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Comparative proteomics of matrix fractions between pimpled and normal chicken eggshells

Zhangguo Liu [a,*,1], Lingzi Song [a,1], Lizhi Lu [b,1], Xianfu Zhang [a], Fuming Zhang [c,d,e,f], Kehua Wang [g], Robert J. Linhardt [c,d,e,f]

[a] College of Animal Science and Technology, Zhejiang Agriculture & Forestry University, Lin'an 311300, Zhejiang, PR China
[b] Institute of Animal Husbandry and Veterinary Science, Zhejiang Provincial Academy of Agricultural Science, Hangzhou 311000, Zhejiang, PR China
[c] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy 12180, NY, USA
[d] Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy 12180, NY, USA
[e] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy 12180, NY, USA
[f] Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy 12180, NY, USA
[g] Poultry Institute, Chinese Academy of Agricultural Sciences, Yangzhou 225000, Jiangsu, PR China

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ABSTRACT

Eggshell matrix can be dissociated into three matrix fractions: acid-insoluble matrix (M1), water-insoluble matrix (M2) and acid-water facultative-soluble matrix (M3). Matrix fractions from pimpled and normal eggshells were compared using label-free proteomic method to understand the differences among three matrix fractions and the proteins involved with eggshell quality. A total of 738 and 600 proteins were identified in the pimpled and normal calcified eggshells, respectively. Both eggshells showed a combined proteomic inventory of 769 proteins. In the same type of eggshell, a high similarity was present in the proteomes of three matrix fractions. These triply overlapped common proteins formed the predominant contributor to proteomic abundance in the matrix fractions. In each matrix fraction and between both eggshell models, normal and pimpled eggshells, a majority of the proteomes of the fractions were commonly observed. Forty-two common major proteins (iBAQ-derived abundance ≥0.095% of proteomic abundance) were identified throughout the three matrix fractions and these proteins might act as backbone constituents in chicken eggshell matrix. Finally, using 1.75-fold as up-regulated and using 0.57-fold as down-regulated cutoff values, twenty-five differential major proteins were screened and they all negatively influence and none showed any effect on eggshell quality. Overall, we uncovered the characteristics of proteomics of three eggshell matrix fractions and identified candidate proteins influencing eggshell quality. The next research on differential proteins will uncover the potential mechanisms underlying how proteins affect eggshell quality.

Biological significance: It was reported that the proteins in an eggshell can be divided into insoluble and soluble proteins. The insoluble proteins are thought to be an inter-mineral matrix and acts as a structural framework, while the soluble proteins are thought as intra-mineral matrix that are embedded within the crystal during calcification. However, the difference between matrix fractions is unknown. Cross-analysis of proteomic data of three matrix fractions from the same type of eggshell, uncovered triply overlapped common proteins formed the predominant contributor to proteomic abundance of any matrix fraction, and we suggested that abundance variance of some common proteins between the three matrix fractions might be an important cause of their solubility differences. Moreover, eggshell is formed in hen's uterus, and uterus tend to be considered as unique organ determining eggshell quality. By cross-analysis on proteomic data of three matrix fractions between two eggshell models, normal and pimpled eggshells, the differential proteins were screened as candidates influencing eggshell quality. And we suggested that the liver and spleen or lymphocytes might be the major organs influencing eggshell quality, because the most promising candidates are almost blood and non-collagenous proteins, and originated from above organs.

1. Introduction

Eggshells efficiently protect eggs against physical damage and against bacterial contamination. During embryogenesis in breeding

* Corresponding author.
E-mail address: liuzg007@163.com (Z. Liu).
[1] These authors contributed equally to this work.

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eggs, the shells can even provide a barrier of water loss, a primary source of calcium, and a regulator of gas interchange for embryonic development [1]. The avian eggshell is a highly specialized structure, which is comprised of bilayered membranes, a calcified extracellular matrix and a cuticle. The calcified extracellular matrix, also called the calcified eggshell, is ultrastructurally comprised of mammillary cones, column palisade and a vertical crystal layer [2].

The calcified eggshell is a porous bioceramic, which is comprised of 95% $CaCO_3$ calcite crystals and a pervading organic matrix. The calcified eggshell, the major portion of avian eggshell, acts as the predominant contributor to the mechanical properties of the eggshell. The size, shape and orientation of the calcite crystals can significantly influence the structure and quality of an eggshell [3]. Chicken eggshells consisting of highly oriented crystals of larger sizes are significantly weaker than eggshells with smaller and less-oriented crystals [3,4]. In the cases of similar thickness, the guinea fowl eggshell, formed by the intricate interlacing of crystals, is much tougher than that of chicken eggshells formed by straight columnar crystals [5].

In chicken eggshells, the organic matrix is primarily comprised of proteins (70%) and polysaccharides (11%) [6,7]. Over the years, it has been well established that the nucleation, growth and calcite crystal shape in eggshells is intimately associated with the organic matrix [8,9]. For instance, the matrix precursors in the uterine fluid, secreted at various phases of eggshell formation, can significantly control crystal size or morphology *in vitro* [10]. The organic matrix extracted from eggshells can *in vitro* regulate calcite crystal formation and its polymorphism [11]. Individual matrix proteins, such as lysozyme [12], ovocleidin-17 (OC-17) [13], ansocalcin (the goose homologue of chicken ovocleidin-17) [14,15] and osteopontin [16], can participate *in vitro* in the nucleation and growth of calcite crystals. Furthermore, genetic association studies have shown that ovalbumin and ovotransferrin correlate with crystal size, and ovocleidin-116 and ovocalyxin-32 are associated with crystal orientation [17].

Over the last decade, abundant proteomic studies have been carried out for various eggshell matrices extracted using different strategies. In the whole matrix from calcified eggshells, 466 proteins have been determined in chicken eggshells after 20% acetic acid decalcification [18], 697 proteins were uncovered after decalcification of turkey eggshells by 10% acetic acid [19]; and 622 proteins were identified in quail eggshells after 50% acetic acid decalcification [20]. In the partial matrix from chicken calcified eggshell or its ultrastructures, 520 proteins were determined in a 10% acetic acid-soluble matrix fraction [21]; in the 0.6 M EDTA-insoluble matrix fraction, 16 proteins were determined in the palisade layer and 23 in the mammillary layer [22]; following 0.1 N HCl decalcification, 18 mammillary cone-specific proteins, and additional 18 proteins enriched in the mammillary cones were identified [23]; and 216 shell matrix proteins were identified at the key stages of shell mineralization [24]. Compared with the uterine fluid or global shell matrix, from hens with different eggshell breaking strength, some proteins involved with normal eggshell quality were determined [18].

The pimpled eggshell is usually characterized as containing many redundant calcified granules scattered on one or both eggshell apexes and even spreading to the equator of shells, a lighter-color cuticle, and exhibiting especially weak breaking strength (Fig. 1). In the current study, pimpled eggshells and normal eggshells were used as objects. The eggshell organic matrix was first dissociated as acid-insoluble fraction (M1) and acid-soluble fraction using 10% acetic acid decalcification, then the acid-soluble matrix was further separated into water-insoluble fraction (M2), and both acid and water facultative-soluble fraction (M3). Using label-free proteomic methods, the proteomics data acquired on the three matrix fractions was cross-analyzed across the three fractions and between eggshell samples. This work improves our understanding the proteinaceous constituents of the various matrix fractions, and uncovers proteins that influence eggshell qualities from the viewpoint of a specific shell malformation.

Fig. 1. Egg specimens. A. Representing pimpled eggs, B. representing normal eggs.

2. Materials and methods

2.1. Experimental eggs

Eggs were from 46-week old of Hy-line Brown commercial layers (Aige Layer Company, Lin'an, Zhejiang, China). Thirty-six eggs with pimpled area >70% of the eggshell surface were sampled as pimpled eggs, and 48 eggs with normal shape and cuticle color were collected as normal eggs (Fig. 1). The eggs were directly sampled from the layer cages on laying day, and each egg was from different layer. According to the breaking strength, 36 pimpled eggshells were equally divided into three groups as biological repetitions; similarity, the normal eggshells were also equally grouped into three biological replicates.

2.2. Measurement of egg or eggshell variables

After sampling, the egg weight and egg shape index (length/width) were measured immediately using balance and caliper, respectively; then the eggshell breaking strength was measured by eggshell strength gauge (FHK, Fujihara Co., Tokyo, Japan). Following breaking strength testing, each egg was broken into halves, the egg content was discarded and the shell was repeatedly washed with tap water. After immersing in 5% EDTA for 25 min, the shell cuticle was removed with a toothbrush and the membranes were manually stripped. The calcified eggshells without both membranes and cuticle were dried at 40 °C for 30 h. The thickness of calcified shell was measured with a digital micrometer; four samples were determined in each area, including both apexes, and blunt, sharp, and equator. Finally, the eggshell samples were stored at −70°C.

