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Antimicrobial properties of avian eggshell-specific C-type lectin-like proteins

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Abstract **C-type lectin-like proteins are major components of the calcified eggshell of multiple avian species. In this study, two representative avian C-type lectin-like proteins, ovocleidin-17 and ansocalcin, were purified from decalcified chicken and goose eggshell protein extracts and investigated for carbohydrate binding activity as well as antimicrobial activity. Purified ovocleidin-17 and ansocalcin were found to bind bacterial polysaccharides, and were bactericidal against *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomona aeruginosa*. Bactericidal activity was found to be enhanced in the presence of calcium but was not dependent on its presence. The results suggest that avian C-type lectin-like proteins may play an important antimicrobial role in defence of the avian embryo.**

Keywords: Antibiotics; Antibacterial; Avian eggshell; Bacterial polysaccharides; C-type lectin

1. Introduction

Ovocleidin-17 (OC-17) and ansocalcin are the major eggshell-specific matrix proteins from chicken and goose, respectively [1–5]. They share significant identity with C-type lectin-like (CTL) proteins, but lack the consensus QPD motif that is required for binding to simple sugars. Similar CTL proteins are present in eggshell of various domesticated birds [6]. Interestingly, two families of CTL proteins have been identified within eggshells of ratite species; the amino acid sequence of one form aligns better with ansocalcin (group I) while the other is more similar to OC-17 (group II) [7–9]. The presence of CTL proteins in the eggshells of multiple avian species suggests a common biologically important role.

The C-type lectin superfamily is a large group of extracellular proteins with diverse functions in multicellular organisms [10]. Recently, RegIIIγ, a mouse epithelial C-type lectin (Group VII CTL) and its human counterpart, HIP/PAP, were shown to inhibit the growth of Gram-positive bacteria [11]. Ansocalcin (33%) and OC-17 (28%) possess significant identity to the *Reg* family of C-type lectins. Given their widespread presence and abundance within the eggshell matrices of various species, we sought to investigate the antimicrobial properties of avian eggshell CTL proteins. Our strategy was to study the binding of ansocalcin and OC-17 to bacterial cell wall polysaccharides, and investigate their antimicrobial properties against Gram-positive and Gram-negative bacteria. The results show that avian eggshell CTL proteins have excellent antimicrobial as well as bacterial polysaccharide binding properties. This is the first report on the antimicrobial properties of eggshell-specific CTL proteins and highlights the multiple roles played by these proteins.

2. Materials and methods

2.1. Protein extraction and purification

Eggshells were ground into a fine powder and decalcified using 20% acetic acid (10 ml of acetic acid/gram eggshell) according to the method of Reyes-Grajeda et al. [5]. Purification of ansocalcin and OC-17 was performed as previously described by Lakshminarayanan et al. [3,4]. The concentration of proteins used for SDS–PAGE, carbohydrate binding and antimicrobial assays was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) using BSA (Bioshop, Burlington, ON) as a standard.

2.2. Carbohydrate binding activity

The ability of ansocalcin and OC-17 to bind to various complex carbohydrates was analyzed by a pull-down assay adapted from Cash et al. [11]. Dry protein samples were suspended in 0.01% acetic acid and further diluted with 0.01% acetic acid and 0.1% bovine serum albumen (BSA). Samples were incubated in the presence or absence of *Micrococcus lysodeikticus* ATCC 4698 cell walls (Sigma–Aldrich, Oakville, ON), *Bacillus subtilis* peptidoglycan (Sigma–Aldrich), crab shell chitin (Sigma–Aldrich), corn starch (Best Foods Canada, Etobicoke, ON) or *Escherichia coli* 0127:B8 lipopolysaccharide (Sigma–Aldrich) suspended in 0.01% acetic acid at 4 °C with gentle shaking for 24 h. After incubation, samples were centrifuged for 5 min (13 000 rpm) at 4 °C, and the supernatant carefully removed. Supernatant and pellet were analyzed by SDS–PAGE.

