Journal of Structural Biology 171 (2010) 207–215

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Stabilization of amorphous calcium carbonate by phosphate rich organic matrix proteins and by single phosphoamino acids

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ARTICLE INFO

Article history:
Received 9 February 2010
Received in revised form 12 April 2010
Accepted 16 April 2010
Available online 21 April 2010

Keywords:
Biomineralization
Amorphous calcium carbonate (ACC)
Crustacea
Crayfish gastroliths
Phosphoproteins
Phosphoamino acids

ABSTRACT

Stable amorphous calcium carbonate (ACC) is a unique material produced naturally exclusively as a biomineral. It was demonstrated that proteins extracted from biogenic stable ACC induce and stabilize synthetic ACC *in vitro*. Polyphosphate molecules were similarly shown to induce amorphous calcium carbonate formation *in vitro*. Accordingly, we tested the hypothesis that biogenic ACC induction and stabilization is mediated by the phosphorylated residues of phosphoproteins. We show that extracellular organic matrix extracted from gastroliths of the red claw crayfish *Cherax quadricarinatus* induce stable ACC formation *in vitro*. The proteinaceous fraction of this organic matrix is highly phosphorylated and is incorporated into the ACC mineral phase during precipitation. We have identified the major phosphoproteins of the organic matrix and showed that they have high calcium binding capacity. Based on the above, *in vitro* precipitation experiments with single phosphoamino acids were performed, indicating that phosphoserine or phosphothreonine alone can induce the formation of highly stable ACC. The results indicate that phosphoproteins may play a major role in the control of ACC formation and stabilization and that their phosphoamino acid moieties are key components in this process.

1. Introduction

Amorphous calcium carbonate (ACC) is a metastable phase which readily transforms to the more stable crystalline phase of calcium carbonate. ACC has been found as a transiently stabilized precursor in the onset of biomineralization in a variety of organisms (Addadi et al., 2003), and is thought to play a role in the biological control over the subsequent crystallization (Gower, 2008). However, various organisms produce stable ACC not as a precursor for subsequent crystalline phases but as a final mineral "of choice". The stable ACC usually serves for hardening skeletal elements and as a temporary calcium storage (Weiner et al., 2003). ACC has several potential biological advantages such as (1) isotropic structure, with no preferred growth directions which enables it to mold into any desired shape, (2) lack of cleavage planes, which makes it less brittle than its counterpart crystalline materials (Aizenberg et al., 2002), (3) high solubility combined with high surface area, which makes it a good temporary storage phase with improved resorption, and (4) ability to incorporate much higher levels of trace elements and impurities than crystalline phases (Bentov and Erez, 2006; Raz et al., 2000). Transient and stable phases of ACC differ in their structure and composition; transient ACC is a dehydrated phase, while stable ACC contains structural water at a 1:1 ratio (Addadi et al., 2003).

Stable ACC is widely used by crustaceans, due to their molting-mineralization cycles, which require intensive mineral mobilization and storage (Lowenstam and Weiner, 1989). ACC produced by crustaceans in the cuticle and storage organs is extremely stable and retains its amorphous character for months under ambient conditions (post mortem). The stabilization of the intrinsically unstable ACC requires the presence of additives in the precipitate that prevent it from transforming into the thermodynamically favored crystalline forms. *In vitro*, ACC precipitation has been achieved using different additives mainly phosphate-containing molecules (Clarkson et al., 1992; Gal et al., 1996; Sawada, 1997; Xu et al., 2005) or negatively charged polymers (Donners et al., 2000; Gower and Odom, 2000; Loste et al., 2003).

Biogenic ACC is probably induced and stabilized mainly by specific macromolecules designed for this purpose (Addadi et al., 2003). It was shown that macromolecules extracted from biogenic ACC skeletons induce the formation of ACC *in vitro* (Aizenberg et al., 1996, 2002; Raz et al., 2003; Shechter et al., 2008b). Aizenberg et al. (1996, 2002) found that such macromolecules extracted from the ACC parts of sponges (*Clathrina*) and ascidians (*Pyura pachydermatina*) are glycoproteins rich in glutamic acid and

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doi:10.1016/j.jsb.2010.04.007

hydroxyamino acids (which are potentially phosphorylated), as opposed to proteins extracted from the adjacent calcite phase, which are rich in aspartic acid. However, it is not known how these macromolecules interact with the ACC to hinder its crystallization.

In this study, we aimed at elucidating the possible roles of phosphorylated proteins in the formation of biogenic stable ACC. For this purpose we used stable ACC from the gastroliths, temporary calcium storage organs of the red claw crayfish *Cherax quadricarinatus*. The gastroliths are formed on both sides of the stomach wall, at the premolt stage, and are made of concentric layers of ACC nanospheres interlaced with α-chitin-protein microfibrils (Shechter et al., 2008a). The organic matrix of the gastroliths (total organic matrix or proteolytically cleaved peptides) was used as an additive for *in vitro* $CaCO_3$ precipitation. In order to test the possible role of the phosphoamino acid moieties in ACC stabilization, we further checked whether single phosphoamino acids can induce stable ACC formation.

2. Materials and methods

2.1. Animals

Cherax quadricarinatus males were grown in and collected from artificial ponds at Ben-Gurion University of the Negev, Beer Sheva, Israel. Animals were anesthetized in ice-cold water prior to dissection.

2.2. $CaCO_3$ precipitation method

Ten milliliters aliquots of 10–50 mM $CaCl_2$ solutions were precipitated in plastic vials, by adding Na_2CO_3 solution to make a 1:1 stoichiometric solution with $CaCl_2$. Precipitation was performed in the presence or absence of additives, as detailed below. Precipitation solutions were shaken vigorously for a few seconds and centrifuged at 4000 rpm for 5 min. The supernatant was decanted, and the precipitate (slurry) was washed once with doubly distilled water. The washed slurry was either smeared on a microscope glass slide and immediately dried by air flow, or freeze-dried by lyophilization. Precipitation of ACC with no additives was preformed as described by Koga et al. (1998).