2.3. Extraction of organic matrix components

The organic matrix components were extracted from each calcified eggshell. Briefly, each eggshell was individually powdered using mortar and pestle, then equal mass of each eggshell in the same biological repetition was sampled and pooled using mortar and pestle. Decalcification

was accomplished by stirring with 10% acetic acid at 15 °C for about 18 h. The amount of 10% acetic acid used was 100 mL per 4 g of shell powder, and the acetic acid was added stepwise according to the proportion of 35:35:30, the first two times of decalcification were individually carried out 3–4 h, and the final decalcification was performed overnight.

After decalcification, the suspension was centrifuged (fixed-angle rotor) at 23,500 ×*g* for 18 min and the deposit was washed twice with distilled water and centrifuged, the pellet was freeze-dried and designated as acid-insoluble matrix. The supernatant (referred as acid-soluble matrix) was repeatedly dialyzed 4-times against 45-volumes of distilled H_2O with 7.6 mM Na azide at 15°C using a Spectra/Por 6 dialysis tubing bag (molecular weight cutoff (MWCO) 8 kDa; Spectrum Labs, Rancho Dominguez, CA, USA). The first 3-times of dialysis were individually carried out 3–4 h, and the final dialysis was performed overnight. The sample was then centrifuged (swinging-tube rotor) at 3500 ×*g* for 40 min to obtain water-insoluble matrix (deposit) and facultative-soluble (both acid and water soluble) matrix (supernatant). The water-insoluble matrix was washed twice with distilled H_2O, centrifuged and freeze-dried; and the facultative-soluble matrix was concentrated using a Millipore spin column (MWCO 10 kDa) and freeze-dried. After freeze-drying for 48 h, each matrix component was individually weighed.

For simplicity, above three matrix fractions, *i.e.* acid-insoluble matrix, water-insoluble matrix and facultative-soluble matrix fractions will be respectively expressed as M1, M2 and M3.

2.4. Protein preparation

The acid-insoluble matrix was firstly and individually powdered using mortar and pestle, then 2 mg of each matrix fraction, such as M1, M2 and M3, was suspended in 500 μL STD buffer (4% sodium dodecyl sulfate, 100 mM 1,4-dithiothreitol (DTT), 150 mM Tris-HCl (pH, 8.0)). After 10 min incubation in boiling water, the sample was sonicated on ice then boiled again for another 5 min, finally, the sample was centrifuged at 16,000 ×*g* at 25 °C for 25 min. The supernatant was collected and protein content was determined with the BCA protein assay reagent (Beyotime).

2.5. Protein digestion

Protein digestion was performed according to the FASP procedure [25]. Briefly, 240 μg of protein from each matrix was sampled, and the detergent of STD buffer, such as DTT and other low-molecular-weight components were removed by twice ultrafiltration (Millipore spin column, molecular weight cut-off (MWCO) 30 kDa) against 200 μL UA buffer (8 M Urea, 150 mM Tris-HCl, pH 8.0). Then 100 μL UA buffer with 0.05 M iodoacetamide was added to block reduced cysteine residues and the sample was incubated in darkness for 30 min. The mixture was washed three-times with 100 μL UA buffer then twice with 100 μL DS buffer (50 mM triethyl-ammonium bicarbonate, pH 8.5). Finally, the protein suspension was digested overnight at 37 °C with 2-μg trypsin (Promega) in 40 μL DS buffer, and the resulting peptides were collected as a filtrate. The peptides of each sample was desalted on C18 Cartridges (Empore™ SPE Cartridges C18, bed inner diameter 7 mm, volume 3 mL, Sigma), then the peptide content was estimated by UV light spectral density at 280 nm and an extinction coefficient of 1.1 for a 0.1% (g/L) solution, which was calculated based on the abundance of tryptophan and tyrosine residues in proteins.

2.6. Liquid chromatography (LC) - electrospray ionization (ESI) tandem MS (MS/MS) analysis by Q exactive

MS experiments were performed on a Q Exactive mass spectrometer (Thermo/Finnigan) that was coupled to Easy nLC1000 (Thermo/Finnigan). The peptide mixture (5 μg) was loaded into a C18-reversed phase column (Thermo Scientific Easy Column SC200, length 10 cm, inner diameter 150 μm) which was balanced by buffer A (2% acetonitrile and 0.1% Formic acid), then separated using a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 400 nL/min controlled by Intelli Flow technology over 120 min. MS data was acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m/z*) for HCD fragmentation. Determination of the target value is based on predictive Automatic Gain Control (pAGC). Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70,000 at *m/z* 200 and resolution for HCD spectra was set to 17,500 at *m/z* 200. Normalized collision energy was 30 eV and the under fill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%.

2.7. Protein identification and relative quantification

The MS data were analyzed using MaxQuant software (version 1.3.0.5). MS data were searched against the Uniprot *Gallus* database (24,408 entries, downloaded on June 2nd, 2016). An initial search was set at a precursor ion mass tolerances of 6 ppm for peptide masses and a mass tolerance of 20 ppm for fragment ions. The search was performed with enzyme specificity trypsin, and two missed cleavages were allowed. Carbamidomethylation of cysteines was defined as fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications for database searching. The cutoffs of global false discovery rate (FDR) for both peptide and protein identification were set to 0.01. The protein identification based on >1 unique peptides, and minimal peptide length was six amino acids. All peptides, derived from keratins (except *Gallus* Keratin) or trypsin (*Sus scrofa*), were excluded as probable contaminations.

The protein abundance was calculated using intensity-based absolute quantification (iBAQ) in MaxQuant software. Compared with proteins from cell samples, proteins from different eggshells were much more heterogeneous biological specimens, therefore the relative abundance of identified protein in each matrix fraction proteome was used and derived from the following formula: relative abundance (%) = protein iBAQ * 100 / total iBAQ of the proteome.

2.8. Statistical analysis

The SPSS 19.0 software was used to analyze the differences of proteins in fraction proteomic inventories between the pimpled and normal eggshells. The data were subjected to an analysis of variance (one-way ANOVA), the means of protein abundances were compared using Independent Samples *t*-tests. It was set 1.75-fold for up-changed and 0.57-fold for down-changed cutoff values and the differential changes were considered significant at $P \le 0.05$.

2.9. Verification of the differential proteins

Since the protein constituents between eggshell matrix were much more heterogeneous than proteins between cells, and there were no internal reference proteins in eggshell matrix; therefore, the results of differential proteins were verified by using iTRAQ (isobaric tagging for relative and absolute quantification) based on M1 samples. Briefly, three biological repetitions of pooled pimpled eggshell powder and normal eggshell powder were respectively prepared, and the M1 was extracted from each eggshell sample. Protein digestion was performed according to the FASP procedure [25], and the resulting peptide mixture was labeled using the 8-plex iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems). The pimpled shell samples were labeled as (Sample A)-113, (Sample B)-114, (Sample D)-115, while normal eggshell samples (Sample E)-116, (Sample F)-117, and (Sample G)-118. iTRAQ labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE

Healthcare), and desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed inner diameter 7 mm, volume 3 mL, Sigma). All fractions were performed LC-MS/MS analysis on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA) against Uniprot *Gallus* database (24,408 entries, downloaded on June 2nd, 2016) and the decoy database.

3. Results

3.1. Comparison of physical and matrix properties of experimental eggs and eggshells

Pimpled eggs and normal eggs were used in this study. The physical properties such as egg weight and breaking strength and matrix content of pimpled and normal eggs were compared (Table 1). The data showed that both weight and thickness of calcified eggshell of pimpled eggs were less than those from normal eggs (Table 1). However, in pooled eggshell powder, the contents of matrix fraction (including M1, M2 and M3) in pimpled eggshells were all much higher than that in normal eggshells (Table 1).

