RESEARCH ARTICLE

Analysis of chicken serum proteome and differential protein expression during development in single-comb White Leghorn hens

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Serum is believed to harbor thousands of distinct proteins that are either actively secreted or leak from various blood cells or tissues. Exploring protein composition in serum may accelerate the discovery of novel protein biomarkers for specific economic traits in livestock species. This study analyzed serum protein composition to establish a 2-DE reference map, and monitored protein dynamics of single-comb White Leghorn hens at 8, 19 and 23 weeks after hatching. A total of 119 CBB-stained and 315 silver-stained serum protein spots were analyzed by MALDI-TOF MS. Of these, 98 CBB-stained and 94 silver-stained protein spots were significantly matched to existing chicken proteins. The identified spots represented 30 distinctive proteins in the serum of laying hens. To compare protein expression during development, expression levels of 47 protein spots were quantified by relative spot volume with Melanie 3 software. Ten protein spots increased and 3 protein spots decreased as hen age increased. Previous research has suggested that some of these proteins play critical roles in egg production. The differentially expressed proteins with unknown identities will be valuable candidates for further explorations of their roles in egg production of laying hens.

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1 Introduction

The goal of egg producers is to grow a pullet to sexual maturity as quickly as possible, expecting her to lay a marketable egg every day of her adult life. Notably, egg production in the primary breeding and multiplication sectors of the poultry industry is also a reproductive trait with important effects on rate of genetic gain and hatchery utilization [1].

Egg production is a trait that has low to moderate heritability [2] and, depending on the period involved, may require a significant amount of data to be informative. With complex sex-limited traits, such as egg production, evaluating new molecular approaches must be multifaceted. Given that time-tested approaches to layer-selection programs historically have been multifaceted, the prospects for developing a suitable mixture of time-tested and molecular techniques seem good, providing leverage for improving performance levels already close to biological thresholds in some environments [1].

Bulfield [3] described two ways in which future molecular genetics could impact poultry breeding: (i) marker-assisted selection (MAS) using cloned genes closely linked to quanti-

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Abbreviations: HDL, high-density lipoprotein; **MAS**, marker-assisted selection; **RVol**, The ratio of volume of each spot to the total volume of all quantitated spots; **VLDL**, very low-density lipoprotein

tative trait loci; and, (ii) genetic manipulation of "trait-genes". Given that transgenic techniques are still in development [4], an appropriate option in the near future will likely be MAS.

Proteomics, now defined as large-scale analysis of gene functions, is central to functional genomics [5]. Parallel quantitative display of proteins is considered the most promising strategy for biomarker discovery. One core technology, 2-DE, established in the 1970s, has been employed to display blood plasma proteins [6, 7]. A second core technology, MS, more recently revolutionized the proteomics field [8, 9]. The study of proteomics will enhance comprehension of protein functions and will have valuable applications in clinical and biomedical fields [10].

Body fluids, such as serum, plasma and urine, are the most convenient materials to collect for serial analysis, diagnostic purposes and following the time course of a disease [11]. Changes in protein profiles as well as levels in body fluids may be of diagnostic, prognostic, and therapeutic significance. Human serum and plasma proteins have already been mapped for clinical applications [12–14]. In animal species, analysis and characterization of serum proteome are still hindered by incomplete genome information. A few studies have characterized the serum proteome for bovines [15], rats [16] and horses [17]. However, there are no published works that explore the proteome of chicken serum. This study applied a proteomic approach to analyze serum protein composition, to construct a 2-DE protein reference map for an egg laying strain (the single-comb White Leghorn) and also to compare the changes in proteome of hens at different developmental stages as a base for exploration the physiology of development or reproduction of laying hens.

2 Materials and methods

2.1 Source of serum sample

Blood samples were obtained by venipuncture from the wing vein of four single-comb White Leghorn chickens at 8, 19, and 23 weeks of age. The chickens were reared at a traditional breeding farm, in the same house and under natural lighting. The birds were managed and treated humanely following the guidelines established by the National Science Council, Republic of China [18]. Blood samples were allowed to clot for 2 h at room temperature. The clotted material was removed by centrifugation at $1700 \times g$ for 10 min. The supernatant sera obtained from the blood samples were stored in a −80°C deep freezer.