2.3. Antimicrobial activity of purified ansocalcin and OC-17

Antimicrobial activity was evaluated by the micro-broth dilution assay adapted from Steinberg and Lehrer [12]. Vehicle was BSA (0.1%) in acetic acid (0.01%), and positive control was 100 μg/ml bovine lactoferricin B (Sigma–Aldrich). *M. lysodeikticus* ATCC 4698 cell walls or *E. coli* 0127:B8 lipopolysaccharide (0.5–5 mg/ml) in the presence and absence of ansocalcin or OC-17 was also investigated for potential inhibition of antimicrobial activity. Antimicrobial activity was evaluated against two Gram-positive (*B. subtilis* ATCC 19659 and *Staphylococcus aureus* ATCC 6538) and two Gram-negative (*Pseudomona aeruginosa* ATCC 15442 and *E. coli* D31) bacteria. Each antimicrobial assay was conducted in triplicate for two independent trials ($N = 2$).

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Antimicrobial activity, in the presence and absence of 1 mM $CaCl_2$ (bacteria suspended in 25 mM HEPES buffer pH 7.3), was also evaluated in order to investigate the effect of calcium on the activity of the avian CTL proteins. Data were analysed using SYSTAT Version 8.0 (SPSS, Chicago, IL). *T*-tests were conducted to identify any significant differences between bacterial populations in the presence and absence of protein samples. For morphological experiments, 200 μg/ml of ansocalcin or OC-17 was incubated in the presence of *B. subtilis* bacterial suspension for 1 h as described for the antimicrobial assay. Following the incubation period, samples were individually centrifuged (3000 × *g*, 4 °C, 10 min), washed and resuspended in an equal volume of 10 mM sodium phosphate buffer (pH 7.3). Bacterial pellets (20 μl) were heat fixed to microscope slides and Gram-stained for examination under oil-immersion light microscopy.

3. Results and discussion

SDS–PAGE analysis of the crude domestic chicken and domestic goose eggshell protein extracts revealed that OC-17 (17 kDa) and ansocalcin (15 kDa), respectively, are major proteins of the shell matrix as we have previously reported and were greatly purified by RP-HPLC [2,3] (Fig. 1).

We investigated the ability of highly purified ansocalcin and OC-17 to bind to bacterial polysaccharides. The binding of OC-17 and ansocalcin to peptidoglycan (PGN, a polymer that forms the thick layer of Gram-positive bacteria and a thin layer in Gram-negative bacteria), lipopolysaccharide (LPS, the outer cell membrane of Gram-negative bacteria), chitin, and *M. lysodeikticus* cell wall was examined. Since complex polysaccharides are insoluble in aqueous solution, a pull-down assay was used to evaluate the polysaccharide binding ability of the avian CTL proteins [11]. Fig. 2 shows the results obtained from the pull-down assay. OC-17 and ansocalcin remained in the supernatant solution of crab shell chitin. Similar results were obtained with corn starch (not shown) indicating lack of binding to these polysaccharides. However, both OC-17 and ansocalcin were greatly retained in the pellet when incubated in the presence of Micrococcus cell wall, lipopolysaccharide or peptidoglycan, indicating their strong bind-