3.2. Protein identification in matrix fractions from pimpled and normal eggshells using label-free proteomic analysis

In the pimpled eggshells, 601, 486 and 443 proteins were identified in M1, M2 and M3 fractions, respectively. These proteins were combined into a proteomic inventory of 738 proteins for pimpled eggshells (Fig. 2A and Table S1). Based on the combined proteomics data, it is apparent that: i) there were 155 (21.0%), 25 (3.4%) and 71 (9.6%) unique proteins identified in M1, M2 and M3 respectively; ii) 420 (56.9%) common proteins were shared between M1 and M2, 331 (44.9%) proteins shared between M1 and M3, and 346 (46.9%) proteins overlapped between M2 and M3; iii) 305 (41.3%) common proteins were exist among the three fraction of matrix (Fig. 2A and Table S1); iv) 50.7% (305/601) of M1 proteome, 62.8% (305/486) of M2 proteome, and 68.8% (305/443) of M3 proteome belong to a triply shared common protein group. These data demonstrate that the majority of proteome in each fraction are shared the common proteins.

In normal eggshells, using label-free proteomic analysis, 464, 406 and 409 proteins were identified in M1, M2 and M3 fractions, respectively. These proteins were combined into a proteomic inventory of 600 proteins in the normal eggshell matrix components (Fig. 2B and Table S2). Based on the combined proteomics data, i) there were 85 (14.2%), 25 (4.2%) and 77 (12.8%) unique proteins in M1, M2 and M3; ii) there were 347 (57.8%) common proteins shared between M1 and M2, 298 (49.7%) proteins shared between M1 and M3, and 300 (50.0%) proteins shared between M2 and M3; iii) 266 (44.3%) proteins were triply shared among the three fractions of matrix (Fig. 2B and Table S2); and iv) 57.3% (266/464) of M1 proteome, 65.5% (266/406) of M2 proteome and 65.0% (266/409) of M3 proteome belonged to the triply shared common protein group. Similarly, in the normal eggs the majority of the proteome of each fraction were triply shared common proteins.

Based on the combined proteomic inventories of both types of eggshells, a total of 769 proteins were identified for chicken eggshells in the current study (Fig. 2C and Table S3). The cross-analysis showed that most of proteins were common to both the pimpled and normal proteomes. For example, 428 proteins (67.2%) were overlapped between both M1 fraction proteomes (Fig. 2D and Table S4), 364 proteins (68.9%) were common between M2 proteomes (Fig. 2E and Table S5), 363 proteins (74.2%) were common between M3 proteomes (Fig. 2F and Table S6), and 569 proteins (74.0%) were overlapped between combined proteomic inventories of both eggshells (Fig. 2C and Table S3). Moreover, the unique proteins in the pimpled eggshells, in the combined proteomic inventory or in various matrix fraction proteome, were more than those of the normal eggshells (Fig. 2C–F and Tables S3–S6).

3.3. The major proteins in various matrix fractions from pimpled or normal eggshells

Using the threshold of ≥0.1% abundance of the total identified proteome, the proteins in quail eggshells were grouped into major and minor proteins [20]. In the current study, the major proteins in the various matrix fractions of chicken eggshells were grouped by using the threshold value of 0.095% proteomic abundance to have more accurate classifying major proteins or minor protein. It was observed that 65 major proteins, covered 94.5% abundance of the pimpled M1 proteome, and 48 major proteins, covered 95.5% abundance of normal M1 (Fig. 3 and Table S7). There were 62 major proteins covering 95.3% abundance of the pimpled M2, and 47 major proteins covering 96.2% abundance of the normal M2 (Fig. 3 and Table S8). The 78 major proteins covered 95.8% abundance of the pimpled M3, and 65 major proteins covered 95.8% abundance of the normal M3 (Fig. 3 and Table S9). Overall, in the three matrix components, the major proteins covered up to 95% abundance of the proteome, which was similar to major proteins in quail shells (94% proteomic abundance) and turkey shells (95% proteomic abundance) [20].

Major proteins enriched were in the shared common protein lists. In pimpled eggshells, 98.5% (64/65) of major proteins in M1 proteome, 100% (62/62) of major proteins in M2, and 92.3% (72/78) of major proteins in M3 belonged to the triply shared protein group (Table S1). Similarly, in normal eggshells, 97.9% (47/48), 100% (47/47) and 98.5% (64/65) of major proteins in M1, M2, and M3, respectively, belonged to the triply shared protein group (Table S2). The results suggested that the major proteins in all matrix fractions were enriched in the triply shared protein lists of both pimpled and normal eggshells. Moreover, the cross-analysis of each matrix fraction proteomes between pimpled and normal eggshells showed that, the major proteins were also enriched in the dual matrix shared common protein group. For example, in both M1 matrix proteomes, 98.5% (64/65) of pimpled major proteins and 100% (48/48) of normal major proteins were in the common protein group (Table S4). In M2 matrix proteomes, 100% (62/62) of pimpled major proteins and 100% (47/47) of normal major proteins were in the common protein group (Table S5). In M3, 98.7% (77/78) of

Table 1
Variables of each repetition of pimpled and normal eggs.

Egg group	Size of specimen	Egg weight (g)	Calcified shell weight (g)	Shell breaking strength (kgf)	Calcified shell thickness (mm)	M1 content (%)[a]	M2 content (%)[a]	M3 content (%)[a]
Pimpled 1	12	65.3 ± 1.4	3.90 ± 0.27	1.77 ± 0.22	0.263 ± 0.013	2.05	0.708	0.228
Pimpled 2	12	64.9 ± 2.2	4.08 ± 0.33	2.00 ± 0.23	0.277 ± 0.014	2.18	0.485	0.261
Pimpled 3	12	65.4 ± 1.9	4.28 ± 0.27	1.99 ± 0.27	0.280 ± 0.014	1.94	0.478	0.219
Normal 1	16	68.1 ± 1.2	5.83 ± 0.12	3.96 ± 0.15	0.348 ± 0.007	1.10	0.395	0.146
Normal 2	16	66.9 ± 1.0	5.70 ± 0.17	3.96 ± 0.14	0.344 ± 0.007	1.10	0.400	0.144
Normal 3	16	68.7 ± 1.0	5.74 ± 0.11	3.95 ± 0.12	0.341 ± 0.006	1.14	0.409	0.153

[a] Matrix content; percentage content of matrix fraction in the calcified eggshell.

