Influence of eggshell matrix proteins on the precipitation of calcium carbonate (CaCO₃)


Abstract

To understand the role of eggshell organic matrix on the biomineralization process, we have tested the influence of different purified fractions of the eggshell organic matrix on calcium carbonate (CaCO₃) precipitation. Purification was carried out after successive anion-exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography of two different prepurified eggshell extracts (A) and (B); the purified fractions (named g, h, n and r) and (c₀, g₀, i₀, k₀) respectively were diluted to 50 μg/ml before being tested in vitro and analysed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedure and mass spectrometry. The precipitation experiments were carried out by the method of vapour diffusion on crystallization mushrooms. Each purified fraction showed a different effect on CaCO₃ precipitation. Some of them exhibited a strong inhibitory effect on nucleation, thus suppressing the precipitation of CaCO₃ almost totally while the others did not produce any notable effect. However, all fractions favoured the precipitation of calcite over the other CaCO₃ polymorphs. Additionally, all fractions modified in a different manner the size and morphology of the precipitated calcite crystals.


1. Introduction

The hen eggshell is a protective barrier for the egg content which allows the extrauterine development of the chick embryo [1]. It consists mainly of a mineral part (> 95%) made of columnar calcite crystals and a pervading organic matrix (1–3.5% of the remaining material), making a composite structure with excellent mechanical properties [1,2]. It is deposited while the egg is residing in the hen’s uterus, or shell gland, in less than 20 h. Three different stages during eggshell formation can be differentiated, namely (a) initial, (b) fast growth and (c) termination.

Eggshell deposition starts with calcium carbonate (CaCO₃) spheruliths nucleating on the eggshell membranes at the initial stage. The growth continues until adjacent spheruliths fuse together. Then, columnar crystals (palisades) emerge from the spherules during the fast growth stage. Columnar crystal growth proceeds until eggshell calcification is terminated with the deposition of the cuticle layer (termination stage). Fig. 1 shows the texture of the final hen eggshell.

Calcite crystals forming the eggshell precipitate in the uterine fluid, an acellular milieu containing ionized calcium and bicarbonate greatly in excess of the solubility product of calcite, as well as the native and soluble organic precursor of the shell matrix [3]. Eggshell organic matrix is composed by proteins, glycoproteins and proteoglycans in the calcified layers and by different types of collagens in the eggshell membranes. Three groups of molecules have...
been found in eggshell: ubiquitous components such as osteopontin and clusterin [4–6], egg white proteins such as ovalbumin, lysozyme, ovotransferrin, and organic constituents unique to the process of shell calcification. The last group comprises dermatan and keratan proteoglycans [2,7] as well as ovocleidins and ovocalyxins [4,5,8]. Some of these organic components are thought to regulate the shell mineralization and particularly crystal growth. There is a variety of experimental evidence for these assumptions [1,8–11].

The aim of this study is to evaluate the effects of different purified fractions containing an array of eggshell organic matrix components on CaCO₃ precipitation, in particular on the nucleation process, polymorphism and morphology of the CaCO₃ crystals. This information is useful to identify active components participating in the eggshell mineralization and to elucidate their role in eggshell calcification.

2. Experimental procedure

2.1. Preparation and purification of eggshell extracts

Organic matrix proteins were extracted by demineralization of the eggshells of 50 hen eggs using acetic acid as a demineralizing agent [5]. Two different prepurified fractions corresponded to (A) supernatant of eggshell extract and (B) pellets solubilized in 10 mM diethanolamine DEA/HCl, 1 M NaCl, were extracted from the initial crude eggshell extract. Characteristics of these fractions are the following: at low ionic strength, fraction A is composed of proteins soluble at pH 5 and 9.5, and fraction B is composed of proteins insoluble at pH 5 at low ionic strength but soluble at pH 9.5 at high ionic strengths.