2.3. Insoluble matrix (ISM) extraction and its use in $CaCO_3$ precipitation

Endocrinologically induced (Shechter et al., 2008b) premolt crayfish were dissected to extract their gastroliths. The gastroliths were weighed, rinsed with distilled water, and kept at −20 °C. The gastrolith external layer was mechanically scraped to remove residual organic material and ground to a powder using a mortar and pestle in liquid nitrogen to increase brittleness. One gram of gastrolith powder was dissolved in 20 ml of ammonium acetate (0.02 M) and EGTA (ethylene glycol tetraacetic acid, 0.5 M) solution, pH 7.0, on ice. After 12 h. the solution was centrifuged (2000 rpm, 15–20 min, 4 °C), and the supernatant containing the soluble fraction was collected. Two hundred microliters of residual ISM (pellet) were added to 10 ml precipitation solution as an additive, termed '*ISM-total*'.

2.4. ISM-peptides production and use in $CaCO_3$ precipitation

Two milliliters aliquots of '*ISM-total*' were subjected to proteolytic hydrolysis by trypsin (1.9 mg/ml) or protease (0.3 mg/ml) or papain (0.13 mg/ml) (Sigma) in 1.8 mm ammonium acetate (2 h; 4 °C; gentle mixing), followed by centrifugation (5 min; 4000 rpm). 1 ml of supernatant from each of the proteolytic peptide solutions, termed '*ISM-peptide*' was added to the $CaCO_3$ precipitation solution as described. The controls included proteolytic enzymes alone in equivalent concentration.

2.5. Phosphoprotein detection

SDS–PAGE analysis of ISM proteins: *ISM-total* aliquot was incubated overnight with 6 m urea (Sigma) at 4 °C with gentle agitation. The solution was dialyzed (12–14 kDa cutoff) for 24 h and lyophilized overnight. Protein content was determined by Bradford method (Bradford, 1976).

Qualitative phosphorylation detection of the gastrolith ISM proteins was performed using the fluorescent stains PhosDecor (Sigma) which selectively label phosphoproteins in SDS–PAGE. The experiment was performed according to the manufacturer's protocol. Calibration to total protein was done by Coomassie blue. Images were acquired using a 300 nm UV transilluminator ChemiGenius (Syngene, MD, USA). Band intensities were quantified using ImageJ software (NIH).

2.6. ^{45}Ca overlay

Detection of Ca^{2+} binding by gastrolith ISM proteins was performed by the calcium overlay assay (^{45}Ca autoradiography) (Maruyama et al., 1984). Samples were separated on SDS–PAGE and then electrophoretically transferred to a nitrocellulose membrane. The membrane was then incubated with ^{45}Ca, and autoradiography was used to detect calcium binding proteins as radioactive bands. The gel was stained with Ponceau's total protein staining (Sigma).

2.7. Raman spectroscopy

Raman spectra were acquired on a Jobin–Yvon LabRam HR 800 micro-Raman system equipped with a liquid-nitrogen cooled detector. Excitation was done with He–Ne laser line (633 nm). A 50× microscope objective, a 100-μm confocal hole, and a 600 grooves/mm grating were used, giving a resolution of 2–4 cm^{-1}.

2.8. Electron microscopy

TEM imaging and electron diffraction were carried out using an FEI Tecnai 12 TWIN operated at 120 keV. ACC precipitates were applied on TEM grids (Cu, 300 mesh, SPI Inc.).

2.9. SEM and energy dispersive spectrometry (EDS) analysis

Dried samples were sputter-coated with platinum or gold and examined by Jeol JSM-7400f.

2.10. XRD

Analysis was performed on precipitate samples spread on a quartz plate with no Bragg peaks (*The Gem Dugout*, USA). A Philips model PW-1050/70 diffractometer was used.

2.11. ICP-AES

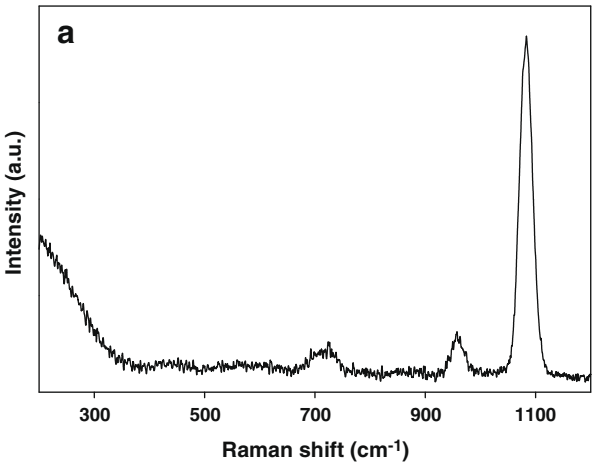
Calcium and phosphorous element content of the gastrolith were determined with inductively coupled plasma-atomic emission spectrograph (ICP-AES – Perkin-Elmer Optima 4300DV) elemental analysis. Powdered gastrolith samples (10 mg) were dissolved in 15 ml 0.5 M HCl solution and diluted to 50 ml with doubly distilled water.

3. Results

3.1. $CaCO_3$ precipitation with insoluble organic matrix and extracted peptides

$CaCO_3$ precipitation in the presence of gastrolith insoluble matrix (*ISM-total*) yielded stable ACC. This was confirmed by Raman spectra showing the v_1 vibration of carbonate at 1080 cm^{-1} as a broad band (Fig. 2), typical of ACC (Tlili et al., 2002). The resultant ACC appears as 50–250 nm spheres with broad size distribution (Fig. 3a). Precipitation in the presence of *ISM-peptides* also resulted in the formation of stable ACC (Fig. 2 and 3b). ACC obtained in both experiments (*ISM-total* and *ISM-peptides*) was stable and showed no sign of crystallization after more than four months under ambient conditions (Fig. 4a). ACC produced with no additives (Koga et al., 1998) was less stable and started transforming to vaterite within 2 weeks (Fig. 4e–f).

