Identification of Some Active Proteins in the Process of Hen Eggshell Formation
Downloaded from http://pubs.acs.org on December 11, 2008

More About This Article
Additional resources and features associated with this article are available within the HTML version:

• Supporting Information
• Access to high resolution figures
• Links to articles and content related to this article
• Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML
ABSTRACT: The hen eggshell is formed in a three-stage sequential process, namely, the initial stage (nucleation of calcite crystals), the active growth phase (linear deposition), and the terminal stage (inhibition of crystal formation). During these phases, different proteins are sequentially expressed in the uterine fluid. Some of them are thought to regulate the shell mineralization and particularly crystal growth. To identify proteins that are actively involved in this process, we have analyzed and compared the effects on CaCO₃ precipitation in vitro of some commercial egg white proteins, some noncommercial purified fractions of the eggshell organic matrix and, also of uterine fluids extracted from the hen uterus during each one of the stages of calcification. Uterine fluids strongly increased precipitation in vitro of some commercial egg white proteins, some noncommercial purified fractions of the eggshell organic matrix. Identifying some active acidic proteins (ovocleidin-17, ovocalyxin-21, osteopontin and ovocleidin-116) showed a strong affinity for the CaCO₃ surfaces and were selectively removed from the solution during its precipitation. These proteins may have an active role on CaCO₃ growth, aggregation, and inhibition. Other proteins, with very different isoelectric points, seem to regulate the chemical environment in which the precipitation takes place, that is, by buffering the pH favoring crystal growth as the couple ovocleidin-17 and ovocalyxin-21.

Introduction

The hen eggshell is a calcified structure which functions as a protective barrier for the egg content and allows the extraterine development of the chick embryo.² It consists mainly of a mineral part (>95%) made of calcite crystals and a pervading organic matrix (1–3.5%), making a composite material with excellent mechanical properties.²–⁴ It is deposited in a very short time (less than 20 h), while the egg passes through the hen’s uterus (shell gland). The eggshell formation is a very well regulated spatio-temporal process, resulting in a material with very well defined compositional and ultrastructural/microstructural characteristics which are common to all avian species. It is also a useful model to understand biomineralization processes in other biological systems. Three different stages during eggshell formation can be differentiated: (a) initial, (b) active-fast growth, and (c) terminal.

It is normally assumed, based on morphological features, that the avian eggshell is composed of six layers. However, only the two innermost non-calcified layers (the inner and outer shell membranes made of a network of organic fibers) are well differentiated. In the calcified part, there is continuity across its thickness as a result of the continuous mode of eggshell deposition. The inner zone of the calcified shell is made of irregular cones (mammillary knob layer), the tips of which are penetrated by the outer membranes fibers. During the initial stage of eggshell formation (stage I) the deposition of the mammillary knobs on the outer shell membrane and the subsequent nucleation of calcite spheruliths take place. The growth continues until adjacent spheruliths fuse together and from there columnar calcite units arise as the lateral growth of crystals is constrained. This type of process in a restricted space is denominated competitive crystal growth as crystals compete for the limited available space.⁵ Columnar calcite crystals extend beyond the bases of the cones during the fast growth stage (stage II) forming the palisade layer. Columnar crystal growth proceeds until the arrest of calcification, which is characterized by the deposition of a thin vertical crystals layer and the cuticle (stage III). The vertical crystal layer is composed of small crystallites aligned in a perpendicular form to the shell surface. The cuticle is a rich organic coating containing hydroxyapatite in its inner zone⁶ and pigments.²,⁷

The mineral part of the shell is made of calcite crystals precipitating from the uterine fluid, which is an acellular milieu secreted by the distal part of the oviduct. This fluid contains calcium and bicarbonate ions greatly in excess of the solubility product of calcite, as well as other ionic species such as magnesium and phosphate and the organic precursor components of the eggshell organic matrix.⁸ Organic components comprise proteins, glycoproteins, and proteoglycans. Among the numerous components identified are egg white proteins as ovalbumin, lysozyme and ovotransferrin, ubiquitous components such as osteopontin and clusterin⁹–¹² and organic constituents unique to the process of shell calcification. This last group comprises dermatan and keratan proteoglycans,¹³ ovocleidins and ovocalyxins.⁹,¹⁴,¹⁵ A recent proteomic survey of the acid-soluble organic matrix of the calcified chicken eggshell layer has allowed the identification of several hundred proteins in the shell.¹⁶ The number of these organic components and their concentration change in the uterine fluid along the different stages of eggshell deposition in a well defined way.¹⁷ In each stage specific organic components are expressed at a given concentration. Also, these organic components are secreted at specific times and locations in the oviduct and incorporated at specific substructural regions of the eggshell. For instance, type X collagen is mostly found at the eggshell membranes, keratan
sulfate proteoglycans are the main components of the mammillary knobs, and osteopontin has been found in eggshell associated with particular crystallographic faces of calcite in the palisade region. On the other hand, in vitro precipitation tests show that some of these components influence calcium carbonate precipitation. In particular, they affect the nucleation flux, the polymorph selection, the crystal size and the morphology. For instance, eggshell membrane collagen and osteopontin are known to inhibit calcite crystal growth, whereas mammillary keratan sulfate proteoglycan is involved in calcite nucleation.

