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Purification, crystallization and preliminary X-ray analysis of struthiocalcin 1 from ostrich (*Struthio camelus*) eggshell

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The purification, crystallization and preliminary X-ray analysis of struthiocalcin 1 (SCA-1), a protein obtained from the intramineral part of ostrich (*Struthio camelus*) eggshell, is reported.

1. Introduction

The eggshell is the only rigid part of an avian egg and consists of calcium carbonate and biological macromolecules (proteins, nucleic acids or polysaccharides). It is a mineral structure with a highly specialized function (Nys *et al.*, 2004). The role of individual matrix proteins in avian eggshell calcification is poorly understood and is an important subject for investigation (Ahmed *et al.*, 2005). Lakshminarayanan *et al.* (2005) performed a comparative study of the structure–function relationships of the avian eggshell matrix proteins ansocalcin and ovocleidin-17. These two proteins belong to the C-type lectin family and share a high degree of similarity, although they interact differently with growing calcium carbonate crystals, suggesting that their roles in eggshell calcification differ. Recently, an increasing number of studies of C-type lectin-like proteins contained in the avian eggshell matrix have been reported (Drickamer, 1999). Mann and Siedler have determined the amino-acid sequences of ovocleidin-17 (OC-17), a major protein in *Gallus gallus* eggshell (Mann & Siedler, 1999), and of struthiocalcin 1 and 2 (SCA-1 and SCA-2), which are present in the ostrich eggshell matrix (Mann & Siedler, 2004). Recently, the amino-acid sequences of two proteins from emu (*Dromaius novaehollandiae*; dromaiocalcin 1 and 2; DCA-1 and DCA-2) and two proteins from rhea (*Rhea americana*; rheacalcin 1 and 2; RCA-1 and RCA-2) have been determined (Mann & Siedler, 2006). It was suggested that the structure of the ostrich eggshell is very similar to those of other avian eggshells. SCA-1 contained 132 residues, the same number as in the goose eggshell orthologue ansocalcin. Its mass determined by electrospray ionization (ESI) mass spectrometry was 15 343.2 $\pm$ 4. SCA-2 contained 142 residues; its mass determined by ESI mass spectrometry was 16 834.1 $\pm$ 2.

2. Methods and results

2.1. Purification

Purification was performed following the method of Mann & Siedler (2004). All purification steps were performed at 277 K. One unfertilized egg from *Struthio camelus* was used for purification. The membrane and cuticle were removed after being treated with 5%(*w*/*v*) EDTA solution for 30 min. Clean pieces of eggshell were subsequently ground up. The proteins were extracted from the mineral powder using 10%(*v*/*v*) acetic acid (20 ml per gram). The solution was stirred for 36 h. The solution was then filtered and concentrated using an ultrafiltration cell (Amicon cellulose filter YM3; 3 kDa molecular-weight cutoff). The supernatant was dialyzed five times against ten volumes of 5%(*v*/*v*) acetic acid. In order to precipitate all proteins, ammonium sulfate was added to saturate the dialyzed solution. After 24 h, the solution was centrifuged at 64 500*g*

for 30 min. The supernatant was discarded and the pellet was resuspended and dialyzed against 5%(*v*/*v*) acetic acid in order to remove the ammonium sulfate. The resulting solution was centrifuged at 29 500*g* for 5 min and the supernatant solution was filtered using 0.2 µm PVDF filters. The solution was then injected onto a Jupiter C18 HPLC reverse-phase column (250 × 10 mm) and eluted using a gradient of acetonitrile (0–100%) with 0.1%(*v*/*v*) TFA for 58 min at a flow rate of 1.0 ml min^{-1}. During the purification step, two proteins were observed: SCA-1 (retention time 56.11 min) at a higher concentration and SCA-2 (retention time 54.26 min) at a lower concentration. Finally, it is important to emphasize the extreme stability of these proteins that allowed them to survive the harsh conditions described above.

2.2. Crystallization

Crystallization conditions were screened using the microbatch method using microbatch plates and Hampton Research Crystal Screen I at 277 K. To prepare the drops, 2 µl reservoir solution was mixed with 2 µl freshly purified protein solution at a concentration of 5 mg ml^{-1} in water and the drops were covered with mineral oil. After a week, small crystals of struthiocalcin 1 were observed with reservoir conditions consisting of 100 m*M* sodium citrate buffer pH 5.6, 30%(*w*/*v*) MPD and 200 m*M* ammonium acetate. However, as these microcrystals were not suitable for X-ray analysis, they were used as seeds. Using the microseeding technique, the crystals were transferred using a cat whisker into droplets that had been pre-equilibrated for 3 d (using the sitting-drop vapour-diffusion method) containing 2 µl protein solution (3 mg ml^{-1} in water) plus 2 µl precipitating agent; the reservoir solution contained 1000 µl precipitating agent. The drops were then equilibrated at 277 K. Single crystals suitable for X-ray diffraction measurement were finally obtained within 3–4 d of seeding (Fig. 1).

