# *In vitro* regulation of $CaCO_3$ crystal polymorphism by the highly acidic molluscan shell protein Aspein

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Keywords: Acidic protein; Aspein; Biomineralization; Calcite; Crystal polymorphism; *In vitro* crystallization; Mollusc shell; Pearl oyster; *Pinctada fucata*; Prismatic layer

Abbreviations: BCA, bicinchoninicacid; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; SEM, scanning electron microscope

## Introduction

Biominerals are generally composed of mineral and organic phases. Organic constituents, known as organic matrix, are essential for elaborated crystal formation and structural organization in biomineralization. In invertebrates, several acidic proteins of the skeletal matrix have been identified [1]. Acidic proteins are believed to play important roles in biomineralization because of their cation binding capacity [2-5], which enables these proteins to interact with particular crystal faces, to regulate crystal growth, and to decide crystal morphology and orientation [6-8].

In molluscs, it is well known that the soluble fraction of the organic shell matrix is rich in aspartic acid [2,3,9]. Recently, complete amino acid sequences of highly acidic proteins from some bivalve molluscs have been established [10-14]. Tsukamoto *et al*. [12] identiflied a novel mantle-specific gene, *aspein*, from the pearl oyster *Pinctada fucata*. This protein is unusually acidic, with aspartic acid residues accounting for up to 60% of mature protein. Gene expression of Aspein is restricted to the mantle edge, suggesting that this protein is specifically involved in the prismatic layer formation [12,15].

Molluscan shells are mainly composed of calcium carbonate ($CaCO_3$), which is the commonest biomineral known. It is distributed throughout diverse hard parts such as

avian eggshells, crayfish carapaces and corals. Calcite and aragonite are the two common polymorphs of $CaCO_3$ observed as biominerals. They differ from each other in lattice structure and stability. Despite these differences, some organisms can form the two polymorphs simultaneously albeit in different positions. One striking example is *P. fucata*, which produces two crystallographically different shell layers, the outer calcitic prismatic layer and the inner aragonitic nacreous layer, in the same shell. The boundary between these layers is distinct, although the crystals of the two layers are deposited from the same solution known as the extrapallial fluid. For the understanding of this *in vivo* mineralization processes, the composition of the extrapallial fluid should be considered. One significant feature of the extrapallial fluids of marine molluscs is their ionic composition, with a high $Mg^{2+}$ content (50 mM) relative to $Ca^{2+}$ (10 mM) [16]. In solution, $Mg^{2+}$ poisons calcite formation, while it hardly affects aragonite precipitation [17,18]. Consequently, aragonite crystals become dominant in the $Mg^{2+}$-rich solutions [19,20]. In the marine bivalve shell crystallization, it is expected that aragonite precipitation is the initial state and some biological processes are required to override this condition to form calcite crystals.

Several *in vitro* experiments demonstrated that the soluble organic matrix extracted from the aragonitic shell layer induces aragonite, while the matrix from the calcitic layer

induces calcite [21-23]. These results suggest that soluble shell proteins are responsible for deciding which crystal polymorph will form. Tsukamoto *et al*. [12] suggested that Aspein operates on the selective formation of calcite just as poly-aspartates influence the $CaCO_3$ polymorphism *in vitro* [24]. To test this hypothesis and elucidate the roles of the acidic proteins in molluscan shell formation, we conducted *in vitro* $CaCO_3$ crystallization experiments in the presence of $Mg^{2+}$ with recombinant Aspein proteins.

## Materials and methods

*Expression and purification of recombinant Aspein proteins*

DNA fragments encoding various length of Aspein were amplified by RT-PCR. PCR products were cloned into the expression vector pGEX-6P-1 (Amersham Biosciences). The plasmids were transformed into *Escherichia coli* BL21.

The colonies with an appropriate plasmid construct were grown in LB/ampicillin medium and GST-Aspein fusion proteins were expressed by induction using 0.1 mM IPTG. The cells were centrifuged and resuspended in B-PER Bacterial Protein Extraction Reagent (PIERCE). After centrifugation, the supernatant was collected and the fusion protein was purified by the affinity matrix Glutathione Sepharose 4B (GE Healthcare). GST protein and GST-Aspein fusion proteins were eluted by glutathion elution buffer. Aspein proteins were separated from GST by PreScission Protease (GE Healthcare). The amount of each protein was estimated by the BCA method.