It is becoming commonly accepted that macromolecules contribute to the regulation of the eggshell mineralization process. However, the mechanisms involved in the interaction of the organic macromolecules with the inorganic phase have not yet been fully elucidated. It is not only the action of one macromolecule in a given condition that must be established, but also the spatio-temporal interactions of a big array of macromolecules. These biological macromolecules differ in molecular weight, isoelectric point (pI), and concentration. It has been demonstrated by in vitro experiments that model globular proteins with different pI and varying concentrations influence CaCO₃ precipitation at different levels, that is, during nucleation, polymorph selection, crystal growth, and crystal morphology.

In order to find evidence of the implication of macromolecules in eggshell formation and to identify some of the macromolecules actively involved in the process, we decided to analyze and compare the effects on calcium carbonate precipitation of macromolecular solutions with different levels of complexity, that is, (1) egg white proteins which are commercially available, (2) purified eggshell matrix extracts (results previously published), and (3) uterine fluids collected at the three main stages of eggshell formation. In particular, we have analyzed the effects on the nucleation, crystal growth, polymorphism and morphology of the precipitated crystals, and then, we have compared the morphology of synthetic crystals obtained in vitro with those present in vivo in various eggshell regions.

Materials and Methods

Organic Macromolecular Solutions. In a first stage, we have used three pure commercial egg white proteins, that is, hen egg white lysozyme (Seikagaku Lot E98301, pI 11.35, MW 14313 Da), hen egg white ovalbumin (Sigma, lot 93H7105, pI 4.5, MW 77000 Da) and ovotransferrin (type I chicken egg white Lot 107F8020, pI 6.1, MW 77000 Da). Stock solutions of the three proteins were prepared and their concentrations were measured by the Layne method, using ovalbumin as standard. Uterine fluid proteins were electrophoretically separated using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) method. Gels were loaded with 40 and 20 mL of each uterine fluid, before and after CaCO₃ precipitation. Proteins were stained with Sypro Ruby Protein Gel Stain. The identification of stained bands displayed in the electrophoretic profile was carried out by comparing them to previously published electrophoretic profiles.

Precipitation Experiments. Precipitation experiments were carried out by CO₂ vapor diffusion using the sitting drop crystallization method on a “crystallization mushroom” at 20 °C and 1 atm total pressure. Calcium carbonate precipitated in aqueous drops of 40 mL containing the corresponding protein bearing solution and 20 mM CaCl₂ (800 µg/ mL Ca), except in the case of the uterine fluids of stages I, II, and III whose Ca concentration were higher. In addition to the added Ca (800 µg/mL) these fluids contain also natural Ca (170 µg/mL in fluid I, 370 µg/mL in fluid II and 480 µg/mL in fluid III). The pH of the drops was raised by the diffusion of NH₃ gas released by the underlying NH₄HCO₃ solution (10 mM) contained in the bottom part of the mushroom. The reagent concentrations of the control experiment (20 mM CaCl₂ and 10 mM NH₄HCO₃) were the same as those reported in previous works. Experiments were finished at 48 h, the mushrooms were opened and both solutions and solids were withdrawn from the microbridges for subsequent analysis.

Analysis. The evolution of the pH along the experiments, the induction times for nucleation (t₀), the crystalline phase and crystalline habit of the precipitated particles have been analyzed following the procedures described in previous papers. The total number of crystals (TNC) in each drop has been determined by counting the crystals with the use of an optical microscope. For this purpose the drops are divided in four quadrants in top view, and the cross-section is divided into three regions: surface of the drops, wall and bottom of the microbridges where drops are deposited. In some cases, prior to FESEM observations, the crystals were treated with proteinase K buffer (PKb) to highlight the inorganic material removing all the proteins, according to the protocol described by Shiao et al. When using the uterine fluids as protein bearing solutions the remaining solutions after the experiments of CaCO₃ precipitation were also analyzed by the SDS–PAGE method. The residual calcium concentration of these fluids was analyzed by atomic absorption spectroscopy.