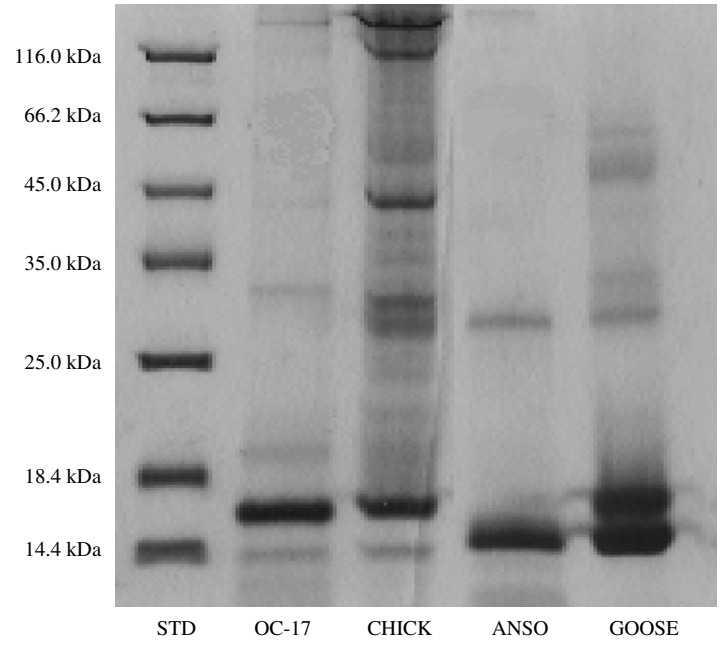

Fig. 1. SDS–PAGE analysis of crude and RP-HPLC purified eggshell protein extracts. Samples (2.5 μg of purified and 20 μg of crude extract) were separated on a 12% SDS–polyacrylamide gel and visualized by Coomassie blue staining. Molecular weight of standards (STD) is indicated on the left. Samples are labelled at the bottom (OC-17: purified ovocleidin-17; CHICK: crude chicken eggshell extract; ANSO: purified ansocalcin; and GOOSE: crude goose eggshell extract).

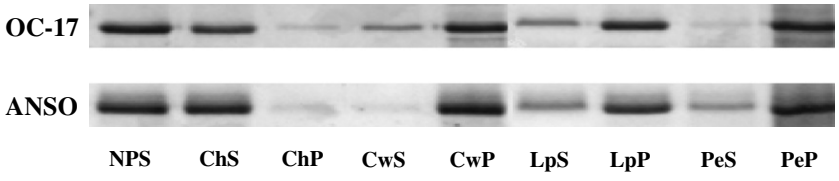

Fig. 2. Eggshell CTL proteins pull-down assay. For clarity, SDS–PAGE of the ansocalcin and OC-17 bands alone are shown. Abbreviations: NPS – no polysaccharide supernatant; ChS – crab chitin supernatant; ChP – crab chitin pellet; CwS – *Micrococcus* cell wall supernatant; CwP – *Micrococcus* cell wall pellet; LpS – *E. coli* lipopolysaccharide supernatant; LpP – *E. coli* lipopolysaccharide pellet; PeS – *B. subtilis* peptidogylcan supernatant; and PeP – *B. subtilis* peptidoglycan pellet.

