Connective Tissue Research
1995, Vol. 31, No. 3, pp. 227-233
Reprints available directly from the publisher
Photocopying permitted by license only

Ovalbumin is a Component of the Chicken Eggshell Matrix

M. T. HINCKE

Department of Anatomy and Neurobiology, University of Ottawa, Ottawa, Canada

(Received November 15, 1994; in revised form March 9, 1995; accepted March 15, 1995)

The protein components of biomineralized structures (matrix proteins) are believed to modulate crystal nucleation and growth, and thereby influence the shape and strength of the final structure. The chicken eggshell contains a complex array of distinct matrix proteins. One of these was found to have similar molecular weight and chromatographic properties as purified egg ovalbumin. A commercially available antibody to ovalbumin was utilized for western blotting to demonstrate that ovalbumin is one of the matrix proteins this is extracted from decalcified eggshell. Immunohistochemistry revealed that ovalbumin is found only in the mammillary bodies of decalcified shell, and is not distributed throughout the shell matrix. These results indicate that ovalbumin is present during the initial phase of shell formation and becomes incorporated into the protein matrix of the mammillary bodies. However, it is not yet clear whether ovalbumin at this site plays a specific role in shell mineralization.

ABBREVIATIONS: BSA, bovine serum albumin; kDa, kiloDalton; OC-17, ovocleidin-17; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis; TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone; TLCK, N-—tosyl-L-lysine chloromethyl ketone

Key Words: *ovalbumin, eggshell, matrix protein, calcification, biomineralization*

INTRODUCTION

The protein components of biomineralized structures (matrix proteins) are thought to influence the strength and shape of the final structure of calcium phosphate (carbonated apatite or dahlite) or calcium carbonate (calcite, aragonite, vaterite, monohydrocalcite or amorphous $CaCO_3$) by modulating crystal nucleation and growth.[1-3] The mechanisms by which macromolecules influence calcite crystallization are not well understood, but may involve acceleration or inhibition of crystal growth on specific crystallographic faces, due to specific interactions between binding sites and calcium carbonate.[4-6] This view is based on experiments with simple organic molecules, and with mixtures of proteins from decalcified molluscan shell.[3,7-10] Further testing of this hypothesis requires that the properties of individual matrix proteins be studied.

The chicken eggshell is a readily available and interesting model system for studying the role of matrix proteins in biomineralization. Biochemical studies have demonstrated that the eggshell matrix, which comprises about 2% of the total eggshell by weight, is predominantly made up of proteins (70%) and polysaccharides (11%) (summarized in).[11] The soluble matrix components of the eggshell have been studied by different laboratories (reviewed by),[12] but without purifying any specific protein to homogeneity for further study. Since calcium carbonate exhibits different crystallization patterns between the mammillary and palisade layers, we believe that valuable insights into biomineralization may be obtained by studying the properties of the isolated proteins. Our earlier study demonstrated that the mineralized layers of the chicken eggshell possess a complex array of distinct proteins, some of which may be differentially localized between the palisade and mammillary layers.[13] More recently, we purified one of the most abundant soluble matrix proteins (ovocleidin—17, OC-17), and demonstrated that it is localized to both palisade and mammillary layers of the shell.[14] In this communication, data is presented which demonstrates that another of the eggshell matrix proteins in fact corresponds to ovalbumin.

Address for correspondence: Dr. Max Hincke, Department of Anatomy and Neurobiology, University of Ottawa, Ottawa, Canada, K1H 8M5. (E-mail: mhincke@labsun1.med.uottawa.ca)

MATERIALS AND METHODS

Chromatography of Shell Matrix Proteins

Unfertilized eggs were obtained from a flock of White Leghorn hens. After washing out the egg white and yolk

with copious amounts of demineralized water, the shell membranes were removed by treating the shell with dilute bleach or HCl as before,[13] or by mechanical stripping (see results). Dried shell was ground to a fine powder and decalcified with 0.65 M EDTA, pH 7.8, 10 mM 2-mercaptoethanol, 3 mM Na azide. In preliminary experiments, the addition of protease inhibitors (PMSF, TLCK, TPCK) did not modify the SDS-PAGE profile of the shell matrix proteins. The decalcified extract was centrifuged (Beckman JA-14 rotor, 115,000 rpm, 1 hr) to obtain the soluble matrix proteins. This supernatant was dialysed against buffer A (10 mM Tris-HCl, 0.05% Na azide, 1 mM 2-mercaptoethanol), and then subjected to anionic exchange chromatography (see Results for details). In some cases, fractions were pooled, dialysed against 10 mM ammonium carbonate and lyophilized.