The protein concentration of serum was quantified by a modified Bradford method [19], using BSA as the standard. The protein sample was split into aliquots of 1 mg or 400 μg and then stored at −80°C for 2-DE analysis.

2.2 Analysis of proteins by 2-DE

Analysis of proteins by 2-DE was performed following the procedure developed by Görg *et al.* [20] with some modification [21]. The IEF was carried out using 18- or 24-cm IPG strips (pH 3–10 or pH 4–7 linear, Amersham Biosciences, Uppsala, Sweden) with the IPGphor system (Amersham Biosciences). Of the total proteins, 1 mg (for spot excision) or 400 μg (for analyzing expression variation) were mixed with 175 μL IEF sample buffer (9.5 M urea, 2% NP40, 2% pharmalyte 3–10, and 65 mM DTT) [6]. These proteins were mixed well with equal volume of rehydration buffer (8 M urea, 2% CHAPS, and 0.5% pharmalyte 3–10), then loaded onto the strip holder and covered with IPG strip (Amersham Biosciences). The strip was rehydrated with sample at 30 V for 12 h, and focused for 64 000 Vh.

After focusing, the strips were first equilibrated for 15 min in 15 mL SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 0.002% bromophenol blue) containing 100 mM DTT, followed by equilibration in 15 mL SDS equilibration buffer containing 250 mM iodoacetamide for 15 min. The IPG strip was layered on top of a vertical 12.5% SDS-polyacrylamide gel for the second dimension separation using the Daltsix Vertical electrophoresis system (Amersham Biosciences). The separation was run at 15°C with a condition of 2.5 W/gel for 25 min followed by 9 W/gel until the dye front reached the bottom of the gel (typically 7–7.5 h). The 2-DE standards (Bio-Rad, Hercules, CA, USA) used for M_r and p*I* calibration were as follows: hen egg conalbumin type I (76 kDa; p*I* 6.0, 6.3, 6.6); BSA (66.2 kDa; p*I* 5.4, 5.6); bovine muscle actin (43 kDa; p*I* 5.0, 5.1); rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kDa; p*I* 8.3, 8.5); bovine carbonic anhydrase (31 kDa, p*I* 5.9, 6.0); soybean trypsin inhibitor (21.5 kDa; p*I* 4.5); and equine myoglobin (17.5 kDa; p*I* 7.0).

2.3 Staining and imaging of the 2-D gels

After separation, the gels were subjected to CBB or sliver staining. For CBB staining, the gels were stained with colloidal CBB (Serva Electrophoresis GmbH, Heidelberg, Germany) for at least 14 h [22]. Following staining, the gel was neutralized with 0.1 M Tris-phosphoric acid (pH 6.5) for 1–3 min then destained with 25% methanol. After destaining, the gels were scanned with a laser densitometer (Amersham Bioscience; software was ImageQuant) and saved as a TIFF image file for further image analysis.

Sliver staining was performed by the method of Shevchenko *et al.* [23] with some modification. Briefly, the gels were fixed over night with 50% methanol and 5% acetic acid. They were then washed with three changes of 7% acetic acid and 5% methanol for 3 min each. The gels were incubated 1 min with 0.02% sodium thiosulfate for sensitizing, and were then rinsed with three changes of distilled water for 1 min each. After rinsing, the gels were incubated with 0.1% silver nitrate for 20 min. The silver nitrate solution was

discarded, and the gels were rinsed twice with distilled water for 1 min and developed in 0.04% formalin (35% formaldehyde in water) in 20% sodium carbonate with intense shaking. After the desired intensity of the staining was achieved, the development was terminated by discarding the solution, followed by washing the gels with 5% acetic acid. The silver-stained gels were stored in distilled water for scanning or further spot picking.

2.4 Analysis of protein expression levels during development

Protein spots on the 2-D gels were detected and analyzed using Melanie 3 software (GeneBio, Geneva, Switzerland). To present protein expression variations in serum at different developmental stages, 47 spots on all 2-D gels from the four chickens at each stage were quantified. The ratio of volume of each spot to the total volume of all quantitated spots (RVol) was generated by the Melanie 3 software to correct for differences in gel staining [21, 24]. The RVol was used to present the expression level of each protein spot.

