Journal of Proteomics 126 (2015) 140–154

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

# Quantitative proteomics provides new insights into chicken eggshell matrix protein functions during the primary events of mineralisation and the active calcification phase

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ARTICLE INFO

*Article history:*
Received 23 March 2015
Received in revised form 11 May 2015
Accepted 29 May 2015
Available online 3 June 2015

*Keywords:*
Chicken
Eggshell
Biomineralisation
Quantitative proteomics
Eggshell matrix

ABSTRACT

Eggshell is a bioceramic composed of 95% calcium carbonate mineral and 3.5% organic matrix. Its structural organisation is controlled by its organic matrix. We have used quantitative proteomics to study four key stages of shell mineralisation: 1) widespread deposition of amorphous calcium carbonate (ACC), 2) ACC transformation into crystalline calcite aggregates, 3) formation of larger calcite crystal units and 4) development of a columnar structure with preferential calcite crystal orientation. This approach explored the distribution of 216 shell matrix proteins found at the four stages. Variations in abundance according to these calcification events were observed for 175 proteins. A putative function related to the mineralisation process was predicted by bioinformatics for 77 of them and was further characterised.

We confirmed the important role of lysozyme, ovotransferrin, ovocleidin-17 and ovocleidin-116 for shell calcification process, characterised major calcium binding proteins (EDIL3, ALB, MFGE8, NUCB2), and described novel proteoglycans core proteins (GPC4, HAPLN3). We suggest that OVAL and OC-17 play a role in the stabilisation of ACC. Finally, we report proteins involved in the regulation of proteins driving the mineralisation. They correspond to numerous molecular chaperones including CLU, PPIB and OCX21, protease and protease inhibitors including OVM and CST3, and regulators of phosphorylation.



## 1. Introduction

Eggshell (including the eggshell membrane) is a biomineral composed of 95% calcium carbonate (in the form of calcite) and 3.5% organic matrix (proteins, polysaccharides and proteoglycans). It is a highly organised mineral layer constituted by columnar calcite crystals arising from nucleation sites, or mammillary cores, anchored on organic membranes [1–5]. This natural envelope of the egg constitutes a protective physical barrier against microbial penetration as long as it remains intact and ensures a harmonious development of the chicken embryo in this closed chamber. Avian eggshell is a porous mineral layer resulting from the sequential deposition of its different zones during precisely defined phases in the distal oviduct. The forming egg, with the egg white surrounding the yolk, enters the white isthmus region of the oviduct where the eggshell membranes are deposited. Five hours after the ovulation of the yolk, eggshell calcification is initiated in the red isthmus/uterus part, and lasts about 19 h[5,6]. The initiation of shell formation is followed by the linear deposition of mineral until the process is inhibited. These distinct phases of calcification result in the formation of different layers: the inner mammillary cones, the palisade layer and the cuticle.

The mineralisation process starts by heterogeneous nucleation on specific sites (organic cores also called mammillary knobs or cones), located in a quasi-periodic array on the surface of the outer eggshell membrane, and deposited on the eggshell membranes during the passage of the forming egg through the red isthmus. These organic cores are the seeding sites where calcium carbonate crystal deposition is initiated, beginning with a spherulitic deposition of microcrystals of calcite (the calcium carbonate polymorph of the shell) and leading to a hemispherical nucleation centre for each mammillary knob. These events represent the "birth" of the calcified shell. During the shell formation, as calcite crystals grow in size, they impinge on each other and continue to grow outward, developing into columnar crystal units. As crystal units grow outward, they compete for the available space and only those favourably oriented, that is with the *c*-axis (their faster growth direction) nearly perpendicular to the shell, are selected and contribute to the outer shell surface developing a preferential orientation of crystals. This 'competitive growth' mechanism explains the development of a

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http://dx.doi.org/10.1016/j.jprot.2015.05.034
1874-3919/

columnar ultrastructure of preferentially oriented crystals starting from randomly oriented nuclei [7]. However, amorphous calcium carbonate (ACC) is now recognised as an early and transient non-crystalline precursor phase of calcite or aragonite in the $CaCO_3$ calcified structures produced by many invertebrates. It represents a common biological mechanism for the fabrication of certain biominerals and allows the growth of single crystals with very complex shapes, for example, sea urchin spicules [8]. In the case of the eggshell, the presence of metastable mineral phases (*i.e.*, ACC) was reported recently and has constituted a major advance to explain how these biomineralisation events are temporally/spatially nucleated and regulated [9]. The ACC mineral first accumulates on eggshell membranes and on specific nucleation sites (mammillary knobs). ACC deposited around these sites dissolves rapidly, providing a continuous supply of ions to form calcite crystals on specific nucleation sites. These units coalesce to form larger crystals in the mammillary layer, and then during the following rapid growth phase they form the compact shell palisade layer made of columnar crystals with preferred orientation. Calcite crystals are consequently formed by the aggregation of ACC particles that support the rapid mineralisation of the eggshell and there is evidence that this non-crystalline form of calcium carbonate is present throughout the phases of shell formation [9].

