

Partial biomimetic reconstitution of avian eggshell formation

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Abstract

The avian eggshell is a biocomposite ceramic consisting of minute amounts of organic matrix and a crystalline calcium carbonate (calcite) filler. It is formed by a well regulated spatio-temporal assembling process, where extracellular matrix proteins, especially the sulfated glycosaminoglycan anionic sites of specific proteoglycans, have been involved in nucleation and growth of the inorganic crystalline phase. Together with such extracellular matrix molecules, the activity of carbonic anhydrase, is crucial for the normal eggshell formation. Here, we studied the effect of dermatan sulfate and carbonic anhydrase on the *in vitro* calcification of non-mineralized eggshell membrane–mammillae substrate at different pH and incubation times. Crystal morphology was analyzed by scanning electron microscopy. Crystal nucleation and growth was delayed at lower pH. Dermatan sulfate modified crystal morphology producing aggregates of large calcite crystals exhibiting a columnar morphology, contributing to the eggshell texture development. Carbonic anhydrase increased the velocity of crystal growth and eventually contributed to the fusion of the crystal aggregates to each other. Although, the effect of other macromolecules could not be ruled out, the combinatory effect of proteoglycans and carbonic anhydrase seems to be important for the control of eggshell formation.

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1. Introduction

The avian eggshell is a biocomposite ceramic consisting of minute amounts of organic matrix and a crystalline calcium carbonate (calcite) filler (Arias et al., 1993; Heuer et al., 1992). It is composed of a non-mineralized bilayered fibrillar membrane and a calcified extracellular matrix which are sequentially assembled during the 22 h the egg moves along the oviduct (Arias and Fernandez, 2001; Fernandez et al., 1997). From the inside to the outside, the eggshell is structurally composed of shell membranes, mammillary layer (formed by mammillary knobs also known as calcium reserve bodies), calcified layer proper or palisade, and finally the cuticle (Arias et al., 1993; Solomon, 1991) (Fig. 1). Among other molecules described elsewhere (Ajikumar

et al., 2003; Fernandez et al., 2003a; Gautron et al., 1997, 2001a,b; Hincke, 1995; Hincke and St. Maurice, 2000; Hincke et al., 1995, 2000, 2003; Lakshminarayanan et al., 2002, 2003; Lavelin et al., 1998; Mann, 1999; Mann and Siedler, 1999; Mann et al., 2002, 2003; Minksik et al., 2003; Nys et al., 1999; Panheleux et al., 2000; Pines et al., 1994), whose role in eggshell formation has not yet been well established, the eggshell organic matrix contains collagen and proteoglycans (Arias et al., 1991a, 1992, 1997; Carrino et al., 1996, 1997; Dennis et al., 2000; Fernandez et al., 1997; Nakano et al., 2001, 2002; Wang et al., 2002). Proteoglycans are constituted of a protein core to which long chains of sulfated glycosaminoglycans (such as keratan, dermatan or chondroitin sulfate) are attached.

Together with the non-confirmed report on the occurrence of type I and V collagen (Wong et al., 1984), the eggshell membranes contain type X collagen and osteopontin (Arias et al., 1991a, 1997; Fernandez et al., 2003a), which once secreted by the isthmus region of the

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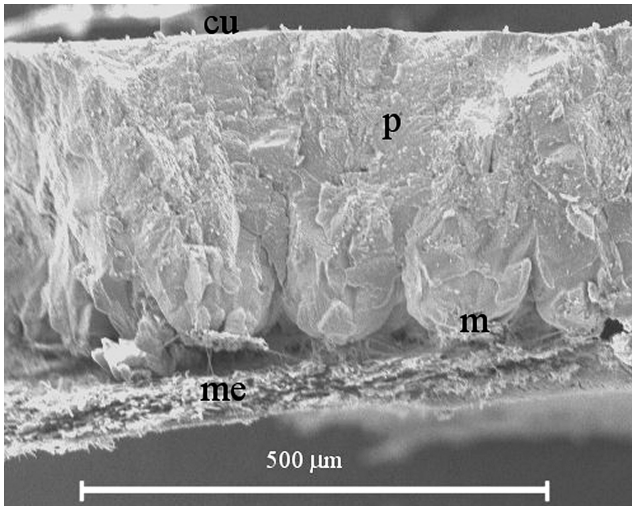


Fig. 1. Scanning electron microscopy of an eggshell, m: mammillae; me: membranes; p: palisade; cu: cuticle, 170 \times .

oviduct (Arias et al., 1991b; Wang et al., 2002), constitute the shell membrane fibrils core (Fernandez et al., 2001, 2003a). Type X collagen and probably osteopontin act inhibiting the mineralization of the eggshell membranes (Arias et al., 1997; Fernandez et al., 2003a). On the outer side of the eggshell membranes, discrete masses of organic material, the mammillae, are deposited. The base of each mammillae contains osteopontin, but its surface contains mammillan, a calcium binding keratan sulfate-rich proteoglycan, which has been involved in the nucleation of the first calcite crystals (Arias et al., 1992; Fernandez et al., 1997, 2001). Carbonic anhydrase, an enzyme which drives carbonate ion concentration, has been described in the cells of the last regions of the oviduct (Balnave and Muheereza, 1997; Bernstein et al., 1968; Ciperá, 1979; Commons, 1941; Diamantstein and Schlüns, 1964; Gutowska and Mitchell, 1945; Hodges and Lorcher, 1967; Holm et al., 2001; Lorcher and Hodges, 1969; Nys, 1990). In addition, carbonic anhydrase has been localized extracellularly in the mammillae (Diamantstein et al., 1964; Krampitz et al., 1974; Robinson and King, 1963). Growing of the calcite crystalline columns is accompanied by a concomitant secretion of ovoglycan, a dermatan sulfate-rich proteoglycan (Arias et al., 1992; Carrino et al., 1996, 1997; Dennis et al., 2000; Fernandez et al., 1997, 2001; Hincke et al., 1999), which has noticeable effects on the morphology of calcite crystals (Arias et al., 2002, 2004). Eggshell is finished by a cuticle deposition, which among other molecules, also contains osteopontin and ovocalyxin-32, contributing to the shell calcification arrest (Dennis et al., 1996; Fernandez et al., 2003a; Hincke et al., 2003). Thus, eggshell formation is regulated by a precise spatio-temporal arrangement of sequentially deposited macromolecules controlling calcite crystal growth, morphology, and texture (Arias et al., 2003).