2.5 Protein identification by MALDI-TOF MS

2.5.1 In-gel digestion

Gel plugs containing protein spots were excised from CBB- or silver-stained gels and placed in Eppendorf tubes. The silver-stained gel plugs were first washed with silver destain solution (1% potassium ferricyanide and 1.6% sodium thiosulfate in distilled water). In-gel trypsin digestion was performed according to the procedure developed by Havlis *et al.* [25] with minor modifications. Gel plugs were washed twice with double-distilled water followed by 50% ACN in 50 mM ammonium bicarbonate and pure ACN. The gel plugs were dried in a SpeedVac evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) then subjected to in-gel digestion or storage at −20°C. For in-gel digestion, gel plugs were reswollen with 20 ng/μL trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 4°C for 30 min. Digestion was continued for 1 h at 56°C. After digestion, peptide products were recovered by adding 40% ACN and 1% TFA.

2.5.2 MALDI-TOF analysis

Digested samples were spotted directly onto a 400 μm/384 well AnchorChip sample target (Bruker Daltonics, Bremen, Germany), and then added with an equal volume of 1 mg/mL solution of CHCA in 0.1% TFA/50% ACN. The MALDI mass spectra were obtained using a Bruker autoflex TOF mass spectrometer equipped with a 384-sample Scout source (Bruker Daltonics). An external peptide calibration standard containing the following fragments was used to calibrate the instrument: angiotensin II ($[M+H]^+$ 1046.54); angiotensin I ($[M+H]^+$ 1296.68); substance P ($[M+H]^+$ 1347.74); bombesin ($[M+H]^+$ 1619.82); ACTH clip 1–17 ($[M+H]^+$ 2093.09); ACTH clip 18–39 ($[M+H]^+$ 2465.20); and, somatostatin 28 ($[M+H]^+$ 3147.47) (Bruker Daltonics). Spectra were acquired in reflectron mode. Peptide masses were searched against NCBInr database employing the MASCOT program [26] for protein identification with search conditions of taxonomy of all entries, fixed modification of carbamidomethyl modification, mass accuracy between 50 and 200 ppm and maximally one missed cleavage site. Positive identification was achieved with at least five peptides matched with the set mass accuracy and modification, and when the score matched in significant probability to the protein or mixture of proteins identified.

2.6 Statistical analysis

The effect of developmental stages on the expression of serum proteins was analyzed by the *t*-test procedure in Statistical Analysis System software [27]. A probability of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Separation of serum proteins of the single-comb White Leghorn chicken by 2-DE

The chicken serum reference map was obtained using data from colloidal CBB- and silver-stained gels. Figure 1 shows a typical 2-D protein reference map of chicken serum proteome obtained from a pH 3–10 IPG strip and a 12.5% SDS gel. Following staining with CBB, over 110 protein spots were detected covering p*I* 3–10 and M_r of 10–200 kDa. The protein profile reveals that a small number of proteins comprise most of the protein masses in the chicken serum.

To enhance the completeness of the reference map, serum 2-D profile was also obtained with a pH 4–7 IPG strip and silver staining (Fig. 2). The result indicates that more than 300 spots had the p*I* of 4–7 and M_r of 10–200 kDa could be detected.

3.2 Identification of chicken serum proteins by MS

A total of 119 CBB-stained and 315 silver-stained protein spots were excised for identification. Suppl. Table 1 presents the identified chicken serum proteins. A total of 98 (82.4%) CBB-stained spots matched chicken proteins, either as experimentally determined protein sequences or cDNA sequences. The other two spots (1.7%) were matched to equivalent proteins in *Homo sapiens*. Of the silver-stained spots, 99 (31.4%) were further identified, of which 22 proteins had different identities or accession numbers from those identified in the CBB-stained gel, as listed in Suppl. Table 1. The number of peptides matching a candidate protein sequence ranged from 6 to 37, and sequence coverage range was 8–97%. Nineteen CBB-stained spots (16.0%) and

