Analysis of the Low-Molecular Weight Protein Profile of Egg-White and its Changes during Early Chicken Embryological Development

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Many low-molecular weight (LMW) proteins in egg-white are potentially bioactive, but the mass range and number of these are not yet fully characterized. The aim of the present study was to map the LMW protein profile in egg-white and provide the basis for further understanding of the physiological function of these proteins. For this purpose, six time points (days 0, 1, 2, 3, 4, 5 of incubation) were selected in an attempt to delineate the LMW proteomic profile in egg-white and its changes during early chicken embryological development. Samples were pretreated using gel chromatography techniques prior to analysis by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Protein search focused on the mass range *m/z* 1,000 to 8,000. One hundred and fourteen mass signal peaks of LMW proteins ranging from *m/z* 1,035.88 to 7,112.91 were detected at all six time points. The observed changes in the LMW protein profile during development were highly dynamic. Eighty six novel mass signal peaks of LMW proteins were generated during incubation, the origin of which could be assigned to the high-molecular weight protein fractions.The list of egg-white LMW proteins provided in this paper is by far the most comprehensive and is intended to serve as a starting point for the isolation and functional characterization of interesting LMW proteins which may play a crucial role in early embryo nutrition and immunity.

Key words: Egg-White, Low-Molecular Weight Protein, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Introduction

It is no surprise that egg-white has been the target of proteomic studies previously, because of its importance in human nutrition, its importance as a source of easily accessible model proteins, and its potential use in biotechnological processes (Mann and Mann, 2011). The arsenal of tools used to study the protein components of egg-white have been complemented by mass spectrometry-based proteomic technologies. Application of these fast and sensitive methods has already enabled the identification of a large number of new egg-white proteins (D'Ambrosio *et al*., 2008; Guerin-Dubiard *et al*., 2006; Mann, 2007). The most comprehensive list of egg-white components has been reported by Mann and Mann (2011) who identified 158 proteins in chicken egg-white with two or more sequence-unique peptides using a dual pressure linear ion trap orbitrap instrument. Nevertheless, it appears that these authors did not include low-molecular weight (LMW) proteins (molecular weight less than 10,000 Da) in their study. However, the search for these minor components in egg-white remains of importance, because very low-abundance small proteins and peptides may have an important biological role, for instance in early embryonic development, such as gallin, a recently identified member of a family of peptides, which is potentially antimicrobial (Gong *et al*., 2010).

Egg-white LMW proteins analysis is challenging, because the protein content of egg-white is dominated by a handful of proteins such as ovalbumin, ovotransferrin, ovomucoid, while the mass signals of low-abundance proteins and peptides in the egg-white are strongly suppressed by these high-abundance proteins. Recently, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has served as a path-breaking tool to open up peptidomic analy-

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sis in the mass range from 1,000 to 7,000 Da. This mass range might be considered as an approximate, operationally defined mass range (within optimal detection by MALDI-TOF MS but below usual detection by two-dimensional gel electrophoresis) for the peptidome (Hortin, 2006). Another important characteristic is that molecular ions formed by MALDI-TOF MS show fewer charges than those formed by liquid chromatography (LC)-TOF MS, resulting in the acquisition of more easily interpreted mass spectrum information (Zeng *et al.*, 2007).

To the best of our knowledge, no proteomic study has been conducted on naturally occurring LMW proteins in egg-white. Here, we firstly used the off-line peptidomics approach combining gel chromatography and MALDI-TOF MS to map the LMW protein profile of egg-white. Furthermore, we determined the changes of egg-white LMW proteins during early chicken embryological development to further understand the physiological function of these proteins.

Material and Methods

Materials

HPLC grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Company (St. Louis, MO, USA); a Sephadex G-50 gel filtration resin was from Pharmacia (Uppsala, Sweden); sinapinic acid (SA) and 2,5-dihydroxycinnamic acid were from Sigma Chemical Company for MS analysis without further purification. Other analytical grade inorganic and organic chemicals were from commercial sources in China (Sinopharm Chemical Company, Beijing, China).

Egg-white sample preparation

Fresh fertilized eggs from Lohmann White Single Comb White Leghorn laid within 24 h were collected in the morning from the poultry research centre farm of Huazhong Agricultural University, Wuhan, China. The chick eggs were incubated at 38 °C and 65–75% relative humidity in a forced air incubator for up to 5 d. Eggs were randomly selected during sampling. At each experimental time point – 0, 1, 2, 3, 4, and 5 d post laying – ten eggs were removed and opened. In order to avoid discrepancy of protein/peptide types among different eggs, the egg-whites of 10 eggs were pooled and homogenized for 5 min, then diluted in an 1:9 ratio with water. All sample solutions were filtered by a 0.45-μm membrane filter before chromatographic analysis.