Western Blotting

Samples were prepared for SDS-PAGE, and their concentrations determined with the Pierce BCA assay, as described previously.[15] After SDS-PAGE on 10% or 12.5% gels, the proteins were electro-blotted to nitrocellulose overnight in 25 mM Tris, 192 mM glycine, 20% methanol (4°C, 20 V).[16] The membrane was washed in TPBS (phosphate-buffered saline, 0.1% tween-20) and blocked for 1 hr (5% blotto in TPBS). The membrane was washed, incubated with anti-ovalbumin (Cappel, raised in goat) for 1 hr (1:100,000 in 0.1% BSA, TPBS), washed again, and then incubated with anti-goat Fab-peroxidase (1:20,000) in TPBS, 0.1% BSA for 1 hr. The enhanced chemiluminescence method (Amersham) was utilized to reveal immunoreactive bands.

Immunohistochemistry

Tissues from two hens were fixed in ice-cold 4% paraformaldehyde in phosphate buffered saline for 24 hr and then equilibrated in sucrose before dehydration and embedding in paraffin wax. Sections (7 micron) were dewaxed in xylene, rehydrated through an ethanol series into TBS (100 mM Tris-HCl, pH 7.7, 0.15 M NaCl) and then incubated with antiserum to chicken egg albumin (Cappel, raised in goat) (1:2000 in TBS, 0.3% triton X-100) for 3 hr at room temperature. In some cases, the antiserum was preabsorbed with purified egg white ovalbumin (Sigma), or was omitted. Sections were washed (2 × 10 min; TBS, 0.3% triton X-100) and then incubated for 1 hr at room temperature with secondary antibody (1:80, donkey anti-goat—FITC conjugate (Cappel)). After washing, the immunoreactivity was visualized by fluorescence microscopy.

Pieces of shell were fixed and decalcified at room temperature in a 1:1 mixture of 150 mM sodium EDTA, pH 7.7 with either 4% para-formaldehyde in phosphate buffered saline or Lana's fixative.[17] Similar immunohistochemistry was observed in either case. After decalcification was complete (3–4 days), shell was dehydrated and embedded in LR white (J. B. EM Services, Inc). Resin was polymerized with catalyst (15 µl / 10 ml resin) at 0°C overnight,[18] and one micron sections were cut for immunohistochemistry. Sections were incubated with antiserum or preabsorbed antiserum (1:200 to 1:2000) overnight in the cold room. Sections were washed 3 × 30 min, TBS, 0.3% triton X-100, and then incubated with secondary antibody (1:80, donkey anti-goat—FITC conjugate (Cappel) for 3 hr, room temperature. After washing, the immunoreactivity was visualized by fluorescence microscopy.

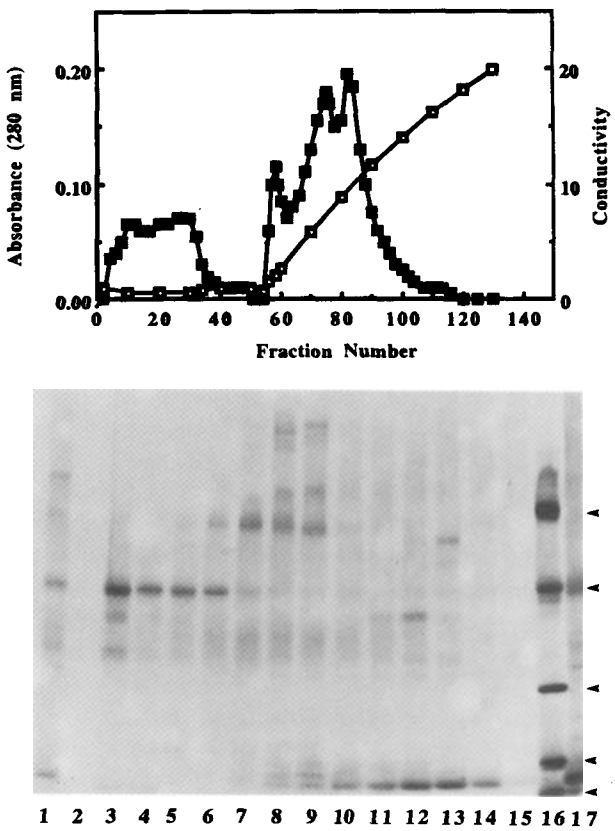

FIGURE 1 Anionic exchange chromatography of soluble eggshell matrix proteins. The shell membranes were removed manually prior to decalcification of 100 g of eggshell (methods). Soluble shell matrix proteins were applied to a column of DEAE cellulose 52 (1.5 × 20 cm) that was equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT. After washing, a linear gradient of NaCl to 0.85 M was developed at a flow rate of 20 ml / hr. 25 ml fractions were collected. A. Column profile. Absorbance (■ —— ■, Conductivity (□ —— □). B. Analysis of the columns fractions by SDS-PAGE (coomassie blue staining). Lane 1, soluble shell matrix proteins; Lanes 2–15, sample from fraction 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85, 88, 91; Lane 16, low molecular weight standards (BioRad) that are also indicated by the arrowheads (lysozyme, 14.4 kDa; soybean trypsin inhibitor, 21.5 kDA; carbonic anhydrase, 31 kDa; ovalbumin 45kDa; BSA, 66.2 kDa); Lane 17, insoluble shell matrix proteins. The 45 kDa protein is particularly evident in lanes 3–6 and the soluble and insoluble matrix. Equal volumes of the column fractions (25 ul) were analysed.

