

# Designer peptides to understand the mineralization of calcium salts

Parayil Kumaran Ajikumar<sup>1</sup>, Rajamani Lakshminarayanan<sup>2</sup>, Suresh Valiyaveetil<sup>\*1,2</sup>, R. Manjunatha Kini<sup>3</sup>

**Abstract:** Recently, we reported the extraction, purification and amino acid sequence of ansocalcin, the major goose eggshell matrix protein. *In vitro* studies showed that ansocalcin induces spherical calcite crystal aggregates. We designed two peptides using the unique features of the sequence of ansocalcin and the role of these peptides in CaCO<sub>3</sub> crystallization was investigated. The peptides showed similar activities as compared to ansocalcin, but at a higher concentration. The full characterization of the peptides and a rationale for the observed morphology for the calcite crystals are discussed in detail.

**Key Words-**Biomineralization, calcium carbonate, crystallization, oligopeptides

## I. INTRODUCTION

Avian eggshells are excellent model systems for biomineralization. The eggshell formation takes place in an extracellular milieu containing mineralized and non-mineralized regions. The mineral deposition in eggshell (~ 5 g per day) is about 100 to 1000 times faster than in nacre (few grams per year) making it one of the fastest bioceramic produced.<sup>1,2</sup> The mineral columns (calcite) grow from a predisposed nucleating sites and is stopped by contact with the adjacent column resulting in spherulite-type crystal lattice.<sup>3,4</sup> Recently, we reported the amino acid sequence of ansocalcin, a major extracellular protein purified from goose eggshell matrix.<sup>5</sup> One of the salient features of ansocalcin sequence is the existence of acidic and basic amino acid multiplets. Although such multiplets are present in other proteins extracted from calcium carbonate biominerals,<sup>6,9</sup> their role in mineralization have not yet been fully understood. To understand the role of such multiplets, we designed two peptides and investigated their influence in CaCO<sub>3</sub> crystallization (Table 1). The incorporation of proline residue in REWDP17 is intended to introduce a turn and to investigate the role of oligomerization of the peptide<sup>10</sup> in biomineralization. Here we discuss the full characterization of the primary and secondary structures of the peptides in solution and their role in biomineralization.

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\* To whom the correspondence should be addressed

<sup>1</sup>Singapore-MIT Alliance National University of Singapore, Singapore 119260.

<sup>2</sup>Department of Chemistry, National University of Singapore, Singapore 117543

<sup>3</sup>Department of Biological Sciences, National University of Singapore, Singapore 117543

## II. RESULTS AND DISCUSSION

Both peptides were synthesized using a peptide synthesizer (Applied Biosystems) employing Fmoc-chemistry. The solubility of the peptide REWD16 was poor in water, especially at higher concentrations (>2 mg/mL) compared to the soluble peptide REWDP17. However both peptides were

Sequence	Observed Mass <sup>†</sup>	Theoretical Mass
RREEWWDDRREEWWDD (REW16)	2364.20± 0.01	2364.43
RREEWWDDPRREEWWDD (REWDP17)	2462.16± 0.02	2461.55

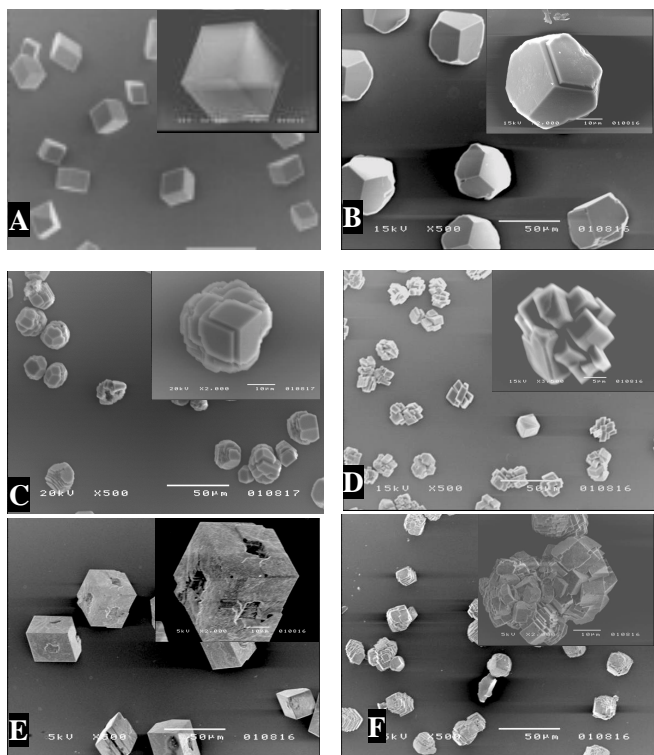
<sup>†</sup>Determined by electrospray mass spectrometry