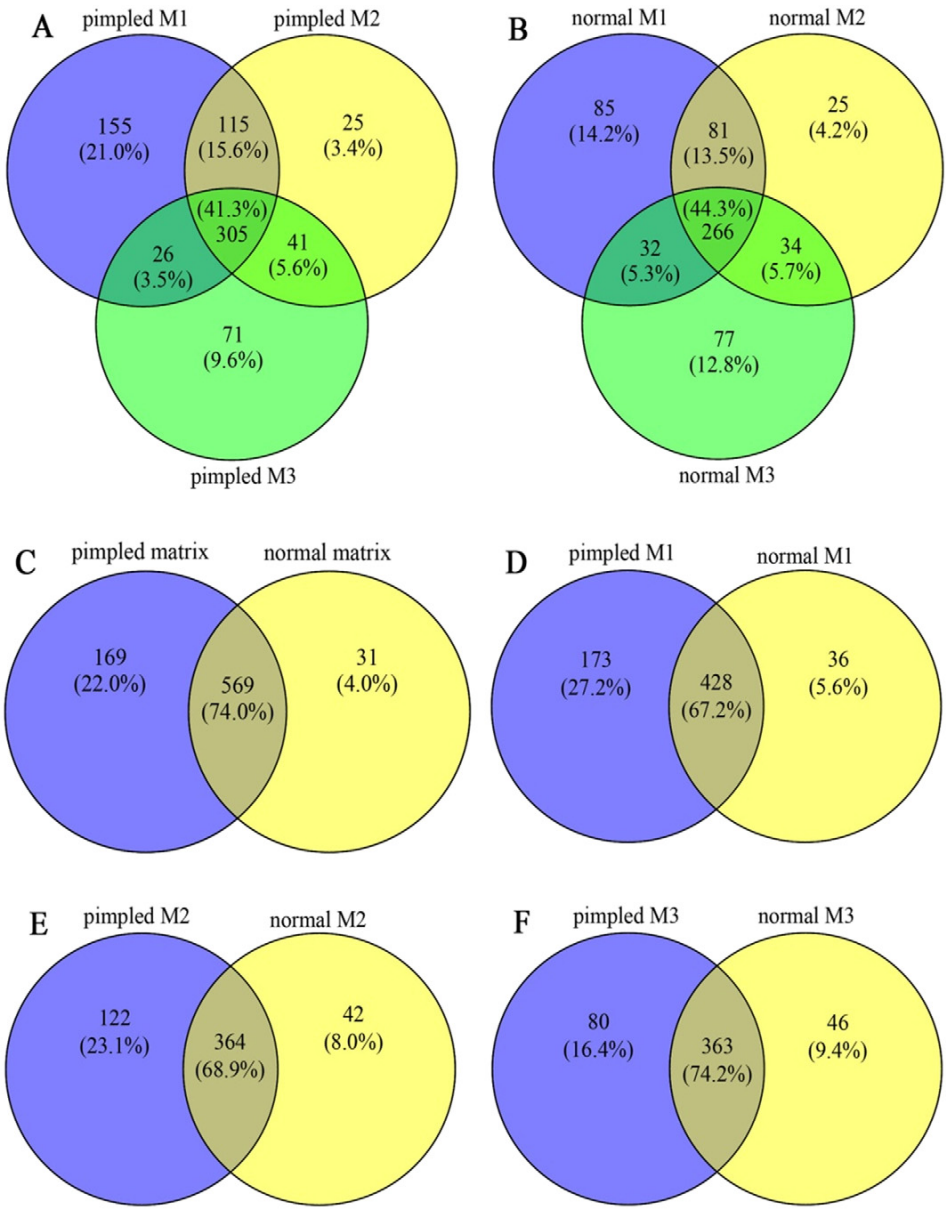

Fig. 2. Venn diagrams representing the overlapped common proteins and unique proteins. A. Representing overlapped proteins and unique proteins between three matrix fractions from pimpled calcified eggshells; B. representing overlapped proteins and unique proteins between three matrix fractions from normal eggshells; C. representing overlapped proteins and unique proteins between the combined proteomic inventories of both eggshells; D. representing overlapped proteins and unique proteins between the M1 proteomes of both eggshells; E. representing overlapped proteins and unique proteins between the M2 proteomes of both eggshells; F. representing overlapped proteins and unique proteins between the M3 proteomes of both eggshells.

pimpled major proteins and 100% (65/65) of normal major proteins were in the overlapped protein group (Table S6). Considering all of the major proteins in both pimpled and normal matrix components, 42 common major proteins (Ovotransferrin shared 2 different UniProt accession numbers) were found to triply overlap among the three matrix components (Table 2), they might act as backbone constituents in chicken eggshell matrix. In the common major protein list, Sentan (Accession No. B7FF67) was a novel identified major protein in chicken eggshell. Among vertebrates, the respiratory and oviduct cilia have conserved and specific apical structures, and Sentan is the protein localized exclusively at the ciliary tips and showed affinity for the membrane protrusions and phosphatidylserine [26]. In current results, Sentan shows negative effects on the eggshell quality but its functional mechanism is unknown.

3.4. Differential proteins between pimpled and normal shell matrix fractions

Next, we applied the cutoff values of 1.75-fold for up-regulated and 0.57-fold for down-regulated proteins. We found 69 significantly up-regulated proteins and 27 down-regulated proteins in the pimpled M1 fraction (Fig. 4A and Table S10); 74 proteins significantly up-regulated and 40 proteins down-regulated (Fig. 4A and Table S11) in pimpled M2 fraction, and 56 up-regulated proteins and 62 down-regulated

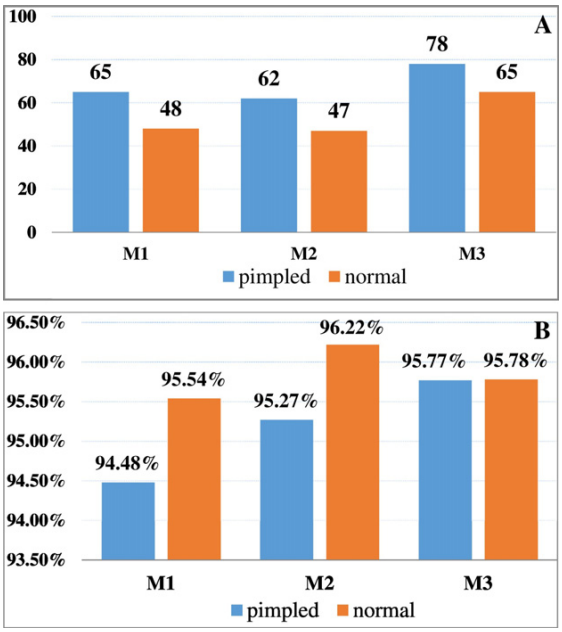

Fig. 3. Size and abundance of major protein group in each matrix fraction. A. Size of major protein group in each matrix fraction from pimpled and normal eggshells. B. Abundance of major protein group in each matrix fraction proteome.

proteins in the pimpled M3 fraction (Fig. 4A and Table S12). Moreover, in M1 fractions, up-regulated proteins covered 11.2% abundance of pimpled proteome and 1.7% of normal proteome, and down-changed proteins covered 0.4% of pimpled proteome and 1.0% of normal proteome (Fig. 4B and Table S10); in M2 fractions, up-regulated proteins covered 22.6% abundance of pimpled proteome and 8.1% of normal proteome, and down-regulated proteins covered 0.5% of pimpled proteome and 1.1% of normal proteome (Fig. 4B and Table S11); in M3 fractions, up-regulated proteins covered 27.7% abundance of pimpled proteome and 7.3% of normal proteome, and down-regulated proteins covered 13.1% of pimpled proteome and 26.1% of normal proteome (Fig. 4B and Table S12). Overall, in M1 and M2 fractions the covered abundance of up-regulated protein group were much higher than that of down-regulated proteins, which suggested the abundance disequilibrium between up- and down-changed differential proteins might majorly be present in M1 and M2 fractions.

3.5. The cross-analysis on major differential proteins in the M1, M2 and M3 fractions

Based on the major protein lists, cross-analysis on differential proteins was performed across the three matrix fractions. 23 common differential major proteins were identified (Table 3). Among these, 9 proteins were commonly major in all three-matrix fractions, and other proteins were major at least in one matrix fraction (Table 3). Since these proteins were commonly differential in all matrix fractions and with high abundance, they might be important candidates for eggshells quality. Moreover, another two proteins, Protein S100-A6 and Transthyretin, consistently fold-changed several times across three matrix fractions (Table 3), we think they might also be candidates for eggshell quality although their significant values in M1 fraction were $P > 0.05$ (Table 3).

The results of differential proteins between the both M1 fractions were verified by using iTRAQ (isobaric tagging for relative and absolute quantification) proteomic method. Among the differential proteins, except the proteins not-detectable, the fold-change directions of other proteins in iTRAQ results were all consistent with that of Label free determination (Table 3) suggesting that the current results are credible.

4. Discussion

4.1. Proteomic inventory of global matrix in avian calcified eggshells

Using high-throughput MS-based proteomics, the global matrices from several species of calcified eggshell have been reported. In chicken eggshell matrix, 466 proteins have been determined [18]; 697 proteins were identified in turkey eggshell matrix [19]; and 622 proteins in quail eggshells [20]. Furthermore, by combining several studies about chicken eggshell matrix, including global matrix and partial matrix [18,22,24,27–29], an assembled proteomic inventory of 699 proteins could be established [24]. The current study identified 738 proteins for the pimpled shell matrix (Fig. 1A and Table S1), and 600 proteins for the normal matrix (Fig. 1B and Table S2). In summary, both results when combined provide a proteomic inventory of 769 proteins (Fig. 1C and Table S3), which further extends the proteomic inventory of chicken eggshells.

4.2. Comparison of proteomics between three matrix fractions from the same eggshells

The proteins in an eggshell can be divided into insoluble and soluble proteins. The insoluble proteins are thought to be an inter-mineral matrix and acts as a structural framework, while the soluble proteins were thought as intra-mineral matrix that are embedded within the crystal during calcification [30], however, the solvent involved is unknown. The current study relies on 10% acetic acid for decalcification and the eggshell matrix was dissociated into three matrix components, *i.e.* acid-insoluble matrix (M1), water-insoluble matrix (M2) and facultative-soluble matrix (M3). However, the proteomic results of both pimpled and normal eggshells show that, for the same eggshells, the majority of any matrix fraction proteome belonged to triply overlapped common protein lists, which account for >40.0% of the combined proteomic inventory (Fig. 1A and B and Tables S1 and S2). Furthermore, proteomic quantification results show that, the major proteins of any matrix fraction almost enriched in the triply overlapped common protein lists (Tables S1 and S2), and the major proteins of any matrix component covered about 95% of the proteomic abundance (Tables S7–S9). Overall, above results suggest that, in the same eggshell sample, high similarity exists among the proteomes of three matrix fractions regardless of their solubility characteristics, and the triply overlapped common proteins formed a predominant contributor for the proteomic abundance of any matrix components. The shell matrix is a meshwork of vesicles connected by fine fibers or fibrous sheets, and even in the case of the ultrasonic disruptor, the structure of the vesicles is very stable [11]; therefore, the relative abundance (or constitutive proportion) varieties of some triply overlapped common proteins among three matrix fractions might be the key cause of their solubility differences.