Results

Compared Precipitation of CaCO₃ in Macromolecules Bearing Solutions. In Table 1 are summarized the main results of the precipitation experiments using commercial egg white proteins and uterine fluids collected at the stages I, II, and III. The results of the control experiment are also included. Figure 2 shows a general view of the drops containing CaCO₃ precipitates when using the uterine fluids and the control sample.

In lysozyme, ovalbumin and ovotransferrin bearing experiments, t₀ were typically around 1000 min, while TNC ranged from 50 to 130. There was always a mixture of the three main CaCO₃ polymorphs (calcite, aragonite and vaterite), though with increasing protein concentration, the percentage of calcite tends to increase. In contrast, in the uterine fluid bearing experiments, t₀ was extremely short (a few seconds), the TNC was at least 2 orders of magnitude higher than in the other protein bearing experiments using commercial proteins or purified eggshell fractions (over 10 000 crystals per drop) and the crystalline phase was always calcite.

pH Evolution in the Control and Uterine Fluid Bearing Experiments. Figure 3 shows the pH profiles of uterine fluids bearing experiments. It can be observed that the pH evolution
in experiments using the uterine fluids of stages I and III displayed a profile similar to that of the control experiment. In these experiments the pH increased sharply until reaching the plateau. However, the pH profile recorded during the experiment in which we used fluid of the stage II was notably different during the first 30 min, thus indicating a peculiar behavior of fluid II during precipitation (Figure 3b). This different behavior consisted of an initial decrease in the pH and then a slow increase. Later, the pH increases sharply until reaching the plateau. The different behavior of uterine fluid II was also manifested when adding 2 \( \mu \text{L} \) of 400 mM CaCl\(_2\) to 38 \( \mu \text{L} \) of fluid II during the preparation of the uterine fluid bearing samples. This addition did not affect significantly the initial pH of the fluid. However, the addition of the same calcium concentration to uterine fluids I and III induced a pH decrease of 0.38 and 1.15 units, respectively. Therefore, we suspect that fluid II acted as a strong buffer. The buffering capacity of the uterine fluid of stage II was also shown when using uterine fluids of the same stage but extracted on two different days (Figure 3b'). The protein concentrations (97 and 479 \( \mu \text{g/mL} \)) were very different on both days, but in both experiments the pH oscillated to compensate the strong pH increase that the diffusion of NH\(_3\) would produce. From a chemical engineering point of view, the uterine fluid II acted as a pH-stat system favoring the growth of calcite at this stage.

Electrophoretic Profile (SDS-PAGE) of the Uterine Fluids. Figure 4 shows the electrophoretic profile of uterine fluids, before and after CaCO\(_3\) precipitation. In Figure 4a (uterine fluid I) the more intense stained bands (higher concentration) correspond to egg white proteins such as ovalbumin (45 kDa) and ovotransferrin (80 kDa). Additionally, a nonassigned band at around 240 kDa was observed in one of the samples. At a much reduced staining intensity, we observed a band at 15 kDa (assigned to lysozyme). Eggshell matrix proteins specific to the uterine tissue, such as ovocleidin-17 (OC-17), ovocalyxin-32 (OCX-32), and ovocalyxin-21 (OCX-21), were also observed. At around 50 kDa, there is a smear of bands that could correspond to osteopontin. After CaCO\(_3\) precipitation the bands below 45 kDa completely disappeared, with the exception of the lysozyme band. In contrast, the bands over 45 kDa associated to ovalbumin, ovotransferrin and the 240 kDa bands remained with the same staining intensity.

In the electrophoretic profile of fluid II (Figure 4b, left-hand side) the following bands from top to bottom are observed: a nonassigned band at 240 kDa, three closely spaced bands corresponding to ovocleidin-116 (OC-116), ovotransferrin (weakly