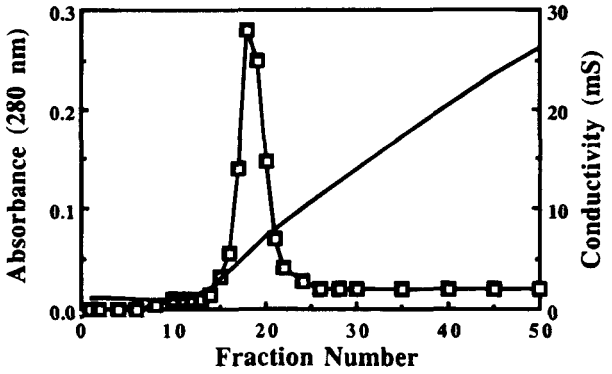

FIGURE 2 Anionic exchange chromatography of egg white ovalbumin. Purified ovalbumin (3.5 mg, Sigma A7641) was dissolved in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT and applied to a column of DEAE Cellulose 52 (5 ml). A linear gradient of NaCl to 0.7 M was developed at 40 ml / hr (2 ml fractions). Absorbance (□ —— □), Conductivity (———).

RESULTS

SDS-PAGE analysis of soluble eggshell matrix proteins separated by chromatography on DEAE-cellulose (Fig. 1A) revealed that one of the major matrix proteins had similar electrophoretic mobility to one of the molecular weight standards (ovalbumin, 45 kDa) (Fig. 1B, lanes 3–6). Authentic egg ovalbumin displayed similar chromatographic behavior as the 45 kDa eggshell matrix protein (Fig. 2). A commercially available antibody to ovalbumin was utilized to further test the possibility that the 45 kDa shell matrix protein corresponded to ovalbumin. Figure 3 demonstrates that the 45 kDa protein which is visualized by coomassie blue staining (Fig. 3A) corresponds in size and relative intensity to the ovalbumin immunoreactivity revealed by western blotting (Fig. 3B). These observations forced us to consider the possibility that washing of the eggshell interior with water and mechanical removal of the shell membranes prior to decalcification had not removed contaminating egg white proteins (the egg white is about 50% ovalbumin).