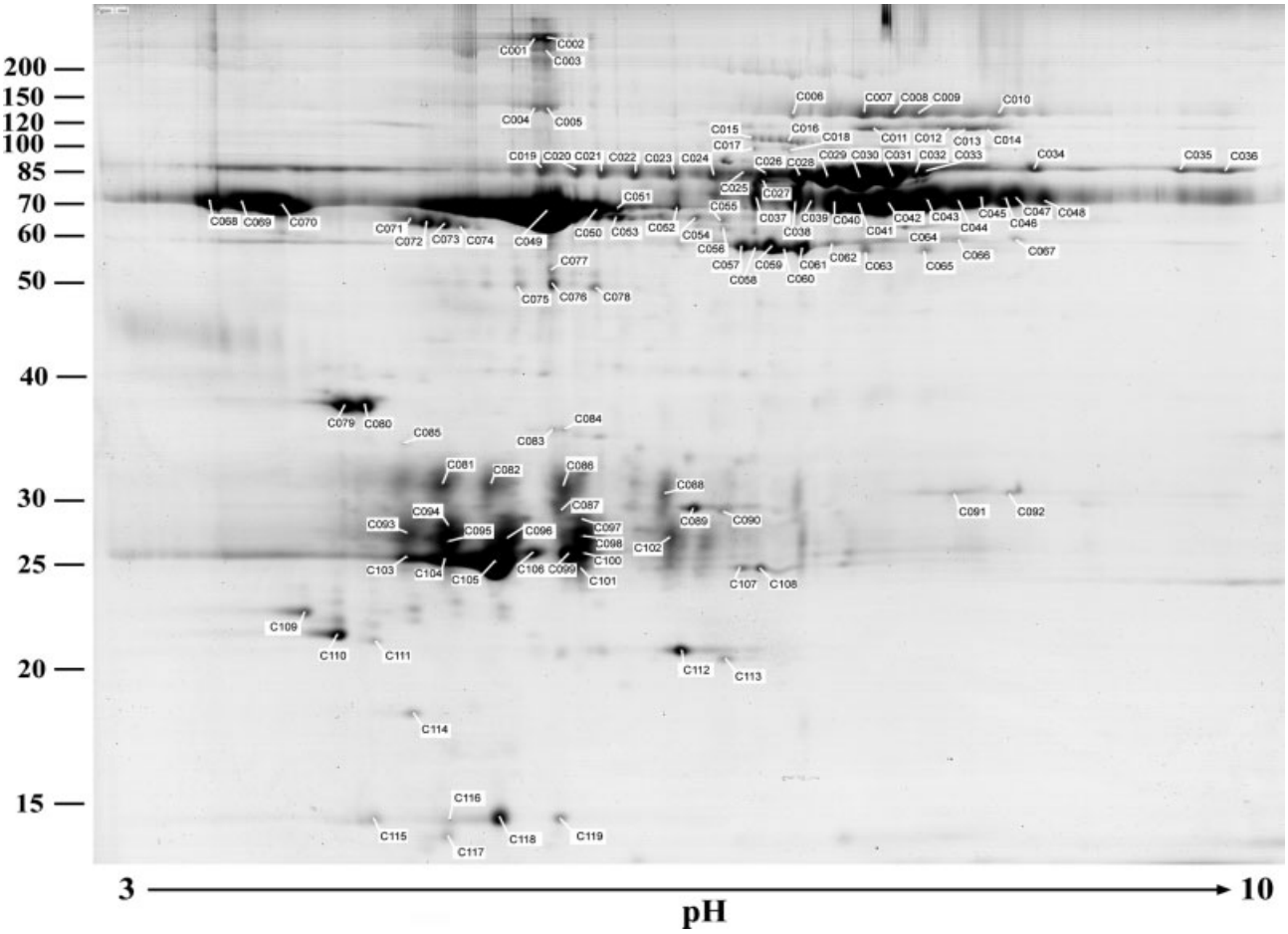

Figure 1. A representative CBB-stained 2-D reference map of chicken serum proteins. Proteins (400 μg loaded) from 23-week-old hens were separated by IEF using 240-mm, immobilized, linear pH gradient strip of pH 3–10, followed by 12.5% SDS-PAGE gels. Spot numbers refer to numbers in Suppl. Table 1. Proteins were identified by MALDI-TOF MS.

