

EGGSHELL MATRIX PROTEINS AND NATURAL DEFENSES OF THE EGG

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ABSTRACT

The chicken eggshell proteins have been largely investigated during the last decade. Proteins and associated genes have been identified and characterized. Various experimental evidences suggest a role for eggshell matrix proteins during mineralization. Indeed, novel specific eggshell matrix proteins are only expressed in tissues where eggshell mineralization takes place and some are highly stimulated during shell formation. These proteins also interact *in vitro* with the growing shell mineral (calcium carbonate) and modify crystal morphology and resulting shell microstructure which influence mechanical properties of the shell. In addition, the matrix composition changes during the various phases of eggshell calcification (initiation, growth and completion) and coincides with some alterations in eggshell mechanical properties that are observed at different physiological states of the hen (aging or molt). Antibacterial proteins (lysozyme, ovotransferrine) are also present in the eggshell. Recently, we also have cloned 2 novel eggshell matrix proteins (ovocalyxin-36 and -25) that could be related to anti-bacterial proteins. Because of their role during eggshell formation and their anti-bacterial properties, eggshell proteins are thought to be involved in natural egg defenses by reinforcing the mineral structure and by expressing antibacterial activities which preserve the hygienic quality of eggs during its formation.

KEY WORDS: Antimicrobial, Eggshell, Hen, Uterus, Matrix proteins, Mineralization.

INTRODUCTION

The egg possesses two major natural defense systems. The first one is the eggshell, together with the cuticle and membranes, which constitute a physical barrier against bacterial penetration. The integrity of this structure is crucial for the protection of the contents of the egg from microbial environment and in the control of water and gases through the pores of the shell during the embryonic development. Defects in the mineralized shell are directly related to increasing risk of bacterial penetration in the egg. The second natural defense of the egg is a chemical barrier composed of proteins that exhibit anti-microbial activity founded in the albumen and at a lesser extend in the other compartment of the egg (yolk and shell). The aim of this manuscript is to review the eggshell matrix proteins that are likely to participate in the natural defense by influencing mechanical properties of the shell and secondly by their anti-microbial activities.

EGGSHELL STRUCTURE AND FORMATION

The chicken eggshell is a highly ordered structure that possesses remarkable mechanical properties (Nys *et al.*, 2004). The eggshell is composed of six layers. The innermost two layers are the non-calcified shell membranes made of a network of organic fibers. The inner calcified layer (cone layer) is composed of the basal parts of calcified columns that penetrate the outer eggshell membranes. The palisade layer starts when the columns amalgamate to form a compact shell. The thin vertical crystal layer is deposited on surface of the palisade layer. The cuticle is the most external layer of the egg and is constituted of organic matter and of the eggshell pigments.

The mineral phase of the shell is mainly composed of calcite, the most stable polymorph of calcium carbonate. The organic and inorganic eggshell precursors are secreted by the distal part of the oviduct. The eggshell formation is initiated in the red isthmus (tubular shell gland), where the first calcite crystals nucleate on specific sites (mammillary knobs) composed of organic aggregates. After entering the uterus, calcite crystal growth continues outward to give rise to the mammillary and the palisade layers. The process of shell mineralization takes place in the uterine fluid, a non cellular milieu, which contains the mineral and organic precursors of the shell (Gautron *et al.*, 1997; Nys *et al.*, 2004). The shell is calcified according to a sequence process consisting of three stages: a) the initial stage when the first calcite crystals are deposited around the mammillary knobs to form the cone layer, b) the active growth phase when there is a rapid calcification during the formation of the palisade layer, and c) the terminal phase (the last two hours of the egg formation process) when there is an arrest of shell calcification and the deposition of the most external layer, the cuticle.

EGGSHELL MATRIX PROTEINS

The avian eggshell is made of an organic matrix (3.5%), comprising the eggshell membranes, the cuticle and some constituents embedded in the layer of calcium carbonate. Since 1990, numerous efforts have been carried out to identify and characterize the protein components of the calcified shell. These previously identified matrix proteins can be divided in 3 groups according to their characteristics.

i.) The major egg white proteins have been identified in the eggshell. They are ovalbumin (Hincke 1995), lysozyme (Hincke *et al.*, 2000) and ovotransferrin (Gautron *et al.*, 2001a). These proteins are synthesized and secreted by the uterus (organ of eggshell calcification) and are mainly localized in the basal layer of the shell.

ii.) The second group is made of ubiquitous proteins widely expressed in other tissues. Osteopontin is a phosphorylated glycoprotein present in the bone, kidney. This protein was shown to be expressed in the chicken uterus and localized in the shell (Pines *et al.*, 1994). A widely secretory protein, clusterin, was also identified as an eggshell and egg white protein (Mann *et al.*, 2003). It is thought to function as an extracellular chaperone.