Precipitation of $CaCO_3$ in the presence of the residual chitinous material (after *ISM-peptides* extraction), yielded calcite (Fig. 2). Control experiments of precipitation with the proteolytic enzymes alone produced calcite (data not shown).



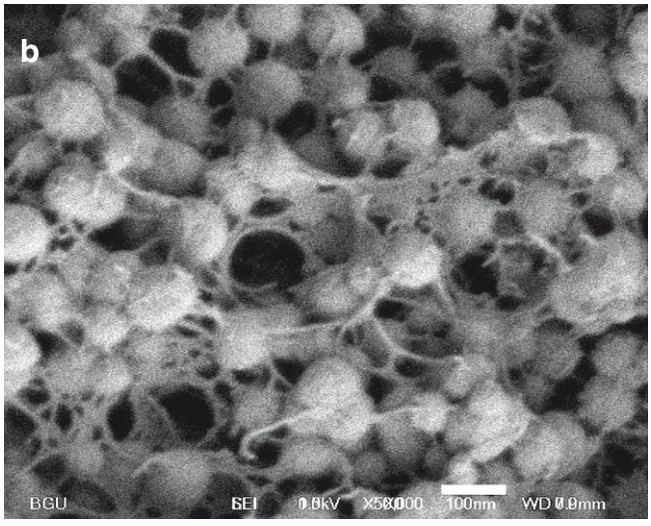


Fig. 1. Biogenic ACC from the gastrolith of *C. quadricarinatus*. (a) Raman spectra of the ACC showing the v_1 vibration of carbonate as a broad band at 1080 cm^{-1}, typical of ACC, and the v_4 vibration of carbonate at 720 cm^{-1}. In addition, note the significant broad band at 955 cm^{-1} which corresponds to the v_1 vibration of phosphate. (b) SEM micrograph of gastrolith section showing chitinous matrix fibrils interwoven with the ACC nanospheres (bar = 100 nm).

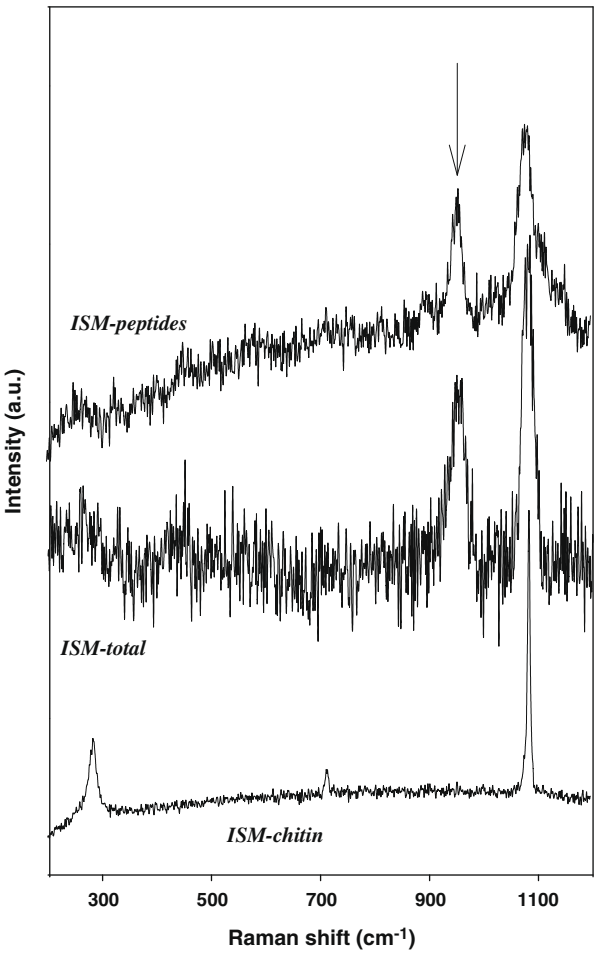


Fig. 2. Raman spectra of $CaCO_3$ precipitated in the presence of insoluble matrix fractions. *ISM-total* and *ISM-peptides* induced the precipitation of ACC while in the presence of the residual chitin (*ISM-chitin*), calcite was precipitated. v_1 vibration of carbonate is located around 1080 cm^{-1} in ACC, and at 1085 cm^{-1} in calcite. v_4 vibration is located at 712 cm^{-1} in calcite. The broad band at 955 cm^{-1} (arrow), corresponds to v_1 vibration of phosphate.

In addition to the main Raman shift of carbonate centered at 1080 cm^{-1}, a broad band at 955 cm^{-1} (Fig. 2 arrow) assigned to the v_1 vibration of phosphate is observed in ISM containing samples. This phosphate represents organic phosphate which is incorporated into the synthetic ACC.

3.2. Detection of phosphoproteins

Specific fluorescent staining of phosphoproteins in SDS–PAGE with PhosDecor has indicated the presence of several phosphoproteins in the ISM, with a prominent phosphoprotein doublet band at ~70–75 kDa (Fig. 5a and b). Semi-quantitative comparison of phosphate content of this doublet and β-casein, (2.4% phosphoresidues) showed that the 70–75 kDa ISM doublet is approximately 5 times richer in phosphoresidues (~12% phosphoresidues). A calcium overlay assay demonstrated that these doublet proteins also have the highest calcium binding capacity among gastrolith proteins (Fig. 5c and d).