Sample preparation by column separation

Protein separation was performed using a 250 mm × 10 mm column packed with Sephadex G-50 gel at a flow rate of 0.2 mL/min. The column was equilibrated with 0.1% TFA solution, and then 1 mL of sample mixture was loaded on the column and eluted with 0.1% TFA solution at a flow rate of 0.2 mL/min. A collector equipped with a 280-nm monitor and a recorder was used to collect fractional samples at a controlled sample volume. A total of 30 protein fractions were collected from each egg-white sample for analysis by MALDI-TOF MS. Fraction volumes were 2.0 mL each.

Analysis by MALDI-TOF MS

MALDI-TOF MS experiments were performed on a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). This system allows the identification and quantification of low-abundance putative protein biomarkers, down to attomole range. The mass spectra of proteins/peptides were obtained in the positive ion reflectron mode, and the instrument was calibrated using Applied Biosystems calibration standards. A volume of 0.3 μL of the sample was subsequently transferred to the steel target plate and mixed with 0.3 μL of matrix, a saturated solution of alpha-cyano-4-hydroxycinnamic acid in acetone. Spectra were recorded in the MS mode within a mass range from *m/z* (mass to charge ratio) 700 to 8,000, and the search of LMW proteins was focused on the mass range *m/z* 1,000 to 8,000. All samples were assayed in duplicate. The analytical procedure for protein/peptide mass signals as described by Wang *et al.* (2003) was employed to ascertain the numbers of unique ion signals measured by MALDI-TOF MS. The data in each mass spectrum (*i.e.*, the relative signal intensity vs. *m/z*) were visualized using the software Data Explorer V 4.0 (Applied Biosystems) provided by the manufacturer. All mass spectra were smoothed using a 19-point Gaussian smoothing routine prior to subsequent peak picking analyses. Peak tables were generated by noting the *m/z* of the salient peaks in each mass spectrum.

Results

Six time points (days 0, 1, 2, 3, 4, 5 of incubation) were selected in an attempt to delineate the LMW proteomic profile in the egg-white during early chicken embryological development. Firstly, the egg-white samples were fractionated by a column packed with Sephadex G-50 gel. Fig. 1 shows four major absorbance peaks (A–D) of proteins/peptides from the egg-white (day 0), and each peak consists of several components, suggesting that the gel column still lacked the ability to effectively separate the complex proteome in egg-white into single purified proteins within the 30 fractions. This separation technique enabled us to dilute, as well as remove from the egg-white, the high-abundance proteins and the various salts having signal suppression characteristics.

All fractionated egg-white samples were then subjected to MALDI-TOF MS analysis. Fig. 2A shows a representative chromatogram displaying LMW proteins detected in egg-white (day 1, fraction 15). Fig. 2B shows a comparison of the MALDI-TOF mass spectra of the LMW proteins in fraction 18 of different samples collected at different incubation times. It is clear that the LMW protein profile of the egg-white is always changing as the incubation progresses.

In total, 114 mass signal peaks of LMW proteins in the mass region of *m/z* 1,035.88 to 7,112.91 were detected at all of the six time points (Table I). Among them, 28, 26, 35, 35, 20, and 17 peaks of proteins were present specifically in the egg-white on days 0, 1, 2, 3, 4, 5 of incubation. Three proteins (with *m/z* 4,483.98, 2,242.59, 1,743.89) were found at all time points (Table I). We speculated that they were digested and absorbed in the late development stages. Proteins with *m/z* 4,749.40, 4,612.81, 4,600.43, 4,596.19, 4,482.05, 3,013.37, 2,893.04, 2,683.98, 2,408.53, 2,405.82, 2,245.79, 1,972.65, 1,929.99, 1,645.41, 1,609.41, 1,481.43, 1,410.39, 1,300.75, 1,231.38, and 1,195.37 disappeared during early embryological development. The reasonable explanations for this phenomenon could be that they are digested by the hatching enzyme in the early development stages, absorbed by the cells of embryo or transferred into the egg yolk.

