# Mollusk Shell Acidic Proteins: In Search of Individual Functions

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*Acidic proteins play a major role in the biomineralization process. These proteins are generally thought to control mineral formation and growth. Thus, characterization of individual acidic proteins is important as a first step toward linking function to individual proteins, which is our ultimate goal. In order to characterize the protein(s) responsible for the assemblage of biominerals, a new gel electrophoresis fixing and staining protocol was developed and many, if not all of the acidic proteins were visualized on the gel for the first time. In an in vitro assay we show that proteins extracted from an aragonitic shell layer induce the formation of amorphous calcium carbonate prior to its transformation into the aragonitic crystalline form. This study removes some major obstacles in the characterization of acidic proteins and sheds more light on the functions of these proteins in the biomineralization process.*

**KEYWORDS:**

aragonite · biomineralization · electrophoresis · glycoproteins · mollusk shell

## Introduction

One of the most remarkable attributes of the mineralized tissues formed by many different phyla is that they contain a most unusual assemblage of proteins and glycoproteins that are very acidic.[1] Most of the unusually acidic proteins found in aragonite- or calcite-containing mineralized tissues of invertebrates are rich in aspartic acid.[2] In the case of the proteins from scleractinian corals, for example, Asp can constitute between 40 and 50 mole percent of the protein assemblage.[3] In mollusks, Asp usually makes up around 30 mole percent of the aragonitic layer and more than 50 mole percent of some calcitic layers.[4, 5] A second group of acidic proteins discovered more recently in invertebrates are rich in glutamic acid and/or glutamine.[6] These proteins are associated with amorphous calcium carbonate rather than with the crystalline forms of calcium carbonate. Very little is understood about the specific functions of either group of unusually acidic proteins, which is the topic of this study.

There are several circumstantial observations that indicate that the acidic proteins are directly involved in controlling mineral formation. In many vertebrate and invertebrate mineralized tissues the acidic proteins can only be extracted if the mineral is dissolved.[7, 8] These proteins are thus intimately associated with the mineral phase. In some cases it has been shown that a subset of the acidic proteins is actually occluded within the mineral phase, where the proteins influence the mechanical properties of the mineral.[9] There is very little direct in vivo evidence to prove that one or more of these macromolecules actually function as nucleators of mineral formation or as modulators of mineral growth. There is, however, a large amount of literature on in vitro experiments, which show that these macromolecules are indeed able to specifically modulate mineral formation.

In many of the in vitro functional assay systems, acidic macromolecules are added to saturated solutions and their effect on crystal nucleation and growth is monitored.[10] It is, however, difficult to differentiate between the contributions of general parameters such as charge density and specific effects due to structural matching of the protein or associated polysaccharide moieties to the mineral. Another approach is to observe changes in the morphologies of crystals grown in the presence of one or more acidic macromolecule in solution.[11–13] These changes, if specific, show that the macromolecule can interact with some crystal faces and not others. One functional assay for assessing specific nucleating effects of mollusk-shell-associated proteins, which in our hands has proved to be reliable, involves the addition of the acidic macromolecules to an in vitro assembly of $\beta$-chitin and silk fibroin, the two major components of the mollusk shell organic matrix. Falini et. al.[14] showed that if the assemblage of unusually acidic proteins from an aragonite layer is added, then aragonite is formed, and if the proteins are derived from a calcitic layer, then calcite forms. This assay thus demonstrates that within the assemblage there are components that nucleate the mineral phase such that polymorph specificity is maintained. Another assay for polymorph specificity was developed by Belcher et al.[15] The Falini et al. assay has been used to identify groups of macromolecules separated by ion exchange chromatography that are responsible for aragonite nucleation.[16] The assay can of course also be used to determine the nucleating capability of individual macro-

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molecules, and can thus serve as the link between structure and function.

A major problem is that it is very difficult to purify and characterize these macromolecules. Some of the reasons are as follows: As these macromolecules are intimately associated with the mineral phase and are highly charged, they are very difficult to extract from the mineralized tissue in such a way that they remain intact and functional. Once extracted the molecules readily aggregate and are thus difficult to separate chromatographically. Many of these macromolecules are also glycosylated,[7] which further complicates their chromatographic fractionation. Furthermore, we and others[17] have repeatedly observed that when the assemblages of acidic macromolecules from various mineralized tissues are subjected to separation by gel electrophoresis, some of the observed bands are diffuse and smeared, whereas others are sharp. The sharp bands can usually be stained with Coomassie Blue and silver, commonly used protein stains, whereas the diffuse bands require special conditions for effective staining, or appear to be weakly stained. Thus, purification and characterization at the protein level has proved to be a major challenge.