3.3. Total phosphorus content (inorganic + organic) in the gastrolith

Chemical analysis of gastrolith composition with ICP-AES showed that the total (organic + inorganic) P/Ca molar ratio in the gastrolith is in the range of 10–15 mol%. EDS analyses of the

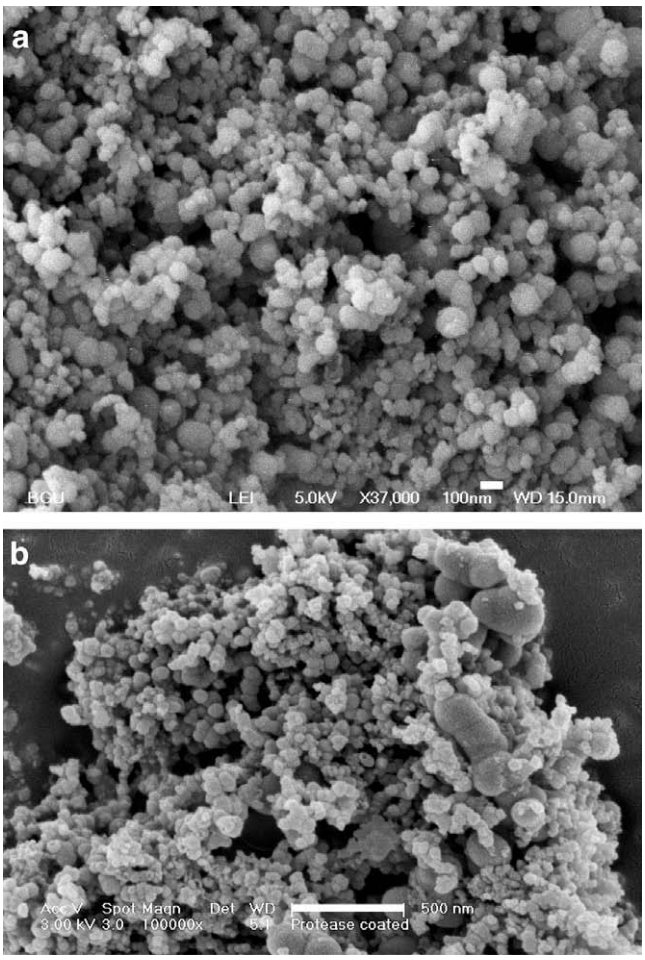


Fig. 3. SEM micrographs of synthetic ACC. (a) ACC comprising 50–250 nm nanospheres produced with the addition of *ISM-total* (bar = 100 nm). (b) ACC comprising 50–250 nm nanospheres produced with *ISM-peptides* extracted by proteases (bar = 500 nm).

gastrolith indicated that the phosphorous content is highly variable with P/Ca ratio ranging from 2 to 25 mol%.

3.4. $CaCO_3$ precipitation with phosphoamino acids

Motivated by the high phosphoprotein content in the ISM, its incorporation into synthetic ACC and the known inhibitory effect of phosphorylated molecules on $CaCO_3$ crystallization (Burton and Walter, 1990; House, 1987; Lin and Singer, 2006; Reddy, 1977), we tested the inhibitory effect of single phosphoamino acids on $CaCO_3$ crystallization.

$CaCO_3$ precipitation in the presence of phosphoamino acids shows that P-Ser and P-Thr induce the formation of stable ACC. In contrast, precipitation in the presence of phosphotyrosine or non-phosphorylated serine resulted in the formation of calcite and vaterite, respectively (Fig. 6). The amorphous nature of the precipitates was determined by Raman, SEM, XRD, and electron diffraction analysis. The Raman spectra (Fig. 6) show a broad v_1 carbonate vibration band at 1080 cm^{-1}. SEM micrographs (Fig. 7) demonstrate that the resultant ACC particles are aggregates of 20-nm to 300-nm polydispersed nanospheres. XRD spectra show that the samples were entirely amorphous with no sign of crystallized phases (Fig. 8a). Selected area electron diffraction analysis (SAED) also confirms the amorphous nature of these precipitates (Fig. 8b and c). The resultant ACC was stable for more than four