iii.) The third group is constituted of specific eggshell matrix proteins which have not previously been characterized. These proteins are only synthesized by oviduct regions

where eggshell takes place (red isthmus and uterus) and are unique to the eggshell. Ovocleidin-17 was the first eggshell protein purified from the shell (Hincke *et al.*, 1995). Ovocleidin-116 (OC-116), another eggshell-specific protein was cloned (Hincke *et al.*, 1999) and found to be the protein core of a 120-/200- kDa eggshell dermatan sulfate proteoglycan (Carrino *et al.*, 1997), which has also been termed ovoglycan (Fernandez *et al.*, 2001). Ovocalyxin-32 (OCX-32), a 32 kDa uterine specific protein is predominately found in the upper part of the calcified shell (outer palisade layer and cuticle) (Gautron *et al.*, 2001b). Ovocalyxin-36 corresponds to a 36 kDa component of the uterine fluid, eggshell and eggshell membrane. This protein is secreted by the uterine tubular gland cells (Gautron *et al.*, 2006). OCX-36 mRNA expression was 17-fold higher in uterine tissue collected during eggshell formation, compared to the period when no shell was in formation. Finally, two additional proteins (Ovocalyxin-25 and Ovocalyxin-21) have also been cloned and identified as novel eggshell matrix proteins (Gautron, Murayama, Hincke, Nys, in preparation).

The recent publication of 90-95% of the chicken genome makes it possible to explore the egg proteome using mass spectrometry-based high-throughput methods as shown recently for the acid-soluble organic matrix of the chicken calcified eggshell layer (Mann *et al.*, 2006). Using these methods 520 different proteins were identified as constituents of the eggshell matrix, including all matrix proteins known before. The highly abundant group corresponded to 32 proteins including all previously identified specific eggshell proteins, known as ovocleidins and ovocalyxins. It is noteworthy that the range of proteins relative to their previous identification and function was very large. The complex mixture contained egg white proteins, extracellular growth factors and many other signal transduction chain components, lipid-binding proteins, immune system-related and antimicrobial proteins, proteins also occurring in body fluids, such as serum albumin, hemopexin or vitamin D-binding protein, and also some previously uncharacterized proteins of unknown origin and function. This observation suggests that the matrix protein mixture might partly correspond to components which derived from decaying cells and basement membranes lining the oviduct and left over from secretion processes. These proteins may have been included into the mineralized shell just because they were present in the uterine fluid during mineralization.

PUTATIVE FUNCTION OF MATRIX PROTEINS

Eggshell matrix proteins are involved in the fabric of the eggshell and consequently influence its resulting mechanical properties. This hypothesis was confirmed by experimental observations: i.) Presence in eggshell of novel proteins only secreted by tissues where eggshell calcification takes place and that are highly stimulated during the calcification process. ii.) Different profile of eggshell matrix proteins in the uterine fluid at each stage of eggshell calcification process that demonstrated an adaptation of the matrix composition depending of the calcification process (Gautron *et al.*, 1997). iii.) *In vitro* modification of calcite crystal morphology when crystals are grown in presence of matrix components showing an interaction of mineral and organic compounds (Gautron *et al.*, 1996; Dominguez-Vera *et al.*, 2000). A similar effect was observed when isolated matrix

proteins (lysozyme and ovotransferrin) were added into the milieu (Hincke *et al.*, 2000; Gautron *et al.*, 2001a). iv.) Furthermore a relationship between changes in eggshell mechanical properties induced by hen aging or molt and level of matrix components were established *in vivo* (Ahmed *et al.*, 2005). This relation was confirmed by genetic association studies between polymorphism in gens coding for eggshell matrix proteins and measurement of eggshell quality (Dunn *et al.*, 2006).

Some egg white proteins (ovotransferrin and lysozyme), well known for their antimicrobial properties, have been identified in egg shell (Gautron *et al.*, 2001; Hincke *et al.*, 2000). We have cloned Ovocalyxin-36, a novel eggshell specific matrix protein (Gautron *et al.*, 2006) that showed significant identities with lipopolysaccharide binding proteins (Schumann *et al.*, 1990), bactericidal permeability increasing proteins (Gray *et al.*, 1989) and the PLUNC family of proteins (Bingle and Craven, 2002). These proteins are often described as "first-line host defence proteins" and could be involved in the innate immune response.

CONCLUSION

These data demonstrates that the chicken eggshell is a sophisticated structure which contains a number of proteins that have been identified and characterized during the past decade. Our experimental data shows that these proteins regulate shell mineralization and may influence its mechanical properties. These matrix proteins could also act as anti-bacterial agents to protect the egg contents against microbial challenge.