Only recently have the sequences of some of the unusually acidic proteins from mineralized tissues been determined. Examples include a protein from vertebrate dentin (DMP-1)[18] and one from mollusk shells (MSP-1).[19] In some cases sequencing has been achieved by working at the DNA level and not with the proteins per se. This approach aggravates the problem of linking structure to function. Various proteins have been isolated and sequenced from mineralized tissues. However, almost all of these are not the unusually acidic ones, but those that are readily stained with Coomassie Blue and/or silver stain. Nine proteins of this type have been sequenced from mollusk shell organic matrices.[19–25] Purification, characterization, and functional determination of the unusually acidic proteins from mineralized tissues is fraught with numerous technical problems.

In this study we address several related aspects of this problem with the ultimate aim of relating the structures of individual acidic macromolecules to their functions. We focus on the problem of mollusk shell formation, and in particular the formation of the nacreous layer of the bivalve *Atrina*. The overall strategy is to optimize extraction, purification, and characterization procedures that are compatible with the use of the Falini et. al. nucleation assay system. We have further investigated the function of the total protein assembly extracted from the mollusk shell with the Falini et. al. system itself. We used this assay in order to distinguish between different protein functions in the assembly and focused on aragonite-nucleating proteins, which were further purified and characterized. We concentrated on aragonite nucleation rather than calcite nucleation since aragonite is the less stable polymorph and the requirements for its formation are more stringent. Aragonite nucleation was monitored at each purification step. Thus, the specificity of the purified proteins was assured and we could determine whether or not more than one component is required for specific nucleation. In this way, we purified and characterized proteins with a well-defined function.

## Results

### Protein extraction from the mollusk shell

Proteins from the aragonitic nacreous layer of the mollusk *Atrina rigida* are extracted by using an ion-exchange resin for dissolution of the mineral.[12] With this method, demineralization is performed in the absence of any chelating agent or acidic reagent. Therefore, the proteins are extracted in a relatively well-preserved state without any additives. The average yield of proteins is 0.01 weight% (calculated as a proportion of mineral weight), according to amino acid analysis. Amino acid compositional analysis shows that these proteins are very acidic (45% Asx + Glx) and contain almost no aromatic residues (Table 1). Falini et. al.[14] proved that only the acidic macromolecules extracted from mollusk shells by using the ion exchange demineralization method are able to specifically control calcium

***Table 1.*** *Amino acid compositions of the soluble proteins from* Atrina rigida *in different stages of purification.*[a]

| Amino acid | Soluble protein assembly extracted from nacre | Nucleating fraction purified on an anion-exchange column | Nucleating fraction purified on a gel-filtration column |
|---|---|---|---|
| **Asx**[b] | **29.7** | **36.2** | **35.6** |
| **Glx**[b] | **14.8** | **13.4** | **5** |
| **Ser** | **13.6** | **19.3** | **24.2** |
| **Gly** | **18.2** | **14.3** | **13.7** |
| His | 0.6 | 0.5 | 0 |
| Thr | 3.3 | 2 | 1.4 |
| Ala | 9.5 | 6.2 | 3.8 |
| Arg | 1.8 | 0.5 | 0 |
| Tyr | 1.9 | 0.8 | 0.5 |
| Cys – Cys | 1.2 | 0 | 0 |
| Val | 2.8 | 1.7 | 1.6 |
| Met | 1.2 | 0.4 | 0.3 |
| Ile | 1.9 | 0.8 | 2.3 |
| Phe | 2 | 1 | 0.7 |
| Leu | 3 | 1 | 0.7 |
| Lys | 1.9 | 0.8 | 1.8 |
| Pro | 3.7 | 1 | 8.4 |

[a] The values shown are average mole%. [b] Asx = Asp or Asn; Glx = Glu or Gln.

  

carbonate polymorphism when they are adsorbed on a chitin and silk assembly. Accordingly, the assembly of proteins extracted from the nacreous layer, once adsorbed on the chitin – silk scaffold induced only aragonite formation (as detected under dehydrated conditions). When proteins were extracted by using acetic acid or ethylenediaminetetraacetate (EDTA), the ability to specifically form aragonite in the chitin – silk assembly was lost.