Table 1. Precipitation of CaCO\(_3\) in Macromolecule Bearing Solutions

<table>
<thead>
<tr>
<th>macromolecular solution</th>
<th>( \text{Ca}<em>a + \text{Ca}</em>{ad} ) (( \mu \text{g/mL} ))</th>
<th>protein (( \mu \text{g/mL} ))</th>
<th>( t_i ) (min)</th>
<th>TNC and polymorphs (%)</th>
<th>crystal size ( L ) (( \mu \text{m} ))</th>
<th>pH(_i)</th>
<th>pH(_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>800</td>
<td>0</td>
<td>( \approx900 )</td>
<td>40 (55% C 22% V 23% A)</td>
<td>100–200</td>
<td>5.3</td>
<td>7.3</td>
</tr>
<tr>
<td>lysozyme</td>
<td>800</td>
<td>128</td>
<td>( \approx700 )</td>
<td>52 (84% C 5% V 11% A)</td>
<td>65, 113</td>
<td>5.8</td>
<td>7.2</td>
</tr>
<tr>
<td>ovalbumin</td>
<td>800</td>
<td>512</td>
<td>( \approx900 )</td>
<td>60 (84% C 6% V 10% A)</td>
<td>68, 113</td>
<td>5.5</td>
<td>7.1</td>
</tr>
<tr>
<td>ovotransferrin</td>
<td>800</td>
<td>128</td>
<td>1080</td>
<td>56 (58% C 26% V 16% A)</td>
<td>120, 180</td>
<td>5.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Uterine fluid I</td>
<td>970</td>
<td>92</td>
<td>( &lt;0.2 )</td>
<td>( \approx10,000 ) (100% C)</td>
<td>( \approx6–20 )</td>
<td>7.6</td>
<td>9.03</td>
</tr>
<tr>
<td>Uterine fluid II</td>
<td>1170</td>
<td>455</td>
<td>( &lt;0.2 )</td>
<td>( \approx10,000 ) (100% C)</td>
<td>5, 10, 24, 52</td>
<td>7.43</td>
<td>8.25</td>
</tr>
<tr>
<td>Uterine fluid III</td>
<td>1280</td>
<td>163</td>
<td>( &lt;0.2 )</td>
<td>( \approx10,000 ) (100% C)</td>
<td>( \approx5–22 )</td>
<td>6.5</td>
<td>8.44</td>
</tr>
</tbody>
</table>

\( ^a \) TNC, \( t_i \), C, A, and V stands for total number of precipitated crystals per drop, induction time, calcite, aragonite, and vaterite, respectively. pH\(_i\) (initial pH); pH\(_f\) (final pH). \( ^b \) Refers to aggregates of crystals.
stained) and an intensely stained band at around 70 kDa. Below this band, a smear of bands at around 50 kDa appear that possibly correspond to osteopontin. Three bands can also be observed that have been assigned to ovocalyxin-36 (OCX-36), ovocalyxin-32 (OCX-32), and ovocleidin-17 (OC-17). Below these bands we found that of lysozyme (weakly stained) and another band at 13 kDa. After CaCO3 precipitation, the intensely stained bands of OC-116, the nonassigned band at 240 kDa, that assigned to osteopontin and the less intense band of OCX-32 disappeared, thus indicating that these proteins were completely removed from the fluid during the precipitation process.

The electrophoretic profile of fluid III (Figure 4b, right-hand side) displays all the bands observed in the previous fluid samples except those corresponding to OC-116 and lysozyme. The most intensely stained bands are the following four: two of them within the interval from 160 to 240 kDa, one at around 70 kDa and another situated around 30 kDa, which seems to correspond to OCX-32. An additional band was also observed at very low molecular weight (around 10 kDa). After CaCO3 precipitation most of the bands below 66 kDa including OCX-32 together with those placed at the top of electrophoretic profile disappeared.

**Effect on Calcite Morphology of Macromolecule Bearing Solutions.** Figure 5 compares the morphology of the calcite crystals found at structurally different eggshell regions, that is, the mammillary knobs, palisade and vertical crystal layer (Figure 5a–c) with the morphology of calcite crystals obtained from uterine fluid bearing precipitation experiments. Most representative morphologies of calcite crystals precipitating in uterine fluids I, II and III bearing solutions are displayed in Figure 5d–f. FESEM observation of crystals grown in vitro in the uterine fluid bearing experiments (Figure 5) shows the presence of globular agglomerates and modified rhombohedral crystals (Fluid I) which are similar to those crystals collected from the mammillary knob at the initial stage of the eggshell formation (Figure 5d). Aggregates of calcite obtained when using uterine fluid of the stage II are quasi-spherical or rods made of microcrystals elongated along the c-axis (Figure 5e). Those crystals obtained with Fluid III are elongated along the c-axis, compact (they do not show any holes) and are similar to crystals observed at the final stage of eggshell formation which are also elongated (Figure 5f). Some of them revealed peanut-like morphology.

The treatment of crystals and eggshell samples with proteinase K buffer (PKb, a nonspecific protease), allowed us to observe the presence of 300–450 nm diameter holes on these crystals. This finding indicates that most probably the holes contained aggregates of proteins occluded during eggshell formation or during the growth of crystals on in vitro experiments. The occurrence of these holes is very common in crystals obtained from uterine fluids collected at stage I and II which is also in agreement with a high frequency of holes found in the mammillary and palisade layer. In contrast, the holes are neither
found at the upper part of the eggshell in the vertical layer nor in the crystals obtained from the uterine fluid at stage III, which produced compact aggregates of crystals.