**Table 1.** Amino acid sequence, observed and theoretical masses of the synthetic peptides.

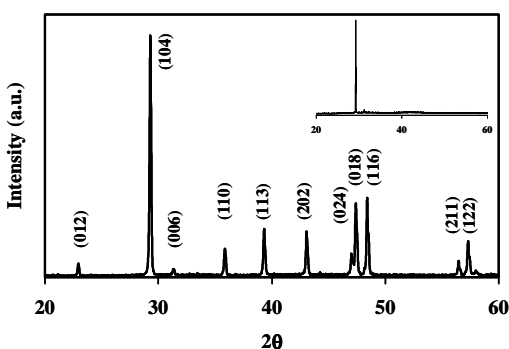
readily soluble in 7.5 mM CaCl<sub>2</sub> solution. The peptides were dissolved in 7.5 mM CaCl<sub>2</sub> solution and the crystallization experiments were performed using a previously reported procedure.<sup>11</sup> A thorough investigation of the influence of ansocalcin in *in vitro* nucleation of calcite crystal aggregate was reported elsewhere.<sup>5</sup> The shape and morphology of CaCO<sub>3</sub> crystals grown in presence of the synthetic peptides changed with the concentration. In the case of peptide REWD16, at low concentrations (0.050, 0.1, 0.25, 0.5 and 1 mg/mL), regular rhombohedral morphology of calcite crystals was observed. As the concentration was increased, the crystals exhibited macroscopic steps and aggregation properties (Fig. 1 B, C). At the highest concentration, 2 mg/mL, large crystal aggregates were observed (Fig. 1D). In presence of peptide REWDP17, up to 0.5 mg/mL, rhombohedral calcite crystals exhibiting screw dislocations were formed (Fig. 1E). The surfaces of the crystals were severely roughened and heavily corrugated as the concentration of the peptide was increased. As seen in the case of REWD16, at the highest concentration (2 mg/mL) of REWDP17, large crystal aggregates were formed (Fig. 1E). At 2 mg/mL, both peptides reduced the crystal aggregate size from ~38 ± 5 μm (0.5 mg/mL) to 17 ± 3 μm (2 mg/mL).

X-ray diffraction studies of the crystals showed a strong diffraction maximum at 2θ ≈ 30, which corresponds to {104}-faces of the rhombohedral calcite crystallites (Fig. 2 inset). In the powdered form, diffractions from all other calcite crystal

planes were observed (Fig. 2). This shows that the crystal aggregates are truly single crystalline in nature.



**Figure 1.** Representative SEM images of the calcite crystals grown (A) in the absence of any protein or peptides (control), in presence of synthetic peptides REWD16 [B (0.05 mg/mL), C (0.5 mg/mL) and D (2 mg/mL)] and REWD17 [E (0.5 mg/mL) and F (2 mg/mL)]. The scale bar indicates 5  $\mu$ m.

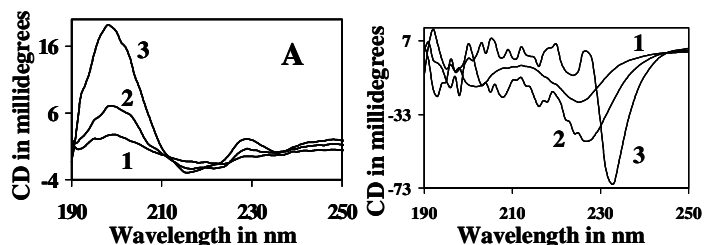


The **Figure 2.** XRD of the powdered (A) and single crystal (B) aggregates formed in presence of 2 mg/mL of the peptide REWD16.