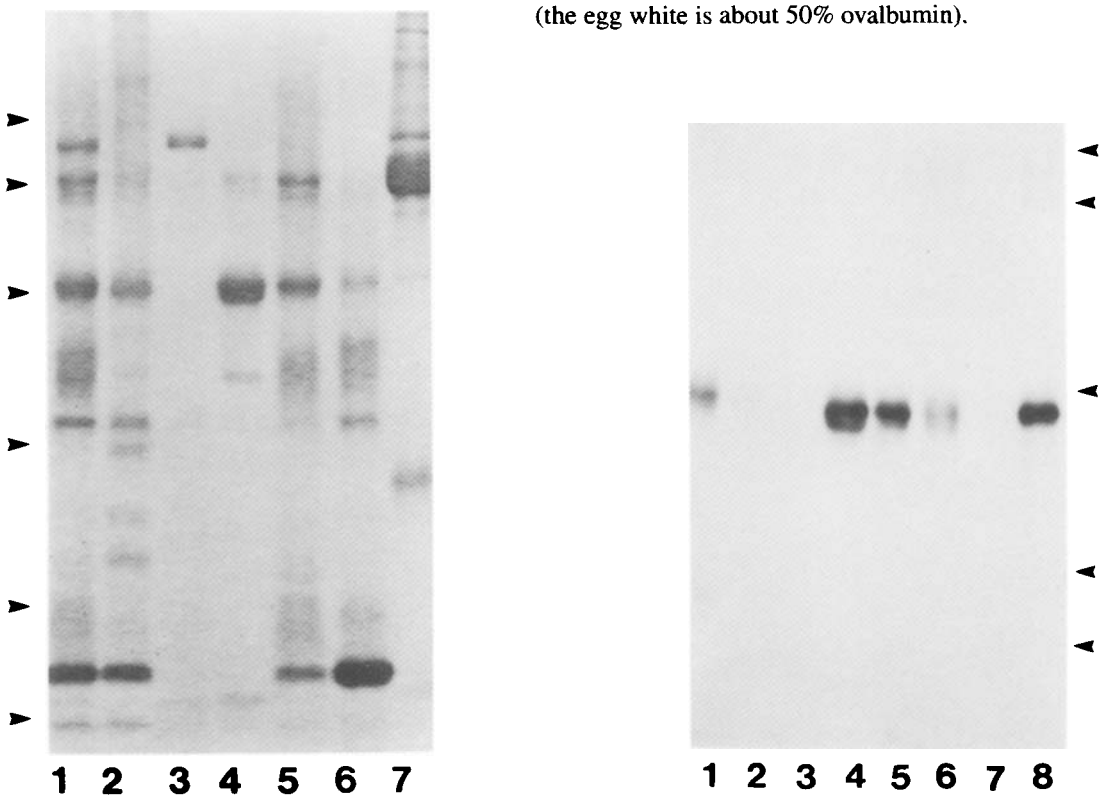

FIGURE 3 SDS-PAGE and western blotting of soluble eggshell matrix proteins after anionic exchange chromatography. Fractions from the experiment presented in Figure 1 were pooled and lyophilized after dialysis, for analysis as follows: A. SDS-PAGE (coomassie blue staining). Lane 1, total soluble eggshell matrix proteins (56 ug); Lane 2, total insoluble matrix proteins (28 ug); Lane 3, fractions 4–34 (void volume) (9 ug); Lane 4, fractions 55–62 (peak 1) (16 ug); Lane 5, fractions 63–78 (peak 2) (30 ug); Lane 6, fractions 79–96 (peak 3) (33 ug); Lane 7, chicken serum (31 ug). The positions of molecular weight standards (BioRad) are indicated at the left (bottom to top, kDa): 14.4, 21.5, 31, 45, 66.2, 97.4. B. Western blot analysis for ovalbumin. Lane 1, total soluble eggshell matrix proteins (14 ug); Lane 2, total insoluble matrix proteins (7 ug); Lane 3, fractions 4–34 (void volume) (2 ug); Lane 4, fractions 55–62 (peak 1) (4 ug); Lane 5, fractions 63–78 (peak 2) (8 ug); Lane 6, fractions 79–96 (peak 3) (8 ug); Lane 7, chicken serum (8 ug). Lane 8, egg white protein (Sigma E0500) (0.5 ug). The positions of *prestained* molecular weight standards (BioRad) are indicated at the left (bottom to top, kDa): 27.5, 32.5, 49.5, 80, 106. (Note "smiling" of gel that is evident in lanes 1 and 2, resulting in slightly slower apparent mobility of the ovalbumin band. Compare to Figure 7, lanes 7 and 8).

The possibility that shell ovalbumin represented surface contamination was addressed by treating the washed eggshell with dilute bleach to chemically degrade and remove all surface proteins before decalcification. Under these conditions, NaClO completely removes the shell membranes and other proteins which are not physically protected within the mineral, as previously demonstrated by Scanning Electron Microscopy.[13] Figure 4 demonstrates that under these conditions the 45 kDa protein is still present as a soluble eggshell matrix protein that is liberated by decalcification, as revealed by chromatography and SDS-PAGE analysis. The chromatographic elution profile of the 45 kDa protein (Fig. 4A, coomassie blue staining) corresponds to that of 45 kDa ovalbumin immunoreactivity (Fig. 4B, western blotting).

These results suggested that the ovalbumin was within or between crystal of mineral which compose the shell, and protected from harsh cleaning procedures. It might therefore be considered a matrix protein. This possibility was more directly investigated by immunohistochemistry with sections of fixed, decalcified shell. Figure 5 demonstrates that anti-ovalbumin solely stains the mammillary bodies of the eggshell, and this immunoreactivity was almost completely abolished by preabsorption of antibody with purified ovalbumin (Sigma). The tissue source of the eggshell ovalbumin was investigated. Figure 6 reveals that only the cells of the magnum (tubular glands) are immunopositive for ovalbumin. The mucosal cells of the shell gland were completely negative (not shown). This distinction was verified by western blotting (Fig. 7), where magnum was revealed to be a rich source of ovalbumin while it could not be demonstrated in shell gland.

DISCUSSION

The developing oocyte sequentially acquires each of its layers as it passes through the oviduct. The proteins of the egg white are mainly secreted by the tubular glands of the magnum region of the oviduct. The inner and outer shell membranes are then deposited as this structure passes through the isthmus. Finally, mineralization and shell formation occurs within the uterus, or shell gland. The mineralized portion of the avian eggshell is subdivided into an outer palisade layer and an inner mammillary knob