The calcite morphologies obtained when using the commercial egg white proteins lysozyme, ovalbumin and ovotransferrin at two different concentrations, 128 and 512 µg/mL, are displayed in Figure 6a–c and Figure 6d–f, respectively. Figure 6g shows the morphologies of calcite crystals precipitated in the presence of a purified fraction of the eggshell organic matrix which contain mainly OC-116.
When using lysozyme and ovalbumin at low protein concentration (128 µg/mL), calcite crystals displayed rhombohedral habits bounded by the {104} face (Figure 6a,b). These crystals are similar to those found in the control experiment. In contrast, the use of ovotransferrin at 128 µg/mL modified the rhombohedral habit of calcite by expressing new though not very well defined faces (Figure 6c,f). The symmetry of the newly expressed faces in the case of ovotransferrin corresponds to that of the {110} first-order prismatic form of calcite and to those of second-order prismatic faces {100} as deducted by comparison with calcite crystal model built using SHAPE V 7.1 (Shape Software). Those new faces were not flat and showed a terraced morphology with well-defined microsteps advancing parallel to {104} rhombohedral crystal faces. At higher concentration of ovotransferrin (512 µg/mL), the edge between rhombohedral faces were inhibited and new faces with the symmetry of {108} rhombohedral faces were expressed. The expression of these new faces reduced the size of the rhombohedral {104} faces of calcite crystals. In the experiments using lysozyme and ovalbumin a morphological effect could only be observed in the case of ovalbumin when its concentration increased to 512 µg/mL (Figure 6d,e). However, in the later cases the identification of the specific crystallographic faces could not be possible because of the large modification of the calcite morphologies. In Figure 6e, the calcite crystal morphology is almost spherical and only the remnant of {104} faces are shown. Finally, the most marked effect on crystal morphology was found when using a purified fraction of the eggshell organic matrix containing OC-116, which yielded calcite crystals as agglomerates of nanocrystals (Figure 6g).

**Discussion**

To understand the role of organic components during eggshell formation, we have used macromolecule bearing solutions with different levels of complexity, from the simplest containing only one type of macromolecular species to the most complex that could simulate the natural process. In particular, we have analyzed the effects on in vitro calcium carbonate precipitation of (1) commercial egg white proteins also present in eggshell matrix, and (2) uterine hen fluids extracted at the three main stages of eggshell formation. In the first group we used three commercially available proteins which are lysozyme, ovalbumin and ovotransferrin. Also, we have compared our results with previous results obtained in our laboratory about the influence on CaCO₃ precipitation of purified extracts of the eggshell organic matrix. This strategy and the resulting information give us insights into the role and effects of individual organic components as well as their combined effects and mutual interactions during the process of eggshell formation.

Our results reveal that individual commercial eggwhite proteins (lysozyme, ovalbumin, ovotransferrin) at concentrations comparable to those found in the uterine fluid have a relatively weak effect on calcite selection, nucleation and growth. In contrast, the purified eggshell organic matrix fractions and the uterine fluid bearing solutions were very effective and, for instance, selected exclusively calcite as precipitating polymorph. Additionally, some purified fractions of the eggshell organic...
matrix studied by Hernandez-Hernandez et al.\textsuperscript{23} (i.e., fractions $g$ composed of ovotransferrin, ovomucoid, quiescence-specific protein and ovocleidin-116, and the fraction $r$ composed of quiescence-specific protein, ovocleidin-17, chain A, and some nonidentified sequences) showed a remarkable ability to inhibit the nucleation. The fraction $g'$, which was composed mainly of OC-116, was very active in modifying the growth mechanism from a spiral mechanism to an aggregation-agglomeration growth mechanism. However, uterine fluids with highly complex organic and inorganic composition (those from which calcite crystals forming the eggshell precipitate in vivo)\textsuperscript{2,16} have a much stronger effect in each one of the precipitation stages (i.e., nucleation, crystal growth and morphology modification). The stronger effect of uterine fluids compared to egg white proteins or eggshell extracts could be due to the following reasons: (1) there is not a significative alteration of the natural state of the original components, (2) the possible cooperative or even synergistic interactions between the wide array of organic components present in these fluids, and (3) the specific chemical and environmental scenario, namely, pH, partial pressure of CO$_2$ and biologically available calcium that only exist in this latter system.$^{22}$

It is important to understand the evolution of the chemical environment (i.e., uterine fluid) in which eggshell forms. During the different stages, the composition of the uterine fluid changed in the number and concentration of the diverse organic components.$^{2,13,17,31}$ At the same time the concentration of calcium and the partial pressure of CO$_2$ supplied throughout the uterus varied in a regulated manner to produce the mineralization of the shell in a very short time (around 20 h).