The secondary structures of both peptides were studied using CD spectroscopy at 25° C in 7.5 mM CaCl<sub>2</sub> solution (Fig. 3). Both the peptides exhibited strong changes in the secondary structures of both peptides were studied using CD spectroscopy at 25° C in 7.5 mM CaCl<sub>2</sub> solution (Fig. 3). Both the peptides exhibited strong changes in the secondary structure with change in peptide concentration. For REWD16 peptide, a negative minimum around 216 nm and a positive maximum at 198 nm are indicative of high degree of  $\beta$ -sheet

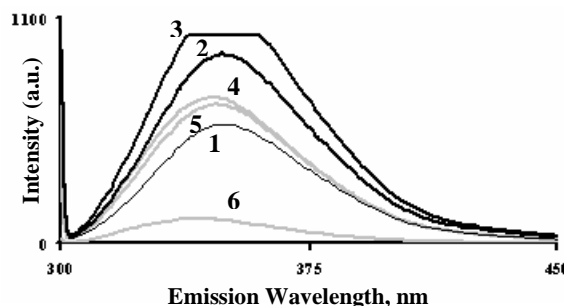
conformation. As the concentration was increased the amplitude of the negative minimum and the positive maximum increases. Incorporation of proline into the sequence (i.e., REWD17) resulted in appearance of negative band at 227 nm indicative of  $\beta$ -turn conformation.<sup>12,13</sup>

In the case of the peptide REWD17 as the concentration was increased up to 0.5 mg/mL, intensity of negative minimum at 227 nm increases. Upon further increase in concentration of the peptide, the features of the CD spectrum changes significantly. The negative band around 227 nm shifted to 233 nm. This might be due to the tryptophan-tryptophan interactions caused by aggregation of the peptide.



**Figure 3.** Circular dichroism of the synthetic peptides, REWD16 (A) and REWD17 (B). The peptide concentration used are (1) 0.5 mg/mL; (2) 1 mg/mL; (3) 2 mg/mL in 7.5 mM CaCl<sub>2</sub>

In order to understand the self-assembly of the peptide and the changes around the tryptophan in native and aggregated state of the peptides, we carried out fluorescent emission studies. Both peptides showed distinct emission characteristics. The emission maximum for REWD16 was observed around 348 nm (Fig. 4). This was red shifted to 357 nm for the 17-mer peptide REWD17 indicating that incorporation of proline exposes the Trp residues more strongly to outside environment. The emission spectra showed changes with change in peptide concentration. For REWD16, the fluorescence intensity increases with peptide concentration up to 0.25 mg/mL and decreases. The decrease was accompanied by blue shift in the emission maxima to 341 nm at the highest concentration of the peptide (2 mg/mL).



**Figure 4.** Intrinsic tryptophan fluorescent emission spectrum of the synthetic peptides REWD16 (A) at various concentrations. The decrease in intensity is indicated by gray lines. (1) 0.05 mg/mL; (2) 0.1 mg/mL; (3) 0.25 mg/mL; (4) 0.5 mg/mL; (5) 1 mg/mL (6) 2 mg/mL

For REWDP17, emission intensity increases up to 0.25 mg/mL, remained same at 0.5 mg/mL and decreases. The emission maximum was blue shifted to 347 nm at 2 mg/mL. For both peptides, a remarkable reduction (~ 85%) in emission intensity was observed at 2 mg/mL. This large decrease may be due to the quenching of the tryptophan by arginine residues, which were brought into close proximity by the aggregation of peptide molecules.<sup>14</sup> Data collected from both CD and fluorescence studies confirmed the concentration dependent self aggregation of peptides. Moreover, calcium ions may also induce the self-assembly of the peptides.<sup>15</sup> This was not the case with the parent protein, in which calcium ions had no effect on the aggregation. Since such assembly of peptide encompasses more molecules, it is reasonable to expect that they accelerate the nucleation of crystal aggregates and thereby reducing the overall size and increasing the nucleation density of the crystals. Thus the calcite crystal aggregates observed at higher concentration might be due to the self-assembly of peptides. Altogether, these observations confirm the important role played by the acidic and basic amino acid residues towards nucleating polycrystalline aggregates of calcite crystals. It is also remarkable that the activity of the peptides at higher concentration is comparable with the parent protein ansocalcin. As expected the effectiveness of the peptides is about 5 to 10 times lower. We are pursuing these studies and further details will be reported elsewhere.

