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Modification of $CaCO_3$ precipitation rates by water-soluble nacre proteins

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ABSTRACT

Mineral growth in nacre and other $CaCO_3$-containing biominerals is controlled by biopolymers. Water-soluble proteins were extracted from nacre of the sea snail *Haliotis laevigata* by dissolving the mineral phase with 6% acetic acid. The influence of this protein mixture on $CaCO_3$ precipitation rates was investigated at different concentrations. A well-established assay for measuring the pH-value during $CaCO_3$ precipitation with and without protein additives was extended by calculating maximum precipitation rates from the pH-values. It could be shown that precipitation rates are greatly influenced by the mixtures of water-soluble nacre proteins. At very low protein concentrations (0.02 μg/ml) a rate enhancement in comparison to the pure supersaturated calcium carbonate solution by a factor of 1.4 was observed. At higher protein concentrations, a strong inhibitory effect occurred, with total inhibition at concentrations of 1.0 μg/ml and higher. Two unspecific proteins (bovine serum albumin and lysozyme) showed little or no modification of precipitation rates. *In vivo,* the function of the strong inhibition of $CaCO_3$ precipitation by nacre proteins at higher concentrations is presumably to prevent uncontrolled crystallization in the extrapallial fluid. The rate-enhancing capability of proteins at low concentrations may be explained by the presence of acidic and/or hydrophilic moieties.

1. Introduction

In biominerals, such as the polymer/mineral composite nacre, proteins and other biopolymers are supposed to play a major role in inducing self-organized growth. Such biopolymers affect the mineralization process in various ways.

For example, the proteins AP7, AP24 [1,2], perlwapin [3] and perlinhibin [4], isolated from gastropod nacre, act as inhibitors of crystal growth *in vitro,* possibly preventing uncontrolled crystal formation during the growth of the natural material. On the contrary, other proteins found in nacre are known to enhance crystal formation *in vitro,* such as the C-type lectin perlucin [5,6] or the acidic proteins AP8-α and AP8-β [7]. A role for such enhancer proteins could be the initiation of crystallization in the correct spatial alignment (when attached to a pre-oriented substrate) and the correct crystal polymorph.

Besides these proteins, which are without doubt involved in $CaCO_3$ nucleation and growth, there are others which seem to have different functions and are not yet known to interact with $CaCO_3$ during crystallization. While it is highly interesting to consider how individual proteins modify $CaCO_3$ nucleation and growth, we must also ask how these proteins collectively alter the mineralization process during biomineral formation.

One method used to characterize proteins and protein mixtures with respect to $CaCO_3$ crystallization is precipitation. In 1981, an assay was published [8], in which a supersaturated $CaCO_3$ solution was created and the pH-value was recorded over time. Due to the chemistry of the system, $CaCO_3$ precipitation is accompanied by a pH-value drop, which can be summarized in the following reaction:

$$HCO_3^- + Ca^{2+} \rightarrow CaCO_3(\downarrow) + H^+.$$

The authors of this initial article found that in the presence of the soluble organic matrix from oyster shells the pH-drop and therefore the precipitation was decreased or completely inhibited. This offered strong support for the hypothesis that soluble organic compounds regulate the growth of the oyster shell.

The work presented here aims to measure the $CaCO_3$ precipitation modulating (inhibiting, enhancing) effect, depending on the concentration of soluble matrix proteins. Therefore, we performed precipitation experiments with mixtures of soluble proteins extracted from nacre of the gastropod *Haliotis laevigata* over a wide concentration range. The results were compared with experimental results of precipitation studies in the presence of the proteins bovine serum albumin (BSA) and lysozyme. This allowed us to appraise the general strength of the precipitation modulating capability in comparison to proteins not involved in biomineralization.

The original precipitation assay from 1981 relied on the comparison of pH-courses, which makes it not well suited for comparative measurements, especially when targeting small effects. Here, we describe an extension of this more qualitative assay, towards a

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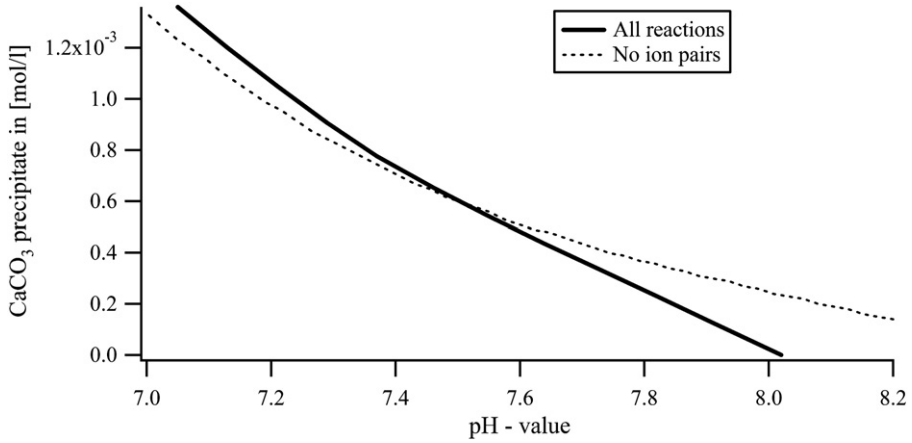

Fig. 1. The pH-value during $CaCO_3$ precipitation experiments can be used to calculate the amount of $CaCO_3$ precipitate. The graph shows the calculated function for the mixing of 60 ml of 20 mM $NaHCO_3$ with 0.5 ml of 0.05 M NaOH and 60 ml of a 20 mM $CaCl_2$ solution at 25 °C. It is important to include ion pair formation, as the result of a complete calculation (solid line) deviates greatly from calculations without ion pairs (dashed line).

method that allows quantification and comparison of the influence of proteins on $CaCO_3$ precipitation. Starting with the precipitation assay described above, the observed pH-value course is used to calculate the amount of precipitated $CaCO_3$ and the precipitation rate $d[CaCO_3]/dt$. From each experiment (e.g., with a protein present), the maximum precipitation rate was chosen as a characteristic parameter.