To have a further insight on the role of such extracellular matrix molecules in eggshell formation and organization, we studied the effect of dermatan sulfate and carbonic anhydrase on the *in vitro* calcification of non-mineralized eggshell membrane–mammillae substrates.

2. Materials and methods

Eggs were obtained from commercial White Leghorn laying hens which were placed in individual wire cages, with artificial light provided for 16 h a day and food and water *ad libitum*. Pieces of non-calcified eggshells containing recently formed mammillae were obtained from eggs at 5:30 h post-oviposition, where no calcium had already been deposited (Fernandez et al., 1997). Nine millimeter squared eggshell strips were used as substrate for *in vitro* mineralization. Additionally, for comparison with normal eggshell formation, eggshells obtained from eggs at different times post-oviposition were analyzed.

The crystallization assays was based on a variation of the sitting drop method developed elsewhere (Dominguez-Vera et al., 2000). Briefly, it consists of a chamber built with a 85 mm plastic petri dish having 18 mm in diameter central hole in its bottom, glued to a plastic cylindrical vessel (50 mm in diameter and 30 mm in height) (Fig. 2). The bottom of the petri dish was divided in 16 radii to assure an equidistant settling of odd number of polystyrene microbridges (Hampon Res., Laguna Niguel, CA). The microbridges were filled with 35 μ l of 200 mM calcium chloride dihydrate solution in 200 mM Tris buffer, pH 7.4 or 9.0. The cylindrical vessel contained 3 ml of 25 mM ammonium carbonate. One strip of eggshell was deposited on the bottom of each microbridge with the mammillary side facing up. Control samples contained only calcium chloride solution, while 64 μ g/ml of dermatan sulfate (generously provided by Dr. G. Zoppetti, Glycores, Milan) and/or 10 μ g/ml bovine erythrocytes carbonic anhydrase (Sigma, St. Louis) were added to the experimental ones. Ten repli-

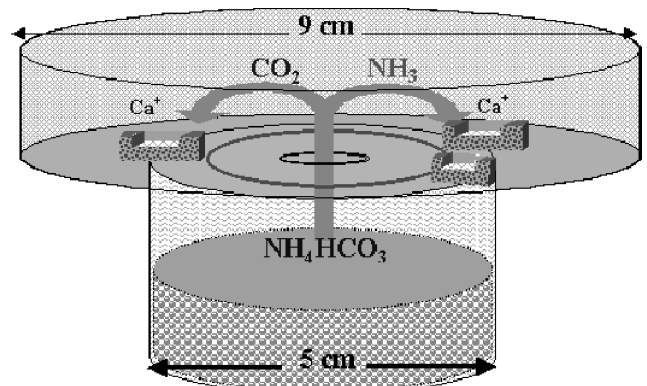


Fig. 2. Crystallization assays chamber scheme.

cates of each experiment were carried out inside the Petri dish chambers at 20 °C for variable periods of time (0–72 h). Precipitation of calcium carbonate results from the diffusion of carbon dioxide vapor into the buffered CaCl₂ solution. Additional control experiments of crystallization were done without shell membrane substrate but in the presence of active or inactivated carbonic anhydrase. Inactivation was done by heating the carbonic anhydrase solution at 80 °C for 60 min. After the experiments, eggshell strips were taken out of the microbridges, air-dried at room temperature, mounted on copper stubs with scotch double-sided tape, and coated with gold. Crystals were observed in a scanning electron microscope TESLA BS 343A at 15 kV. Crystallographic planes were estimated by measuring some angles of the visible faces of the obtained crystals.

3. Results

Eggshells obtained at 5:30 h post-oviposition consist of discrete aggregations of non-calcified organic material termed mammillae which are randomly deposited on the most external fibers of the shell membranes (Fig. 3A). At this stage mammillae do not contain calcium detected by EDS (data not shown, but well established in Fernandez et al., 1997). However, eggshells obtained at 6:45 h post-oviposition showed large aggregations of calcite crystals growing as columns on each mammilla, which depending on their vicinity, started to fuse with the nearest ones, and eventually all fused together given a continuous calcified surface, except at pores sites (Figs. 3B and C).

When non-calcified 5:30 h post-oviposition eggshells are incubated in calcium chloride, without any additive at pH 7.4, small calcite crystals growing on each mammilla were visible at 72 h of incubation and even smaller at 24 h of incubation (Figs. 4A and B). However, incubation under the same conditions but at pH 9.0, showed the formation of noticeable bigger calcite crystals on the mammillae at any studied time (24–72 h) (Figs. 5A and B). They start as small crystals (2–3 μm) on the surface of each mammilla, becoming aggregates of randomly oriented larger crystals (10–20 μm) outward. They showed predominantly {104} faces.

When non-calcified eggshells are incubated in the presence of carbonic anhydrase at pH 7.4, aggregates of small crystals (5–7 μm) are formed on each mammilla at every incubation time (Figs. 6A and B). Again, they showed {104} faces, but also {hk0} and {0kl} faces are exhibited. However, when the same experiment is done at pH 9.0, the aggregations of almost regular calcite crystals grown on each mammilla appeared to fuse each other, showing a continuous flat upper surface (Figs. 7A and B).