There is very limited information regarding the specific role of the individual organic components involved in eggshell mineralization.$^{2,32}$ In this respect, comparison of electrophoretic profiles SDS–PAGE of the uterine fluids before and after CaCO$_3$ precipitation (Figures 4a,b) with their respective pH profiles when the uterine fluids were used as protein bearing solutions in CaCO$_3$ precipitation experiments is specially relevant in order to shed light to determine which are the active proteins regulating eggshell growth and those active in regulating the chemistry of the system. We propose that those proteins that have been removed by the precipitate in successive stages (and do not show up in the electrophoretic profiles after CaCO$_3$ precipitation) have a strong affinity toward calcite surfaces and should have an active role in controlling calcite growth or its aggregation behavior.$^{23}$ Among these proteins are the acidic proteins OC-17, OCX-32 and osteopontin (see Table 2), which are selectively removed by the precipitate. These proteins should display a net negative charge during the precipitation process (which takes place within the pH range from 7.6 to 9.03) whereas calcite crystals at this pH range are neutral or positively charged. Calcite point of zero charge, pH$_{pzc}$, is around 8.3 and the concentration of Ca$^{2+}$ in the uterine fluid bearing solutions is slightly higher than in the control experiment. Under these conditions electrostatic interactions can take place between these

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fesem_images.png}
\caption{FESEM images of (a) calcite grown in the presence of 128 $\mu$g/mL lysozyme; (b) calcite grown using 128 $\mu$g/mL ovalbumin, (c) calcite grown using 128 $\mu$g/mL ovotransferrin; (d) calcite grown using 512 $\mu$g/mL lysozyme; (e) calcite grown using 512 $\mu$g/mL ovalbumin; (f) calcite grown in the presence of 512 $\mu$g/mL ovotranferrin; (g) calcite crystals grown in the presence of a purified fraction of the eggshell containing mainly ovocleidin-116. Most representative calcite faces are indexed.}
\end{figure}
proteins and calcite leading to their adsorption to calcite surfaces during crystal growth. The adsorption of proteins to calcite could occur through deprotonized negatively charged -COO\(^{-}\) groups of the proteins and the positively charged surface complexes, that is, \(\text{CO}_3\text{Ca}^{+}\). As a result of this process, the calcite crystals show modified rhombohedral morphologies as was found in our previous work\(^{22}\) using an acidic model globular protein. Additionally, calcite crystals treated with proteinase-K buffer reveal holes that indicate the previous presence of adsorbed and/or occluded proteins.

In fluid II, OC-116, osteopontin and lysozyme (a minority component) are removed after CaCO\(_3\) precipitation. OC-116, or occluded proteins. reveal holes that indicate the previous presence of adsorbed and/or occluded proteins. Additionally, calcite crystals treated with proteinase-K buffer reveal holes that indicate the previous presence of adsorbed and/or occluded proteins.

On the other hand, proteins remaining in solution after CaCO\(_3\) precipitation should not be so active in the control of mineralization. However, they could also influence calcium carbonate precipitation by modifying the chemical environment (i.e., pH, supersaturation, level of Ca). For instance, in fluid I, ovalbumin, ovotransferrin and lysozyme, proteins that are not removed by CaCO\(_3\) precipitation, slightly favors calcite nucleation in in vitro experiments. Moreover, OCX-21 and OC-17, which have very different isoelectric points (pI 9.3 and pI 4.1 respectively), may be responsible for the high buffering capacity of fluid II in spite of being minority components of the complex mixture of proteins. The fact that OCX-21 and OC-17 are minority components and have different isoelectric points could explain the similar buffering capacity of the two fluids of the stage II tested (those collected in different days, whose concentrations were 97 and 479 \(\mu\)g/mL, respectively). In the formation of this buffer other basic proteins like lysozyme could have participated. However, because of the peculiar pH behavior described above was only found when using uterine fluid of the stage II as protein bearing solution, we can discard the presence of this protein in the buffer. This pH-stat chemical scenario favors the growth of calcite\(^{22}\) and could explain the fast growth stage during eggshell formation in which large columnar calcite crystals (palisades) are deposited. Also, in fluid II bearing experiments, lysozyme is removed by the precipitate, probably adsorbed because of the surface charge reversion caused by the previous adsorption of OC-116 and osteopontin. Interestingly, lysozyme, though as a bactericidal, has been found in the palisade layer of eggshell.\(^{10}\)