*SDS-PAGE*

The purified GST and GST-Aspein fusion proteins were electrophoresed on 10% SDS-polyacrylamide gels, and then stained with CBB or Stains-all staining.

*In vitro crystallization*

The solution for calcium carbonate crystallization was prepared according to [19]. Calcium carbonate was dissolved into $CO_2$-aerated water and excess precipitates were removed by filtration (0.22 μm). The $Ca^{2+}$ concentration in the solution was checked by EDTA titration, and adjusted to 10 mM by adding an appropriate amount of water. A magnesium chloride solution (1 M) was added to the calcium bicarbonate solution to the final concentration of 50 mM. The solution was transferred to a 24-well plate. Subsequently GST protein in glutathion elution buffer or Aspein protein in PreScission Protease cleavage buffer was added into each solution. The same volume of glutathion elution buffer or cleavage buffer without protein was also used for control experiments. Glass slides were set on the bottom of each well to grow and collect crystals on the glass surfaces. The plate was placed into a sealed box with a sheet of wet paper to avoid evaporation. Crystallization was carried out at room temperature for one week.

*Characterization of $CaCO_3$ crystals*

Crystals on the glass slides were lightly rinsed with water, air-dried and then observed with an optical microscope. Raman spectra of the crystals were measured with a Photon-Design Mars micro-Raman spectrometer equipped with a monochromator (JOBIN YVON HR-320) and a CCD detector (ANDOR DU-401). The Raman spectra were excited by the laser line having a wavelength of 514.5 nm from an Argon laser. The laser output was fixed at approximately 100 mW and the acquisition time was 10 s.

After the Raman experiments, the samples were coated with gold and observed using a SEM (JEOL JSM-5500LV).

## Results

*Recombinant Aspein proteins*

The primary structure of Aspein [12] can be subdivided into four regions: a putative signal peptide ($M_1$ to $T_{19}$), followed by an uncharged N-terminal region ($F_{20}$ to $S_{46}$) and two domains rich in aspartic acids, the DA domain ($D_{47}$ to $N_{77}$) and the poly-D domain ($D_{78}$ to $Q_{413}$), as shown in figure 1. Four kinds of DNA fragments that encode partial amino acid sequences of various length of Aspein were designed. Each DNA fragment encoded a different partial Aspein sequence without the signal peptide (Fig. 1). Aspein-ΔDAΔD was devoid of both the DA and poly-D domains. Aspein-ΔD was composed of the N-terminal region and the DA domain. Aspein-D1 possessed all three regions but a short poly-D domain, while Aspein-D2 had full-length poly-D domain. Their molecular characteristics are summarized in Table S1 in supplementary materials. The plasmid constructs of all the four recombinant *aspein* fragments were successfully transformed into the host, an observation confirmed by PCR and sequencing (data not shown). However, the longer polypeptide, GST-Aspein-D2 was not obtained. Therefore, experiments described below were conducted using the remaining recombinant proteins, Aspein-ΔDΔDA, ΔD and D1.

*SDS-PAGE analyses for Aspein proteins*

The recombinant proteins were purified and analyzed by SDS-PAGE (Fig. 2). Values of the molecular mass estimated from SDS-PAGE for GST and GST-Aspein-ΔDAΔD were nearly the same as those predicted from their primary structure (26 kDa and 28.5 kDa, respectively). On the other hand, the size of GST-Aspein-ΔD was estimated at 39 kDa and GST-Aspein-D1 at 47.5 kDa on gels, being obviously larger than predicted (31.2 kDa and 36.7 kDa, respectively). GST-Aspein-ΔD and GST-Aspein-D1 were stained blue by Stains-all (Figs. 2B, 3), while GST and GST-Aspein-ΔDAΔD were stained purple.

*Influence of Aspein on calcium carbonate crystallization*

To investigate the effect of Aspein on $CaCO_3$ crystal formation, *in vitro* crystallization experiments were carried out. In every experiment, Raman spectra of at least 10 crystals with the same morphology observed under optical microscope were individually measured. All crystals similar in shape were of the same polymorph. We therefore took into consideration that the crystal morphology reflected the crystal polymorph.