### Identification of nacre water-soluble proteins by gel electrophoresis

The most widely used technique for determining the purity of fractions obtained from chromatographic columns is gel electrophoresis. Purified bands from gels can also be used for obtaining the protein sequence. As noted above, many problems have been encountered when using gel electrophoresis to fractionate acidic proteins from mineralized tissues. We subjected total extracts from *Atrina* shell, fractions from the anion exchange column, and fractions from the gel filtration column to standard electrophoresis conditions, with Coomassie Brilliant Blue or silver nitrate as staining agents. No proteins were observed when Coomassie Blue was used, and only a few bands were poorly visualized with silver nitrate. Several other staining methods and agents were tested, such as fluorescent staining, Stains-All, and Alcian Blue. None of these approaches showed any significant improvement. This result raised the possibility that staining is not the problem, but that these proteins may have a pronounced tendency to diffuse out of the gel because they are very acidic.

We therefore developed new fixing conditions that involve the simultaneous use of two reagents, formaldehyde and glutaraldehyde, to trap the proteins in the gel before staining. Notwithstanding this extreme fixation, when the fixation procedure was performed overnight on the total assembly of proteins, only two major bands were visualized (Figure 1 a). When the fixation was reduced to a few hours, however, protein detection was dramatically improved (Figure 1 b). By performing this short and massive fixation procedure on tris(hydroxymethyl)aminomethane (Tris)-tricine gradient gels (10 – 20 %), tens of different soluble proteins with a broad range of molecular weights were visualized in the total extract. To our knowledge, these are the best results ever obtained by using gel electrophoresis to fractionate these elusive matrix proteins.

When the same fixation procedure was used and the gels were stained with Coomassie Blue, only a few weak, almost undetectable bands were observed (Figure 1 c). This result is in sharp contrast to that seen when staining was carried out with silver nitrate (Figure 1 b). This outcome shows that Coomassie Blue does not bind to these acidic proteins, which is consistent with the observation that the Coomassie dye interacts with basic and hydrophobic amino acids in polypeptides chains.[26, 27] Note that with these gradient gels, the molecular weight markers do not migrate according to their true molecular weights (compare standards in Figure 1 and Figure 3).

### Isolation and purification of aragonite-inducing proteins

We followed the same approach as Levi et al.[16] for purifying aragonite-inducing fractions of macromolecules. The total protein assembly extracted from the *Atrina* nacre was fractionated by fast performance liquid chromatography (FPLC) on an anion-exchange column, with a 20-minute linear elution gradient of 0.2 M – 0.5 M NaCl. Figure 2 shows a typical chromatogram of the eluting fractions. We noted that the resolution of the peaks is clearly dependent on the freshness of the shells. The chromatograms of extracts from beach-collected shells were less well resolved as compared to those from shells frozen when fresh. Each fraction isolated from the FPLC was assayed separately by using the Falini et. al. system. Only Fraction I (Figure 2), which eluted at 0.44 M NaCl, induced massive crystallization of pure aragonite in the form of spherulites inside the chitin framework. Other fractions induced either a mixture of calcite and aragonite or only calcite formation. In particular, the