### Table 2. Molecular Weight, Isoelectric Point and Localization of Some Selected Proteins Associated to the Process of Eggshell Formation\(^{16-28,30}\)

<table>
<thead>
<tr>
<th>protein</th>
<th>localization</th>
<th>MW (kDa)</th>
<th>pI (theoretical)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ovalbumin</td>
<td>egg white, uterine fluid of the stage I</td>
<td>45.0</td>
<td>4.5</td>
</tr>
<tr>
<td>ovotransferrin</td>
<td>eggshell membranes, uterine fluid of the stage I</td>
<td>77.0</td>
<td>6.05</td>
</tr>
<tr>
<td>lysozyme</td>
<td>eggshell membranes, uterine fluid of the three stages specially at the growth phase</td>
<td>14.3</td>
<td>9.3</td>
</tr>
<tr>
<td>ovomucoid</td>
<td>the bases of the mammillary cores, and palisade</td>
<td>21.2</td>
<td>52</td>
</tr>
<tr>
<td>clusterin</td>
<td></td>
<td>21</td>
<td>9.3</td>
</tr>
<tr>
<td>ovocalyxin-25</td>
<td></td>
<td>25</td>
<td>8.84</td>
</tr>
<tr>
<td>ovocalyxin-32</td>
<td>the external part of the calcified shell (palisade, vertical crystal layer and cuticle)</td>
<td>32</td>
<td>6.81</td>
</tr>
<tr>
<td>ovocalyxin-36</td>
<td>eggshell membranes and basal parts of the mammillary cores, and palisade matrix</td>
<td>38</td>
<td>5.62</td>
</tr>
<tr>
<td>ovolecidin-116</td>
<td>mammillary and palisade layers</td>
<td>77.3</td>
<td>6.62</td>
</tr>
<tr>
<td>ovolecidin-17 (chain A) cystatin</td>
<td></td>
<td>15.7</td>
<td>4.03</td>
</tr>
<tr>
<td>ovoglican</td>
<td></td>
<td>15.6</td>
<td>8.4</td>
</tr>
<tr>
<td>osteopontin</td>
<td>in the core of the eggshell membranes, at the bases of the mammilla, in the outer of and throughout the palisade layer</td>
<td>207</td>
<td>4.5–4.6</td>
</tr>
</tbody>
</table>

OCX-32 was selectively removed after CaCO\(_3\) precipitation. In general, OCX-32 shows a high absorption affinity to calcite and has a high inhibitory effect of the precipitation of CaCO\(_3\). This result is consistent with previously reported work that describes the inhibition of the precipitation that fluid III has on metastable solutions formed by calcium chloride and sodium bicarbonate. The authors believe that this protein could have an important role in the arrest of eggshell mineralization.\(^9\)

Regarding the control over the crystal growth processes, we have found that all organic components tested affected calcium carbonate precipitation at three fundamental levels: (a) selecting or at least favoring the precipitation of a particular mineral phase, (b) controlling the number of crystals or the nucleation flux, and (c) controlling crystal morphology and the aggregation behavior.

At the first level, calcite formation is favored over the other calcium carbonate polymorphs (e.g., aragonite and vaterite). This study shows that eggshell extracts and uterine fluids were especially effective in selecting calcite and suppressing totally the appearance of vaterite and aragonite, despite the fact that the system is supersaturated with respect to the three main calcium carbonate polymorphs (e.g., calcite, aragonite and vaterite) as well as other common calcifying minerals (i.e., hydroxyapatite). Only in the terminal stage hydroxyapatite is found. This superior capacity for selecting a specific polymorphic mineral phase is well described in other biological mineralization processes. For instance, some mollusc shell proteins are able to switch from calcite to aragonite and back.\(^{33,34}\)

Macromolecules also have an important effect on the nucleation flux. We have observed that some individual egg white proteins and purified fractions of the organic matrix are active in controlling calcite nucleation. Nevertheless, uterine fluids are much more active and induced a more intense nucleation. These phenomena occurred irrespectively of the stage of eggshell formation at which these fluids were extracted. In our experimental system, we have added calcium to the uterine fluid to reproduce the control conditions. This added calcium could explain partly a more intense nucleation. However, the small difference in calcium concentration in the uterine fluid bearing solution compared to that of the control solution could not explain by itself such a large increase in nucleation density. The uterine fluid bearing solutions produced a total number of calcite crystals per drop which was at least 2 orders of magnitude more intense than any other individual commercial proteins or purified extracts of the eggshell organic matrix tested. In our opinion, the presence of charged proteins and other
organic compounds facilitate adsorption of calcium ions to their surface therefore favoring the nucleation of calcite by a mechanism known as the ionotropic effect.35