First, the influence of $Mg^{2+}$ on $CaCO_3$ polymorphism was checked. Without $Mg^{2+}$, typical rhombohedral calcite crystals were generated (Fig. 4A,B and L). On the other hand, in the presence of $Mg^{2+}$ (50 mM), large needle-like crystals were preferentially formed (Fig. 4C indicated by an arrow, D). A low amount of small polyhedral crystals were also observed (Fig. 4C arrowhead, E). Raman measurements demonstrated that the former crystals consisted of aragonite and the latter of calcite (Fig. 4M). The blank experiments using buffer solutions without protein showed no effect on $CaCO_3$ polymorphism (data not shown). GST protein served as a negative control, and also had no effect on $CaCO_3$ polymorphism (data not shown).

Figure 4F-K show SEM images of $CaCO_3$ crystals grown in the presence of Aspein-D1 protein, at a higher and a lower concentration, 10 and 2 μg / ml, respectively. Of 10 μg / ml, a number of dumbbell-like crystals were exclusively induced (Fig. 4F-H). Raman measurements showed that all crystals formed under this condition were calcite (Fig. 4N). The lower concentration of Aspein-D1 (2 μg / ml) caused the formation of spherical aragonite crystals (Fig. 4I arrow, J and O) and smaller calcite crystals (Fig. 4I arrowhead, K and O). Other modified Aspein proteins, Aspein-ΔDAΔD and Aspein-ΔD did not accelerate calcite formation, and needle-like aragonite crystals were preferentially formed even at a high concentration (10 μg / ml) (Fig. 5). These proteins, therefore, had

no influence on $CaCO_3$ polymorphism.

A quantitative account of calcite and aragonite crystals formed from these experiments is shown in Table S2. Exclusive precipitation of calcite from $Mg^{2+}$ containing solutions was observed only when Aspein-D1 was added at a concentration of 10 μg / ml.

## Discussion

The unusually acidic proteins of bivalves reported to date exist mainly in the calcitic layer [10,13,14]. The Aspein gene is expressed only in the outer edge of mantle epithelia [12]. These findings imply that the acidic matrix proteins play a role in the formation of the calcitic shell.

To investigate the property of each region of the Aspein protein, truncated *aspein* gene fragments of various length were used. In our *E. coli* expression system, the full length Aspein protein without the signal peptide (Aspein-D2) could not be obtained, presumably due to its excessively biased amino acid composition. However, we presume that the truncated Aspein protein with a short poly-D region (Aspein-D1) may have properties similar to that of the native Aspein, because Aspein-D1 and native Aspein are different only in the length of the poly-D region, which is made up of a simple repeat of SG(D)n peptides. Thus, the results of the experiments using Aspein-D1 must be meaningful for consideration of the functions of the native Aspein protein.

In SDS-PAGE, GST-Aspein-ΔD and GST-Aspein-D1 exhibited a higher molecular mass than expected (Fig. 2). Overestimation of the molecular mass of acidic proteins may be due to high contents of negatively charged amino acids that prevent sufficient binding of SDS [25]. Stains-all gave an intensely blue color with GST-Aspein-D1 (Fig. 2B, 3),

indicating that this protein can bind cations. Removal of the aspartic acid rich domains resulted in the loss of this property. Thus, we infer that the aspartic acid rich domains can bind $Ca^{2+}$.

Calcium carbonate crystallization experiments demonstrated that aragonite crystals are dominantly formed under $Mg^{2+}$-rich conditions in the absence of any additives (Fig. 4C; [20,24]). Aspein-D1 has the capability of calcite induction in the solution with a high concentration of $Mg^{2+}$ (Fig. 4F, Table 2). On the other hand, the proteins that lack the poly-D domain do not have this property (Fig. 5, Table 2). Falini *et al*. [24] demonstrated that poly-aspartate molecules preferentially induce calcite formation in solutions with a high $Mg^{2+}$ / $Ca^{2+}$ molar ratio. We therefore conclude that the poly-D domain of Aspein is crucial for calcite precipitation.

In *P. fucata*, *aspein* transcripts are localized only in the mantle edge, and this expression of *aspein* coincides with expression of other shell matrix protein genes involved in prismatic layer formation [12,15]. In the prismatic layer, highly acidic macromolecules exist in intraprismatic crystals [26]. These soluble matrix proteins would be connected to insoluble matrix proteins and located in a precise position within the shell layer. Aspein is likely to be one of the major constituents of this fraction and to work for crystal formation in prisms. Tsukamoto *et al*. [12] hypothesized that Aspein is a