In complementary experiments, polymorph and morphology of crystals precipitated in the presence of nacre proteins were analyzed by X-ray powder diffraction (XRD) and scanning electron microscopy (SEM). The results were compared with precipitates obtained without added proteins.

2. Materials and methods

2.1. Precipitation assay

60 ml of a 20 mM carbonate solution was prepared in a 200 ml Duran glass vessel (Schott, Mainz, Germany) by dissolving $NaHCO_3$ (Sigma-Aldrich, St. Louis, USA) in ultra pure[1] water and adding 0.5 ml of a 0.05 M NaOH (Sigma-Aldrich, St. Louis, USA) to increase the initial pH-value and $CaCO_3$ supersaturation so that precipitation began in a reasonable amount of time. In some experiments bovine serum albumin (SigmaAldrich, St. Louis, USA), Lysozyme (Sigma-Aldrich, St. Louis, USA) or the soluble nacre protein fraction (see below) was added to the carbonate solution. A solution of 60 ml of a 20 mM $CaCl_2$ was prepared by dissolving $CaCl_2 * 2H_2O$ (Sigma-Aldrich, St. Louis, USA) in ultra pure water. To start precipitation, the $CaCl_2$ solution was rapidly poured into the vessel containing the $NaHCO_3$ solution. The vessel was stirred at a constant rate, to achieve good homogeneity of the solution. To reduce the amount of CO_2 exchange with the environment, the solution was covered with aluminum foil. All experiments were performed at room temperature (18–24 °C). The pH-value was measured with a single-rod measuring electrode (WTW SenTix Mic, Weilheim, Germany) connected to a pH-meter (PP-20, Sartorius, Goettingen, Germany). At least six experiments per data point were performed. Data were collected from the pH-meter with a computer using the scientific software package IGORpro (WaveMetrics, Portland, USA) in 3 s intervals over RS232 connection.

2.2. Calculation of precipitation rates

The theoretical function describing the amount of precipitate x for a series of pH-values of the solution was calculated for the known initial concentrations (as described in the results section in greater detail). By using this theoretical dependency, the amount of precipitate could be determined from the measured pH-courses at each pH-value (Fig. 1). To calculate the precipitation rate dx/dt, the calculated amount of precipitate $x(t)$ was smoothed with a cubic spline across 20–50 interpolation-points. This was necessary because of digit jumps in the recorded pH-values (e.g., 7.40 to 7.39), which would cause a high slope at the point of digit jump and no slope in between digit jumps. The smoothing was programmed using the scientific software package IGORpro (WaveMetrics, Portland, USA). From each experiment the maximum precipitation rate (Fig. 2) was chosen as a parameter.

2.3. Extraction of soluble matrix protein mixture

Shells of the gastropod *H. laevigata* (obtained from Abalone Exports, Laverton North, Victoria, Australia) were cleaned with water and a brush. Calcitic outer parts of the shell were removed by blasting with corundum slurry (slurry blaster WA70, Sigg, Jestetten, Germany). The nacreous shells were incubated for 2 min in a solution of 50% sodium hypochlorite (NaOCl, Merck, Frankfurt, Germany) and ultrapure water to remove organic contaminants from the surface. Hypochlorite and dissolved organic compounds were removed by extensive washing with ultrapure water. The remaining nacreous shell was crushed to a coarse powder with a jaw crusher. The powder was dialyzed at 4 °C against 6% (v/v) acetic acid (Sigma-Aldrich, St. Louis, USA) with 0.02% NaN_3 (Merck, Frankfurt, Germany) to prevent bacterial growth. Dialysis tubes (MWCO 3500, Spectrum Labs, Rancho Dominguez, USA) were heated to 100 °C in 5 mM ethylene diamine tetraacetic acid (EDTA) for 15 min, to inactivate microbial proteases and bind heavy metal contaminants. In order to discharge the high amount of developing CO_2, the dialysis tube was placed in a large beaker containing the acetic acid, such that the upper part was outside the solution and could be perforated. After one week the supernatant of the solution was filtered with a 0.22 µm membrane filter. For use in precipitation experiments the solution was dialyzed (MWCO 3500, preparation as described above) against 20 mM $NaHCO_3$ at 4 °C. The solution was changed three times. During the first two volumes 0.02% NaN_3 was present to prevent microbial growth and the release of bacterial proteases. In the last solution change NaN_3 was omitted to avoid possible effects on the precipitation assay. The concentration of the protein mixture was determined with the Bradford method [9]. Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) in 10% gels of the protein solution was performed following established protocols.

[1] Deionized water, further cleaned by a Milli-Q Academic water purification device (Millipore, Billerica, MA, USA).