Alternatively, some proteins have a strong affinity to specific calcite surfaces and could favor the nucleation more actively acting as templates. This template effect is due to a stereo-chemical matching between the disposition of specific functional groups (i.e., COOH–) in the proteins or their motifs and the arrangement of carbonate or calcium ions in specific target calcite faces. The large increase in the nucleation could also be due to a cooperative interaction of different proteins and/or other organic components present in the fluids since the same individual components or eggshell extracts composed of a lower array of organic components show a much more limited effect on nucleation. However, we cannot discard the possibility that the lower effect of individual components on CaCO3 precipitation could be due to an alteration of the natural state of proteins derived from the extraction and purification procedures. If some proteins act as a template for calcite nucleation at their natural state, any alteration of their molecular configuration could significantly decrease their capacity to induce calcite nucleation by a template mechanism.

Finally, in vitro CaCO3 precipitation experiments show that individual commercial egg white proteins, at concentrations similar to those found in the uterine fluids, have very weak effect on crystal morphology. In contrast, purified extracts of the eggshell organic matrix and uterine fluids have a strong and specific effect on calcite crystal morphologies. For instance, fluid I induced the overexpression of specific crystal faces of calcite, that is, the {110} and {100} prismatic forms of calcite. On the other hand, fluid II and III bearing solutions seem to promote aggregation of calcite crystals and modified the calcite morphologies on a less specific way.

Modifications in crystal growth morphology can provide insights regarding protein–mineral surface interactions.35 The type of modification depends on the specificity of the protein–mineral interactions. In the case that there is stereo-chemical compatibility between the protein and a particular crystal face, the protein will be preferentially absorbed to this face and all symmetrically equivalent faces. These faces will be expressed as a consequence of reductions in their normal growth rate and the morphology of the crystals will be modified accordingly. However, in other cases a nonspecific absorption of proteins (to minimize hydrophobic interaction with the solution) can explain a greater modification of calcite crystals in an unspecific way resulting in rounded crystals without well-defined crystal faces.22,36

The above results shed light into understanding what occurs in vivo. Although these results cannot explain how eggshell growth is stopped, we have shown how specific eggshell organic extracts are highly effective in decreasing crystal nucleation. The sum of all these findings suggest the high level of control exerted by the organism over the biomineralization of the shell and, in particular, over the formation of its mineral part, which is composed of calcite crystals of well-defined orientation, size and morphologies. In addition to the control of crystal growth exerted by the organic matrix components,2,3,7 there is a competitive process during the growth of adjacent crystals resulting in a columnar microstructure with a preferential crystallographic orientation.5,37

This precise control over composition and the microstructural characteristics of the eggshell has important consequences in the mechanical properties, integrity and functionality of this biomaterial.26,38

Conclusions

In summary, to understand the role of organic components during eggshell formation, we have used organic bearing solutions with different levels of complexity, from the simplest (individual eggshell proteins) to the most complex and realistic composition (uterine fluids) that could simulate the natural process. Using this approach, we found that the precise control of eggshell formation can be due to the combined action of the components present in the uterine fluid which regulate crystal growth and the chemistry of the system. However, in vitro CaCO3 precipitation experiments reveal that some specific acidic proteins (including OC-17, OCX-32, OC-116 and osteopontin) inside the uterine fluid and in the purified eggshell extracts have a strong affinity for the surface of CaCO3 and are selectively removed from the solution during its precipitation. These proteins must have an active role in CaCO3 growth, aggregation and inhibition. Other proteins regulate the chemical environment in which the precipitation takes place, that is, by buffering the pH favoring crystal growth as for instance the OC-17 and OCX 21. Individual egg white proteins (lysozyme, ovalbumin, ovotransferrin), at concentrations similar to those found in uterine fluids, affected slightly the nucleation density and growth morphology of calcite in in vitro experiments, but did not select exclusively calcite as precipitating polymorph.

Acknowledgment. This work was supported by funding received from the European Community for the project Egg Defense (QLRT-2001-01606). A.H.H., J.G.M. and J.M.G.R also acknowledge the Excellence project RNMI344 of the Junta de Andalucía and to the “Factoría de Cristalización” (Consolider Ingenio 2010).

References


Active Proteins in Hen Eggshell Formation