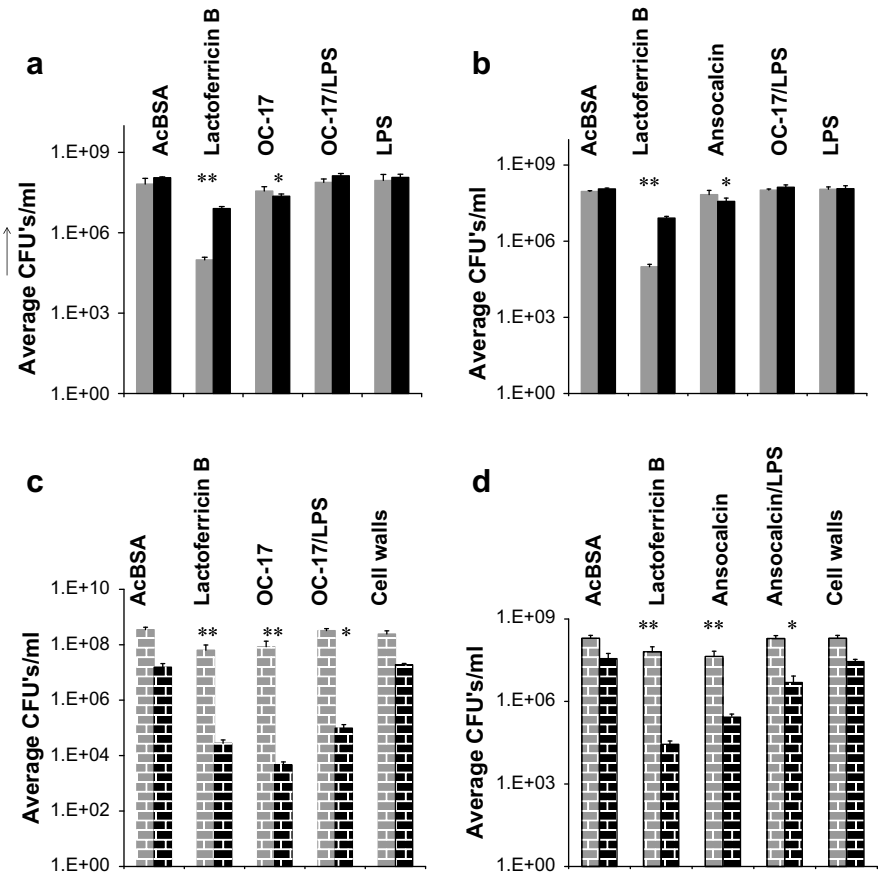

Fig. 3. Antimicrobial properties of eggshell CTL proteins. OC-17 and ansocalcin were tested against selected Gram-negative (a and b) and Gram-positive (c and d) bacteria. The grey and black color in panels a and b represent *E. coli* and *P. aeruginosa*, respectively, whereas the checked grey and black shading in panels c and d represent *S. aureus* and *B. subtilis*, respectively. Bovine serum albumen (0.1%) in 0.01% acetic acid (AcBSA) was used as a negative control and bovine lactoferricin B (100 μg/ml) served as a positive control. The average colony counts were obtained after plating multiple dilutions on LB agar. Each experiment was conducted in duplicate. Significant reductions in bacterial populations ($P < 0.05$) are indicated by an asterisk (*).

Table 1
Effect of lipopolysaccharide and *Micrococcus* cell walls on the bactericidal activity of ansocalcin and ovocleidin-17

Bacteria	Protein sample	(%) Reduction ± S.D.	*P*-value
E. coli D31	Lactoferricin B (100 μg/ml)	99.9 ± 0.03	0.035
	Ovocleidin 17 (200 μg/ml)	*	*
	Ovocleidin (200 μg/ml), lipopolysaccharide (500 μg/ml)	*	*
	Ansocalcin (200 μg/ml)	*	*
	Ansocalcin (200 μg/ml), lipopolysaccharide (500 μg/ml)	*	*
P. aeruginosa	Lactoferricin B (100 μg/ml)	92.9 ± 0.02	0.027
	Ovocleidin 17 (200 μg/ml)	75.6 ± 3.8	0.05
	Ovocleidin (200 μg/ml), lipopolysaccharide (500 μg/ml)	*	*
	Ansocalcin (200 μg/ml)	67.8 ± 5.8	0.05
	Ansocalcin (200 μg/ml), lipopolysaccharide (500 μg/ml)	*	*
S. aureus	Lactoferricin B (100 μg/ml)	73.5 ± 9.7	0.024
	Ovocleidin 17 (200 μg/ml)	79.8 ± 3.4	0.031
	Ovocleidin (200 μg/ml), cell walls (2.5 mg/ml)	*	*
	Ansocalcin (200 μg/ml)	78.0 ± 2.4	0.048
	Ansocalcin (200 μg/ml), cell walls (2.5 mg/ml)	*	*
B. subtilis	Lactoferricin B (100 μg/ml)	99.9 ± 0.03	0.004
	Ovocleidin 17 (200 μg/ml)	99.9 ± 0.01	0.007
	Ovocleidin (200 μg/ml), cell walls (2.5 mg/ml)	99.4 ± 0.05	0.007
	Ansocalcin (200 μg/ml)	99.2 ± 0.14	0.004
	Ansocalcin (200 μg/ml), cell walls (2.5 mg/ml)	86.4 ± 0.85	0.004

Activity is expressed as the percent reduction of bacterial population (±S.D.) for two independent trials. The absence of significant reductions in the bacterial populations ($P > 0.05$) is indicated by an asterisk (*).