During these distinct phases, matrix proteins probably play a key role to thermodynamically stabilise this transient form of $CaCO_3$[9], but also influence the selection of the calcite polymorph into which it is ultimately converted. They should also control the relative growth of crystals along different directions which determines the preferential orientation of calcite crystals in the eggshell surface [4,10]. This interaction leads to the eggshell ultrastructure and consequently contributes to its resulting mechanical properties [1,2,4]. This study used GeLC–MS/MS analyses combined with label free quantitative analysis to identify and quantify matrix proteins on the same forming eggshell samples as used in our previous study [9]. We assumed that a more abundant protein at a particular stage has a higher probability to be involved in its control. We performed additional bioinformatic analysis to provide a comprehensive report on the potential role of eggshell matrix proteins involved in the primary nucleation (ACC deposition), initiation of mineralisation (calcite crystal formation by aggregation of ACC), formation of larger calcite crystals and then development of the columnar palisade layer with preferred calcite orientation.

## 2. Materials and methods

### 2.1. Ethical statement, animal handling and housing

All experiments, including all animal-handling protocols, were carried out in accordance with the European Communities Council Directives concerning the practice for the care and use of animals for Scientific Purposes and the French Ministry on Animal experimentation under the supervision of authorised scientists (authorisation # 7323, delivered by the DDPP, "Direction Départementale de la Protection des Populations d'Indre et Loire-France"). The experimental unit UE-PEAT 1295, where the birds were kept, has permission to rear birds and for the euthanasia of experimental animals (decree No B37-175-1 of August 28th 2012 delivered by the "Préfecture d'Indre et Loire" following the inspection of the Direction of Veterinary Services). The protocol was approved by an ethical committee (comité d'éthique de Val de Loire, officially registered under number 19 of the French national ethics committee for animal experimentation) under agreement number 00159.02.

### 2.2. Collection of eggshell samples

Sixty brown-egg laying hens (ISA-Hendrix, 48 weeks old at time of sampling) were caged individually and subjected to a cycle of 14 h of light/10 h of darkness. Each cage was equipped with a device for automatic recording of oviposition (time of egg laying). Hens were fed a layer mash *ad libitum* as recommended by the Institut National de la Recherche Agronomique (INRA). Eggs were collected after animal's euthanasia either at the initial phase of eggshell mineralisation (5, 6 and 7 h after ovulation, p.o.) when the nucleation sites appear and early mineralisation starts, or during the linear growth phase of rapid calcification (16 h p.o.). Eggs were broken and forming eggshells were washed with water, air dried and stored at −20 °C until protein extraction.

### 2.3. Extraction of proteins from eggshell samples

Eggshell matrix proteins were extracted as described in [11] with slight modifications. Briefly, the whole eggshell pieces including eggshell membranes were rinsed with 154 mM NaCl solution containing protease inhibitors (2.5 mM benzamidine-HCl, 50 mM amino-n-caproic acid, 0.5 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride) then ground to a fine powder. Eggshell powders were completely demineralised by shaking them with 20% acetic acid overnight at 4 °C. The resulting suspensions were placed in dialysis tubing (cut off 3500 Da), dialysed against demineralised water for 24 h at 4 °C and then lyophilised. Eggshell samples were then shaken overnight at 4 °C using the following solution: 4 M guanidine hydrochloride, 5 mM benzamidine hydrochloride, 0.1 M ε-amino-n-caproic acid, 10 mM EDTA, 50 mM sodium acetate and 1 mM phenylmethylsulfonyl fluoride. Samples were then dialysed (cut off 3500 Da) against 0.5 M sodium acetate pH 7.4 for 24 h at 4 °C. Then, samples were centrifuged for 10 min at 2000 g at 4 °C and supernatants were stored at −20 °C until use.

### 2.4. Sample preparation for MS analyses

Protein concentration in individual samples was determined using the protein DC kit (BioRad, Marnes-la Coquette, France) according to the manufacturer's instructions and using bovine serum albumin as a standard. A total of 24 individual eggshell protein extracts were used. Six samples collected at the same time point were pooled in equal amounts for each time (5 h p.o., 6 h p.o., 7 h p.o. and 16 h p.o.). The four pooled samples 5 h p.o., 6 h p.o., 7 h p.o. and 16 h p.o. (51 μg of proteins/sample) were fractionated on a 4–20% SDS-PAGE gel (8.3 cm × 7.3 cm × 1.5 mm). Proteins were stained with Coomassie blue and the entire SDS-PAGE lanes were sectioned into 15 bands for each individual pooled sample (Fig. 1). Excised proteins were in-gel digested with bovine trypsin (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [12] and analysed by nanoscale liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS).

### 2.5. NanoLC–MS/MS analyses

All experiments were performed using a (FT-MS) LTQ Orbitrap Velos Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex, Amsterdam, The Netherlands) as previously described [12]. Five microlitres of each sample was loaded into a trap column and desalted. The peptide separation was conducted using a nano-column (Acclaim PepMap $C_{18}$, 75 μm inner diameter × 50 cm long, 3 μm particles, 100 Å pores). Mobile phases consisted of (A) 0.1% formic acid, 97.9% water, 2% acetonitrile (v/v/v) and (B) 0.1% formic acid, 15.9% water, 84% acetonitrile (v/v/v). The gradient consisted of 4–55% B for 90 min at 300 nl/min flow rate. Data were acquired using Chromelon Software (version 6.8 SR11, Dionex, Amsterdam, The Netherlands) and Xcalibur software (version 2.1; Thermo Fisher Scientific, San Jose, CA). The LTQ Orbitrap Velos instrument was operated in a positive mode in data-dependent mode. Resolution in the Orbitrap was set to $R = 60{,}000$. In the scan range of m/z 300–1800, the 20 most intense peptide ions with charge states ≥2 were fragmented using Collision Ion Dissociation (CID). Dynamic exclusion was activated for 30 s