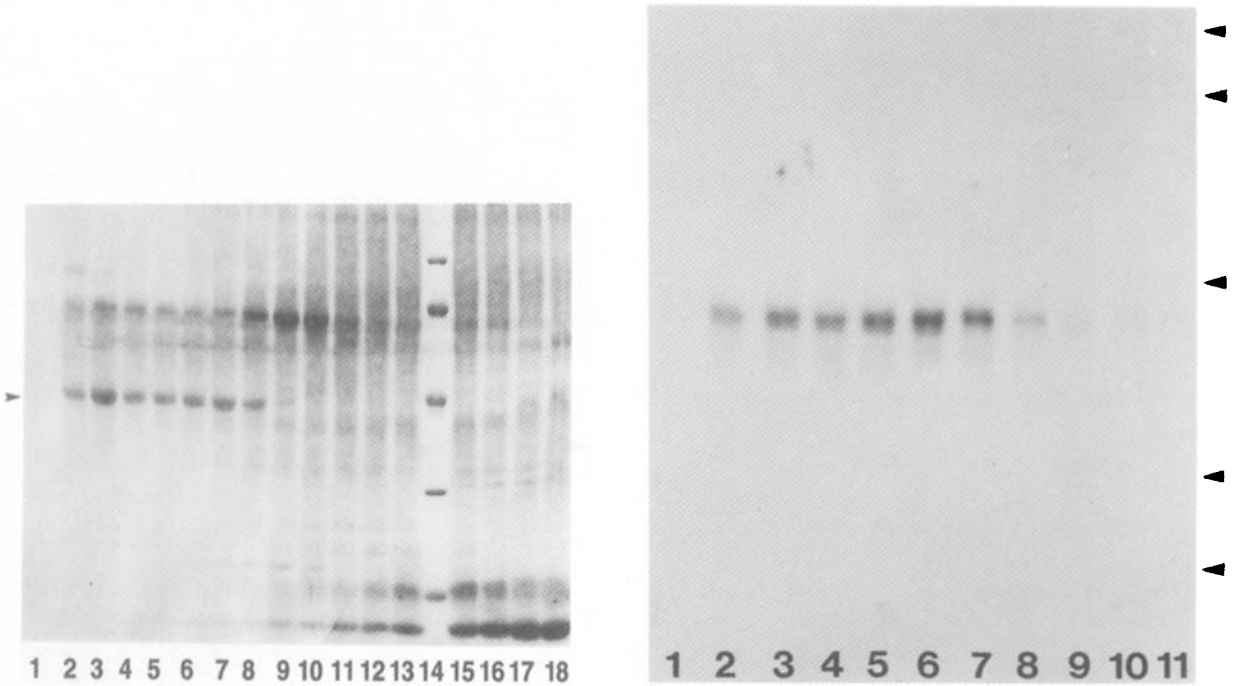

FIGURE 4 Analysis after anionic exchange chromatography of soluble proteins from decalcification of 100 g of bleach treated eggshell. Following dialysis against 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM DTT, 7.6 mM Na azide, the protein solution was applied to a column of DEAE Sephacel (1.5 × 20 cm) which had been equilibated with the same buffer. Proteins were eluted with a linear gradient to 0.8 M NaCl (25 ml / hr, 5 ml fractions). Only the first part of the gradient is shown in this figure (70 fractions in total, see ref (13)). A. Analysis of individual columns fractions by SDS-PAGE (coomassie blue staining). Lanes 1–13 and 15–18, fractions 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32 and 34, 36, 38, 40. Lane 14 contains molecular weight standards (BioRad) which are from bottom to top: soybean trypsin inhibitor (21.5 kDa); carbonic anhydrase (31 kDa); ovalbumin (45 kDa); bovine serum albumin (66.2 kDa); phosphorylase B (97.4 kDa). The arrow indicates the position of the 45 kDa band (Lanes 2–9, fractions 10–24). Equal volumes of the column fractions (25 ul) were analysed. B. Western blot analysis of individual column fractions for ovalbumin. Fractions 8 to 30 from Figure 4A were transferred to nitrocellulose and probed for ovalbumin (primary antibody, 1:100,000). Lanes 1 to 11 correspond to fractions 8, 10, 14, 16, 18, 20, 22, 24, 26, 28, 30. The positions of *prestained* molecular weight standards (BioRad) are indicated at the right (bottom to top, kDa): 27.5, 32.5, 49.5, 80, 106.

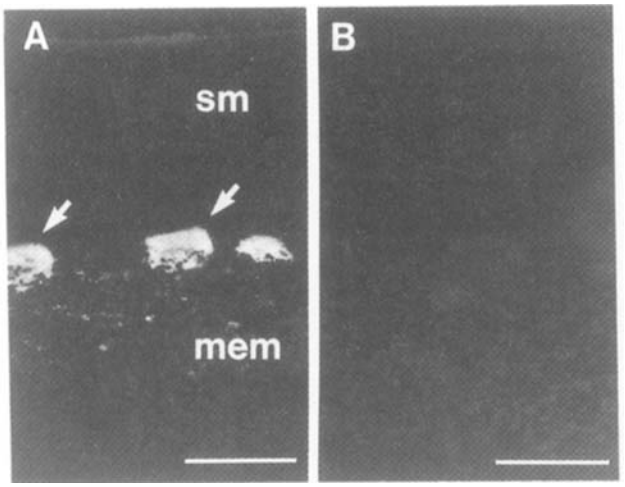

FIGURE 5 Immunohistochemical localization of ovalbumin in decalcified shell. Shell was fixed and decalcified, and embedded in LR white (methods). A. Shell. anti-ovalbumin, 1:2000; B. Shell. anti-ovalbumin, 1:2000, pre-absorbed with purified egg white ovalbumin (Sigma). bar = 50 micron. Abbreviations: sm, shell matrix; mem, shell membranes. The arrows indicate mammillary bodies. Omission of primary antibody resulted in no detectable staining of mammillary knobs.