Table 1. Differentially expressed chicken serum proteins during different developmental stages[a)]

Spot ID on 2-D map[b)]	Protein name	Age (weeks)		
		8	19	23
C013	Predicted: similar to plasminogen	0.323 ± 0.067[d)]	0.510 ± 0.043[d)]	0.768 ± 0.106[c)]
C012	Predicted: similar to plasminogen	0.474 ± 0.061[d)]	0.704 ± 0.092[c)d)]	1.059 ± 0.167[c)]
C014	Predicted: similar to plasminogen	0.572 ± 0.108[d)]	0.698 ± 0.078[d)]	1.203 ± 0.192[c)]
C057	Vitamin-D binding protein precursor	1.688 ± 0.429[d)]	2.491 ± 0.599[d)]	5.137 ± 0.591[c)]
C059	Vitamin-D binding protein precursor	3.757 ± 0.274[d)]	4.860 ± 1.180[d)]	9.070 ± 0.464[c)]
C061	Vitamin-D binding protein precursor	2.573 ± 0.185[d)]	3.174 ± 1.008[d)]	6.412 ± 0.246[c)]
C075	Ig mu chain C region	0.315 ± 0.080[d)]	0.455 ± 0.045[c)d)]	0.634 ± 0.051[c)]
C076	Ig mu chain C region	0.430 ± 0.107[d)]	0.726 ± 0.051[c)]	0.982 ± 0.088[c)]
C080	Apolipoprotein AIV	3.539 ± 0.696[c)]	1.053 ± 0.238[d)]	1.681 ± 0.625[d)]
C085	Unknown	0.103 ± 0.020[c]	0.515 ± 0.059[c)]	0.298 ± 0.033[d)]
C089	Unknown	0.601 ± 0.142[d)]	0.973 ± 0.173[d)]	1.883 ± 0.263[c)]
C099	Apolipoprotein AI	11.881 ± 1.174[c)]	9.564 ± 0.721[c)d)]	7.004 ± 0.535[d)]
C117	Chain B, transthyretin (formerly known as prealbumin)	0.708 ± 0.164[c)]	0.272 ± 0.104[d)]	0.079 ± 0.042[d)]

a) The protein expression was represented the relative volume of a spot to the overall volumes of all the quantified spots generated by the Melanie 3 software and used to present the expression level of a protein spot.

b) The spot numbers refer to the numbers labeled in Fig. 1.

c, d) Means within the same row with different superscript differ significantly.