Dermatan sulfate at pH 7.4 or 9.0, in the absence of carbonic anhydrase, produced large crystals on each

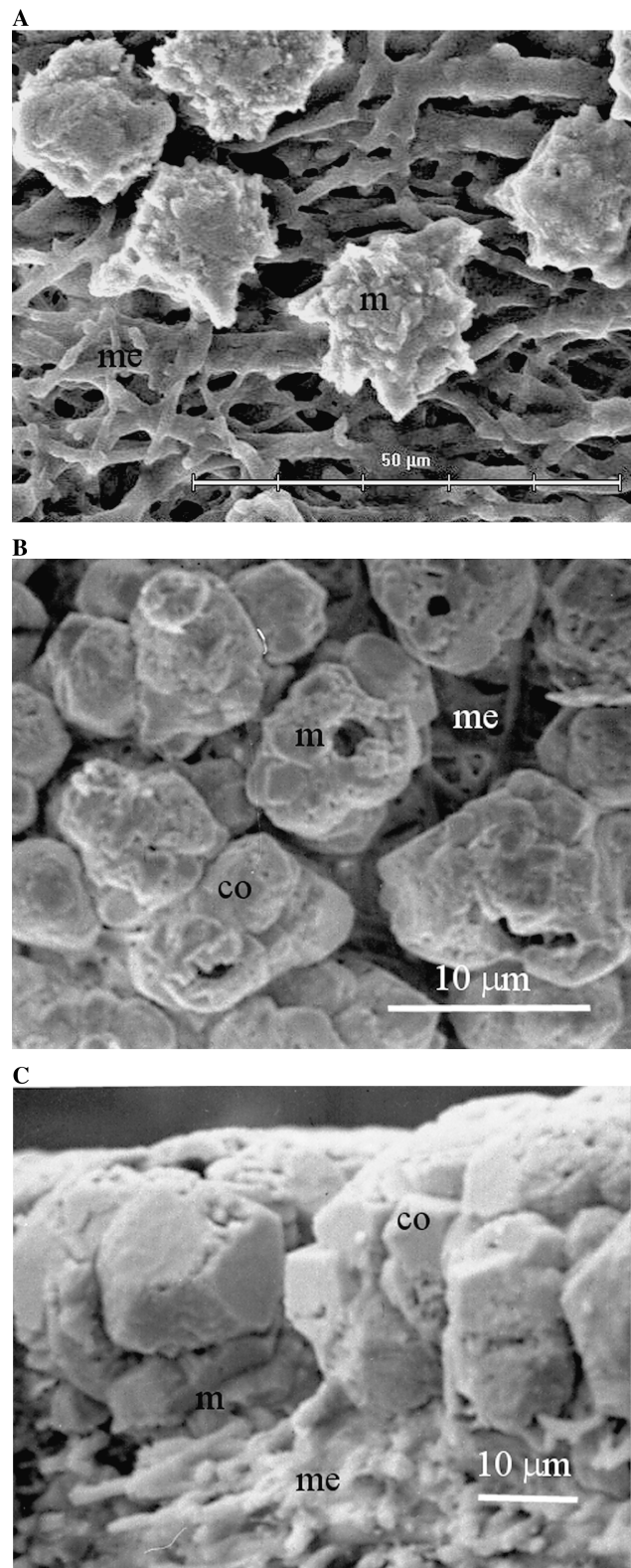


Fig. 3. Scanning electron microscopy of eggshell during normal development: (A) Top view of 5:30 h post-oviposition eggshell showing randomly deposited mammillae on the most external fibers of the shell membranes, m: mammillae; me: membranes, 1700×. (B) Top view and (C) side view of a 6:45 h post-oviposition eggshell showing aggregations of calcite crystals growing as columns on each mammillae, m: mammillae; me: membranes, co: columns, 640× and 320×.

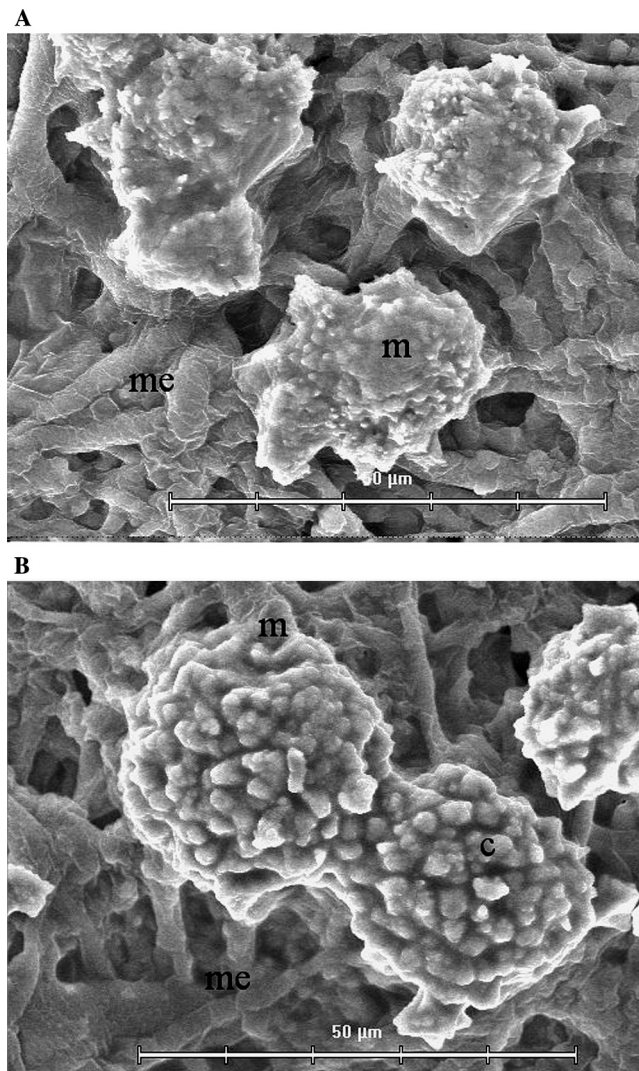


Fig. 4. Scanning electron microscopy of 5:30 h post-oviposition egg-shell incubated in calcium chloride, at pH 7.4 without any additive: (A) 24 h incubation showing mammillae with small calcite crystals deposited, m: mammillae; me: membranes. (B) 72 h incubation showing a little bigger calcite crystals growing on each mammillae, m: mammillae; me: membranes; c: calcite crystals, 1700 \times .

