shown in Table 6, unfertilized and fertilized egg proteins exhibited distinct difference in secondary structure. β-Sheet structure dominated in unfertilized egg proteins and decreased gradually during incubation period. Contrarily, the proportion of unordered structure increased

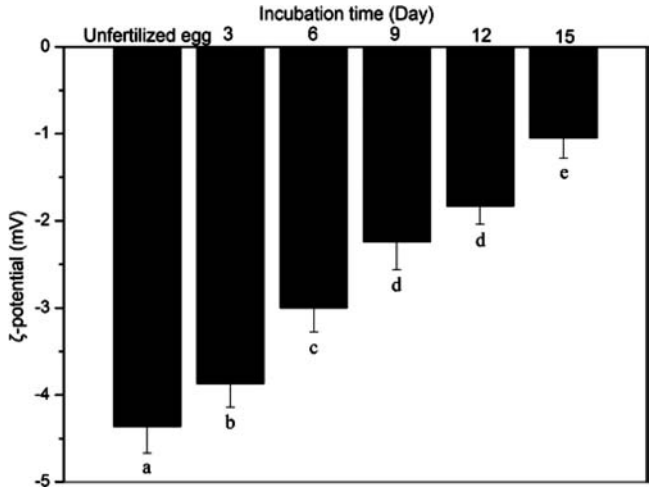

Figure 5. Zeta-potential of proteins of unfertilized egg and fertilized egg at different developmental stages. Different letters (a–e) below columns indicate significant differences ($P < 0.05$).

with increasing incubation time. Meanwhile, α-helix structure increased gradually before day 12 and then began to decrease.

The increase in unordered structure lets more hydrophobic segments buried inside the proteins be exposed to the outside.[37] This was agreement with the result observed in surface hydrophobicity. In addition, recent research reported that increase in β-structures resulted in a more hydrophobic, rigid structure.[38] However, although the hydrophobic property increased during the embryogenesis process, the relative amount of β-structures (sum of β-sheets and β-turns) decreased from 52.2 to 42.0% after 15 days of incubation.

Dielectric constant measurement (ε') is a simple and nondestructive way to characterize agricultural products.[39] Measurement of ε' is required for understanding, explaining, and empirically relating physicochemical properties of materials.[9] In this work, the dielectric constant (ε') for both unfertilized and fertilized egg proteins appeared to almost linearly decrease with increasing frequency over the range from 10 kHz to 15 MHz (Figure 6A). In addition, the fertilized sample had somewhat lower values than the unfertilized sample over the range from 10 to 100 kHz, and the values decreased generally with increasing incubation time (Figure 6B). When embryonic development begins, the proteins start to unfold and

Table 6. Percentage of Secondary Structures of Proteins in Unfertilized Egg and Fertilized Egg at Different Developmental Stages[a]

	secondary structures (%)			
protein sample	α-helices	β-sheets	β-turns	unordered
unfertilized egg	22.7 ± 1.3b	37.7 ± 3.2a	15.5 ± 0.7b	22.7 ± 3.0c
day 3	23.8 ± 2.4b	36.7 ± 1.2a	17.7 ± 2.5ab	20.8 ± 1.5bc
day 6	26.7 ± 2.1b	31.6 ± 0.8b	16.4 ± 2.3b	25.3 ± 2.4bc
day 9	33.3 ± 3.6a	20.3 ± 1.3cd	20.1 ± 0.9a	26.6 ± 3.8ab
day 12	36.0 ± 2.8a	17.1 ± 2.9d	19.2 ± 3.3ab	27.1 ± 1.9ab
day 15	25.7 ± 1.7b	20.9 ± 1.4c	21.1 ± 1.2a	30.9 ± 1.4a

[a]Different letters (a–d) in the same row indicate significant differences ($P < 0.05$).

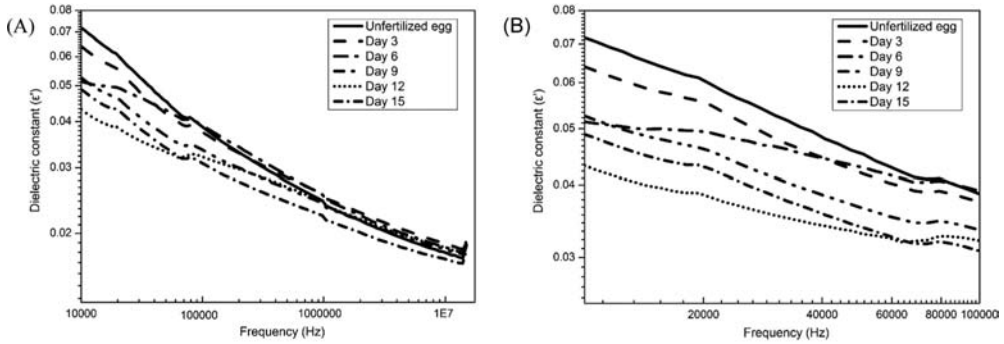

Figure 6. Dieletric constant of unfertilized and fertilized egg proteins at different developmental stages over the ranges from 10 kHz to 150 MHz (A) and from 10 to 100 kHz (B).

the surface hydrophobicity increases because of the exposure of hydrophobic groups. This induced hydrophobicity-driven protein interactions that cause aggregation of proteins. The aggregation of proteins results in a decrease in ion mobility, which is reflected as a decrease in dielectric constant.[39] These findings discussed here suggested that the dielectric constant, which can be easily measured, could be regarded as a simple way to measure conformational change of egg proteins during embryogenesis.

In summary, the present study compared the nutritional composition and protein conformation of unfertilized and fertilized egg. In contrast to the studies containing information on egg white and yolk alterations during embryogenesis, we are interested in the total nutritional composition of fertilized egg from the perspective of food. Compared with unfertilized egg, fertilized egg before day 9 exhibited higher EFAA and MUFA levels, the same amounts of total PLs and PC, and lower cholesterol concentrations. Thus, fertilized egg has the potential of being utilized as an EFAA/MUFA-rich, low-cholesterol dietary supplement for the aged and people with special dietary requirements. Furthermore, this work provided information on changes in secondary structures and conformation of egg proteins during the incubation period. This was necessary for establishing their structure–function relationships and characterizing their functional properties.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +86 510 85917100. Fax: +86 510 85917100. E-mail: xmxu@jiangnan.edu.cn.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ANOVA, one-way analysis of variance; ANS, 1-anilinonaphthalene-8-sulfonic acid; ARA, arachidonic acid; CD, circular dichroism; DHA, docosahexaenoic acid; DTNB, 5,5′- dithiobis-(2-nitrobenzoic acid); EDTA, disodium ethylenediaminetetraacetic acid; EFAA, essential free amino acid; FA, fatty acid; FAA, free amino acid; FAME, fatty acid methyl esters; FID, flame ionization detector; H_o, surface hydrophobicity; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin layer chromatography; LPE, lysophosphatidylethanolamine; MUFA, monounsaturated fatty acid; MW, molecular weight; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLs, phospholipids; PUFA, polyunsaturated fatty acid; SAFA, saturated fatty acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, free sulfhydryl; SM, sphingomyelin; TFA, total free amino acids; ε', dielectric constant

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