### III. EXPERIMENTAL PROCEDURES

*Synthesis and Characterization of the Peptides:* Fmoc-PAL-PEG-PS and all the N<sup>α</sup>-Fmoc-L-amino acid pentafluorophenyl esters were purchased from Novabiochem, San Diego CA. The peptides were synthesized using The Pioneer peptide synthesizer (Applied Biosystems) using the N<sup>α</sup>-Fmoc-L-amino acid pentafluorophenyl ester/HOBt coupling method. The peptides REWD16 and REWDP17 were assembled on an Fmoc-PAL-PEG-PS (0.18 mmole/g) resin. Both the peptides were synthesized on a 0.1 mmole scale using the extended cycle protocol. The completed peptides were deprotected and cleaved by treating with the cleavage cocktail (90% trifluoroacetic acid (TFA), 2.5% phenol, 2.5% water, 2.5% thioanizole, 2.5% ethanedithiol) for 3 hours at room temperature. The mixture was filtered and the filtrate was concentrated under reduced pressure. The peptide was precipitated from ice cooled diethylether. This precipitated peptide was centrifuged on an ultracentrifuge with repeated washing by ice cold ether to remove all contaminants. Finally the peptides were lyophilized with 10% acetic acid solution and obtained as white powder and were purified using the Phenomenex (250 × 10 nm, 10μ) C18 reverse-phase column (on a BioCAD Workstation). The solvent system used for the purification is as follows: solvent A, 0.1% TFA and solvent B, 0.1% TFA in 80% acetonitrile. The linear gradient (flow rate 2 mL/min) 25%-50% B over 50 min was used. The peptide elution was monitored at 215 nm. The purity of the peptides was confirmed by analytical HPLC and ESI MS.

*Circular Dichroism Experiments:* Circular dichroism experiments were done using Jasco J 715 spectropolarimeter. The CD spectra of the peptides (50 μg/mL - 2 mg/mL) in CaCl<sub>2</sub> (7.5 mM) were collected at the wavelength range of 260 to 190 nm using 0.1 mm sample cell. The instrument optics was flushed with 30 L/min nitrogen gas. A total of three scans were recorded and averaged for each spectrum and baseline was subtracted.

*Fluorescence Spectroscopy:* The fluorescence emission spectra were collected on a Shimadzu RF-5301PC spectrofluorophotometer, with the emission and excitation band passes set at 3 nm. The excitation wavelength was set at 295 nm and the spectra were recorded from 300 to 400 nm at a scan rate of 50 nm/min. Spectra of the peptides were recorded in 7.5 mM CaCl<sub>2</sub> solution.

*Crystal Growth Experiments:* CaCO<sub>3</sub> crystals were grown on glass cover slips placed inside the CaCl<sub>2</sub> solution kept in a Nunc dish 4 × 6 wells. Typically, 1 mL of 7.5 mM CaCl<sub>2</sub> solution was introduced into the wells containing the cover slips and the whole set up was covered with aluminum foil with a few pinholes on the top. To study the role of synthetic peptides in the CaCO<sub>3</sub> crystallization, aliquots of peptides (50 μg/mL - 2 mg/mL) dissolved in 7.5 mM CaCl<sub>2</sub> solution was introduced into each well containing the cover slips. Crystals were grown inside a closed desiccator for 2 days by slow diffusion of gases from the decomposition of ammonium carbonate placed at the bottom of the desiccator. After 2 days, the slides were carefully lifted from the crystallization wells, rinsed gently with Millipore water, air dried at room temperature, glued to copper stubs and used for further analysis.

*Scanning Electron Microscopy (SEM):* SEM studies on the CaCO<sub>3</sub> crystals were carried out using JEOL 2200 scanning electron microscope at 15/20 kV after sputter coating with gold to increase the conductivity.

*X-ray Diffraction Studies:* Powder and single crystal X-ray diffraction studies on the crystals were carried out using D5005 X-ray diffractometer with Cu-Kα radiation at 40 kV and 40 mA multisampler system. For the X-ray investigation, the samples were slightly crushed using a mortar, placed on a plastic holder and wetted with a drop of ethanol to form continuous film. Similarly the crystals grown on glass plates were mounted on the plastic holder and carried out the experiments.

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