mammilla exhibiting a columnar morphology as $\{hk0\}$ cylinders with three $\{104\}$ faces forming a cap at both ends. With incubation time, the crystals become larger and tended to form aggregates of 50 μm in diameter (Figs. 8A–D). The same type of crystal aggregates are formed on each mammilla in the presence of dermatan sulfate and carbonic anhydrase at pH 7.4, which start to fuse to neighbor aggregates at 72 h although without showing a flat surface (Figs. 9A and B). However, at pH 9.0, the cylindrical crystal aggregates grew as columns on each mammilla and fused to each other showing a continuous flat upper surface (Figs. 10A–C).

When crystallization assays were done without shell membrane substrate at pH 9.0 in the presence of active carbonic anhydrase, large aggregations of almost

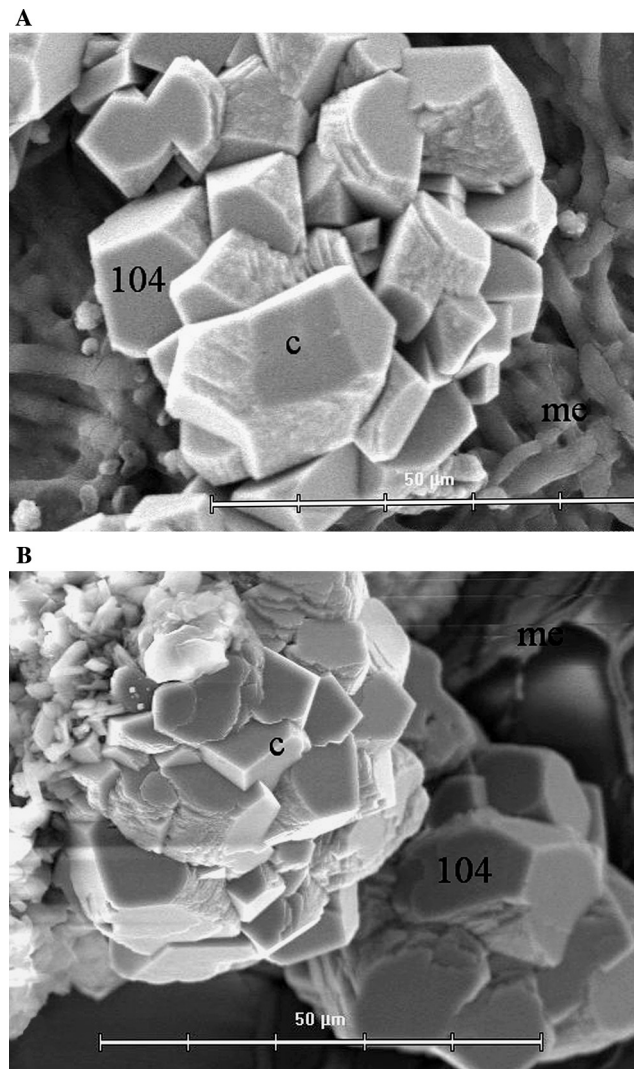


Fig. 5. Scanning electron microscopy of 5:30 h post-oviposition egg-shell incubated in calcium chloride, at pH 9.0 without any additive showing the formation of calcite crystals with predominantly $\{104\}$ faces at any time of incubation: (A) 24 h incubation, me: membranes; c: calcite crystals. (B) 72 h incubation, me: membranes; c: calcite crystals, 1700 \times .

unmodified crystals were formed (Fig. 11A). However, when inactivated carbonic anhydrase was used, isolated unmodified crystals were formed (Fig. 11B).

4. Discussion

Biom mineralization is a widespread phenomenon among living organisms, leading to the formation of precisely controlled inorganic–organic composites, in which the minute organic component exerts substantial control on the mineralization process (Lowentam and Weiner, 1989; Mann, 2001; Simkiss and Wilbur, 1989). More than 20 proteins have been described to be involved in the control of biomineralization of egg- and