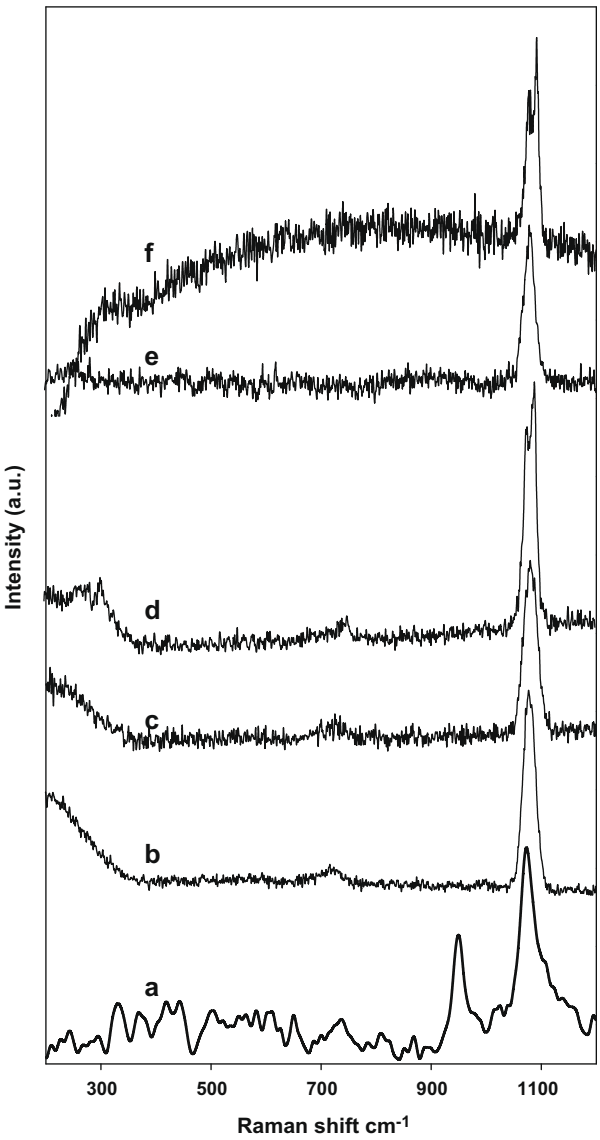


Fig. 4. ACC stability under ambient room conditions. (a) ACC produced with *ISM-peptides*, after 5 months. (b) ACC produced with P-ser, after 4 months. (c) ACC produced with P-Thr, after 4 months. (d) ACC produced with P-Thr after 5 months, partially transformed to vaterite as indicated by the split bands of v_1 and v_4 carbonate modes (around 1080 and 740 cm^{-1}, respectively). (e) Fresh ACC produced with no additives at high pH. (f) The sample of (e) 2 weeks later: the ACC has transformed to vaterite as indicated by the split band of v_1 carbonate mode around 1080 cm^{-1}.

months under ambient conditions, showing no sign of crystallization processes (Fig. 4b and c). Only after 5 months the precipitate partially crystallized to vaterite (Fig. 4d).

The effective concentration of P-Ser and P-Thr required for ACC formation was in the range of 0.1–5 mM (for $CaCO_3$ concentration of 10 mM). Lower concentrations gave $CaCO_3$ mixtures of ACC, calcite and vaterite, while higher concentrations completely inhibited all spontaneous precipitation.

4. Discussion

The results of the precipitation experiments with the *ISM-total* and *ISM-peptides* demonstrate that insoluble proteins play a major role in the formation of stable ACC in the gastrolith. It should be noted that the "soluble"–"insoluble" matrix definition is empirical,

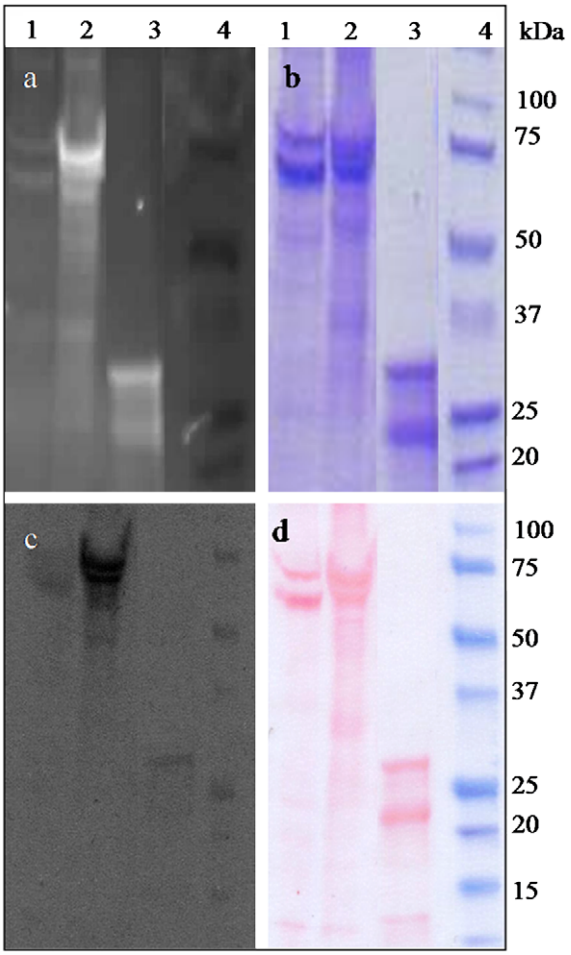


Fig. 5. Phosphoprotein detection and calcium overlay assay of gastrolith proteins. Lane 1, gastrolith soluble proteins; lane 2, insoluble proteins (ISM); lane 3, β-casein (positive control); lane 4, MW ladder. (a) Labeling with the fluorescent probe PhosDecor (Sigma) for phosphoproteins. (b) The same gel stained for total protein with Coomassie blue. (c) ^{45}Ca overlay. (d) The same gel stained for total protein with Ponceau S dye.

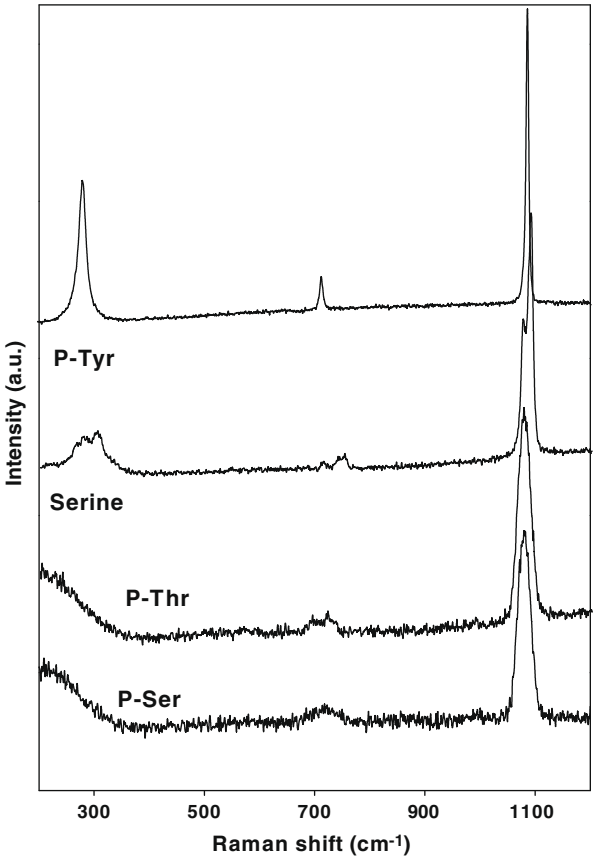


Fig. 6. Raman spectra of $CaCO_3$ in the presence of P-Ser (ACC), P-Thr (ACC), non-phosphorylated serine (vaterite), and phosphotyrosine, (calcite). ν_1 vibration of carbonate appears as a broad band at 1080 cm^{-1} in ACC, as a split band at 1075, 1091 cm^{-1} in vaterite, and as a sharp band at 1085 cm^{-1} in calcite. ν_4 vibration of carbonate appears at 720 cm^{-1} in ACC, as a split band at 740 and 750 cm^{-1} in vaterite and at 712 cm^{-1} in calcite.