Fractions A and B were further purified by anion-exchange chromatography (7.5 × 50 mm² phenyl sepharose high performance HiTrap Amersham column, Amersham Pharmacia Biotech, Uppsala, Sweden) using 50 mM NaP, 1.5 M (NH₄)₂SO₄, pH 7 (buffer A) and 50 mM NaP, pH 7 (buffer B) as the mobile phases, flow rate of 1 ml/min; protein elution performed with a gradient from 1.5 to 0 M of (NH₄)₂SO₄ in 10 min, and finally, selected fractions were separated by gel filtration chromatography (7.5 × 60 TSK-gel G2000SW column, Tosoh Bioscience, Hampton, UK) using 50 mM NaP, 0.3 M NaCl, pH 7 as the mobile phase (flow rate of 0.6 ml/min). Between the various steps of purification fractions were dialysed and concentrated. From soluble eggshell extracts we obtained purified fractions h, g, n and r, and from initially insoluble eggshell extracts, the purified fractions c', g', i' and k'. The total amount of protein in the purified fractions was measured by the Bradford procedure (kit Uptima UPF8640 Coo Protein Assay, Interchim, Montluçon, France). Protein concentration of fractions (700–6500 µg/ml) was adjusted by dilution to 50 µg/ml before being tested in vitro. To identify individual components the apparent molecular weights of components of these fractions were determined by the SDS-PAGE technique [11] and each band was submitted to digestion by trypsin. The amino acid sequences from the different peptides were obtained by LC-MSMS: nanochromatography (CapLC, Waters, Milford, USA) coupled to mass spectrometry (ESI/Q-TOF, Waters, Milford, USA).

2.2. Precipitation experiments

Certified chemical reagents and Milli-Q water (Millipore) were used to prepare stock solutions of CaCl₂ (Sigma, Lot. 110K0226) and NH₄HCO₃ (Sigma, Lot. 78H0114). Precipitation experiments were carried out by vapour diffusion using the sitting drop method on "crystallization mushrooms" (Triana Science & Technology, S.L., Granada, Spain) [13], at 23 ± 2 °C and 1 atm. Two mushrooms containing 10 microbridges were run in parallel. A mushroom for fractions h, g, n, and r, and the other one for c', g', i' and k'. Each mushroom contained two microbridges to allocate 40 µl drops of the control solution (20 mM CaCl₂) and eight microbridges to allocate the protein-bearing solutions. They were composed of 38 µl of the diluted purified fraction (50 µg/ml) plus 2 µl 400 mM CaCl₂. A 3 ml of the NH₄HCO₃ solution (10 mM) was poured into the bottom container of the mushroom. The system was closed and sealed with silicon grease. The precipitation reaction was initiated by the simultaneous diffusion of NH₃gas and CO₂gas released by the underlying NH₄HCO₃ solution. The pH, recorded in a preliminary control experiment, started at 5.2 and increased progressively to 8.3.

The time elapsed from the moment of closing the mushrooms until the appearance of the first precipitate (induction time, t), the total number of crystals precipitated in the microbridges (TNC) and the identification of polymorphs, were all determined using an optical
microscope (SZH10 Olympus, 14×), the latter based on characteristic morphologies of crystals associated to each CaCO₃ phase, i.e. calcite, aragonite and vaterite. These crystals were obtained in preliminary experiments in the absence of protein and conveniently characterized [13]. At the end of the runs the precipitates were harvested from the microbridges having been previously rinsed several times with deionized Milli-Q water, dried at room temperature, and finally coated with gold and inspected with a field emission scanning electron microscope (FESEM, Gemini-1530). The average size of crystals (diameter or length of the characteristic dimension) was averaged by measuring at least 10 crystals and a maximum of 50 crystals in precipitates with a larger number of crystals. X-ray diffraction data were recorded on a Bruker Smart 6000 CCD detector with Kappa configuration (X8 Proteum) using Cu Kα radiation from a Bruker Microstar microfocus (Montel Optics) rotation anode generator, operating at 45 kV and 60 mA.

### 3. Results

The electrophoretic profiles of fractions h, g, n, and r and c', g', κ' and k' as well as the putative identity of the stained bands are shown in Fig. 2a,b. Most of the fractions are composed of several bands, of which the more intensely stained correspond to the following compounds: h (quiescence-specific proteins and ovoleidine-17 chain A), g (ovotransferrin, ovomucoid, quiescence-specific protein and ovoleidine-116), n (ovotransferrin, preproalbumin, hemopoxin, ovoinhibitor precursor, cystatin), r (quiescence-specific protein, ovoleidine-17 chain A and non-identified sequences), c' (clone AGENAE not identified, ovoleidine-17 chain A and lysozyme C precursor), κ' (clusterin, ovocalyxin-116, EGF-like repeats and discoidin I-like domains containing protein 3 [Homo sapiens]), k' (cystatin, ovoleidine-116, glypican 4, ovalbumin, and ovocalyxin-32, glypican 4, clusterin, ovocalyxin-36, lysozyme C precursor, ovotransferrin, no sequence, lysozyme C precursor and no sequence. MQ: molecular weight markers.