layer. The mammillary knobs form an array of rounded cones on the inner surface of the mineralized shell, to the tips of which the fibrous shell membranes attach and penetrate.[11,19,20] The mammillary knobs form upon the surface of the outer shell membrane in the distal part of the isthmus, the so-called 'red region', and their organic material is thought to be secreted by the granular epithelial cells found here.[21] The mammillary knobs develop at sites where small granules containing Ca but not carbonate are initially found. The mineral and matrix proteins of the palisade layer are then deposited during the 20 hr period that the egg remains in the shell gland. The calcite crystals of the eggshell develop from discrete nucleation sites on the shell membranes which correspond to the mammillary knobs, giving rise to the hypothesis that these are epitactic centres which initiate calcification.[22,23] If true, this might be the basis for correlations between uniformity, density and shape of mammillary knobs, and shell quality.[19] More recently, mammillary knobs have been sub-divided into two structures,[24] the outer one is termed the 'calcium reserve assembly' and appears to correspond to the zone which we have found is immunopositive for ovalbumin. This region becomes gradually decalcified during the last half of embryonic development as calcium is mobilized to meet the needs of the growing embryo. It remains to be determined whether ovalbumin localization at this site is relevant to the decalcification process.

In general, the role matrix proteins play in mineralized structures is not known with any certainty. Therefore, the hypothesis that such proteins are important in modifying the crystallization pattern of biominerals must be tested by investigating the properties of purified matrix proteins.

In this study we have demonstrated that one of the matrix proteins of the chicken eggshell is ovalbumin. Therefore, in the hen oviduct it appears likely that ovalbumin is present in the uterine fluid when the proteinaceous cores of the mammillary bodies are formed, and it is incorporated into these structures. Although ovalbumin represents more than 50% of the egg white proteins, a specific function has not yet been defined.[25] One proposed role for ovalbumin is that of metal ion transport and storage,[26] and its ability to bind a variety of di- and tri-valent cations, including Ca^{2+} at a specific site would support this concept.[27] Moreover, ovalbumin may affect calcite mineralization, since it accelerates the nucleation of calcium carbonate in an in vitro precipitation assay.[28] Egg white ovalbumin is heterogeneous with respect to phosphorylation status and the composition of its carbohydrate chain, and also occurs as a conformational variant, S-ovalbumin, and these properties may affect its ability to bind calcium. It is possible that the ovalbumin which is localized to the mammillary bodies is homogeneous with respect to one or more of these parameters, and plays some role in the initiation of calcification during shell formation.

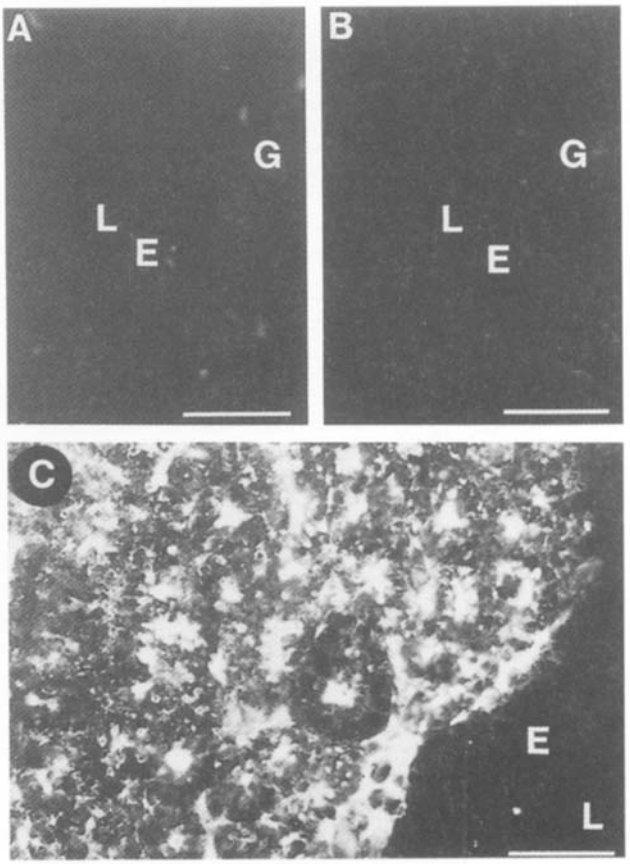

FIGURE 6 Immunohistochemical analysis of ovalbumin localization in hen magnum. A. Magnum. Primary antibody omitted. (scale bar = 100 micron). B. Magnum. anti-ovalbumin, pre-absorbed with purified egg white ovalbumin. (scale bar = 100 micron). C. Magnum. anti-ovalbumin, 1:2000. (scale bar = 50 micron). Abbreviations: G, tubular glands; E, epithelium; L, lumen.