based on the employed extraction procedures. Following demineralization the gastrolith organic matrix is divided into a soluble matrix fraction that contains mainly hydrophilic proteins, and an insoluble matrix (ISM) that contains chitin with associated proteins. In crustaceans, the insoluble 'chitin–protein complex' is quantitatively dominant (Welinder, 1975) and is thought to play a major role in directing the calcification process (Lowenstam and Weiner, 1989; Roer and Dillaman, 1984). The "insoluble" proteins are associated with the chitin matrix through various kinds of bonds (such as covalent, ionic, and hydrogen bonds, or vander-Waal's force (Diaz-Rojas et al., 2006)). It is possible that some of the weakly bound proteins will dissociate from the chitin during demineralization. It is also possible that during demineralization negatively charged "soluble" proteins will associate with amine moieties of chitin (Kato, 2000) and be incorporated to the "insoluble" fraction. For example, the gastrolith protein 65 (GAP 65) which was found to inhibit crystallization (Shechter et al., 2008b) is found in both "soluble" and "insoluble" fractions of the gastrolith matrix.

The fact that a similar effect of ACC stabilization was achieved by intact proteins (*ISM-total*) and protein fragments extracted by proteolysis (*ISM-peptides*), suggests that the proteins' structure and conformations are less crucial for the process. Interactions between specific proteins and structured mineral faces are probably necessary for controlling biominerals' crystal polymorph, orientation, and texture (Berman et al., 1990; Weiner and Addadi, 1997). In contrast, it was shown *in vitro* that ACC is induced and stabilized at surfaces presenting mixed repertoire of chemical interacting moieties (Aizenberg et al., 2003).

Raman spectra of the synthetic ACC precipitated in the presence of ISM proteins and peptides (Fig. 2) show considerable amounts of phosphate associated with the ACC mineral phase. This phosphate could have only originated from the organic matrix phosphoproteins incorporated (entrapped) into the ACC. It is noteworthy that the Raman peak of the organic phosphate associated with the ACC is indistinguishable from the band reported for inorganic ACP (Kazanci et al., 2006; Mitchell et al., 1996; Raz et al., 2002). Therefore, a similar Raman shift which appears in the spectra of gastrolith ACC and in other biogenic ACC (Addadi et al., 2003; Hild et al., 2008; Raz et al., 2003) may represent both organic and inorganic phosphates.

Detection of phosphoproteins in SDS–PAGE has indicated the presence of several phosphoproteins with a heavily phosphorylated doublet band at ~70–75 kDa. The same phosphoprotein bands also show high calcium binding capacity (Fig. 5).

Phosphoproteins from bivalves were also shown to effectively inhibit calcium carbonate crystallization (Borbas et al., 1991; Halloran and Donachy, 1995). Dephosphorylation of these proteins resulted in an almost complete loss of their inhibitory activity (Borbas et al., 1991; Halloran and Donachy, 1995). Two proteins associated with stable crustacean ACC, CAP1 (Inoue et al., 2001) and orchestin (Hecker et al., 2003), were found to be phosphorylated. It was demonstrated that the phosphorylation of CAP1 is