cation carrier which selectively binds $Ca^{2+}$ with a substantially lower affinity to bind $Mg^{2+}$. In contrast to the surrounding extrapallial fluid, it is thought that the $Mg^{2+}$ / $Ca^{2+}$ ratio in the inner part of the prismatic wall is so reduced due to $Ca^{2+}$ transport by Aspein that calcite is preferentially precipitated. The aspartic acid rich region contains apparently random insertions of serine - glycine dipeptides, which may contribute to physical flexibility of the protein. It seems that plasticity of the highly negatively charged region facilitates aggregation of $Ca^{2+}$ and reduces the activation energy for crystal nucleation [27,28]. It is speculated that Aspein acts as a crystal nucleator using the mechanism of ionotropy [28] and simultaneously determines the $CaCO_3$ polymorph to form calcite by decreasing the $Mg^{2+}$ / $Ca^{2+}$ ratio at the sites of crystal nucleation.

According to the theory of ionotropy, it is predicted that the acidic macromolecules accumulate precursor ions in disordered state, and form amorphous calcium carbonate (ACC) in the pre-crystallization stage [29]. Gower and Odom [30] observed that poly-aspartate induced transient ACC, followed by transformation into calcite. Recently, Politi *et al*. [31] performed *in vitro* $CaCO_3$ crystallization experiments using recombinant Asprich protein, which is one of the unusually acidic shell protein identified from *Atrina rigida*. When Asprich protein was added, ACC phase was stabilized prior to calcite formation. Therefore, we infer that Aspein initially deposits ACC, which subsequently

transforms into the stable calcite phase.

The alternative explanation of the effect of Aspein is that Aspein inhibits aragonite formation by binding to specific aragonite crystal faces. This hypothesis is testable by observing whether Aspein can specifically interact with aragonite growth rather than with calcite.

It is most likely that Aspein proteins diffuse throughout the extrapallial fluid after secretion from the epithelial cells of the mantle edge. Then Aspein would aggregate inside the prismatic wall as an “envelope” [26] and accelerate calcite formation.

**Acknowledgements**

We appreciate Drs. K. Sashida and M. Kimata for valuable comments. Dr. P. Westbroek is thanked for kindly reviewing the manuscript. We also thank H. Oniki and T. Kaifu of Tasaki Institute for Marine Biological Research for providing samples of *P. fucata*. Thanks are due to T. Echigo and N. Ishida for technical support during Raman spectroscopy analysis. This work was partially supported by the JSPS Grant-Aid for Scientific Research No. 15104009.

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**Legends of Figures**

Figure 1.

Schematic representation of wild type (wt) and recombinant Aspein proteins.

Figure 2.

SDS-PAGE analyses of the GST-Aspein fusion proteins. Lane 1, GST-Aspein-ΔDAΔD; lane 2, GST-Aspein-ΔD; lane 3, GST-Aspein-D1; lane 4, GST.

Figure 3.

The absorption spectrum of Stains-all solution in the absence and presence of recombinant Aspein proteins. The proteins at 5 μg / ml dissolved in 2 mM MOPS, pH 7.0, 30% ethylene glycol and 5 μM Stains-all were incubated in the dark at room temperature for 30 min then the absorption spectra were measured. Addition of Aspein-D1 most significantly affects spectral feature, suggesting the ability of cation binding.

Figure. 4.

SEM images of $CaCO_3$ crystals and their Raman spectra. (A, B) Crystals grown without

any proteins in the absence of $Mg^{2+}$, showing rhombohedral calcite crystals. (C-E) Crystals grown without any proteins in the presence of $Mg^{2+}$. A needle-like crystal (aragonite) is indicated by an arrow in (C) and enlarged in (D). A polyhedral crystal (calcite) is indicated by an arrowhead in (C) and enlarged in (E). (F-H) Crystals grown in the presence of Aspein-D1 at 10 μg / ml. Dumbbell-like crystals (F, G) are formed. (H) Fused dumbbells are also observed. Under this condition, all the crystals formed are calcite. (I-K) Crystals grown in the presence of Aspein-D1 at 2 μg / ml. A spherical crystal (aragonite) is indicated by an arrow in (I) and enlarged in (J). A polyhedral crystal (calcite) is indicated by an arrowhead in (I) and enlarged in (K). (L-O) Micro-Raman spectra of the crystals formed in these experiments.

Figure 5.

SEM images of $CaCO_3$ crystals in the presence of $Mg^{2+}$ and Aspein-ΔDAΔD (A) or Aspein-ΔD (B) at 10 μg / ml. Large needle-like aragonite crystals are preferentially precipitated. These results are similar to those of blank experiments shown in figure 4C.