Notes to Table 2
[a] "Fold change" and "t-test sig." in bold; significantly changed.
[b] –, lacking information.
[c] emPAI (exponentially modified protein abundance index) in ref.24: abundances of proteins in eggshells sampled at the stage of 16-h shell calcification.
[d] me, membranes; ma, mammillary; p, palisade layer; c, cuticle layer.

Table 2
Common major proteins overlapped throughout M1, M2 and M3 fractions from pimpled or normal eggshells.

UniProt Accession NO.	Protein name	iBAQ(%)/ hierarchy rank in pimpled M1	iBAQ(%)/ hierarchy rank in normal M1	Fold change1/ t-test sig.[a]	iBAQ(%)/ hierarchy rank in pimpled M2	iBAQ(%)/ hierarchy rank in normal M2	Fold change2/ t-test sig.[a]	iBAQ(%)/ hierarchy rank in pimpled M3	iBAQ(%)/ hierarchy rank in normal M3	Fold change3/ t-test sig.[a]	Abundance in ref.18 ($\times 10^3$) [b]	emPAI in ref.21[b]	emPAI in ref.24[b,c]	eggshell localization in ref.22,23[b,d]
F1NSM7	Ovocleidin-116	38.006 / 1	43.619 / 1	0.87 / 0.23	24.664 / 1	29.515 / 1	0.84 / 0.02	15.881 / 1	25.550 / 1	0.62 / 0.00	1224.7	65.3 (high)	7167.8	me,ma,p,c
R9PXP5	Ovalbumin	8.317 / 2	8.4624 / 3	0.98 / 0.93	2.761 / 8	1.451 / 10	**1.90 / 0.04**	2.533 / 9	2.396 / 9	1.06 / 0.79	322.7	85.6 (high)	368.5	me,ma
Q9PRS8	Ovocleidin-17	5.989 / 3	9.292 / 2	0.64 / 0.00	19.579 / 2	25.122 / 2	0.78 / 0.04	1.420 / 15	2.434 / 8	0.58 / 0.10	218.1	64.8 (high)	3315.0	me,ma,p,c
F1NPR2	whey acidic-like isoform X2	5.025 / 4	3.349 / 7	1.50 / 0.33	1.629 / 14	0.807 / 13	**2.02 / 0.01**	1.556 / 14	1.061 / 16	1.47 / 0.04	—	—	42.5	—
F1P1Y2	BPI fold-containing family B member 4-like	3.846 / 5	5.608 / 4	0.69 / 0.02	2.714 / 9	3.212 / 7	0.84 / 0.30	0.478 / 33	0.595 / 26	0.80 / 0.56	—	16.8 (high)	26.3	—
F1NCN3	EGF-like repeat and discoidin I-like domain-containing 3	3.287 / 6	3.947 / 6	0.83 / 0.05	5.095 / 3	7.101 / 3	0.72 / 0.01	1.063 / 20	1.490 / 12	0.71 / 0.10	215.1	83.3 (high)	3022.9	me,ma,p
Q9YGP0	Clusterin	3.222 / 7	4.887 / 5	0.66 / 0.01	2.791 / 7	3.878 / 6	0.72 / 0.05	10.665 / 3	20.539 / 2	**0.52 / 0.05**	723.4	42.7 (high)	347.4	me,ma,p,c
F2Z4L6	Serum albumin	2.856 / 8	0.355 / 18	**8.04 / 0.03**	2.449 / 11	0.198 / 32	**12.39 / 0.00**	12.089 / 2	2.936 / 5	**4.12 / 0.00**	504.6	113.5 (high)	1489.3	me,ma
D3KYT5	Ovocalyxin-32	2.708 / 9	2.314 / 8	1.17 / 0.57	4.835 / 4	4.265 / 5	1.13 / 0.59	0.513 / 32	0.515 / 29	1.00 / 0.99	197.8	71 (high)	23.7	me,ma,c
E1C2G7	gastrokine-2/ Ovocalyxin-21	2.510 / 10	2.303 / 9	1.09 / 0.57	4.293 / 6	4.483 / 4	0.96 / 0.65	0.604 / 26	0.634 / 24	0.95 / 0.88	343.8	176.8 (high)	92.4	me,ma
E1BQC2	Ovotransferrin	2.135 / 11	1.063 / 11	2.01 / 0.11	2.520 / 10	1.099 / 12	**2.29 / 0.04**	2.841 / 7	1.705 / 10	1.67 / 0.00	457.9	22.9 (high)	2514.5	me,ma
Q53HW8	Ovocalyxin-36	0.980 / 14	1.724 / 10	0.57 / 0.13	0.793 / 16	1.268 / 11	0.63 / 0.02	0.112 / 71	0.268 / 43	**0.42 / 0.03**	554.3	23.2 (high)	34.3	me,ma,p,c
P08250	Apolipoprotein A-I	0.991 / 13	0.098 / 48	**10.09 / 0.03**	0.301 / 30	0.013 / 125	**22.53 / 0.01**	0.197 / 50	0.007 / 211	**29.78 / 0.03**	201.4	2.2 (medium)	39.0	me
F1NXM7	prostate stem cell antigen	0.754 / 15	0.586 / 13	1.29 / 0.62	0.132 / 51	0.108 / 44	1.22 / 0.59	0.246 / 46	0.238 / 47	1.03 / 0.92	320.1	0.8 (low)	54.4	—
Q5ZMQ2	Actin, cytoplasmic 2	0.638 / 16	0.187 / 32	**3.41 / 0.00**	0.291 / 31	0.087 / 51	**3.33 / 0.00**	0.125 / 67	0.029 / 110	**4.27 / 0.00**	284.5	9 (high)	—	—
F1NSC7	Ig lambda light partial	0.594 / 18	0.160 / 35	**3.71 / 0.04**	0.970 / 15	0.321 / 24	**3.02 / 0.02**	1.823 / 12	0.715 / 22	2.56 / 0.13	immunoglobulin	immunoglobulin	immunoglobulin	—
F1NBX7	Ig lambda light partial	0.592 / 19	0.054 / 68	**11.06 / 0.02**	0.394 / 25	0.143 / 39	**2.75 / 0.00**	0.960 / 21	0.473 / 30	**2.03 / 0.02**	immunoglobulin	immunoglobulin	immunoglobulin	—
A2N883	VH1 protein	0.501 / 20	0.129 / 40	**3.87 / 0.00**	0.651 / 17	0.327 / 23	**1.99 / 0.05**	0.350 / 39	0.007 / 210	**48.36 / 0.01**	immunoglobulin	immunoglobulin	immunoglobulin	ma
R9PXM5	Ig lambda chain V-1 region isoform X2	0.435 / 21	0.138 / 38	**3.15 / 0.00**	1.876 / 13	0.554 / 17	**3.39 / 0.01**	2.595 / 8	0.844 / 19	**3.07 / 0.01**	immunoglobulin	immunoglobulin	immunoglobulin	
F1NAR5	alpha-2-antiplasmin isoform X2	0.423 / 22	0.410 / 15	1.03 / 0.78	0.422 / 23	0.495 / 18	0.85 / 0.22	0.421 / 34	0.831 / 20	**0.51 / 0.02**	—	Low-medium	65.852	—
F1NC22	Ig lambda partial	0.420 / 23	0.084 / 53	**4.99 / 0.01**	0.316 / 28	0.073 / 56	**4.31 / 0.00**	0.629 / 25	0.215 / 50	**2.93 / 0.00**	immunoglobulin	immunoglobulin	immunoglobulin	—
P27731	Transthyretin	0.385 / 24	0.048 / 70	7.98 / 0.23	0.243 / 34	0.027 / 92	**9.07 / 0.01**	0.538 / 29	0.085 / 69	**6.30 / 0.01**	104.2	2.2 (medium)	4.4935	ma
Q8JGM4	Sulfhydryl oxidase 1	0.380 / 25	0.386 / 17	0.99 / 0.88	0.376 / 26	0.461 / 19	0.82 / 0.05	0.388 / 36	0.681 / 23	**0.57 / 0.04**	—	4.7 (medium)	13.357	me,ma,p
E1C0K5	lactadherin isoform X2/milk fat globule-EGF factor 8 protein	0.334 / 26	0.459 / 14	0.73 / 0.26	0.206 / 38	0.319 / 25	0.65 / 0.06	0.096 / 78	0.269 / 42	**0.36 / 0.01**	—	15.9 (high)	37.01	me,ma
A0A140T8F5	polymeric Ig receptor	0.327 / 28	0.162 / 34	2.01 / 0.06	0.332 / 27	0.185 / 35	1.80 / 0.08	0.555 / 27	0.546 / 28	1.02 / 0.95	286.7	5.4 (medium)	18.175	ma
H9L385	Hemopexin	0.304 / 29	0.277 / 24	1.10 / 0.70	0.180 / 44	0.164 / 37	1.10 / 0.50	1.166 / 17	1.653 / 11	0.71 / 0.10	210.8	11.6 (high)	139.02	ma
E1BSP1	Prosaposin	0.302 / 31	0.295 / 23	1.02 / 0.77	0.124 / 53	0.188 / 34	0.66 / 0.12	0.391 / 35	0.959 / 17	**0.41 / 0.00**	—	9 (high)	29.21	p,c
P01005	Ovomucoid	0.300 / 32	0.240 / 26	1.25 / 0.70	0.421 / 24	0.070 / 60	6.04 / 0.45	4.011 / 6	4.180 / 4	0.96 / 0.92	388	2.2 (medium)	42.6325	me
P00698	Lysozyme C	0.277 / 34	0.155 / 36	1.78 / 0.06	4.420 / 5	2.279 / 9	**1.94 / 0.01**	6.681 / 4	5.323 / 3	1.26 / 0.22	1053.4	128.2 (high)	13537	me,ma
B7FF67	Sentan	0.275 / 35	0.189 / 31	1.46 / 0.29	0.141 / 48	0.095 / 47	1.48 / 0.21	0.355 / 38	0.258 / 45	1.38 / 0.14	—	—	—	—
Q90839	Dickkopf-related protein 3	0.256 / 36	0.407 / 16	0.63 / 0.30	0.214 / 37	0.334 / 22	0.64 / 0.04	0.322 / 43	0.633 / 25	0.51 / 0.13	321.4	14 (high)	83.003	ma
F1NSM8	Osteopontin	0.240 / 39	0.321 / 21	0.75 / 0.27	2.298 / 12	2.336 / 8	0.98 / 0.91	0.191 / 52	0.227 / 48	0.84 / 0.36	867.5	1.89 (low)	7.1359	—
P21611	Beta-2-microglobulin	0.214 / 40	0.105 / 44	2.04 / 0.10	0.237 / 35	0.075 / 52	**3.16 / 0.02**	0.538 / 30	0.366 / 34	1.47 / 0.09	722.6	4.6 (medium)	—	me
F1NVF3	Vitamin-D binding protein	0.184 / 42	0.017 / 127	**10.69 / 0.03**	0.178 / 45	0.018 / 109	**9.89 / 0.02**	0.803 / 23	0.156 / 56	**5.15 / 0.04**	306.9	9 (high)	15.127	—
P01875	Ig mu chain C	0.180 / 44	0.137 / 39	1.32 / 0.41	0.253 / 32	0.236 / 27	1.07 / 0.82	0.162 / 57	0.274 / 40	0.59 / 0.07	185.3	30.6 (high)	17.425	ma
F1NGB1	nucleobindin-2	0.179 / 45	0.342 / 19	**0.52 / 0.01**	0.169 / 46	0.214 / 30	0.79 / 0.12	0.874 / 22	1.140 / 14	0.77 / 0.03	465.6	7.3 (medium)	138.76	ma,p,c
H9KZK6	PIT 54	0.159 / 47	0.021 / 112	7.54 / 0.23	0.198 / 39	0.016 / 117	**12.69 / 0.02**	2.026 / 11	0.280 / 39	7.24 / 0.07	166.8	1.6 (low)	23.419	—
E1C652	proenkephalin-A	0.153 / 49	0.314 / 22	0.49 / 0.09	0.533 / 21	0.587 / 16	0.91 / 0.74	0.152 / 61	0.194 / 53	0.78 / 0.47	495.4	1.5 (low)	6.868	—
E1C004	carbonic anhydrase 4	0.128 / 58	0.186 / 33	0.69 / 0.14	0.187 / 41	0.301 / 26	0.62 / 0.01	0.202 / 49	0.449 / 31	**0.45 / 0.00**	332.1	2.9 (medium)	7.7954	ma
P02789	Ovotransferrin	0.110 / 62	0.079 / 56	1.39 / 0.26	0.253 / 33	0.128 / 40	**1.97 / 0.01**	0.387 / 37	0.263 / 44	1.47 / 0.03	373.9	—	—	me,ma
R4GLT1	Cystatin	0.101 / 63	0.128 / 41	0.79 / 0.21	0.574 / 20	0.408 / 21	1.41 / 0.15	2.362 / 10	2.542 / 7	0.93 / 0.74	401.5	45.4 (high)	358.46	me
F1NSC8	Ig lambda partial	0.095 / 64	0.021 / 113	**4.55 / 0.02**	0.110 / 56	0.028 / 86	**3.96 / 0.03**	0.146 / 63	0.051 / 88	**2.83 / 0.02**	immunoglobulin	immunoglobulin	immunoglobulin	—
F1N9D8	Cathepsin B	0.077 / 76	0.122 / 42	0.63 / 0.04	0.096 / 61	0.124 / 41	0.77 / 0.02	0.173 / 55	0.298 / 37	0.58 / 0.00	242.1	24.8 (high)	7.4772	me