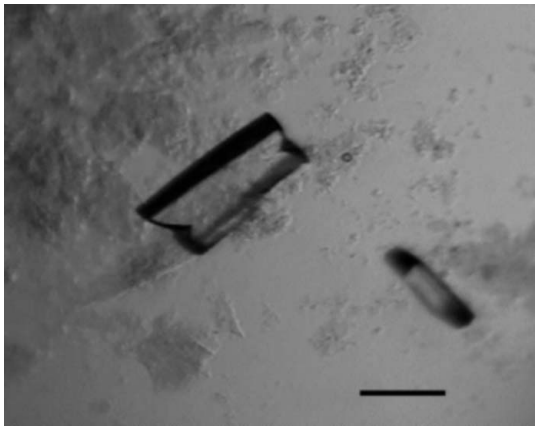

Figure 1
Crystals of struthiocalcin 1 grown by the sitting-drop vapour-diffusion method. The scale bar corresponds to 100 µm.

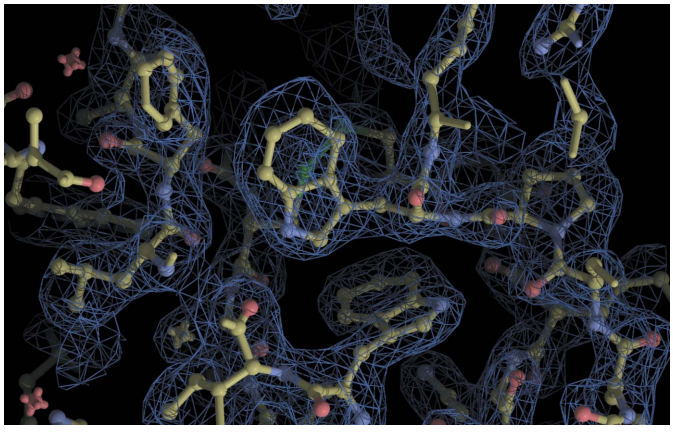

Figure 2
Quality of the electron-density map. The map is contoured at the 1.0σ level.

Table 1
Crystal data statistics.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 55.8, b = 68.81, c = 97.72
Resolution limit (Å)	2.15
Specific volume (cm^3 g^{-1})	0.73
No. of molecules in ASU	3
No. of unique reflections	21060
Completeness (last shell) (%)	100 (99.91)
$R_{\rm merge}$† (%)	5.8

† $R_{\rm merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum I_{hkl}$.

2.3. Data collection and processing

Data collection was performed at 100 K at a wavelength of 0.90 Å on beamline X6A at the National Synchrotron Light Source (Brookhaven, USA); an ADSC Quantum 210 detector was used with a crystal-to-detector distance of 220 mm. For data collection under cryogenic conditions, crystals were soaked for a few seconds in a solution containing a 1:1 mixture of glycerol and 100 m*M* sodium citrate buffer pH 5.6, 30%(*w*/*v*) MPD and 200 m*M* ammonium acetate. Crystals were mounted in a nylon loop and flash-cooled in a liquid-nitrogen stream at 100 K. The data were processed using *MOSFLM* (Leslie, 1999) and reduced with *SCALA* and *TRUNCATE* (Collaborative Computational Project, Number 4, 1994), yielding a unique data set consisting of 21 060 reflections with an $R_{\rm merge}$ of 5.8%. The crystals belonged to space group $P2_12_12_1$ with three monomers per asymmetric unit, corresponding to a solvent content of 48.17%. The data-collection parameters and statistics are shown in Table 1.

Although the SCA-1 protein contains a lectin-like domain, it does not bind calcium as well as lectin-like proteins. The specific selectivity of SCA-1 for carbonate anions (and other intramineral proteins from the eggshell) has been evaluated using electrochemical methods (Marín-García *et al.*, 2007). The selectivity for carbonate was also measured using atomic force microscopy with an electrochemical cell (EC-AFM).

The preliminary crystal structure of struthiocalcin 1 was determined by molecular-replacement methods using the program *MOLREP* (Vagin & Teplyakov, 2000). The atomic coordinates of ovocleidin-17 (PDB code 1gz2) were used as a structural model (Reyes-Grajeda *et al.*, 2004). Refinement and deposition of the structure are in progress (Fig. 2).

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