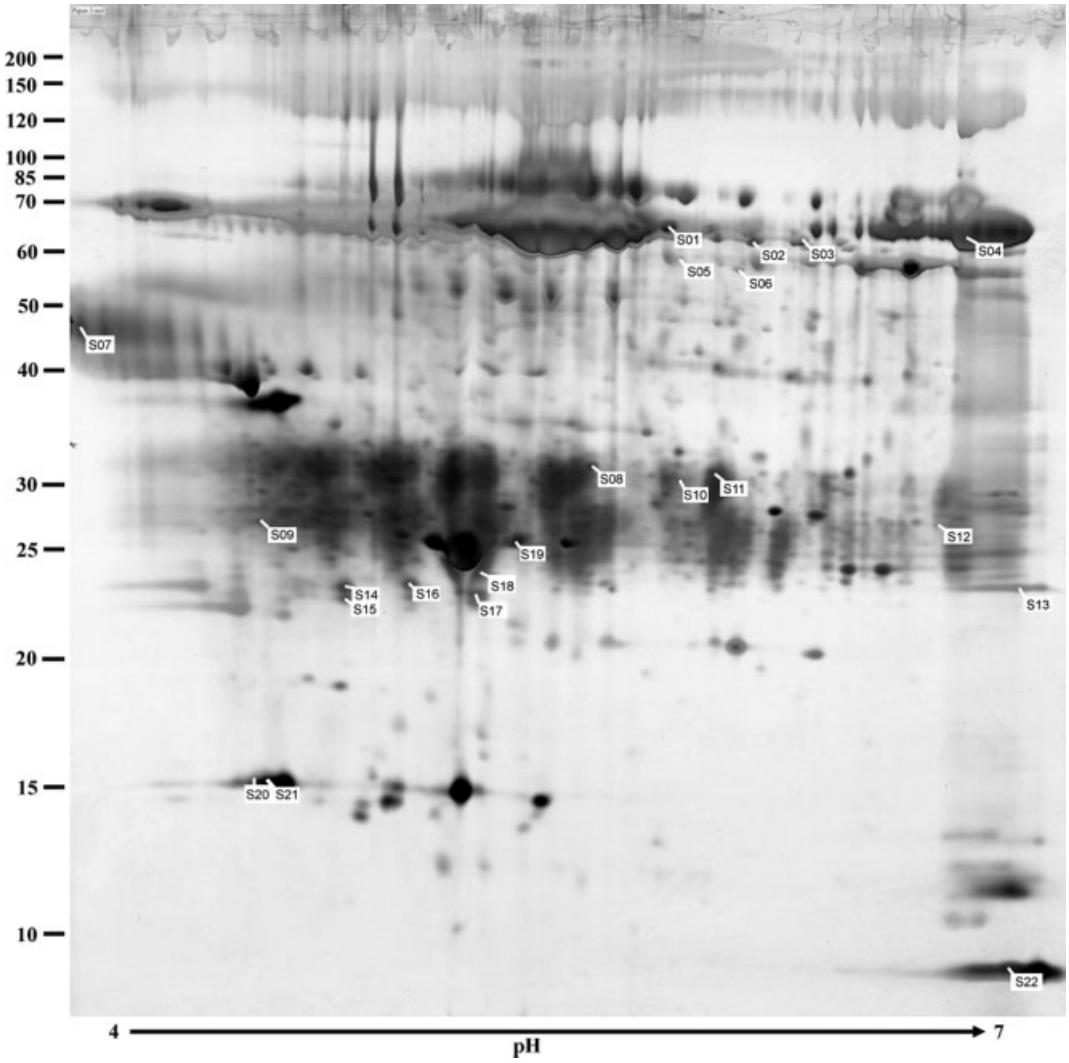

Figure 2. A representative silver-stained 2-D reference map of chicken serum proteins. Proteins (400 μg loaded) from 23-week-old hens were separated by IEF using 180-mm, immobilized, linear pH gradient strip of pH 4–7, followed by 12.5% SDS-PAGE gels. Spot numbers refer to numbers in Suppl. Table 1. Proteins were identified by MALDI-TOF MS.

216 silver-stained protein spots (68.6%) were unsuccessfully identified. Thirty distinctive proteins were identified in the serum of laying hens.

Over 20 proteins were expressed as multiple spots on the reference map, demonstrating that they were either charge isoforms, or fragments of the same protein. These proteins included major serum proteins such as albumin, immunoglobulin, conalbumin and apolipoproteins (Fig. 1 and Suppl. Table 1). The minor proteins were plasminogen, homogenin, ovoinhibitor, vitamin-D binding protein precursor, PIT 54 protein, dTDP-D-glucose 4,6-dehydrogenase, retinol-binding protein, hemopexin, hemoglobin, ovoglycoprotein precursor, serine (or cysteine) proteinase inhibitor, plasma glutathione peroxidase precursor, immunoglobulin J polypeptide, adult alpha D-globin and transthyretin.

3.3 Change of serum protein levels during development

Figure 3 illustrates the representative protein profiles of hens at different ages, and clearly reveals that the pattern of protein expression remained similar throughout the developmental stages. Some protein spots had altered substantially in their serum when the hens reached 19 or 23 weeks of age, which is consistent with a major post-hatch developmental shift.

Table 1 further lists the differentially expressed serum proteins during development. The expression levels increased in ten protein spots, and decreased in three protein spots, as the age of hens increased. The levels of plasminogen, vitamin-D binding protein precursor, Ig mu chain C