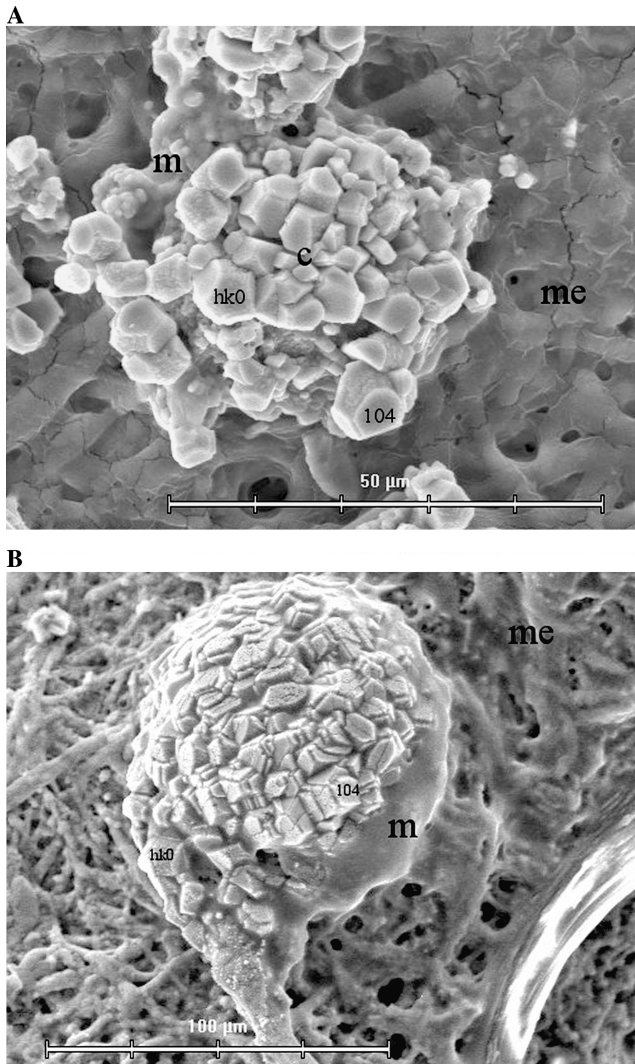


Fig. 6. Scanning electron microscopy of 5:30 h post-oviposition eggshell incubated in calcium chloride, at pH 7.4 in the presence of carbonic anhydrase showing the deposition of calcite crystals with $\{104\}$ faces and $\{hk0\}$ and $\{0kl\}$ at every incubation time: (A) 24 h incubation, me: membranes, c: calcite crystals, 1700 \times . (B) 72 h incubation, me: membranes; c: calcite crystals, 670 \times .

seashells (Nagasawa, 2004; Nys et al., 1999; Samata, 2004). However, not enough similarities among their amino acid sequences or conformation can be found to be implicated in a universal mechanisms of regulation of crystal nucleation and growth. By studying egg- and seashells, we have found the persistent occurrence of specific polyanionic sulfated macromolecules referred to as proteoglycans (Arias and Fernandez, 2003). These proteoglycans have particular calcium affinity and behave as gels. The distribution of these proteoglycans together with their function on *in vitro* mineralization assays, show that they are involved not only in the nucleation but also in the growth of the calcium carbonate crystalline phase (Arias et al., 1993, 2003; Fernandez et al., 2001).

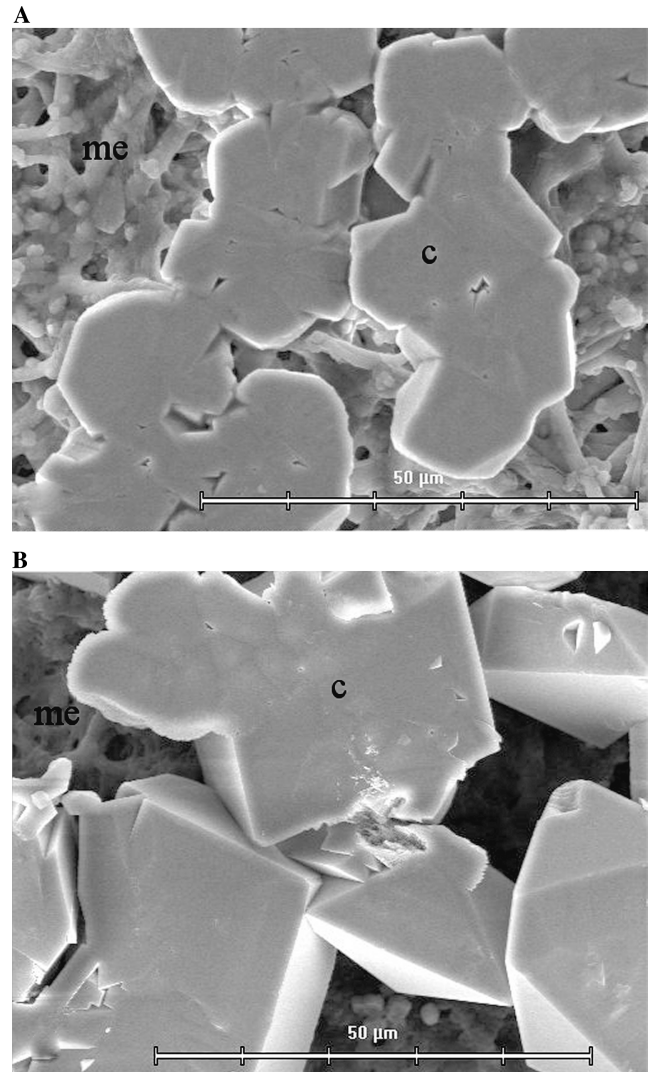


Fig. 7. Scanning electron microscopy of 5:30 h post-oviposition eggshell incubated in calcium chloride, at pH 9.0 in the presence of carbonic anhydrase showing aggregations of calcite crystals on each mammillae that appeared to fuse each other showing a flat upper surface: (A) 24 h incubation, me: membranes, c: fused calcite crystals, 1700 \times . (B) 72 h incubation, me: membranes; c: fused calcite crystals, 1700 \times .