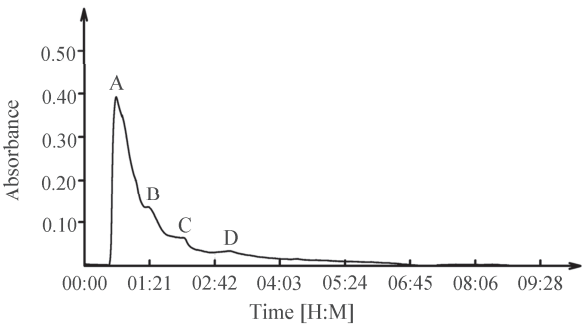

Fig. 1. Representative chromatogram of proteins/peptides from the egg-white eluted with 0.1% TFA solution on a gel column packed with Sephadex G-50. Four major peaks (A, B, C, D) of egg-white proteins are recorded on a monitor equipped with a recorder and collector at 280 nm.

It should be noted that the observed changes in the LMW protein profile of the egg-white from different developmental stages are highly dynamic. During early chicken embryological development, 86 novel LMW proteins were generated, the origin of which could be assigned to the high-molecular protein fractions. Fig. 3 shows the distribution of *m/z* values of LMW proteins in egg-white at days 0, 1, 2, 3, 4, 5 of incubation. Most of the proteins were within the *m/z* range from 1,000 to 2,000. As time elapsed, the number of proteins in the mass range *m/z* 1,000–2,000 in egg-white increased from 13 on day 0 to 20 on day 2, and 21 on day 3. However, after three days of incubation, the total number decreased to 12 on day 4 of incubation and 7 on day 5 of incubation. The number of proteins in the mass range *m/z* 4,000–5,000 decreased after three days of incubation. Little change was observed in the total number of proteins ranging from *m/z* 5,000 to 8,000 and *m/z* 3,000 to 4,000.

Discussion

Egg-white contains many compounds essential for life and represents an extensive source of bioactive compounds such as peptides and proteins. However, 70–80% of egg-white proteins are ovalbumin, ovotransferrin, and ovomucoid (Miguel *et al.*, 2005). The presence of these highly abundant proteins makes the detection of the smaller, less abundant egg-white proteins difficult. In the present study, the application of gel chromatography to separate components in dilutions of egg-white followed by MALDI analysis of the collected fractions resulted in the detection of over 114 peaks in the *m/z* 1,000–8,000 mass range (Table I), an order of magnitude increase in the number