### Table 1

CaCO₃ precipitation in the presence of different purified fractions of eggshell matrix proteins whose concentrations were adjusted to 50 μg/ml, using as reference conditions 20 mM CaCl₂ and 10 mM NH₄HCO₃ at 23 ± 2 °C.

<table>
<thead>
<tr>
<th>Run</th>
<th>tᵢ (min)</th>
<th>TNC average total number of crystals</th>
<th>C</th>
<th>A</th>
<th>V</th>
<th>L (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushroom 1 Controls</td>
<td>1100–1200</td>
<td>60</td>
<td>18</td>
<td>22</td>
<td>~90</td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>1680</td>
<td>12</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>h</td>
<td>1100</td>
<td>147</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>n</td>
<td>1300</td>
<td>112</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>195</td>
</tr>
<tr>
<td>r</td>
<td>&gt;1300</td>
<td>10</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>130–230</td>
</tr>
<tr>
<td>Mushroom 2 Controls</td>
<td>960–1200</td>
<td>77</td>
<td>55</td>
<td>22.5</td>
<td>22.5</td>
<td>~95</td>
</tr>
<tr>
<td>c'</td>
<td>960–1200</td>
<td>59</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>g'</td>
<td>960–1200</td>
<td>40</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>62–118</td>
</tr>
<tr>
<td>κ'</td>
<td>960–1200</td>
<td>77</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>49–138</td>
</tr>
<tr>
<td>k</td>
<td>&gt;1360</td>
<td>11</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>118.5</td>
</tr>
</tbody>
</table>

TNC, average total number of crystals; C, calcite; A, aragonite; V, vaterite; tᵢ, induction time for nucleation measured visually with error ±60 min. In the case of fractions c', g' and κ', tᵢ oscillates between 960 and 1200 min. L, average size.

I-like domains containing protein 3 [Homo sapiens], k' (a mixture of cystatin, ovoleidine-116, glypican 4, ovalbumin, ovocalyxin-32, clusterin, ovocalyxin-36, ovotransferrin and lysozyme). Only fraction g' has a higher level of purity as the stained band correspond to ovoleidine-116. Some of the proteins are known, and their isolation and identification in the eggshell have been reviewed elsewhere [8] while other components have yet to be identified.

The effects of the purified fractions on the CaCO₃ nucleation (TNC, tᵢ), polymorphism and average size of crystals are shown in Table 1. In mushroom 1 the fractions...
h and n did not produce notable effects on the nucleation with respect to the control, as deduced by comparing the TNC and t. However, fractions g and r inhibited clearly the nucleation of CaCO₃ decreasing drastically the number of crystals (less than 12 crystals in each microbridge) and increasing t. In mushroom 2 the fractions c’ and t behaved similarly to h and n in mushroom 1; g’ slightly inhibits the nucleation and k’ behaved as a strong inhibitor. The differences in the effects on nucleation found in the control samples in both mushrooms must be exclusively attributed to differences in the local temperature in their surroundings; although the average laboratory temperature was kept at 25 ± 2 °C. Unfortunately, we cannot replicate the experiments to perform a statistic study because we lack sufficient amounts of the purified fractions.

A notable effect of organic fractions on CaCO₃ polymorphism is observed. Whereas in the control samples mixtures of 60% calcite, 18% aragonite and 22% vaterite (mushroom 1) and 55% calcite, 22.5% aragonite and 22.5% vaterite (mushroom 2) are obtained, in the presence of all purified fractions the only polymorph obtained is calcite. Calcite crystals grown in the control samples are isolated crystals with average size around 90–95 μm while aragonite and vaterite are observed as polycrystalline agglomerates (around 100 μm size) formed by smaller primary nanocrystals. Calcite crystals display the characteristic cleavage rhombohedral habits showing the {104} face. Aragonite precipitates as needle-like agglomerates and vaterite as hexalobulated leaf-like agglomerates (figures similar to those shown in Ref. [13]).