Eggshells are fabricated by a biologically controlled mineralization process through a spatio-temporal regulated secretion and assembly of inorganic and organic moieties (Fernandez et al., 1997, 2001). This “bottom up” regulated assembly process results in the formation of a composite bioceramic of defined structure and organization. Eggshell formation starts with the fabrication of the shell membranes, a net of type X collagen- and osteopontin-containing fibers, which do not mineralize, but are the material substrate on which keratan sulfate proteoglycan-rich nucleation sites (mammillae) are randomly deposited (Fernandez et al., 1997, 2001). Calcite crystals nucleate on the surface of the mammillae and initially grow upward as essentially

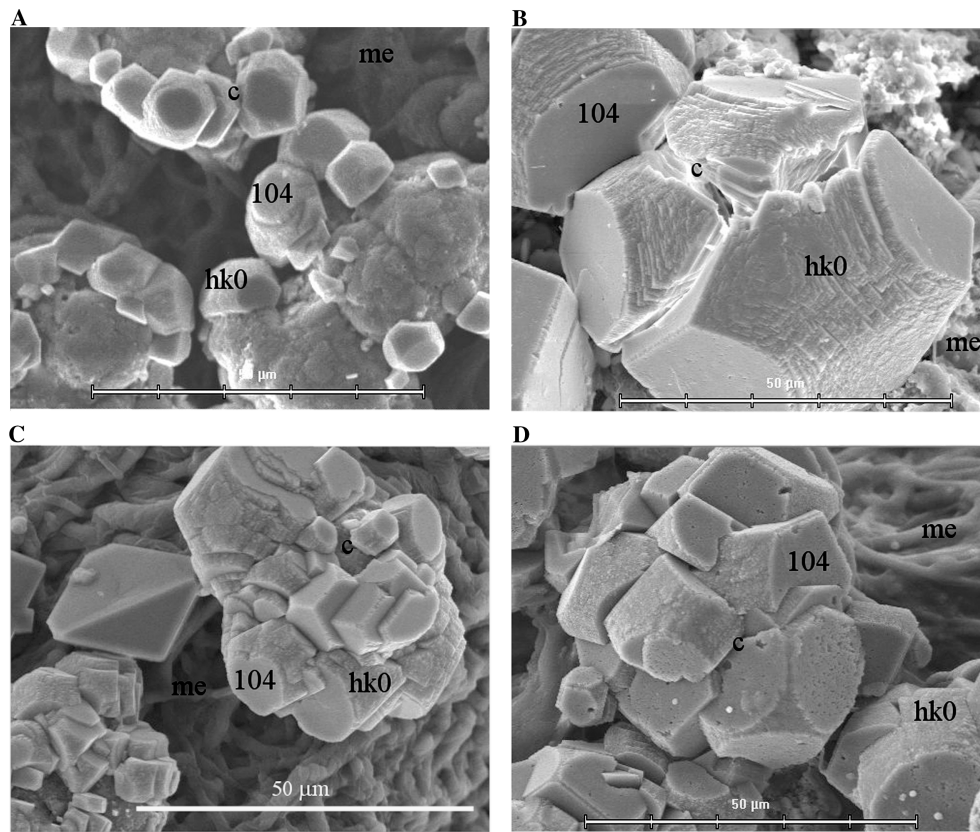


Fig. 8. Scanning electron microscopy of 5:30 h post-oviposition eggshell incubated in calcium chloride, in the presence of dermatan sulfate showing large crystals on each mammillae exhibiting a columnar morphology as $\{hk0\}$ cylinders with three $\{104\}$ faces forming a cup at both ends. At pH 7.4 (A) 24 h incubation, me: membranes, c: calcite crystals, 1700 \times . (B) 72 h incubation, me: membranes; c: calcite crystals, 1700 \times . At pH 9.0: (C) 24 h incubation, me: membranes, c: calcite crystals, 1700 \times . (D) 72 h incubation, me: membranes; c: calcite crystals, 1700 \times .

randomly oriented discrete groups (Arias et al., 1993). It appears that after nucleation, only a few of the initially formed crystals continue to grow, and the shell increases in thickness by individual crystals packed together and growing vertically to the surface, where a moderate crystal orientation is found (Arias et al., 1993). Concomitant with this growing is the occurrence of a dermatan sulfate proteoglycan (ovoglycan), which is secreted during the time of palisade formation (Fernandez et al., 2001).

In this study, we show how dermatan sulfate and carbonic anhydrase interact with growing calcite crystals which have been nucleated on the mammillae. As it has been demonstrated elsewhere (Cölfen and Qi, 2001), pH influences drastically the nucleation rate and crystal size. Although the effects of pH we observed could be related to the degree of protonation of the sulfate groups of the mammillary keratan sulfate, the carbonate supersaturation of the solution, due to the action of carbonic anhydrase, could also be considered. In fact, at pH 7.4 the calcite crystals obtained at a particular incubation time in the absence of eggshell membranes are fewer and smaller than those obtained at pH 9.0. In the presence of eggshell membranes at pH 7.4, there are only small crystals visible

at 24 and 72 h of incubation, while at pH 9.0 there are bigger crystals at any time of incubation. This feature changes drastically when experiments were done in the presence of carbonic anhydrase, which shifts the bicarbonate/carbonate ratio, producing large and numerous crystals on the mammillae as early as 24 h even at pH 7.4.

It has been previously shown that the addition of dermatan sulfate has noticeable effects on the calcite crystals morphology obtained in vitro (Arias et al., 2002). It produced crystals exhibiting a columnar morphology as a $\{hk0\}$ cylinder with three $\{104\}$ faces forming a cap at both ends. Here, we show that dermatan sulfate produced almost the same effect even on the crystals nucleated on the mammillae. Specific interactions of polyanionic proteins with particular sets of crystal faces have been implicated in the regulation of calcite crystal growth and morphology (Aisenberg et al., 1996, 2002; Albeck et al., 1993). Although, we do not know how dermatan sulfate interact with calcite, it specifically inhibits the growth of planes ($hk0$). X-ray diffraction and scanning electron microscopy of eggshell have shown that there is a modest but progressive increase of crystallographic texture (preferential orientation) from the inside to the outside of the shell (Arias