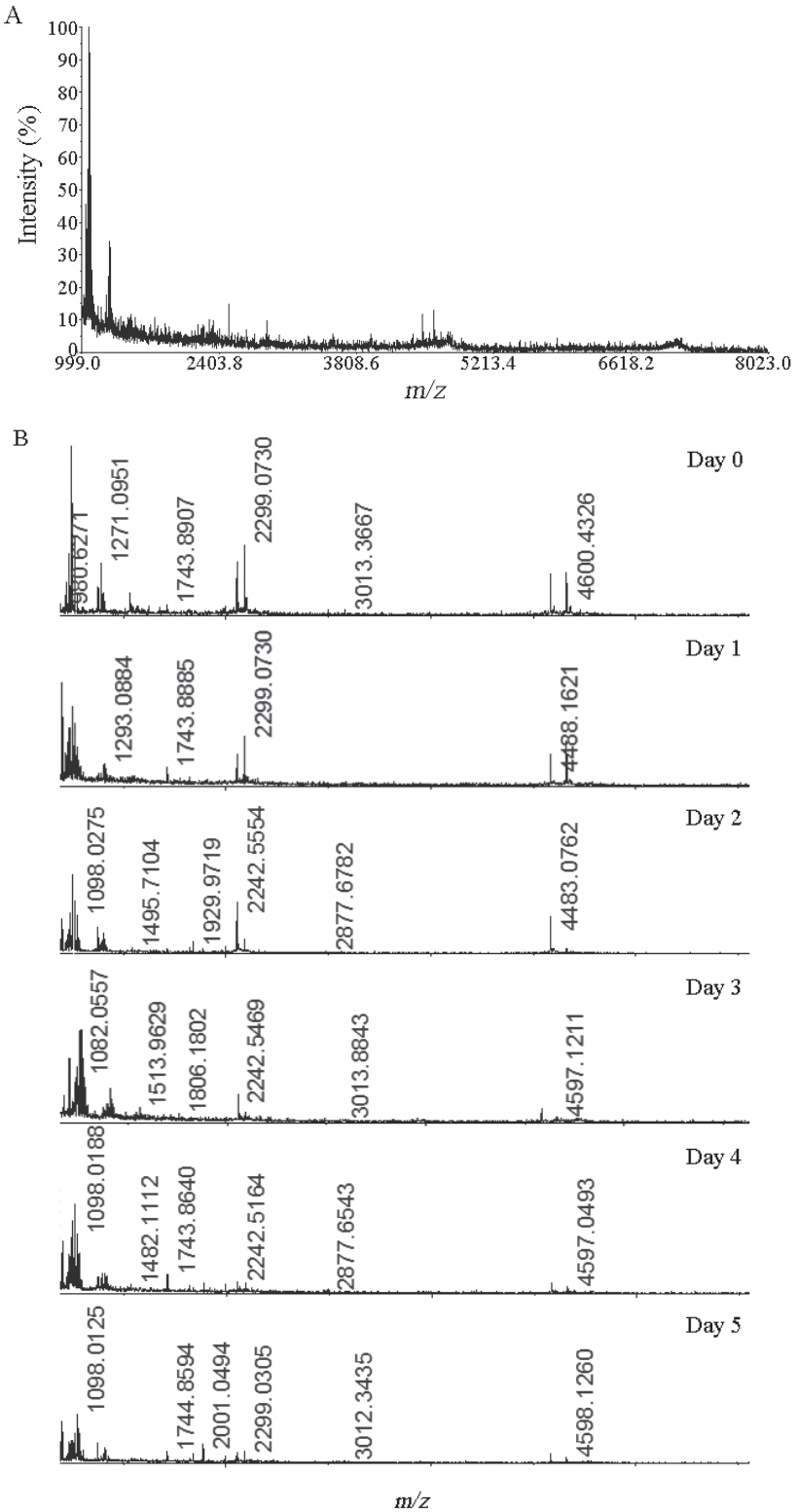

Fig. 2. (A) Representative MALDI-TOF mass spectrum of LMW proteins detected in egg-white (day 1, fraction 15). The ion signals of proteins/peptides are shown in the *m/z* range 999–8,023. (B) A comparison of the MALDI-TOF mass spectra of the LMW proteins in fraction 18 of different samples which were collected at different incubation times. Spectra were recorded in MS mode within a *m/z* range from 1,000 to 8,000.

Table I. Masses of LMW proteins detected in egg-white collected at different incubation times by gel chromatography fractionation/off-line MALDI-TOF MS analysis; the relevant presence is indicated with √.

m/z	Day						*m/z*	Day					
	0	1	2	3	4	5		0	1	2	3	4	5
7,112.91		√					1,929.99	√	√	√			
5,319.35					√		1,903.04				√		
4,775.42						√	1,902.01				√	√	
4,766.02			√				1,892.57				√		
4,756.89				√			1,813.86				√		
4,752.35		√					1,812.89	√	√			√	√
4,749.40	√						1,806.18				√		
4,612.81	√						1,788.66			√			
4,600.43	√						1,745.48				√		
4,598.22		√				√	1,744.89			√	√		√
4,597.12				√	√		1,743.89	√	√	√	√	√	√
4,596.19	√	√					1,732.09					√	
4,594.14				√			1,719.51				√		
4,488.16		√					1,662.06		√				
4,487.05			√				1,645.41	√					
4,485.99				√			1,609.41	√					
4,483.98	√	√	√	√	√	√	1,599.66			√			
4,482.05	√						1,585.99					√	
4,481.10			√	√			1,557.94						√
3,951.48			√				1,544.03		√				
3,782.71			√				1,531.01					√	
3,686.67		√					1,528.01						√
3,587.03				√			1,513.96	√			√		
3,569.10		√					1,496.05		√	√			
3,296.16						√	1482.11					√	
3,014.91		√					1,481.43	√					
3,013.88				√			1,479.76			√			
3,013.37	√						1,469.91					√	
3,012.34						√	1,453.93			√			
2,895.62			√				1,446.55				√		
2,895.09					√		1,437.79			√			
2,893.72		√					1,411.91		√				
2,893.04	√						1,410.39	√					
2,877.68			√		√		1,381.91					√	
2,683.98	√						1,380.35				√		
2,408.53	√						1,353.85				√		
2,405.82	√						1,351.00			√	√		
2,389.08			√	√			1,337.06		√				
2,388.10			√				1,315.06					√	
2,343.23				√	√	√	1,309.05					√	
2,340.99				√			1,300.75	√					
2,299.60	√	√				√	1,299.78	√		√	√		
2,299.07		√					1,297.44				√		
2,245.79	√						1,273.62			√	√		
2,243.54			√	√			1,266.80			√			
2,242.59	√	√	√	√	√	√	1,249.76				√		√
2,241.53	√					√	1,231.38	√					
2,219.81					√		1,225.16		√	√			√
2,156.72				√			1,195.37	√					
2,086.79			√				1,164.06			√			
2,049.69			√				1,130.00		√			√	
2,011.62			√				1,120.05			√			
2,001.06						√	1,115.00			√			

Table I continued.