In the presence of fraction h the crystals precipitated are twinned and elongated (Fig. 3A). In these crystals inhibition always started at corners of {104} faces. The symmetry of the newly expressed faces corresponds to that of the {100} and {110} prismatic forms of calcite. In the presence of purified fractions g and n, we obtain spherulithic calcite crystals (see Fig. 3B and C) displaying terraced morphology with macro steps. These crystals resemble those present in the mammillary knob layer of the eggshell (see Fig. 3I). Both fractions share the presence of ovotransferrin, which has been found to be present in the mammillary knob layer [8]. Another protein found in fraction n is preproalbumin, a precursor of ovalbumin. Ovalbumin has been also described in the mammillae. In fraction r the spheruliths obtained are agglomerates of prismatic nanocrystals displaying self-organized banding. This last observation was found at high magnification (Fig. 3D).

In the presence of purified fractions c’, g’, t and k’ (Fig. 3E-H) we obtained polydisperse precipitates whose
average size oscillated between 60 and 140 μm. They were formed by globular agglomerates composed of spherical (Fig. 3E) or oriented columnar nanocrystals (Fig. 3F,G, and H). It is worth mentioning that fraction \( g' \), which produces these particular agglomerates, is composed exclusively of ovocleidin-116, the core protein of ovoglycan. This molecule which contains dermanatan sulphate glycosaminoglycan chain is polyanionic and acidic with calcium affinity and is likely to module crystal growth during palisade formation. This molecule noticeably affects the morphology of calcite crystals precipitated \( \textit{in vitro} \) [12]. In the agglomerate of Fig. 3G we can see that adjacent arrays of nanocrystals are fused.

In the agglomerates formed in the presence of fraction \( k' \), it was difficult to distinguish the effect of a particular component, since this fraction was very complex; however, the texture of these agglomerates, formed by columnar nanocrystals, is similar to that produced by fraction \( g' \), which contained only ovocleidin-116. It is interesting to note that both fractions (\( g' \) and \( k' \)) contain ovocleidin-116 and inhibit the precipitation.

4. Discussion and conclusions

The effects on CaCO₃ precipitation \( \textit{in vitro} \) of purified fractions composed by arrays of eggshell matrix proteins provide with insights on their possible role on the formation of the eggshell. The biomineralization processes in general are exquisitely modulated by organisms through specialized physicochemical mechanisms and specific organic molecules. These organic molecules are released sequentially at defined times to the growth media playing an important role in the control of the polymorphic phase, size, morphology and orientation of individual crystals. In this way, organisms are able to exert a superior control over mineralization processes [14]. Avian eggshell formation is interesting as it is a relatively simple form of biomineralization. Eggshell microstructure is the result of some degree of control exerted by the organic matrix components affecting the mineralogy, size and morphology of individual growing crystals [1,2,8] and of a competitive process during growth of adjacent crystals resulting in a columnar microstructure with a preferential crystallographic orientation [15]. These characteristics are very well defined and constant within a given avian species indicating the existence of a high degree of control over the eggshell formation.

The purification experiments reveal the great complexity of the eggshell organic matrix, with numerous proteins present in eggshell extract. Some of them have also been identified in the uterine fluid and may have been trapped along the different stages of the process of shell calcification. They represent a broad range of putative functions which are currently explored either by analogy with the role of these proteins in other organs, or by direct test \( \textit{in vitro} \), i.e. analysing their effects on CaCO₃ precipitation.

Fractions affect precipitation at different levels:

(i) All fractions select calcite among three possible anhydrous polymorphs, e.g., calcite, aragonite or vaterite. This result shows that soluble organic components participate in the selection of the eggshell mineral phase. Similar specificity over polymorphic selection has been observed by other authors using soluble organic macromolecules extracted from mollusc shells [16]. They have proved \( \textit{in vitro} \) that these components can specifically select the same polymorphic phase of calcium carbonate as that of shell layer from which they were extracted, i.e., aragonitic or calcitic. Levi et al. [17] showed that macromolecules that promoted calcite nucleation are strongly polyanionic and more strongly acidic than aragonite inducing ones. Acidic proteins could act as chelating agents reducing the available calcium concentration in solution and the effective supersaturation. A reduced degree of supersaturation will favour the precipitation of the most stable polymorph, which is calcite [18,19]. Other mechanism for polymorph selection is the inhibition of the nucleation by selective adsorption of macromolecules on the nascent nuclei of a stable polymorph thus allowing the growth of the metastable one. Because of the presence of soluble poly anionic acidic macromolecules in the eggshell extracts, the first mechanism is probably the one acting during the selection of the polymorph calcite.