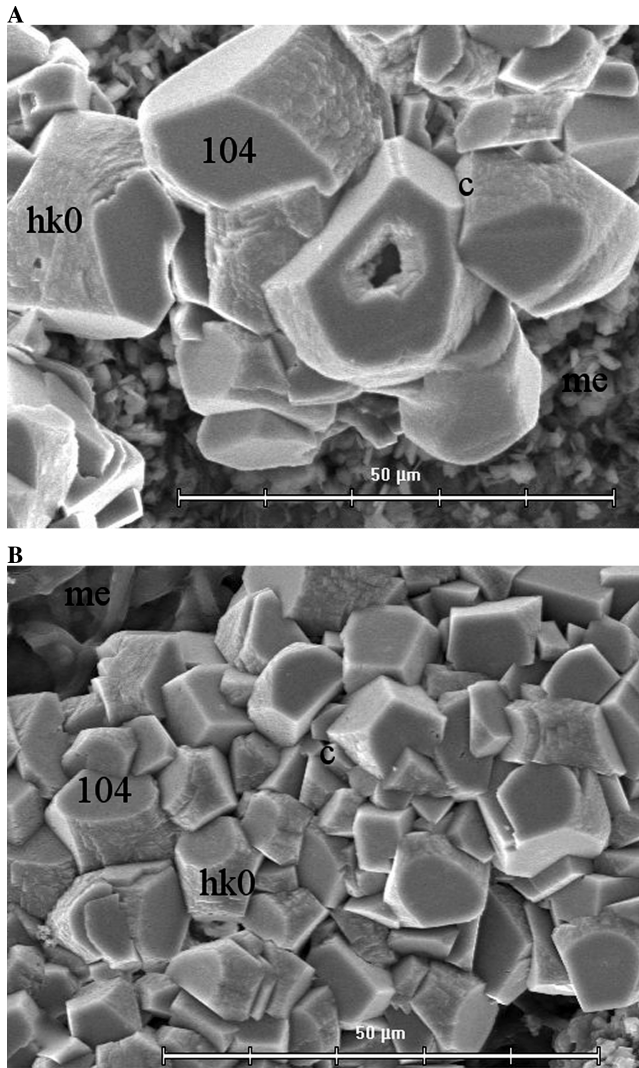


Fig. 9. Scanning electron microscopy of 5:30 h post-oviposition eggshell incubated in calcium chloride, at pH 7.4 in the presence of dermatan sulfate and carbonic anhydrase showing large crystals with columnar morphology as $\{hk0\}$ cylinders with three $\{104\}$ faces forming a cup at both ends: (A) 24 h incubation, me: membranes, c: calcite crystals, 1700 \times . (B) 72 h incubation, large deposits of crystals that start to fuse; me: membranes; c: calcite crystals, 1700 \times .

et al., 1993; Garcia-Ruiz et al., 1995; Wu et al., 1992). Although, the development of texture in layered aggregates could be explained in terms of geometric selection of the orientation of crystals (Rodriguez-Navarro and Garcia-Ruiz, 2000), the modulation of crystal growth direction exerted by dermatan sulfate could contribute to the establishment of the columnar morphology of the calcite aggregates which structures the eggshell.

Although activity of carbonic anhydrase has not been demonstrated in the uterine fluid, it has been found inside uterine cells and in the mammillae. When carbonic anhydrase is added to the mineralization milieu, as we have done here, the enzyme by itself has few effects on