1,972.65	√				1,103.79			√
1,959.65			√		1,042.33			√
1,940.26		√			1,038.06	√		
1,930.54		√	√	√	1,035.88		√	

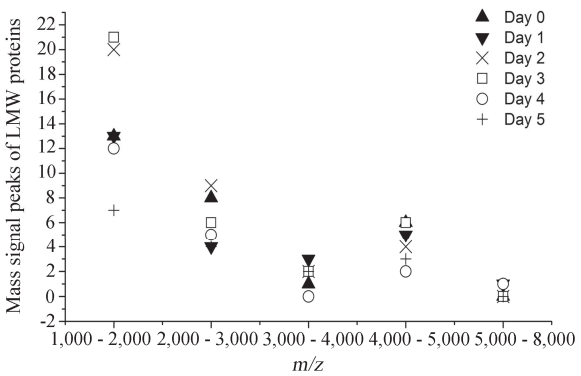

Fig. 3. Distribution of *m/z* values of LMW proteins in egg-white at days 0, 1, 2, 3, 4, and 5 of incubation.

of components observed when compared with direct MALDI analysis of the entire egg-white samples (data not shown). The result has shown that this procedure was effective and allowed for the visualization of small proteins and peptides which have never been reported before.

It can be presumed that the pool of LMW proteins might have contained protein molecules resulting from hydrolysis of the vitelline membrane (VM) or egg-white. An avian egg is surrounded by the VM, egg-white, shell membrane, and eggshell. Avian hatching is considered to involve either total or partial digestion of these envelopes. The avian VM is a multilayered proteinaceous structure separating egg-white from yolk, and 137 proteins were identified in the VM using a high-throughput, high-end LC-MS^n method (Mann, 2008). These proteins or their hydrolysates are possibly released into the egg-white accompanying the breaking down of the VM. However, the embryonic hatching enzyme may also be secreted into the egg-white; it plays a role in digestion of the egg-white (Yasumasu *et al.*, 2005). As development progresses, the egg-white is translocated toward the vegetal pole and enclosed and absorbed by the albumen sac (Yoshizaki *et al.*, 2002). In spite of great potential interest, relatively little is known concerning the uptake of egg-white proteins and peptides by developing embryos. Unquestionably, the digestion of egg-white components facilitates the uptake of the egg-white by the cells during the early chicken embryological development. This work could provide the basis for new biological monitoring methods for gaining insights into the hatching mechanism of avian eggs.

The presence of LMW proteins in egg-white during incubation may be functionally important since structure/function studies of many egg proteins have shown that specific domains within these native proteins retain or even exhibit enhanced biological activities (Kovacs-Nolan *et al.*, 2005). It is well documented that several egg-white proteins, including lysozyme, ovomucin, ovalbumin, and ovotransferrin, which collectively make up around 73% of the total egg-white composition, have demonstrated a number of biological functions including antimicrobial, antiviral, anticancer, protease-inhibiting, and immune-modulating activities (Li-Chan *et al.*, 1995; Kovacs-Nolan *et al.*, 2005; Mine and D'Silva, 2008). The activity of these egg-white proteins has been shown to be enhanced following proteolytic digestion (Mine *et al.*, 2004). During early chicken embryological development, the changes in the LMW protein profile may be due to protein disintegration by the hatching enzyme. We speculate that the LMW proteins produced in the early developmental stages may exert some important bioactive effects to the embryo. For instance, the fact that the egg-white is known for its armoury of antimicrobial proteins and peptides (Mine and Kovacs-Nolan, 2006) that protect the embryo during incubation suggests that this might be a function of these LMW proteins. The data provided in this work present the basis for determining the precise function of these LMW proteins.

On the basis of the above data, we conclude that the number of LMW proteins present in egg-white appears to be large. Further investigations are now required to elucidate the structures of these protein fractions, which are mostly un-

known. It is shown that the dynamic change of the LMW protein profile in egg-white is highly related with the development of the embryo. In-depth analyses will be needed to explore the physiological function of these proteins in the embryological development. However, the identification of these minor (very low-abundance) components in egg-white is an exciting opportunity for detection of important functional proteins in egg-white but also a great challenge, it requires much more manpower and resources to accomplish the purposes.

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