(ii) All fractions modified in a different manner the morphology of the precipitated calcite crystals. The change in the growth morphology is obviously produced by the presence of one or a group of soluble macromolecules with a certain three-dimensional conformation. These macromolecules have a high number of \( -\text{COO}^- \) and \( -\text{NH}_3^+ \) groups on their surfaces, and depending on their isoelectric points and the pH of the experiment, they have a certain net positive, negative or zero surface charge. Alteration in the growth morphology by eggshell organic macromolecules with respect to the equilibrium morphology, the cleavage rhombohedra, seems to be the result of a change of the growth mechanism depending on the fraction used. This change is influenced by the specificity of the protein–surface interaction during the precipitation process. In the absence of macromolecules (control experiment) and at the low supersaturation at which the experiments are made, the parabolic mechanism acts during the formation of rhombohedral calcite. In the presence of fraction \( h \) a preferential adsorption of macromolecules to specific crystal faces, which become expressed as a consequence of the decrease in their growth rate, is the mechanism responsible for the change in growth morphology. In this case crystals expressed new and well defined crystallographic faces \{100\} and \{110\}, in spite of being twinned crystals. Fractions \( g \) and \( n \), however, produce spheruliths with terraced microstructure composed by macrosteps. Our hypothesis is that 2D-nucleation mediated growth could be the most likely mechanism acting during the formation of these spheruliths, and ovotransferrin, which is found in both fractions, may be
the specific macromolecule interacting with the solid phase. Further experiments of CaCO₃ precipitation in the presence of ovotransferrin will be necessary to check this hypothesis. This growth mechanism has been also reported in experiments of CaCO₃ precipitation in the presence of increased amounts of aerosol OT [20]. In the above cases specific protein–surface interactions seem to govern the precipitation process.

In the presence of fractions, a strong and non-specific protein–surface interaction most probably governs the process, which results in the precipitation of globular agglomerates composed of spherical or columnar primary nanocrystals. Such a strong non-specific interaction probably takes place after the nucleation event, once the supercritical nucleus reaches nanometric sizes, at the zone of highest supersaturation in the drops. The big globular agglomerates are thus the result of an aggregation–agglomeration–growth mechanism [21]. The texture of the agglomerates obtained in the presence of fraction g', composed of oriented columnar nanocrystals, is influenced by ovocleidin-116, the only macromolecule composing this fraction (Fig. 3F). Also fraction k', a complex fraction containing ovocleidin-116, produces the formation of agglomerates with microtexture similar to those found in the presence of fraction g'.

(iii) Some fractions exhibit a strong inhibitory effect on nucleation, suppressing the precipitation of CaCO₃ almost completely, while others do not produce any notable effect. Some organic macromolecules present in the fractions can affect CaCO₃ nucleation because they can act as strong chelating agents, reducing the available calcium concentration in solution and the effective bulk supersaturation to values at which the nucleation is greatly reduced. The effect is manifested at very low concentrations (i.e. 50 μg/ml). This behaviour is characteristic of potent crystallization inhibitors that have a strong affinity for target crystal surfaces [22].

In conclusion, the effects of purified fractions of the eggshell organic matrix on CaCO₃ precipitation have been tested. We found that macromolecules of these fractions are active in selecting calcite as the precipitating solid phase and changing the growth mechanisms. This change results in peculiar morphologies which in some cases resemble those found in eggshell. In some cases we have identified putative macromolecules responsible for these effects, although further experiments must be carried out to confirm this. Finally, some of the fractions with different macromolecular composition exhibited a similar strong inhibitory effect on nucleation. This finding indicates that there is more than one specific macromolecule acting as a strong Ca-chelating agent.

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