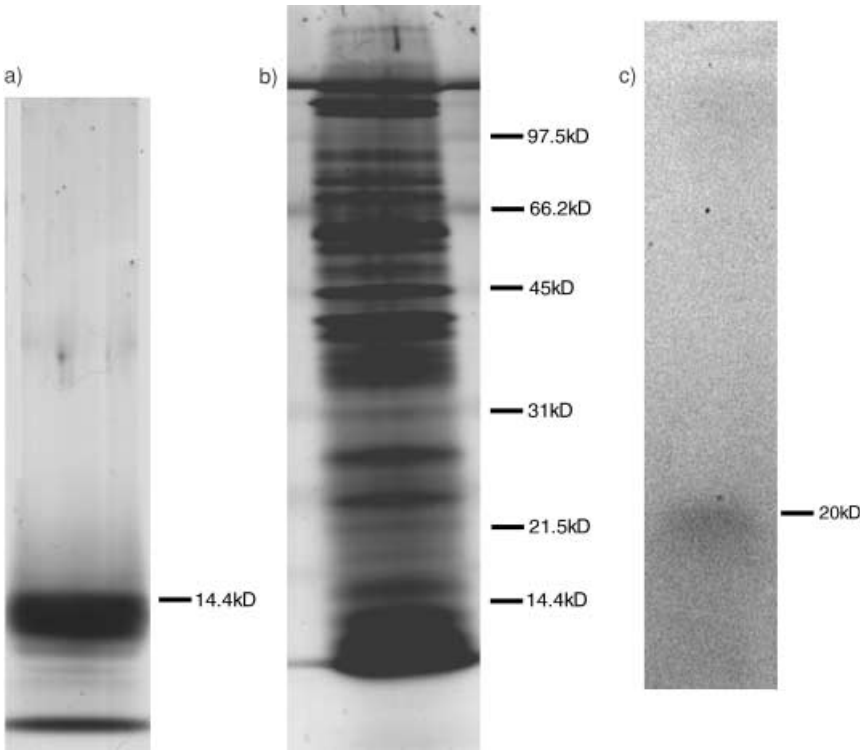


***Figure 1.*** *Tris-Tricin polyacrylamide gradient gel (10 – 20 %) of the total assembly of acidic macromolecules extracted from the aragonitic layer of the mollusk* Atrina *stained with silver nitrate after double fixation with two reagents (formaldehyde and glutaraldehyde). a) Fixed with formaldehyde overnight before staining. b) The fixation stage was shortened to one hour. c) Fixation as in (b), stained with Coomassie Blue. The molecular weight standard mixture used is the broad-range protein standard (Bio-Rad).*

  

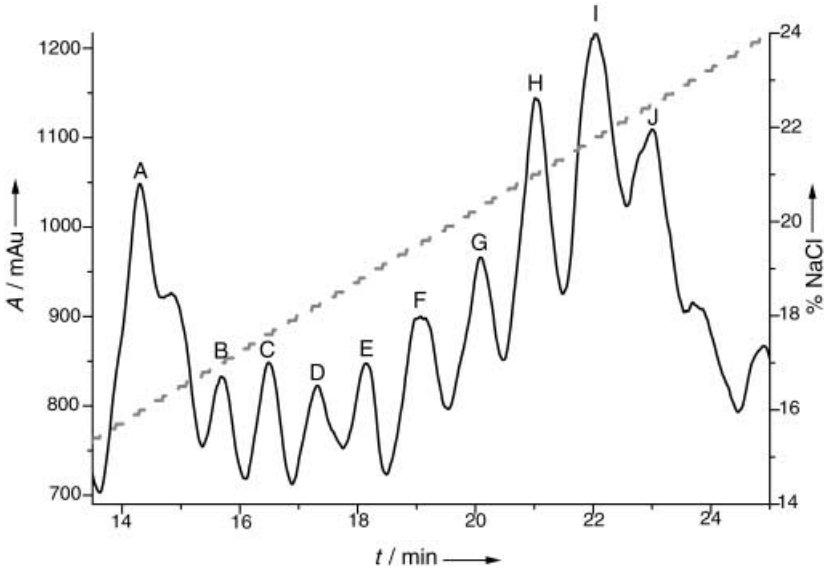


***Figure 2.*** *Mono Q anion-exchange chromatographic separation of the total assemblage of soluble proteins extracted from the nacreous layer of* Atrina. *The dashed line represents the salt gradient applied during the elution (100% NaCl corresponds to a concentration of 2 M). The solid line represents absorbance at 220 nm. Fraction I induced massive crystallization only of aragonite.*

fraction that eluted at the highest concentration of salt (Fraction J) induced only calcite, as was detected by Raman imaging spectroscopy and infra red spectroscopy.

In the absence of adsorbed proteins, no crystallization occurs inside the chitin – silk substrate, which indicates that aragonite formation is due to the presence of specific proteins. The proteins in the aragonite-nucleating fraction were active at a concentration as low as 20 nmol amino acid $mL^{-1}$ (according to amino acid analysis), which is five times less than the concentration used with the total protein assemblage adsorbed on the chitin – silk substrate (100 nmoles amino acid $mL^{-1}$). The amino acid composition of the nucleating fraction shows that the proteins are composed of about 50% acidic residues (Asx and Glx), that is, these proteins are much more acidic than the total protein assemblage (Table 1). We analyzed the constituents of the aragonite-nucleating fraction from the anion-exchange column by gel electrophoresis (Figure 3a). The nucleating fraction contains seven different bands in the range 3 – 30 kD (according to polypeptide molecular weight standards). In the region between 14.5 kD and 3.5 kD the gel does not contain defined bands. Extraction of this region yielded a protein composed of 17% Asx and 10% Glx. In general amino acid analyses of different protein bands extracted from the gel show that the bands that run at the front of the gel are the most acidic (30% Asx residues).