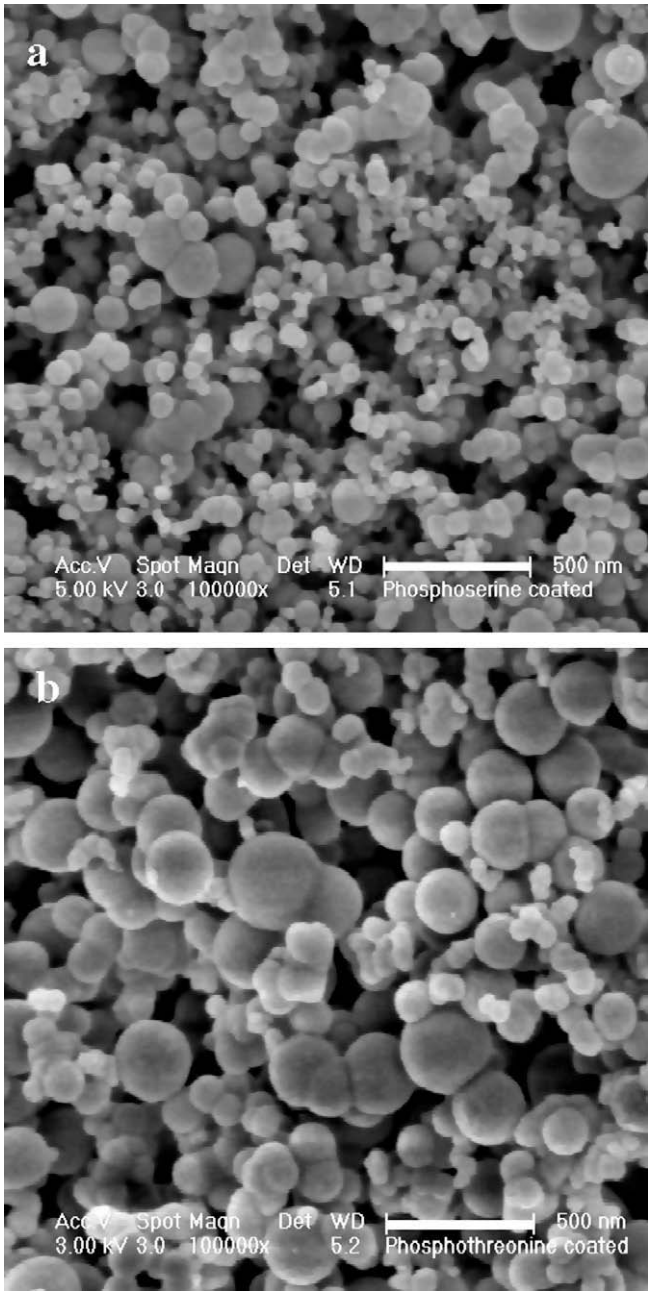


Fig. 7. (a) SEM micrographs of ACC produced in the presence of P-Ser comprising 20-nm to 200-nm nanospheres (bar = 500 nm). (b) ACC induced by P-Thr (1 mM), composed of 50-nm to 300-nm nanospheres (bar = 500 nm).

essential for the inhibition of calcium carbonate precipitation (Inoue et al., 2003) and that of orchestin is essential for its calcium-binding ability (Hecker et al., 2003). *C. quadricarinatus* gastrolith GAP 65 protein was found to induce ACC formation *in vitro*; approximately 4.6% of GAP 65 residues are potential phosphorylation sites (Shechter et al., 2008b).

The role of phosphoproteins is even more pronounced in the biomineralization of calcium phosphate (Veis et al., 1991). Phosphoproteins have been shown to be a prominent constituent of the extracellular matrix of bone and dentin and have been implicated as key participants in the regulation of mineralization, either as crystallization inhibitors or initiators (George and Veis, 2008). The inhibitory effect of most of these phosphoproteins (e.g., osteopontin, DMP1, PRP) was eliminated or sharply decreased after dephosphorylation (Boskey et al., 1993; Hunter et al., 2009; Tartaix et al., 2004). It was further shown that the degree of ACP stabilization by osteopontin is dependent on its phosphorylation degree (Gericke et al., 2005; Holt et al., 2009), indicating a crucial role of the phosphoamino acid residues.

$CaCO_3$ precipitation in the presence of the residual chitinous fraction, after proteolysis, yielded calcite crystals with no observable ACC formation, in agreement with earlier observations (Kato, 2000). Nevertheless, it is possible that in natural biogenic structures such as the gastrolith and the cuticle, the chitinous framework improves the stability of the amorphous phase by the spatial arrangement of the ACC nano-spherules at discrete positions (Fig. 1). Transiently stabilized ACC was reported to crystallize by coalescence and aggregation of hydrated precursor "droplets" (Gower, 2008; Gower and Odom, 2000). The chitinous framework thus may increase ACC stability by inhibiting the interactions between adjacent ACC spherules and hinder their assembly and coalescence.

Although the present study deals exclusively with organic phosphate it should be noted that biogenic stable ACC frequently contains considerable amounts of inorganic phosphate. In the gastroliths of *C. quadricarinatus* the average P/Ca ratio is 10–15 mol%, in agreement with others' estimates of phosphate content in gastroliths from other species (Huxley, 1880; Scott and Duncan, 1967). Similarly, most if not all biogenic stable ACC from various taxa are reported to contain considerable amounts of phosphorous in wide concentration ranges (Aizenberg et al., 2002; Huxley, 1880; Lowenstam and Weiner, 1989; Prenant, 1927; Raz et al., 2002; Scott and Duncan, 1967; Vinogradov, 1953; Weiner et al., 2003). The phosphorus content in biogenic ACC structures is attributed mostly to inorganic phosphate in the form of ACP (Becker et al., 2005; Hild et al., 2008; Neues et al., 2007; Raz et al., 2002). The known retarding effect of phosphate on calcite growth (Burton and Walter, 1990; House, 1987; Lin and Singer, 2006; Reddy, 1977) may suggest that inorganic phosphate plays a cooperative role with the organic matrix, in ACC stabilization (Hild et al., 2008; Weiner et al., 2003).

Stable ACC formation in the presence of P-Ser or P-Thr demonstrates that these phosphoamino acids are efficient additives in ACC stabilization. We assume that these additives mimic the effect of phosphoproteins in biogenic ACC stabilization. Natural phosphoproteins may have additional structural functions, but the inhibition of $CaCO_3$ crystallization probably takes place via phosphoamino acid residues.

Acidic amino acids interact with growing $CaCO_3$ crystals and modify their growth habit through strong, electrostatic interaction with calcium ions (Orme et al., 2001). Phosphoamino acids have a stronger binding affinity to calcium salts due to their highly charged phosphate residue in addition to their α-carboxylate moiety. However, comparison of the effective inhibition exhibited by P-Ser and P-Thr and the inefficient activity of phosphotyrosine (P-Tyr), suggests that the presence of a phosphate moiety alone cannot inhibit crystallization; the nature of the side chain of the amino acid is likely to also play a role in the process.

In addition to the strong binding to calcium salts, phosphoamino acids also have high binding capacity to calcium; studies on phosphoproteins from bivalves and vertebrates (Marsh, 1989; Marsh and Sass, 1984) show that phosphoproteins can bind up to 1.33 calcium ions per organic phosphate residue, which is a very high binding capacity even compared to highly acidic polyaspartate peptides (Gower, 2008). The details of the interactions through which phosphoamino acids stabilize ACC are not yet clear; the different theories about ACC stabilization by additives (reviewed in Addadi et al. (2003) and Gower (2008)) could also be applied to the action of the phosphoamino acids.