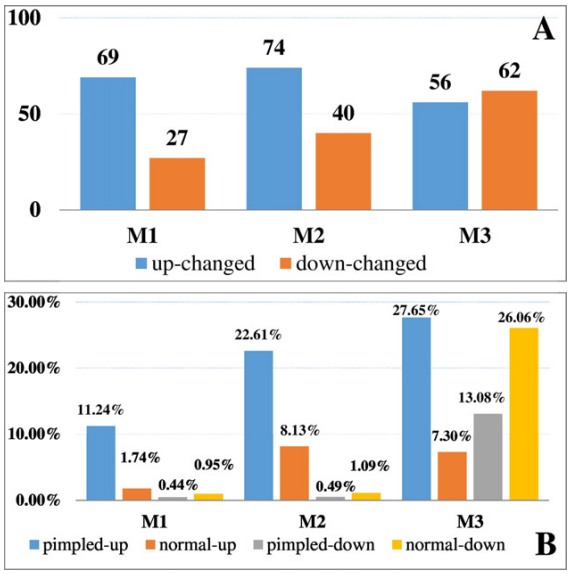

Fig. 4. Size and abundance of differential protein group in each matrix fraction. A. Size of differential protein group in each matrix fractions between pimpled and normal eggshells. B. Abundance of differential protein group in each matrix fraction proteome.

4.3. Proteomic comparison for each matrix fraction between both eggshells

Pimpled eggshell is one class of eggshell abnormality. Compared to normal eggshells, pimpled eggshells are much thinner and have weaker breaking strength (Table 1). Moreover, in pimpled calcified eggshells, not only are the percentage contents (Table 1) but also the proteomic inventories (Fig. 1C–F; and Tables S4–S6) of any matrix fractions much greater than that in normal calcified eggshells. However, between the pimpled and normal eggshells, overlaps in the M1, M2, M3 fraction proteomes, and their combined proteomic inventories are, respectively, 67.2%, 68.9%, 74.2%, and 74.0% (Fig. 1C–F and Tables S3–S6). Nearly all (>98%) of the major proteins of each matrix component are concentrated in the dually overlapped common protein lists (Tables S4–S6). These results are consistent with other related studies. The proteomic study of the whole matrix from normal chicken eggshells with different breaking strength showed that most of proteins were common between strong and weak eggshell proteomes [18]. The overlap of global proteomes between chicken and turkey eggshells was reportedly up to 52%, and the turkey proteins of high-abundance mainly distributed in the common protein group [19]. Overall, the above results suggest that for any matrix components of eggshells with different quality or even from different poultry species, the majority of their proteomes are in common, and the common proteins should be the dominant contributor for their abundance in these proteomes.