The aragonite-nucleating fraction was further purified by gel filtration FPLC (Figure 4) and each fraction was tested separately with the crystallization assay. Fraction 1 induced massive crystallization exclusively of aragonite at concentrations as low as 10 nmol amino acid $mL^{-1}$, while Fraction 2 induced formation of very few aragonite spherulites, and Fraction 4 induced no crystallization. The amino acid composition of Fraction 1 is very similar to that of the nucleating fraction isolated from the anion-exchange column (Table 1). Gel electrophoresis of Fraction 1 (Figure 3b)

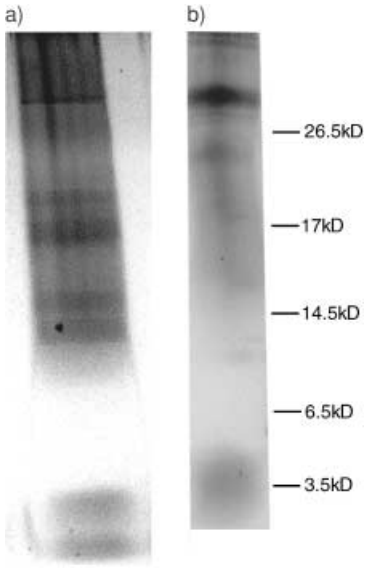


***Figure 3.*** *Tris-Tricin polyacrylamide gradient gel (10 – 20%) of the aragonite-nucleating fraction purified by an anion-exchange column (a) then further purified on a gel-filtration column (b). Note that in these gels the standard is a polypeptide molecular weight (MW) standard. The distribution of MW markers is similar to that of the broad-range standard used in the analysis of the total assemblage (Figure 1).*

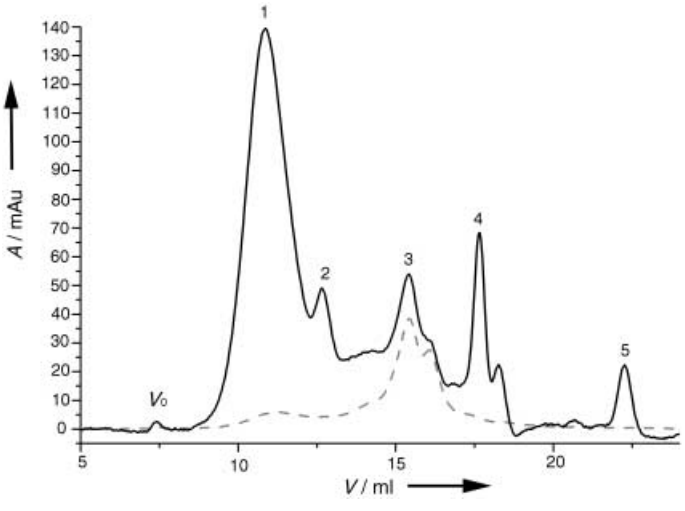


***Figure 4.*** *Superdex 75 gel filtration chromatographic separation of the aragonite-nucleating fraction (Fraction I in Figure 2). The solid line represents the absorbance at 220 nm and the dashed line represents the absorbance at 280 nm. Fraction 1 induced massive crystallization of aragonite alone. Fraction 2 induced very few aragonite crystals. Fraction 3 cannot be considered a protein since the ratio of absorbance at 220 nm to that at 280 nm is 1:1. Fraction 4 induced no crystallization. Fraction 5 is also found in the buffer and is attributed to the salt.* $V_0 = 7$ *ml.*

  

shows that this fraction contains a major band at 30 kD (according to polypeptide molecular weight standard) and some weak bands at lower molecular weight. The gel-filtration step thus dramatically improved the purification of the aragonite-nucleating fraction. We are currently trying to obtain the sequences of the constituents of this unique protein fraction with proven aragonite-nucleating activity.

**Protein function: Characterization of the mineral induced by the soluble macromolecules in the Falini et. al. assay**

The chitin – silk and acidic macromolecule assembly provides a reliable assay for testing the ability of protein mixtures or of isolated protein fractions to induce calcium carbonate deposition with polymorph control. This in vitro microenvironment thus enables us to isolate and distinguish between different components of the organic matrix in terms of their ability to specifically nucleate aragonite.