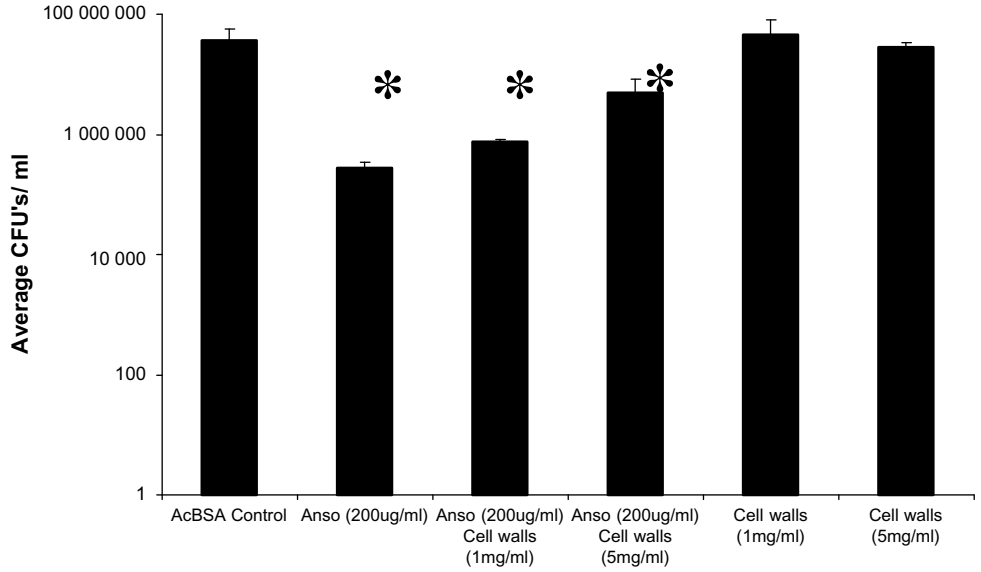

Fig. 4. Inhibition of ansocalcin bactericidal activity in the presence of *Micrococcus* cell walls. *Bacillus subtilis* suspended in 10 mM sodium phosphate buffer (pH 7.3) was incubated 1 h in the presence or absence of the avian C-type lectin-like protein and *Micrococcus* cell walls. Bovine serum albumen (0.1%) in 0.01% acetic acid (AcBSA) was used as a negative control. Bovine lactoferricin B (100 μg/ml) was used as a positive control. The average colony counts were obtained after plating on LB agar. Each experiment was conducted in duplicate. Significant reductions in bacterial populations ($P < 0.05$) are indicated by an asterisk (∗).

ing to the bacterial polysaccharides. This effect was more pronounced for PGN pellet than for LPS. OC-17 is almost completely removed from the supernatant of PGN, indicating that it binds more strongly to this bacterial polysaccharide than ansocalcin.

We evaluated these CTL proteins for their antimicrobial activity against various bacteria. When incubated with Gram-negative bacteria, both OC-17 and ansocalcin exhibited a weak antimicrobial activity against *P. aeruginosa* (Fig. 3 and Table 1). OC-17 exhibits greater bactericidal activity than ansocalcin against *P. aeruginosa* (Fig. 3a, b). The antibacterial activity of both CTL proteins was diminished in the presence of added lipopolysaccharide (Fig. 3a and b and Table 1). However, lipopolysaccharide alone did not demonstrate any antibacterial activity against either of the Gram-negative bacteria. No statistically significant reductions in *E. coli* bacterial populations were detected at a 200 μg/ml concentration of the avian CTL proteins. On the other hand, in accordance with the pull-down assay, OC-17 and ansocalcin exhibited strong antimicrobial activity against the Gram-positive *S. aureus* and *B. subtilis* (Fig. 3c, d and Table 1). For both avian CTL proteins, the antimicrobial activity was higher against *B. subtilis* than *S. aureus*, and the activity against both Gram-positive bacteria was reduced in the presence of *Micrococcus* cell walls (Figs. 3c, d and 4; Table 1). *Micrococcus* cell wall alone had no antibacterial activity against either of the Gram-positive bacteria (Figs. 3c, d and 4).