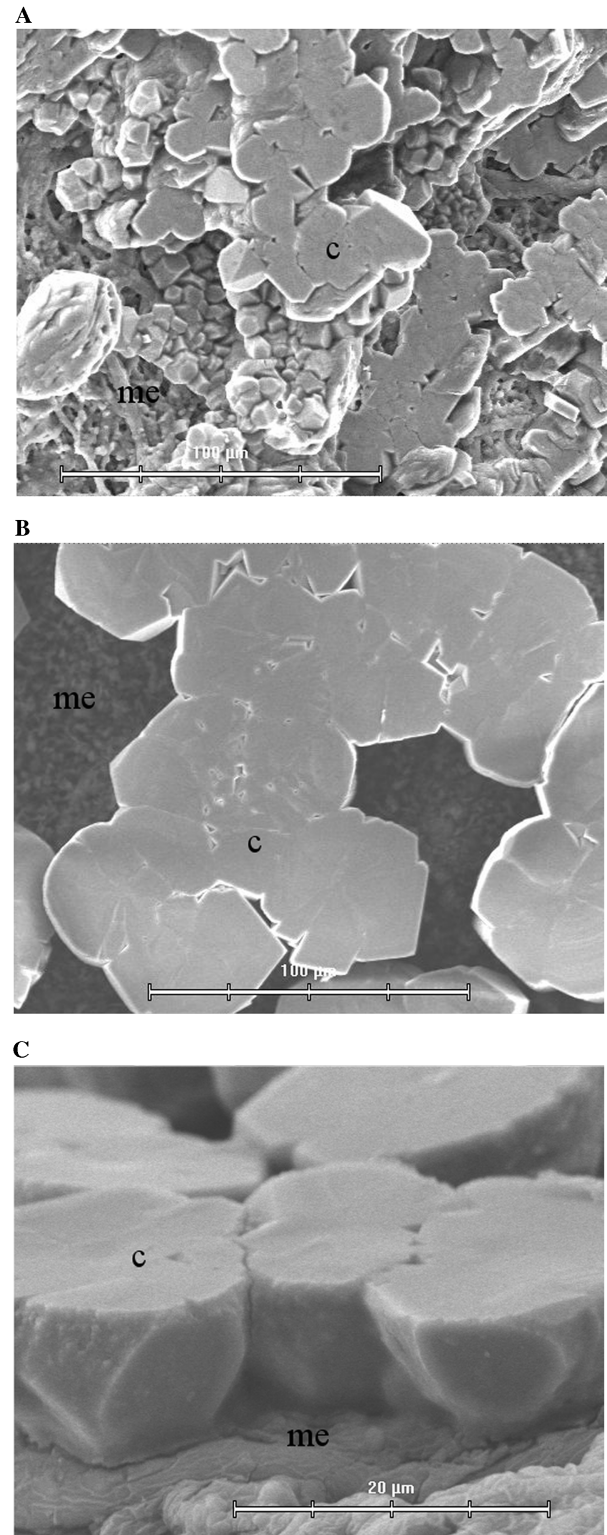


Fig. 10. Scanning electron microscopy of 5:30 h post-oviposition eggshell incubated in calcium chloride, at pH 9.0 in the presence of dermatan sulfate and carbonic anhydrase showing crystals that grew as columns on each mammilla and fused to each other showing a continuous flat upper surface: (A) 24 h incubation, me: membranes, c: fused calcite crystals, 670 \times . (B) 72 h incubation, me: membranes; c: fused calcite crystals, 670 \times . (C) side view of 72 h incubation, me: membranes; c: fused calcite crystals, 3300 \times .

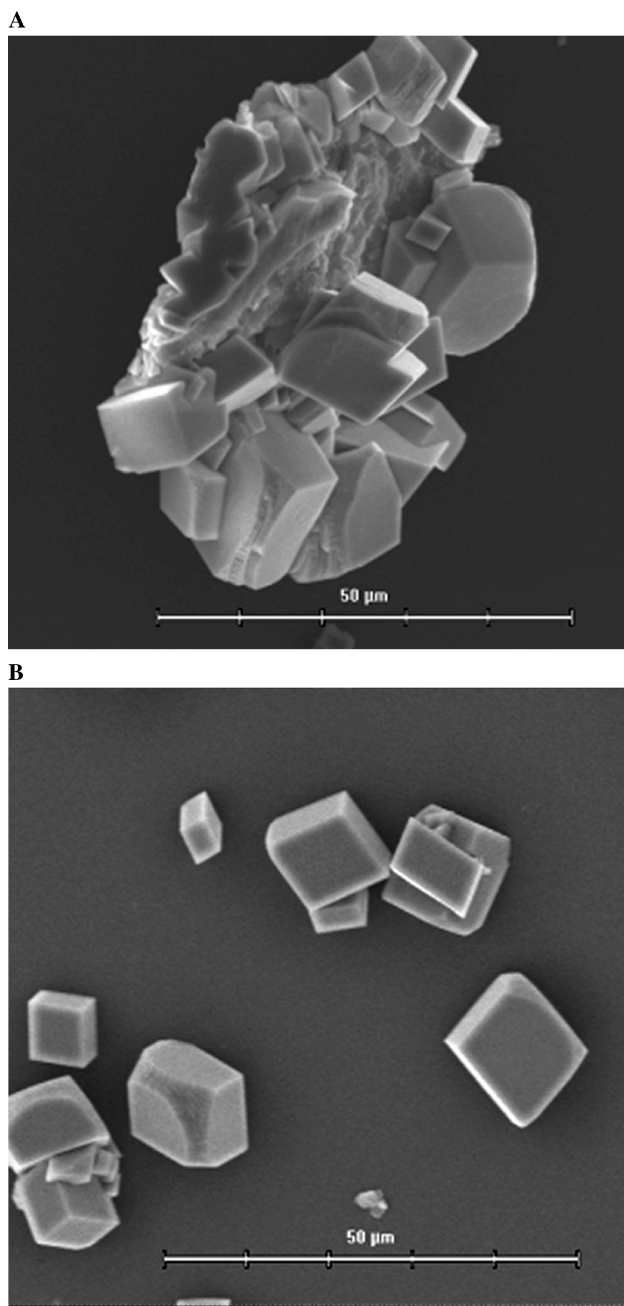


Fig. 11. Scanning electron microscopy of calcite crystals obtained at 24 h in the absence of eggshell membranes at pH 9.0: (A) in the presence of active carbonic anhydrase; (B) in the presence of inactivated carbonic anhydrase.

calcite crystal morphology, but its main effect relates with the increment of calcium carbonate availability, producing early nucleation and growth at pH 7.4 and fusion of the growing columns at pH 9.0. In fact, inactivated carbonic anhydrase does not modify crystal morphology, but the active enzyme induces aggregation of calcite crystals. During the initial stages of normal eggshell formation, individual mammillary calcified bodies can be clearly identified (Fernandez et al., 1997;

Solomon, 1991). However, as calcification proceeds, calcified mammillae fuse together, and eventually all the eggshell surface is organized as a continuous layer of calcium carbonate, except at pores sites. Normal eggshell calcification is completed in 16 h at about pH 7.5, immersed in a milieu containing an almost infinite solution of 10 mM calcium ion and 70 mM carbonate (Thapon and Bourgeois, 1994). Although our experiments were done at a higher calcium concentration (200 mM CaCl_2), the uncontrolled carbon dioxide flux and the small volume of the solution (35 μl), did not allow us to recapitulate the whole process of eggshell formation. However, under these experimental conditions, we were able to get a structural organization resembling the stage of eggshell formation obtained at 6:45–7:15 h post-oviposition (Fernandez et al., 1997).

On the basis of our observations, we believe that eggshell formation starts with the nucleation of calcite crystals on the mammillae, due to mammillan, a keratan sulfate proteoglycan. Ovoglycan, a dermatan sulfate proteoglycan, modulates the morphology of the growing calcitic layer, contributing to the eggshell texture development, while at any time, carbonic anhydrase acts to increase the carbonate availability for a fast crystal growth and columns fusion. Although the effect of other macromolecules could not be discharged, the combinatory effect of proteoglycans and carbonic anhydrase seems to be important for producing one of the most rapidly mineralizing biological systems known, the eggshell.

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