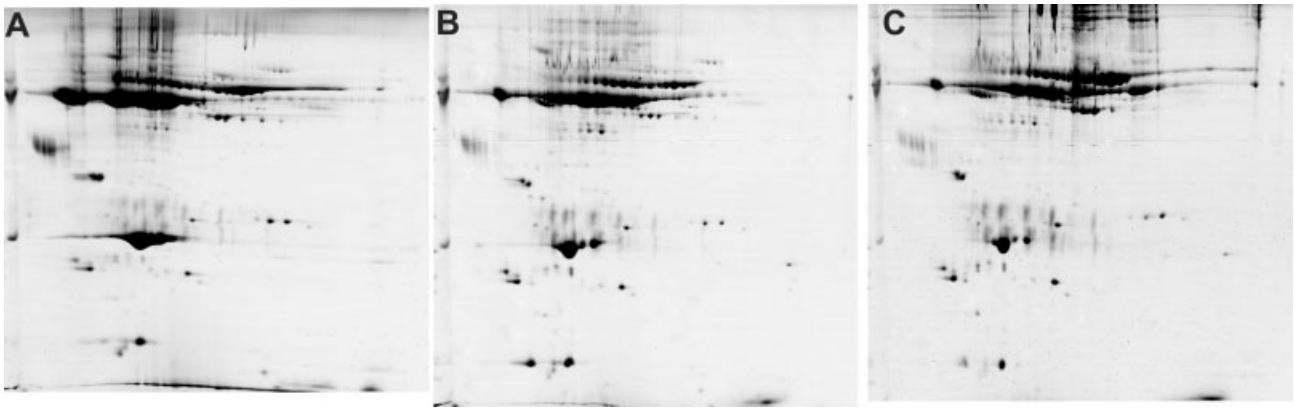

Figure 3. Comparison of 2-DE serum protein profiles of hens at different ages. Proteins (400 μg loaded) from (A) 8-, (B) 19- and (C) 23-week-old hens were separated by IEF using 180-mm, immobilized, linear pH gradient strip of pH 3–10, followed by 12.5% SDS-PAGE gels. Proteins were visualized by CBB staining.

region and an unknown protein rose as the age increased ($p < 0.05$). However, the expression levels of apolipoprotein AI, apolipoprotein AIV and transthyretin fell as development proceeded ($p < 0.05$).

4 Discussion

The single-comb White Leghorn chicken is one of the most highly selected breeds for egg production worldwide. Understanding the serum protein expression in this breed can provide information essential to improving reproduction in other breeds, such as the local chicken breeds in Taiwan. This investigation established a 2-DE reference map for chicken serum proteins (Figs. 1, 2) with more than 100 protein spots identified by PMF (Suppl. Table 1). Thirteen quantified protein spots were differentially expressed during development. These differentially expressed proteins require further study of their roles in the reproductive function of the laying hens.

Although chickens are evolutionarily distant from mammals, most of the identified chicken serum proteins have also been identified in mammals [15–17, 28]. Four proteins were matched to the proteins of *Homo sapiens* and *Sus scrofa*, including TXNDC5 protein, chromosome X open reading frame 31, serine (or cysteine) proteinase inhibitor, immunoglobulin heavy chain variable region and hemoglobin. These proteins might have important function, and their expression has therefore been conserved in phylogenetically distant species. However, a random match is still possible. Therefore, the identity of these spots needs to be verified by sequence tags. The failure to identify the other spots indicates that they may be unidentified proteins.

Among the identified chicken serum proteins, the p*I* and M_r of albumin, immunoglobulin light chain, immunoglobulin mu chain, immunoglobulin gamma chain, plasminogen, transferrin, transthyretin and apolipoproteins AI, H, AIV were similar to those of their mammalian counterparts [15–17, 28]. The hemopexin has also been found in human plasma with a similar p*I* but with different M_r [28]. The retinol-binding protein has also been discovered in bovine serum [15]. The homogenin, ovoinhibitor, vitamin-D binding protein precursor, PIT 54 protein, dTDP-D-glucose 4,6-dehydrogenase and ovoglycoprotein precursor have not been identified in either the mammalian serum or the plasma reference map. These proteins may represent avian-specific serum proteins, and hence may play significant roles in physiology related to egg production.

The apolipoproteins AII, haptoglobin and antitrypsin were not identified in this study. Apolipoproteins AII has not been found to exist in avian serum, whereas haptoglobin has been reported to be present in birds, including chickens [29, 30]. The existence of alpha 1-antitrypsin in chicken serum has not been directly reported previously. However, Leicht *et al.* [31] reported that chicken ovalbumin and human alpha 1-antitrypsin show significant sequence homology and belong to a common protein superfamily. The failure to detect these proteins in this study may result from the low resolution of the 2-D analysis. Future studies for further enhancing the resolution of 2-D analysis will be required.