In the experiments described here, crystallization was induced by soluble macromolecules extracted either from the aragonitic nacreous layer or the calcitic prismatic layer of the mollusk *Atrina* by following the procedures used by Falini et al. The mineral spherulites produced proved to be either calcite or aragonite when detected by infrared spectroscopy under dehydrated conditions.[14] In the present study, the spherulites were analyzed in situ by Raman imaging spectroscopy. The advantage of this technique is that measurements are performed under hydrated conditions while the spherulites are still embedded in the chitin scaffold. While some of these spherulites were already in the form of aragonite or calcite depending on the macromolecule source, others were clearly composed of amorphous calcium carbonate (Figure 5 a). The Raman spectrum of amorphous calcium carbonate shows a relatively sharp peak at 1085 $cm^{-1}$, which is also typical of crystalline calcium carbonate. In contrast, the peak of stable biogenic amorphous calcium carbonate at 1085 $cm^{-1}$ is relatively broad (Figure 5). The peaks in the region 150 – 250 $cm^{-1}$, typical lattice frequencies of the crystalline forms, are however completely absent in the spectrum of amorphous calcium carbonate. These peaks are substituted by a broad peak typical of the amorphous phase. The appearance of these amorphous spherulites changed while the Raman laser beam was focused on them for a few minutes. When Raman measurements were subsequently repeated on the same spherulites that initially produced an amorphous calcium carbonate spectrum, conversion of the amorphous phase to a stable crystalline phase was observed (Figure 5 b). In most cases the conversion was to aragonite even in the presence of proteins from the calcitic layer. The conversion of the amorphous phase into the crystalline phase has often been observed under the light microscope. Amorphous calcium carbonate spherulites are transparent and become opaque with time. Transition of amorphous calcium carbonate spherulites into a crystalline phase was also detected when protein fractions purified by anion-exchange chromatography were adsorbed on the chitin – silk complex. However, when synthetic poly(Asp) was adsorbed on the chitin – silk complex, only the crystalline form of calcium carbonate was detected (as shown by Levi et. al.[16]). Amorphous calcium carbonate was not observed.

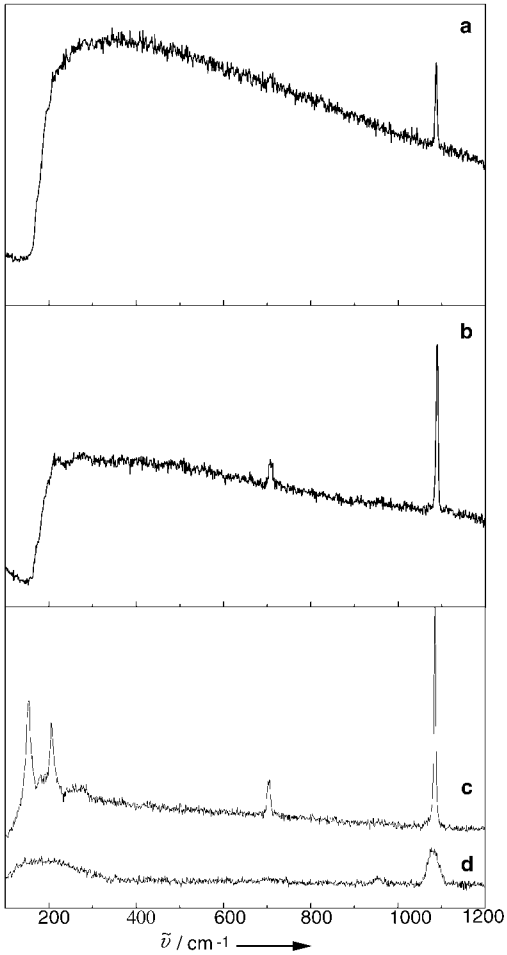


***Figure 5.*** *Raman spectra of the spherulites that form inside the β-chitin and silk scaffold. a) Amorphous calcium carbonate spherulites shortly after the crystallization experiment was completed. The spectrum is characterized by a very broad baseline rise at 150 – 250 $cm^{-1}$ and a sharp peak at 1085 $cm^{-1}$. b) Aragonite spherulites formed after focusing the Raman laser beam on the initially formed spherulites analyzed in (a) for a few minutes. The characteristic peaks of this spectrum are at 206, 703, and 1085 $cm^{-1}$. c) Control aragonite from* Nautilus *shell septa. d) Control stable amorphous calcium carbonate from the antler spicules of* Pyura pachydermatina. *Note the broad peak at 1085 $cm^{-1}$.*

These results indicate that the first mineral deposited in the chitin – silk assemblage is amorphous calcium carbonate, and that this phase transforms into a crystalline phase.

## Discussion

The overall aim of this study is to better understand the function of the acidic proteins during mineral formation in general, and mollusk shell formation in particular. There are tens of acidic proteins in mollusk shells,[28, 29] which in this study were observed in the improved gel electrophoresis results. The functions of these proteins are related not only to their structures, but probably also to their neighboring macromolecules and the microenvironments in which they are located. Testing these

  

functions requires an in vitro assay that bears some relation to the in vivo environment. Thus, relating structure to function for these unusual proteins is extremely difficult. Here, we report results on both the purification and characterization of acidic proteins, as well as results that provide a better understanding of the in vitro assay system devised by Falini et al.[14]