The precipitation of calcium carbonate from a supersaturated solution system seems to obey the "Ostwald rule of stages" (Nyvlt,

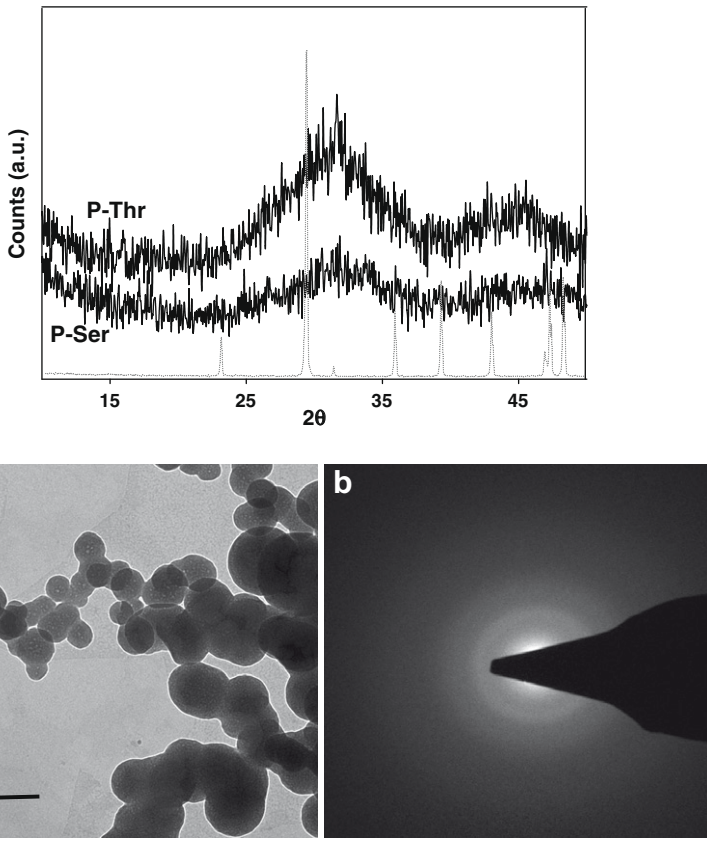


Fig. 8. X-ray and electron diffraction of synthetic ACC. (a) XRD diffractogram of ACC precipitated in the presence of 2 mM P-Ser and in the presence of 1 mM P-Thr; diffractogram of calcite is shown in gray. (b) TEM image of ACC produced in the presence of P-Thr (bar = 200 nm). (c) Selected area electron diffraction acquired from this region, demonstrates the amorphous nature of the precipitate.

1995). According to this rule, in a calcium carbonate system, ACC precipitates first, followed by transformation to vaterite, a metastable polymorph, and finally to the stable form – calcite or aragonite. This transformation sequence is governed by both thermodynamic and kinetic considerations (Navrotsky, 2004). Impurities incorporated into the mineral can alter the normal transformation process. The impurities can alter the solubility of the ACC and/or modify the energy barriers between the reaction steps, thus slowing the kinetics of the transformation from ACC to crystalline phases. The additives may also cap the mineral surface and form internal interfaces that slow down the dissolution–recrystallization process. It was shown that the crystallization of transiently stabilized ACC is associated with the exclusion of impurities from the crystallizing phase (Aizenberg et al., 2003; Dai et al., 2008; Gower, 2008; Gower and Odom, 2000). In contrast, long term stabilization of ACC probably requires permanent incorporation of additives into the mineral phase to prevent dissolution–recrystallization and solid state transformation after ACC formation. It is possible that stable ACC formation is induced by impurities which are strongly bound to the mineral phase and sustain exclusion. The energy required for this bond dissociation, raises the energy barriers between ACC and crystalline phases thus kinetically slowing the crystallization of the ACC. It is plausible that incorporated impurities such as phosphoproteins and phosphoamino acids which have a strong affinity to calcium may serve as ACC stabilizing agents in biological systems.

It should be noted that although ISM and phosphoamino acid induces stable ACC formation *in vitro*, this ACC is still less stable than biogenic stable ACC which maintains its amorphous nature in a wet (physiological) environment as well, as is the case in the crustacean gastrolith (Shechter et al., 2008a). The enhanced stability of the biogenic ACC may be attributed to other factors such as (1) the spatial arrangement of the ACC nanospheres within the chitinous scaffold, as postulated above, (2) the incorporation (by coprecipitation or adsorption) of inorganic ions such as phosphate and magnesium, (3) the composition of the aqueous solution which surrounds the ACC structure (Akhtar et al., 2006), and (4) interactions of other functional groups of matrix proteins with the ACC (e.g., glutamate enriched region (Aizenberg et al., 1996) or hydrophobic sites). As was argued for controlled biomineralization in general (Gebauer et al., 2009), the formation and maintenance of stable ACC may require several different additives and inhibitory agents. Nevertheless, our results suggest that phosphoproteins, via their phosphoamino acid moieties, are central players in this complex mechanism.

In conclusion, we showed that organic matrix extracted from gastroliths of the crayfish *C. quadricarinatus* is highly phosphorylated and that it can induce stable ACC formation *in vitro*. We have identified the major phosphoproteins of the organic matrix and showed that they have high calcium binding capacity. It was further demonstrated that single phosphoamino acids (phosphoserine or phosphothreonine) can induce the formation of highly stable ACC, *in vitro*. The results suggest that phosphoproteins play a key role in the stabilization of ACC, and that their phosphorylated residues per se are central components in this mechanism. The similar effect exhibited by single phosphoamino acids emphasizes the role of the individual residue as the "minimum requirement" for ACC stabilization, rather than protein conformation. The results of this study may advance our fundamental understanding of the biological mechanism of ACC stabilization. The elucidation of a "simplified"

mechanism of biogenic stable ACC formation bears potential for fabrication of biomimetic, amorphous materials of substantial scientific and industrial importance.

Acknowledgments

We would like to thank Mr. Shahar Shimshon and Mr. Roni Nisan for technical assistance. This research was supported in part by a grant from Amorphical Ltd. through B.G. Negev Ltd.

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