The concentration of CTL proteins was varied from 12 to 200 μg/ml to examine the dose-dependency of the antimicrobial activity (Fig. 5). Bacteria incubated with OC-17 exhibited a greater decrease in population compared to ansocalcin, further confirming the more potent antimicrobial action of OC-17. Morphological examination following incubation with avian C-type lectins revealed that few intact bacilli remain following incubation with ansocalcin (200 μg/ml) (Fig. 6). Moreover, in the presence of OC-17, the vast majority of *B. subtilis*

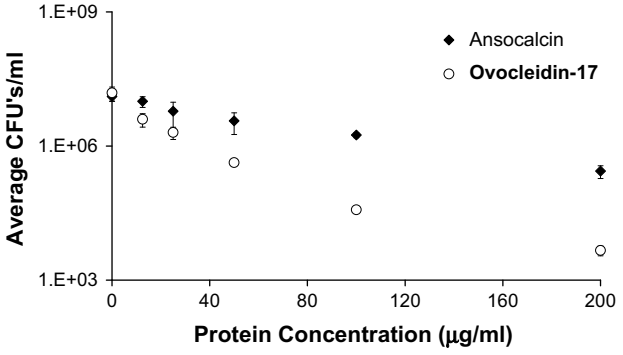

Fig. 5. Dose–response of antimicrobial activity of eggshell CTL proteins. The ability of different concentrations of OC-17 and ansocalcin to reduce bacterial populations of *B. subtilis* was evaluated.

cells were lysed as a result of cell wall damage and only unstained bacterial debris was detected reflecting loss of Gram-staining cellular contents (Fig. 6). Since many CTL proteins bind polysaccharides in the presence of Ca^{2+}, the effect of calcium on the antimicrobial activity of ansocalcin and OC-17 was investigated. For both ansocalcin and OC-17, bactericidal activity against *B. subtilis* was enhanced in the presence of 1 mM $CaCl_2$ (Fig. 7). The antimicrobial activity of ansocalcin showed ~3-fold increase in the presence of Ca^{2+} whereas OC-17 showed ~10-fold increase. In the absence of avian CTL's, $CaCl_2$ did not affect the viability of *B. subtilis* bacterial populations.

Previous studies have investigated the effect of pH and/calcium ion upon the conformation and dimerization of OC-17 and ansocalcin. Circular dichroism and dynamic light scattering measurements reveal that OC-17 conformation is not affected by calcium ion, and that the protein would be essentially monomeric at the concentrations used in our antimicrobial assay [4]. Ansocalcin, however, dimerizes above

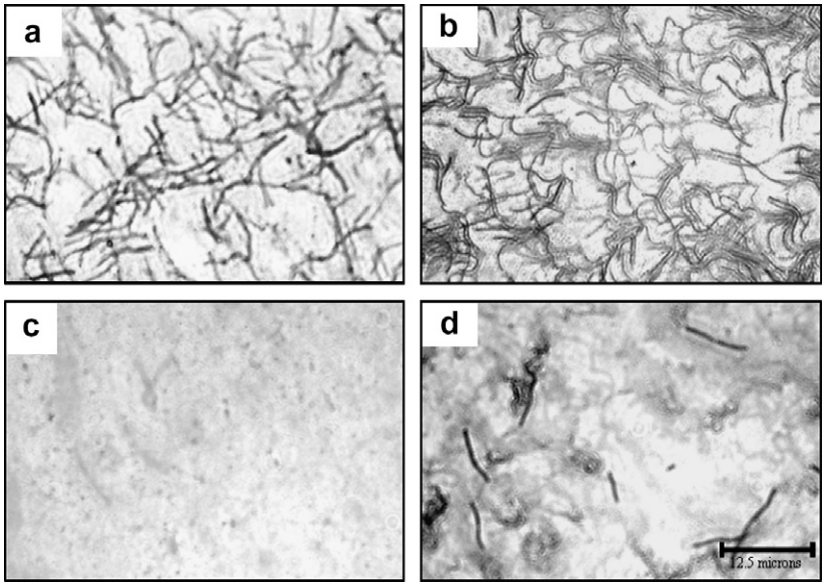

Fig. 6. Bacterial morphology of *B. subtilis* in the presence of eggshell CTL proteins. Bacteria were incubated 1 h in the presence of vehicle alone (a), 100 μg/ml lactoferricin B (b), 200 μg/ml OC-17 (c) or 200 μg/ml ansocalcin (d). Bacterial smears were Gram-stained and photographed under light microscopy. These images are representative of multiple fields of view for triplicate slides for each condition. Scale bar 12.5 μm.