A key method for protein purification in general is gel electrophoresis. We show that only when massive and rapid fixation procedures are used are the acidic macromolecules from mollusk shells detectable by gel electrophoresis. Furthermore, Coomassie Blue does not stain these proteins, unless, presumably, they happen to have nonacidic segments. We also note that the mode of decalcification influences the number of bands obtained. We used the ion-exchange method for decalcification. When acetic acid was used to dissolve the mineral, fewer and less-well-defined bands were observed. The many published studies of mollusk shell proteins that used gel electrophoresis are therefore unlikely to be representative of the true diversity of these important matrix components. Furthermore, in many reports, the gels are massively overloaded, and the discrete bands obtained even by Coomassie Blue staining are probably only minor components.[29] Indeed, most of the sequenced proteins from mollusk shells were stained by Coomassie Blue and are not particularly acidic.[20–25] One acidic protein, MSP-1,[19] has been sequenced. Its sequence contains stretches of nonacidic amino acids.

Gel electrophoresis, with gels fixed under our new conditions, showed that the main aragonite-nucleating fraction obtained by ion-exchange chromatography contained seven bands (not necessarily seven gene products as migration may be affected by post-translational modifications). The same fraction, then run on the gel filtration column, contained one dominant gel electrophoresis band at 30 kD. This result is definitely a significant improvement in the purification process, which initially involved tens of proteins of the total assembly. Thus, this new gel electrophoresis method opens up the possibility of better characterizing the many different shell matrix proteins. Furthermore, the acidic proteins found by chromatographic methods to be present in other mineralized tissues may now be more amenable to characterization by gel electrophoresis. It is interesting to note that, in contrast to almost all other mineralized tissues studied, to date very few unusually acidic proteins have been identified in the EDTA extract of bone matrix.[30, 31] The EDTA extract is the fraction that contains the macromolecules most intimately associated with the mineral phase. It will be very interesting to use this gel electrophoresis method to check whether there are additional hitherto undetected proteins in bone and other mineralized tissues.

With the new gel electrophoresis procedure in hand, we can now confirm that the approach originally used by Albeck et. al.[12] and extended in this study, namely decalcification by use of an ion-exchange resin, followed by purification first by anion-exchange chromatography and then by gel filtration, is an effective means of purification of these proteins. This protocol also maintains the specific nucleating capability of the purified fractions by using the Falini assay system. It is therefore of interest to understand as much as possible about this assay and its relevance to the in vivo situation.

This assay uses the major components of the mollusk shell organic matrix. The $\beta$-chitin was purified from the pen of the squid *Loligo*; this $\beta$-chitin can be considered a nonmineralized analogue of the shell $\beta$-chitin. The silk was obtained from silkworms. The mollusk silk protein has been sequenced and it is in fact closer in sequence to spider silk than to that of *Bombyx mori*.[21] The acidic proteins were obtained from the mollusk shell. The amino acid composition of the aragonite-nucleating fraction obtained during purification shows an increase in Asx residues compared to the amount present initially. Moreover, as the purification process progresses, lower protein concentration is required in order to induce aragonite nucleation, which indicates that the relative concentration of aragonite-nucleating protein(s) increases during this process. The arbitrary manner in which the components are assembled in vitro leaves little doubt that this system does not closely mimic the in vivo environment of mineral formation. However, during the formation of aragonite in vitro, the first mineral formed is amorphous calcium carbonate (ACC) and not aragonite. The ACC then transforms into aragonite. Furthermore, the Raman peak of the transient ACC at 1085 $cm^{-1}$ is narrow, as compared to the same peak of stable biogenic ACC (Figure 5).

Weiss et al.[32] reported that this same series of events occurs during the formation of the larval mollusk shell in vivo. The transient ACC phase of the larval shells also has a narrow 1085 $cm^{-1}$ peak. It is not known, as yet, whether ACC is a transient precursor phase in the formation of the adult shell. ACC has been found in the formation of the sea urchin larval spicule,[33] where it too has a narrow 1085 $cm^{-1}$ peak. It has been shown that this form of ACC in the sea urchin larvae does not contain water.[34] As the echinoderms and mollusks are on two different branches of the animal phylogenetic tree, this could imply that the phenomenon is widespread. If this is indeed the case, then the Falini et. al. assay resembles the in vivo situation to a greater extent than we previously thought. Formation of amorphous calcium carbonate is induced in the chitin – silk assay by Asp-rich proteins. This may imply an additional role for Asp-rich proteins in the formation and stabilization of the transient amorphous phase. However, since all nacre protein purified fractions induce the formation of ACC, this phenomenon cannot be attributed to a specific fraction.