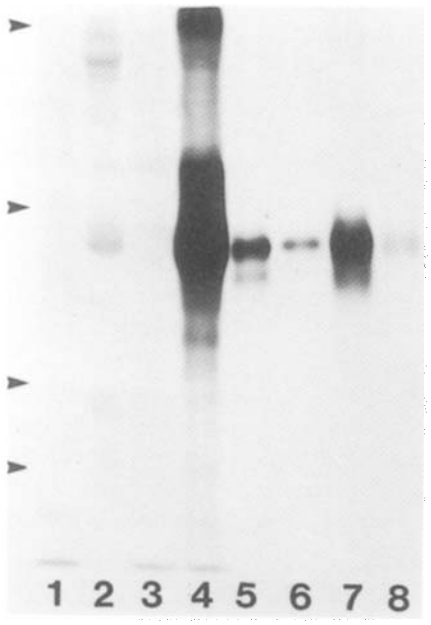

FIGURE 7 Western blot analysis for ovalbumin in hen tissues. Lane 1, hen liver (100 ug); Lane 2, yolk (100 ug); Lane 3, shell gland (100 ug); Lane 4, magnum (100 ug); Lane 5, Egg whites (Sigma, 0.5 ug); Lane 6, Purified ovalbumin (Sigma, 0.1 ug); Lane 7, total soluble eggshell matrix proteins (14 ug); Lane 8, insoluble matrix proteins (7 ug). The positions of *prestained* molecular weight markers (BioRad) are indicated by arrows, and are from bottom to top (kDa): 27.5, 32.5, 49.5, 80. The higher molecular weight bands evident in lanes 2 and 4 are possibly aggregate dimers of ovalbumin.

Our observation that ovalbumin is a component of the mineralized eggshell is reminiscent of a class of proteins that can be extracted from developing bovine molar enamel, termed 'enamelins', to which a critical role in calcification had been ascribed.[29] More recently, however, it has become evident that the enamelins are in fact composed of mainly serum albumin plus other serum proteins (alpha-HS glycoprotein, gamma-globulin, fetuin, and a proline-rich salivary protein), in addition to bone fide enamelin proteins such as tuftelin.[30–33] The non-collagenous proteins of bone also comprise both serum proteins and bone-specific proteins.[34] These examples suggest that the hypothesis that ovalbumin plays a specific role in mineralization of the mammillary bodies of the chicken eggshell must be investigated experimentally.

ACKNOWLEDGEMENTS

This study was supported by the Ontario Egg Producers' Marketing Board. I am grateful to Richard Figueras (paraffin) and Vijay Kapal (plastic) for embedding and sectioning the blocks for immunohistochemistry, Dr. William Staines for providing FITC—labelled secondary antibody and assistance with preparation of the fluorescence micrographs, and Drs. May Griffith, Robert Langille and Roberto Narbaitz for a critical reading of the manuscript.