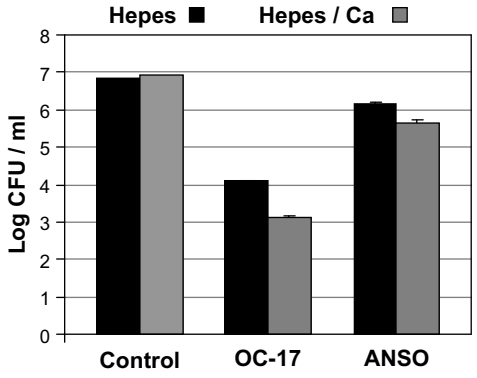

Fig. 7. Effect of calcium on the antimicrobial properties of OC-17 and ansocalcin. The presence of 1 mM $CaCl_2$ did not affect the bacterial populations in the absence of avian CTL proteins. Average colony counts were obtained after plating on LB agar in triplicate. Each experiment was conducted in duplicate.

100 μg/ml and this property is independent of the calcium and pH; below this concentration it is monomeric [3,4]. It is possible that the enhanced antimicrobial activity of OC-17 is due to its monomeric state at the assay concentration, compared to dimeric ansocalcin (Fig. 5).

Structure–function studies of broad-spectrum antimicrobial peptides reveal that key requirements are cationic charge and an ability to fold into amphiphilic or amphipathic conformations [13]. The crystal structure of OC-17 and the molecular model of ansocalcin (modeled from the crystal structure of OC-17) indicate that the former has widespread distribution of positive charges compared to the latter (Fig. 8) [5]. On one side of the OC-17 molecule the surface of the protein displays an extended solvent exposed basic stretch consisting of 17 of the 21 arginine/lysine residues. This feature is reminiscent of the multiple arginine and lysine residues that cationic antimicrobial peptides exhibit [13], and probably contributes to the increased activity of OC-17 compared to ansocalcin.

In addition to the mineral phase, which provides a primary physical barrier against microorganisms, the avian egg is equipped with a number of chemical defenses, such as lysozyme, avidin, ovotransferrin, ovomucoid, and α-*N*-acetylglucosaminidase that exhibit varying degree of antimicrobial activities [14]. These components are abundant in the egg albumen and scarce in the eggshell. During embryonic development, selective decalcification of the inner eggshell occurs due to protons secreted by the cells of the chorioallantoic membrane. This process would concomitantly release occluded

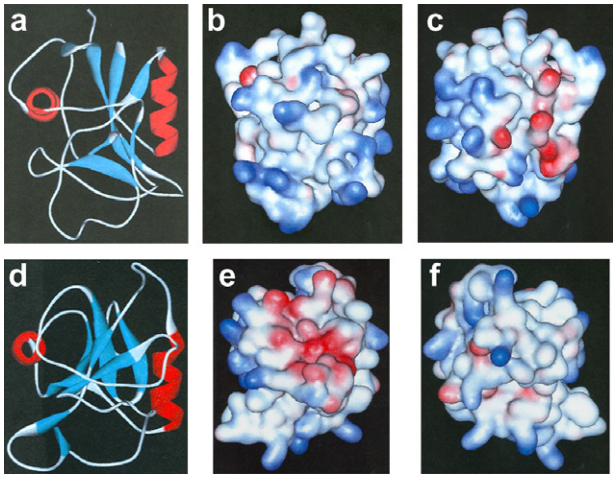

Fig. 8. Three-dimensional structures of avian eggshell CTL proteins. (a) Crystal structure of OC-17. (b) Surface structure of OC-17 in the same orientation as in (a). (c) Surface structure of OC-17 that is rotated 180° clockwise around the *Y*-axis of (a). (d) Molecular model of ansocalcin. (e) Surface structure of ansocalcin model in the same orientation as in (d). (f) Surface structure of ansocalcin that is rotated 180° clockwise around the *Y*-axis of (d). In figures (a) and (d) protein secondary structures are indicated in blue (β-strand) and red (α-helix). The blue and red colors in the surface plot indicate the distribution of positive and negative charges, respectively.

avian CTL proteins from the inner shell into a calcium-rich environment and thereby up-regulate antimicrobial defenses surrounding the embryo as the eggshell becomes progressively weaker in preparation for hatching. We mimicked this situation by conducting the pull-down assay in an acidic milieu, where an interaction with bacterial cell wall polysaccharides was observed.