A comparison of proteomic change in serum proteins at different developmental stages shows that the levels of three plasminogen isoforms, three vitamin-D binding protein precursor isoforms and two Ig mu chain C region isoforms increased significantly during the laying period (Table 1). The plasminogen/plasmin system contains a proenzyme, plasminogen, which is converted to the active enzyme plasmin by a tissue-type or urokinase-type plasminogen activator [32, 33]. The tissue-type plasminogen activator-mediated plasminogen activation is primarily involved in the dissolution of fibrin in circulation [34], whereas the urokinase-type plasminogen activator-mediated plasminogen activation appears to be mainly involved in pericellular proteolysis [35, 36]. However, the physiological function of the differentially

expressed plasminogen in the laying hens remains unclear. The 25-hydroxyvitamin D3 binding protein is associated with eggshell formation. During the egg laying process, 25-hydroxyvitamin D3 is induced under the action of estrogen, increasing its level in the serum. The 25-hydroxyvitamin D3 directly binds to the 25-hydroxyvitamin D3 binding protein and is then transported to the oviduct and shell gland for use in eggshell formation. The rise in vitamin-D binding protein precursor levels is consistent with its role in egg production. Two Ig mu C region isoforms increased as the bird matured (Table 1). The frequency of IgM-positive cells in the mucosal epithelium of all oviductal segments, and in the subepithelium of infundibulum, uterovaginal junction and vagina, has been reported to be significantly higher in laying hens than in immature birds [37]. The role of Ig mu C region increase in the development and egg production of laying hens necessitates further investigation.

The levels of apolipoprotein AI, apolipoprotein AIV, and transthyretin decreased as the hens reached 19 weeks of age (Table 1). Apolipoprotein AI and apolipoprotein AIV have been shown to activate the enzyme lecithin:cholesterol acyltransferase, and play an important role in transporting high-density lipoprotein (HDL) by transporting cholesteryl esters from various tissues to the liver [38, 39]. High-density lipoproteins are the most lipid-rich particles in growing chicks [40]. The levels of lipoproteins, triglycerides, phospholipids and cholesterol rose significantly in the plasma of laying hens [41]. The serum level of very low-density lipoprotein (VLDL) was high, whereas the level of HDL was low during laying [42]. These observations imply that most plasma lipids were absorbed by the oocytes rather than transported to the liver. Some HDL particles were also incorporated into the *bona fide* cytoplasm of the oocyte [43, 44]. The changes in apolipoprotein levels in this study also indicate that, consistent with an earlier report [45], the plasma composition in laying hens differed from those of growing chicks.

Transthyretin binds to T3 or T4 to aid thyroxin transportation and works with retinol binding protein, indirectly facilitating vitamin A transportation [46, 47]. Recent reports also demonstrate that some transthyretin can be transported in blood by binding to apolipoprotein AI and cutting the C terminus of apolipoprotein AI. Thus, transthyretin is considered an important protease in plasma. Transthyretin was recently defined as an estrogen-independent class of yolk precursor proteins [48]. The oocytic transthyretin is thought to be derived from the circulation, where it is a constitutive component, and deposited into yolk as a result of endocytosis mediated by a specific receptor [48]. However, the exact role of transthyretin in egg production or developmental biology remains unknown.

The limited dynamic range of proteins covered in any proteomic approach is a common challenge to marker discovery. Both plasma and serum show significant variations in individual protein abundances, for example, albumin is 10^9-fold more abundant than troponin T in serum. Fractionation should expand the dynamic range of protein measurements in serum. Further enhancement of detection and analysis of low abundant proteins in requires electrophoretic prefractionation methods, such as narrow-range pH gradient 2-D techniques [49], depletion of highly abundant proteins [50], free-flow electrophoresis [51] and LC approaches [52, 53]. These methods will be applied in the analysis of chicken serum proteins.

In conclusion, this study is the first to establish a serum protein database for chickens. This database provides essential prerequisite information for further investigation of levels and patterns of chicken serum proteins under various physiological situations and at different production levels. The change in protein levels during development reveals that they may be involved in the maturation and/or egg production. These proteins are currently being evaluated for use as candidates for further investigation of markers for egg production in our laboratory.

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