REFERENCES

1. Mann, S. (1988). Molecular recognition in biomineralization. *Nature* **3**, 119–124.
2. Lowenstam, H. A. and Weiner, S. (1989). "On Biomineralization." New York: Oxford University Press.
3. Addadi, L., Weiner, S. (1992). Control and design principles in biological mineralization. *Angew. Chem. Int. Ed. Engl.* **3**, 153–169.
4. Krampitz, G., Graser, G. (1988). Molecular mechanisms of biomineralization in the formation of calcified shells. *Angew. Chem. Int. Ed. Engl.* **2**, 1145–1156.
5. Weiner, S., Addadi, L. (1991). Acidic macromolecules of mineralized tissues: the controllers of crystal formation. *Tr. Biochem. Sci.* **1**, 252–256.
6. Mann, S., Archibald, D. D., Didymus, J. M., Douglas, T., Heywood, B. R., Meldrum, F. C., Reeves, N. J. (1993). Crystallization at inorganic-organic interfaces: biominerals and biomimetic synthesis. *Science* **2**, 1286–1292.
7. Addadi, L., Moradian, J., Shay, E., Maroudas, N. G., Weiner, S. (1987). A chemical model for the cooperation of sulfates and carboxylates in calcite crystal nucleation: relevance to biomineralization. *Proc. Nat. Acad. Sci. USA* **8**, 2732–2736.
8. Mann, S., Heywood, B. R., Rajam, S., Birchall, J. D. (1988). Controlled crystallization of $CaCO_3$ under stearic acid monolayers. *Nature* **3**, 692–695.
9. Furedi-Milhofer, H., Moradian-Oldak, J., Weiner, S., Veis, A., Mintz, K. P., Addadi, L. (1994). Interactions of matrix proteins from mineralized tissues with octacalcium phosphate. *Connective Tiss. Res.* **3**, 251–264.
10. Fritz, M., Belcher, A. M., Radmacher, M., Walters, D. A., Hansma, P. K., Stucky, G. D., Morse, D. E., Mann, S. (1994). Flat pearls from biofabrication of organized composites on inorganic substrates. *Nature* **3**, 49–51.
11. Burley, R. W. and Vadehra, D. V. (1989). "The Avian Egg, Chemistry and Biology." Toronto: J. Wiley & Sons.
12. Leach, R. M. (1982). Biochemistry of the organic matrix of the eggshell. *Poultry Sci.* **6**, 2040–2047.
13. Hincke, M. T., Bernard, A. -M., Lee, E. R., Tsang, C. P. W., Narbaitz, R. (1992). Soluble protein constituents of the domestic fowl's eggshell. *British Poultr. Sci.* **3**, 505–516.
14. Hincke, M. T., Tsang, C. P. W., Courtney, M., Hill, V., Narbaitz, R. (1995). Purification and Immunochemistry of a soluble matrix protein of the chicken eggshell (Ovocleidin 17). *Calcified Tissue Int.* (in press).
15. Hincke, M. T., Nairn, A. C. (1992). Phosphorylation of elongation factor 2 during Ca^{2+}-mediated secretion from rat parotid acini. *Biochem. J.* **2**, 877–882.
16. Towbin, H., Staehelin, T., Gordon, S. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **7**, 4350–4354.
17. Staines, W. A., Meister, B., Melander, T., Nagy, J. I., Hokfelt, T. (1988). Three-colour immunofluorescence histochemistry allowing triple labelling with a single section. J. Histochem. *Cytochem.* **3**, 145–151.
18. Singhrao, S., Cole, G., Henderson, W. J., Newman, G. R. (1990). LR white embedding allows a multi-method approach to the analysis of brain tissue from patients with Alzheimer's disease. *Histochem.* **2**, 257–268.
19. Parsons, A. H. (1982). Structure of the eggshell. *Poultr. Sci.* **6**, 2013–2021.
20. Hamilton, R. M. G. (1986). The microstructure of the hens eggshell—a short review. *Food Microstruct.* **5**, 99–110.
21. Wyburn, G. M., Johnston, H. S., Draper, M. H., Davidson, M. F. (1973). The ultrastructure of the shell forming region of the oviduct and the development of the shell of Gallus domesticus. Q. *J. Exp. Physiol.* **5**, 143–151.
22. Creger, C. R., Phillips, H., Scott, J. T. (1976). Formation of an eggshell. *Poultr. Sci.* **5**, 1717–1723.
23. Stemberger, B. H., Mueller, W. J., Leach, R. M. (1977). Microscopic study of the initial stages of eggshell calcification. *Poultr. Sci.* **5**, 537–543.

24. Dieckert, J. W., Dieckert, M. C., Creger, C. R. (1989). Calcium reserve assembly: a basic structural unit of the calcium reserve system of the hen egg shell. *Poultry Sci.* **6**, 1569–1584.
25. Stevens, L. (1991). Egg White Proteins. *Comp. Biochem. Physiol.* **1**, 1–9.
26. Taborsky, G. (1974). Phosphoproteins. *Adv. Protein Chem.* **2**, 1–187.
27. Goux, W. J., Venkatasubramanian, P. N. (1986). Metal ion binding properties of hen ovalbumin and S-ovalbumin: characterization of the metal ion binding site by ^{31}P NMR and water proton relaxation rate enhancements. *Biochemistry* **2**, 84–94.
28. Burgess, S. K., Carey, D. M., Oxendine, S. L. (1992). Novel protein inhibits in vitro precipitation of calcium carbonate. *Arch. Biochem. Biophys.* **2**, 383–387.
29. Slavkin, H. C., Snead, M. L., Zeichner-David, M., MacDougall, M., Fincham, A., Lau, E. C., Luo, W., Nakamura, M., Oliver, P., Evans, J. (1988). Factors influencing the expression of dental extracellular-matrix biomineralization. *Ciba Found. Symp.* **1**, 22–41.
30. Strawich, E., Seyer, J., Glimcher, M. J. (1993). Note on Tooth Enamelins. Immunoidentification of two non-amelogenin proteins of developing bovine enamel isolated by affinity chromatography. *Connective Tiss. Res.* **2**, 163–169.
31. Menateau, J., Dajean, S., Laboux, O., Aubry, J. (1990). Proteins of the mineral compartment of bovine fetal enamel share common antigenic determinents with serum proteins. *Calcif. Tissue Int.* **47**, 251–255.
32. Strawich, E., Glimcher, M. J. (1990). Tooth 'enamelins' identified mainly as serum proteins. Major 'enamelin' is albumin. *Eur. J. Biochem.* **191**, 47–56.
33. Deutsch, D., Polman, A., Fisher, L. W., Termine, J. D., Young, M. F. (1991). Sequencing of bovine enamelin ("Tuftelin") a novel acidic enamel protein. *J. Biol. Chem.* **266**, 16021–16028.
34. Termine, J. D. (1985). "The tissue-specific proteins of the bone matrix." In: The Chemistry and Biology of Mineralized Tissues, edited by W. T. Butler) pp, 94–97. Birmingham: Ebsco Media Inc.