We have found that the percentage contents of the three matrix components in the calcified eggshells are not linearly correlated with eggshell quality [31]. In the current study, the results show that the major proteins covered up to 95% abundance of any matrix component, and the major proteins of each matrix component were enriched in the triply overlapped common protein list (Tables S7–S9). Therefore, we hypothesized that compared with the content of matrix component, the constitutive proportions (relative abundance) of some proteins in the common lists might be much more important for the eggshell quality. The post-translation modification of some matrix proteins might be considered as another important regulator for the eggshell quality, however, the contents of glycosaminoglycans in the calcified eggshells are not linearly correlated with eggshell quality [32].

4.4. Major proteins common throughout the three matrix fractions

The current study reveals 42 common major proteins overlapped among the three matrix components (Table 2). A series of immunoglobulins were identified in the common major protein list (Table 2), although most Accession numbers of these cannot be found in reported chicken eggshell proteomes [18,21,24]. However, immunoglobulins are encoded by a large and complex gene family, and many immunoglobulin proteins with different protein ID or gene ID have been previously identified as major shell proteins [18,21,24]. Except for immunoglobulin, most of the common major proteins were also identified as major proteins or proteins with high abundance in previous reports (Table 2) [18,21,24]. Therefore, it is possible that the current common major proteins might act as backbone constituents in the chicken eggshell matrix. Among these major proteins, Ovocleidin-116, Ovalbumin, EDIL3 and Ovocalyxin-36 were also determined and defined as the most abundant proteins (>1.0% proteomic abundance of the whole eggshell matrix) in both quail and turkey calcified eggshells, respectively accounting for 71.8% abundance of the quail matrix and 53.5% of the turkey matrix [19,20]. There were two proteomic studies that showed the localizations of certain eggshell proteins. Among the current most major proteins with abundances >1.0% in the M1 proteome, ovocleidin-116, ovocleidin-17, clusterin and ovocalyxin-36 distributed throughout the whole eggshell, including membranes, mammillary, palisade and cuticle layers. Ovalbumin, serum albumin, ovocalyxin-21 and ovotransferrin were mainly distributed in the membranes and mammillary layer, EDIL3 was mainly distributed in the membranes, mammillary and palisade layers, and ovocalyxin-32 was mainly distributed in the membranes, mammillary and cuticle layers (Table 2) [22,23].

Matrix precursors in the uterine fluid can reportedly affect the nucleation or crystal growth in opposite directions [10,33]. This functional heterogeneity also exists among the proteins in the eggshells. By taking the most major proteins in current study, since their abundances were very high, few of these meet the differential standards (fold-changes >1.75 or <0.57, $P < 0.05$). However, when pimpled eggshells are compared with normal eggshells, ovocleidin-116, ovocleidin-17, BPI fold-containing family B member 4-like, EDIL3, clusterin and ovocalyxin-36, tend to positively regulate the eggshell quality (Table 2). Whey acidic-like isoform X2, serum albumin, ovocalyxin-32 and ovotransferrin show the opposite effects (Table 2) and the effect of ovocalyxin-21 was intermediate (Table 2). Overall, above results suggest that the proteins in eggshells also affect eggshell quality in different directions, and it is suggests that there may be some equilibrium between matrix proteins during eggshell mineralization to ensure eggshell texture and quality. The proportions of some proteins in eggshells might decrease when other proteins increase, which might change the equilibrium and result in the variation seen in eggshell quality.

4.5. Differential major proteins

Based on cross-analysis on both differential proteins and major proteins through the three matrix components, 25 differential major proteins are revealed (Table 3). These are all up-regulated in pimpled eggshells. It is known that proteins play an important role in inducing Ca-P mineralization *in vivo*, among which, some proteins affect the biomineralization in dual directions, promoting or inhibiting the nucleation and growth of Ca-P precipitates, depending on the type, concentration and conformation of proteins *in vitro* or *in vivo* [34]. This increases the likelihood that when the abundances of some differential proteins enhanced over the levels in normal eggshells, they would have a negative impact on eggshell calcification. Serum albumin is a blood major protein and is also identified in egg yolk and is named as α-livetin [21]. Serum albumin is important for *in vivo* Ca-P biomineralization and shows multiple, concentration dependent functions in biomineralization. Serum albumin acts as a promoter for the growth rate of

Table 3
Common differential major proteins.

UniProt Accession No.	Protein name	iBAQ (%)/hierarchy rank in pimpled M1[a]	iBAQ (%)/hierarchy rank in normal M1[a]	Fold change1/*t*-test sig.[b]	iBAQ (%)/hierarchy rank in pimpled M2[a]	iBAQ (%)/hierarchy rank in normal M2[a]	Fold change2/*t*-test sig.[b]	iBAQ (%)/hierarchy rank in pimpled M3[a]	iBAQ (%)/hierarchy rank in normal M3[a]	Fold change3/*t*-test sig.[b]	Verification of FC1/*t*-test sig. by iTraq[a]
F2Z4L6	Serum albumin	2.856/8	0.355/18	8.04/0.03	2.449/11	0.198/32	12.39/0.00	12.089/2	2.936/5	4.12/0.00	1.83/0.01
P08250	Apolipoprotein A-I	0.991/13	0.098/48	10.09/0.03	0.301/30	0.013/125	22.53/0.01	0.197/50	0.007/211	29.78/0.03	1.93/0.00
Q5ZMQ2	Actin, cytoplasmic 2	0.638/16	0.187/32	3.41/0.00	0.291/31	0.087/51	3.33/0.00	0.125/67	0.029/110	4.27/0.00	n.d.
F1NBX7	Ig lambda light partial	0.592/19	0.054/68	11.06/0.02	0.394/25	0.143/39	2.75/0.00	0.960/21	0.473/30	2.03/0.02	1.16/0.02
A2N883	VH1 protein	0.501/20	0.129/40	3.87/0.00	0.651/17	0.327/23	1.99/0.05	0.350/39	0.007/210	48.36/0.01	2.05/0.00
R9PXM5	Ig lambda chain V-1 region isoform X2	0.435/21	0.138/38	3.15/0.00	1.876/13	0.554/17	3.39/0.01	2.595/8	0.844/19	3.07/0.01	1.89/0.00
F1NC22	Ig lambda partial	0.420/23	0.084/53	4.99/0.01	0.316/28	0.073/56	4.31/0.00	0.629/25	0.215/50	2.93/0.00	1.28/0.01
F1NVF3	Vitamin-D binding protein	0.184/42	0.017/127	10.69/0.03	0.178/45	0.018/109	9.89/0.02	0.803/23	0.156/56	5.15/0.04	n.d.
F1NSC8	Ig lambda partial	0.095/64	0.021/113	4.55/0.02	0.110/56	0.028/86	3.96/0.03	0.146/63	0.051/88	2.83/0.02	1.12/0.26
P02659	Apovitellenin-1	1.060/12	0.015/136	70.18/0.00	0.228/36	0.003/245	88.88/0.00	0.063/93	n.d.	Infinity	2.65/0.00
F1NFL6	Vitellogenin-2	0.329/27	0.021/114	15.85/0.01	0.109/57	0.019/107	5.68/0.00	0.004/260	0.00009/404	40.17/0.00	2.21/0.00
E1BV78	Fibrinogen gamma chain	0.140/53	n.d.	Infinity	0.035/99	n.d.	Infinity	0.001/350	n.d.	Infinity	n.d.
P02112	Hemoglobin subunit beta	0.304/30	0.005/220	62.79/0.05	0.094/63	n.d.	Infinity	0.053/97	n.d.	Infinity	2.99/0.00
P62801	Histone H4	0.163/46	0.001/391	294.65/0.01	0.035/99	n.d.	Infinity	0.001/350	n.d.	Infinity	1.69/0.02
P02552	Tubulin alpha-1 chain	0.252/37	0.091/50	2.77/0.03	0.066/78	0.019/108	3.49/0.00	n.d.	n.d.	–	1.37/0.09
Q9YGW6	Ezrin	0.136/55	0.034/86	3.96/0.00	0.093/64	0.022/97	4.21/0.00	0.076/88	0.016/145	4.78/0.00	1.29/0.01
O93532	Keratin, type II cytoskeletal cochleal	0.150/50	0.024/105	6.35/0.00	0.024/121	0.005/202	5.34/0.00	0.017/156	0.008/189	2.09/0.01	1.69/0.01
P27731	Transthyretin	0.385/24	0.048/70	7.98/0.23	0.243/34	0.027/92	9.07/0.01	0.538/29	0.085/69	6.30/0.01	1.67/0.00
Q98953	Protein S100-A6	0.072/80	0.009/173	8.32/0.12	0.589/19	0.075/53	7.85/0.03	4.500/5	1.309/13	3.44/0.00	1.32/0.03
P02752	Riboflavin-binding protein	n.d.	n.d.	–	n.d.	n.d.	–	0.185/53	0.068/75	2.73/0.01	n.d.
Q6WEB3	Thymosin beta 4	n.d.	n.d.	–	n.d.	n.d.	–	0.350/40	0.052/85	6.70/0.03	n.d.
X1WI10	Non-histone chromosomal protein	n.d.	n.d.	–	0.007/204	n.d.	Infinity	0.098/74	0.001/298	66.34/0.02	n.d.
Q7LZS1	12 K serum protein, beta-2-m cross-reactive	0.071/81	n.d.	Infinity	n.d.	n.d.	–	0.264/45	n.d.	Infinity	1.40/0.13
P62149	Calmodulin	0.007/238	n.d.	Infinity	0.065/80	0.013/129	5.18/0.00	1.074/19	0.346/35	3.10/0.00	n.d.
P08629	Thioredoxin	0.006/252	n.d.	Infinity	0.011/179	0.001/307	10.96/0.01	0.114/70	0.035/106	3.23/0.00	1.43/0.01

[a] n.d., not detectable.
[b] –, lacking information.