REFERENCE

- Ahmed, A. M., A. B. Rodriguez-Navarro, M. L. Vidal, J. Gautron, J. M. Garcia-Ruiz, and Y. Nys. 2005. Changes in eggshell mechanical properties, crystallographic texture and in matrix proteins induced by moult in hens. *Br. Poult. Sci.* 46:268-79.
- Bingle, C. D., and C. J. Craven. 2002. PLUNC: a novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. *Hum. Mol. Genet.* 11:937-943.
- Carrino, D. A., J. P. Rodriguez, and A.I. Caplan. 1997. Dermatan sulfate proteoglycans from the mineralized matrix of the avian eggshell. *Connect. Tissue Res.* 36:175-193.
- Dominguez-Vera, J. M., J. Gautron, J. M. Garcia-Ruiz, and Y. Nys. 2000. The effect of avian uterine fluid on the growth behavior of calcite crystals. *Poult. Sci.* 79:901-907.
- Dunn, I. C., N. T. Joseph, P. Milona, M. Bain, A. Edmond, P. W. Wilson, Y. Nys, J. Gautron, M. Schmutz, R. Preisinger, and D. Waddington. 2006. Polymorphisms in eggshell organic matrix genes are associated with eggshell quality measurements in pedigree Rhode Island Red hens. Submitted
- Fernandez, M. S., A. Moya, L. Lopez, and J. L. Arias. 2001. Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. *Matrix Biol.* 19:793-803.

- Gautron, J., M. Bain, S. Solomon, and Y. Nys. 1996. Soluble matrix of hen's eggshell extracts changes in vitro the rate of calcium carbonate precipitation and crystal morphology. *Br. Poult. Sci* 37:583-866.
- Gautron, J., M. T. Hincke, and Y. Nys. 1997. Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens. *Connect. Tissue Res.* 36:195-210.
- Gautron, J., M. T. Hincke, M. Panhéleux, J. M. Garcia-Ruiz, T. Boldicke, and Y. Nys. 2001a. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. *Connect. Tissue Res.* 42:255-267.
- Gautron, J., M. T. Hincke, K. Mann, M. Panheleux, M. Bain, M. D. McKee, S. E. Solomon, and Y. Nys. 2001b. Ovocalyxin-32, a novel chicken eggshell matrix protein. isolation, amino acid sequencing, cloning, and immunocytochemical localization. *J. Biol. Chem.* 276:39243-39252.
- Gautron, J., E. Murayama, A. Vignal, M. Morisson, M. D. McKee, S. Réhault, M. L. Vidal, Y. Nys Y and M. T. Hincke. 2006. Cloning of Ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins (LBP) bactericidal permeability-increasing proteins (BPI), and Plunc family proteins. Submitted.
- Gray, P. W., G. Flaggs, S. R. Leong, R. J. Gumina, J. Weiss, C. E. Ooi, and P. Elsbach. 1989. Cloning of the cDNA of a human neutrophil bactericidal protein. Structural and functional correlations. *J. Biol. Chem.* 264:9505-9509.
- Hincke, M. T. 1995. Ovalbumin is a component of the chicken eggshell matrix. *Connect. Tissue Res.* 31:227-233.
- Hincke, M. T., C. P. Tsang, M. Courtney, V. Hill, and R. Narbaitz. 1995. Purification and immunochemistry of a soluble matrix protein of the chicken eggshell (ovocleidin 17). *Calcif. Tissue Int.* 56:578-583.
- Hincke, M. T., J. Gautron, C. P. Tsang, M. D. McKee, and Y. Nys. 1999. Molecular cloning and ultrastructural localization of the core protein of an eggshell matrix proteoglycan, ovocleidin-116. *J. Biol. Chem.* 274:32915-23.
- Hincke, M. T., J. Gautron, M. Panhéleux, J. M. Garcia-Ruiz, M. D. McKee, and Y. Nys. 2000. Identification and localization of lysozyme as a component of the eggshell membranes and shell matrix. *Matrix Biol.* 19:443-453.
- Mann, K., J. Gautron, Y. Nys, M. D. McKee, T. Bajari, W. J. Schneider, and M. T. Hincke. 2003. Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white. *Matrix Biol.* 22:397-407.
- Mann, K., B. Macek, and J. V. Olsen. 2006. Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. *Proteomics.* 6:3801-3810.
- Nys, Y., J. Gautron, J. M. Garcia-Ruiz, and M. T. Hincke. 2004. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *C R Palevol.* 3:549-562.

- Pines, M., V. Knopov, A. Bar. 1994. Involvement of osteopontin in egg shell formation in the laying chicken. *Matrix Biol.* 14:765-771.
- Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science.* 249:1429-1431.