In conclusion, this study reports that OC-17 and ansocalcin demonstrate strong binding preference for bacterial polysaccharides and especially peptidoglycan. Both OC-17 and ansocalcin are potently bactericidal against Gram-positive bacteria such as *B. subtilis* and *S. aureus*, and exhibited enhanced activity in the presence of calcium. CTL proteins are major eggshell matrix components in multiple avian species and have been proposed to play a role in eggshell calcification [1,2,4,5]. Avian CTL proteins may therefore be involved in both eggshell calcification and antimicrobial defence. It will be interesting to compare the antimicrobial properties of eggshell CTL proteins from diverse avian species that are subject to a variety of microbial environments. Our results further indicate that proteins of potential pharmaceutical interest can be obtained from the avian eggshell; an inexpensive and readily available source of bioactive molecules.

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References

[1] Mann, K. and Siedler, F. (1999) The amino acid sequence of ovocleidin 17, a major protein of the avian eggshell calcified layer. Biochem. Mol. Biol. Int. 47, 997–1007.

[2] Hincke, M.T., Tsang, C.P.W., Courtney, M., Hill, V. and Narbaitz, R. (1995) Purification and immunochemistry of a soluble matrix protein of the chicken eggshell (ovocleidin 17). Calcif. Tissue Int. 56, 578–583.

[3] Lakshminarayanan, R., Valiyaveettil, S., Rao, V.S. and Kini, R.M. (2003) Purification, characterization, and in vitro mineralization studies of a novel goose eggshell matrix protein, ansocalcin. J. Biol. Chem. 278, 2928–2936.

[4] Lakshminarayanan, R., Joseph, J.S., Kini, R.M. and Valiyaveettil, S. (2005) Structure–function relationship of avian eggshell matrix proteins: a comparative study of two major eggshell matrix proteins, ansocalcin and OC-17. Biomacromolecules 6, 741–751.

[5] Reyes-Grajeda, J.P., Moreno, A. and Moreno, A.A. (2004) Crystal structure of ovocleidin-17, a major protein of the calcified Gallus gallus eggshell: implications in the calcite mineral growth pattern. J. Biol. Chem. 279, 40876–40881.

[6] Panheleux, M., Bain, M., Fernandez, M.S., Morales, I., Gautron, J., Arias, J.L., Solomon, S.E., Hincke, M.T. and Nys, Y. (1999) Organic matrix composition and ultrastructure of eggshell: a comparative study. Brit. Poult. Sci. 40, 240–252.

[7] Mann, K. and Siedler, F. (2004) Ostrich (*Struthio camelus*) eggshell matrix contains two different C-type lectin-like proteins. Isolation, amino acid sequence, and posttranslational modifications. Biochim. Biophys. Acta 1696, 41–50.

[8] Mann, K. (2004) Identification of the major proteins of the organic matrix of emu (*Dromaius novaehollandiae*) and rhea (*Rhea americana*) eggshell calcified layer. Brit. Poult. Sci. 45, 483–490.

[9] Mann, K. and Siedler, F. (2006) Amino acid sequences and phosphorylation sites of emu and rhea eggshell C-type lectin-like proteins. Comp. Biochem. Physiol. B 143, 160–170.

[10] Zelinsky, A.N. and Gready, J.E. (2005) The C-type lectin-like domain superfamily. FEBS J. 272, 6179–6217.

[11] Cash, H.L., Whitham, C.V., Behrendt, C.L. and Hooper, L.V. (2006) Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 313, 1126–1130.

[12] Steinberg, D.A. and Lehrer, R.I. (1997) Designer assays for antimicrobial peptides. Disputing the ''one-size-fits-all'' theory. Meth. Mol. Biol. 78, 169–186.

[13] Powers, J.P. and Hancock, R.E. (2003) The relationship between peptide structure and antibacterial activity. Peptides 24, 1681–1691.

[14] Wellman-Labadie, O., Picman, J. and Hincke, M.T. (2007) Avian antimicrobial proteins: structure, distribution and activity. World's Poultry Sci. J. 63, 421–438.