Ca-P precipitates at low levels but as an inhibitor at higher concentrations [35–37]. Apolipoprotein A-I (ApoA-I) is the major component of high-density lipoproteins (HDL) particles in plasma and has an anti-biocalcification effect on human aortic valves. ApoA-I was much more abundant in normal aortic valves than in stenotic aortic valves [38]. Vitamin-D binding protein (DBP) belongs to the albumin gene family and is a multifunctional protein in plasma. It is capable of binding iron or vitamins inhibiting bacteria and activating macrophages and osteoclasts [21]. DBP is present at both growth and terminal phases of eggshell calcification [39] and it is in higher abundance in the weak eggshells than in strong eggshells [18]. The three proteins described above are in high-abundance compared with other differential major proteins, however, except these three proteins, to our knowledge have no known effect on the eggshell crystal nucleation and growth.

Biomineralization-associated proteins are mainly comprised of collagens, amelogenins, non-collagenous proteins and proteins in the blood [37]. In the current study few of the differential proteins belonged to collagens or amelogenins, instead they belonged to proteins of the blood and non-collagenous proteins.

Serum albumin, ApoA-I, and vitamin-D binding protein are blood proteins and synthesized by liver. Furthermore, some other differential proteins identified in this study were also derived from plasma. Apovitellenin-1 (Apov1) is a major component of not only very low-density lipoprotein (VLDL) particles, but also the granules of egg yolk [40]. Apov1 acts as a potent lipoprotein lipase inhibitor, preventing the loss of triglycerides from VLDL on their way from the liver, blood to the follicles. Apov1 was previously identified in chicken eggshells [18,21] and turkey eggshells [19]. Vitellogenins, normally synthesized in liver and presenting in blood, are precursor proteins of egg yolk. Vitellogenin-2 (VTG2) is antibacterial because of its capacity to chelate iron ions [41] and VTG2 is abundantly present in the uterine fluid at the active growth phase of eggshells [39]. Fibrinogen-γ chain (FGG) is synthesized in the liver, together with fibrinogen-α (FGA) and fibrinogen-β (FGB), FGG polymerizes to form an insoluble fibrin matrix, and participates in hemostasis as one of the primary components of blood clots. Riboflavin-binding protein (RBP) is synthesized by the liver and secreted into blood. It was detected in yolk, egg white, and eggshell membranes and has the capability of inhibiting bacterial growth [23]. Transthyretin (TTR) is also expressed in the liver and secreted into blood, and functions as transporters of thyroxine and retinol. TTR was abundant in yolk of fertilized egg [40], and is also distributed in the shell mammillary cones [23]. Hemoglobin subunit-β (HBB) is a globin protein, which is synthesized by immature erythrocytes and located in red blood cells. Hemoglobin has a potent oxygen-binding capacity, and also carries the body's respiratory carbon dioxide [42]. To our knowledge, 12 K serum protein, β-2-m cross-reactive (Accession No. Q7LZS1) is a novel protein determined in eggshells. Additionally, a series of immunoglobulins (Accession No. F1NBX7, A2N883, R9PXM5, F1NC22, F1NSC8) were also identified in the current differential protein group and members of the immunoglobulin class have also been previously identified in chicken eggshells [18,21,24]. Immunoglobulins are secreted into plasma after being synthesized in the spleen or in lymphocytes. Immunoglobulins are the tools to tackle all foreign invaders by virtue of molecular recognition. Crystalline materials can form inside organisms under both physiological and pathophysiological conditions and it has been demonstrated that these crystal surfaces can act as antigens [43]. It is possible that an immune response promotes immunoglobulins to join in eggshell crystal formation. The blood differential proteins identified in this study are mainly derived from liver and immune organs.

The remaining differential proteins identified in this study were non-collagenous. Eggshell formation requires the synthesis of extracellular matrix (ECM) proteins and the releasing matrix vesicles (MVs) to initiate matrix mineralization. These MVs contains actin and actin regulating proteins [44] and the actin microfilament network has a critical role in MV budding and release [45]. Cytoplasmic 2 (Actg1) and ezrin belong to a family of actin regulating proteins and serve as a cross-linker between plasma membrane and actin cytoskeleton [46]. Thymosin-β 4 (Tβ4) is an actin sequestering protein, and regulates actin polymerization [47]. These actin proteins may be involved in the release of matrix vesicles. Additionally, tubulin α-1 chain (TUBA1) is one major constituent of microtubules. Just as actin and ezrin, tubulin is also detected in eggshell matrix [19]. Keratin, type II cytoskeletal cochleal (K2CO) is abundant in the cochlea of the inner ear, and is the major component of intermediate filaments, which are primordial constituents of the cytoskeleton and the nuclear envelope. Histone H4 (H4) is a nuclear histone protein that binds DNA to form histone-DNA complexes. Both histone H4 and hemoglobin have been detected in urinary stones [48]. Non-histone chromosomal protein belongs to the family of nucleoproteins that are involved in chromosomal functions, such as selectively binding to DNA, resulting in tissue-specific RNA synthesis. Calmodulin (CALM) acts as a calcium sensor that subcellularly localizes in the cytoplasm or within organelles. This protein has two nearly symmetrical globular domains each containing a pair of EF-hand motifs. The EF-hand motifs can bind to Ca^{2+} ions and allow calmodulin to sense intracellular calcium levels. S100 proteins are structurally similar to calmodulin, and functions in regulating of Ca^{2+} homeostasis and dynamics of cytoskeleton constituents. Thioredoxin belongs to a family of small redox proteins which act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Thioredoxin mainly localizes in the cytoplasm or in the mitochondria, but also can be found in plasma. Recently, thioredoxin was detected in low abundance in avian eggshells [18,19,21]. Overall, the non-collagenous differential proteins belong to cellular constituents, including the cytoskeleton, chromosomal proteins and calcium sensors.

Overall, twenty-five differential proteins were screened (Table 3) and these all showed negative effects on eggshell quality when their relative abundances were significantly elevated over normal levels. Almost all differential proteins were blood and non-collagenous proteins, but few of these belonged to collagens and amelogenins. Based on their abundance, the blood proteins were the predominant differential proteins and mainly originated from liver and immune organs (Table 3). Furthermore, these differential proteins were major proteins in eggshell matrix fractions, therefore, in terms of matrix proteins, the liver and spleen or lymphocytes appear to be the major organs influencing eggshell quality.

5. Conclusions

In the same eggshells, a high similarity exists among the proteomes of three matrix fractions regardless of their solubility and the triply overlapped common proteins formed a predominant contributor for the proteomic abundance of any matrix fraction. The variation in the relative abundance of some common proteins among three matrix fractions might be the key cause of their solubility differences. In each matrix fraction and between both eggshells of different quality, a majority of the proteomes of the fractions were commonly observed. These common proteins should be the dominant contributor for the abundance of each proteome and the constitutive proportions of some of these common proteins within the eggshell matrix might be important for the eggshell quality. Forty-two common major proteins were identified throughout the three matrix fractions and these might act as backbone constituents in chicken eggshell matrix. Finally, twenty-five differential major proteins were screened, they all showed negative effects on eggshell quality; and they were blood and non-collagenous proteins, which suggests that in terms of matrix proteins, the liver and spleen or lymphocytes are the major organs influencing eggshell quality.

Conflict of interests

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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