Levi-Kalisman et. al.[35] suggested that the silk in the nacre organic matrix is in the form of a hydrated gel. In this form, the silk could conceivably be involved in the formation of the amorphous calcium carbonate precursor phase since it could create the microenvironment, including the supersaturation, which is essential for the formation of the amorphous phase. Stable amorphous calcium carbonate is known to be associated with Glu-rich macromolecules.[6]

## Concluding Remark

The challenge of linking function to specific macromolecular constituents of the organic matrix of mineralized tissues is daunting. On the one hand, demineralization and purification

  

can affect function, and on the other hand, function needs to be assessed in a complex microenvironment that resembles the in vivo environment. Here we show one approach whereby, for the mollusk shell at least, these difficult and often contradictory requirements can be met.

## Experimental Section

**Materials and equipment**: The water used in all experiments was ultrapure water filtered through a Milli-Q column supplied by Millipor (Bedford, MA, USA). Fresh shells of the bivalve *Atrina rigida* from the east coast of Florida were purchased from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA). Dialysis tubes (Cell-Sep, T1, MWCO = 3500, diameter = 46 mm) were purchased from Membrane Filtration Products, Inc. (Seguin, TX, USA). Protease inhibitor cocktail tablets, Cimplete, were obtained from Roch Molecular Biochemicals (Mannheim, Germany). Cation-exchange resin, Dowex 50 × 8, mesh 50 – 100, was purchased from Sigma – Aldrich (Rehovot, Israel). Polyacrylamide Tris-Tricine 10 – 20% gradient gels were bought from Bio-Rad Laboratories (Hertfordshire, UK). Gels were run on a Mini-protean 3 apparatus from Bio-Rad Laboratories. Gel standards were either polypeptide SDS-PAGE molecular weight standard or broad-range prestained SDS-PAGE standard purchased from Bio-Rad Laboratories. All the solutions for gel staining were prepared with materials purchased from Sigma – Aldrich (Rehovot, Israel). Gel drying films were obtained from Promega (Madison, WI, USA). $CaCl_2$ was obtained from Merck (Darmstadt, Germany).

Raman experiments were performed with a Renishaw Raman Imaging microscope (New Mills, UK). Protein purification was performed on an Akta-FPLC system purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Anion-exchange chromatography separation was performed on a Mono Q HR 5/5 column obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Gel filtration separation was performed on a Superdex 75 HR 10/30 column from Amersham Pharmacia Biotech. Trizma base reagent for chromatographic separation was purchased from Sigma – Aldrich (Rehovot, Israel). NaCl purified for HPLC was purchased from BDH (Poole, UK). Buffers were filtered through disposable 0.22-μm filters from Corning Incorporated (Acton, MA, USA). Proteins were concentrated before loading onto gels by using a PAGEprep kit purchased from Pierce (Rockford, IL, USA).

**Shell preparation**: Shells of the bivalve mollusk *Atrina rigida* were stored dry at −20 °C. The outer calcitic prismatic layer was mechanically separated from the inner aragonitic layer (nacre). Each layer was washed over night with 10% ammonium hydroxide solution followed by extensive washes with water. The shell layers were then ground to a fine powder with an electrical grinder.

**Protein extraction**: In general, the protein extraction procedure described by Albeck et. al. was followed.[12] Specifically, the ground shell powder was poured into dialysis tubes and suspended in water (50 mL per 2 g ground shell). One tablet of protease inhibitor cocktail was added to 50 mL water. The sealed dialysis membranes were placed in glass cylinders (34 × 4.5 cm). About one quarter of the cylinder, outside the dialysis membrane, was filled with prewashed cation-exchange resin and water was added to fill the cylinder. The cylinders were closed with rubber stoppers at both ends and rotated slowly and continuously in a propeller-like mode at room temperature to keep the resin and the shell in suspension. The water outside the dialysis bag was changed twice a day. Complete decalcification of 2 g shell was achieved in 4 days and the absence of mineral was confirmed by infrared spectroscopy. The contents of the dialysis tube were then extensively dialyzed against water for an additional 2 days. The resulting soluble and insoluble materials inside the dialysis tube were separated by centrifugation (ultracentrifuge 45 000 rpm, 1.5 h, 4 °C). The soluble material (30 mL) was concentrated by lyophilization to a final volume of 2 mL. This protein extraction process was performed in parallel with as many as 9 cylinders, each containing 2 g ground shell. The resulting lyophilized material from all the cylinders was combined and diluted with water (to a total volume of 40 mL) in order to proceed directly to protein purification and characterization. Protein activity and purification are dependent on the freshness of the proteins as well as on the freshness of the shells. The protein concentrations of the macromolecule solutions were determined by amino acid analysis.

**Amino acid analysis**: Aliquots of the soluble protein solution were lyophilized and hydrolyzed under HCl (6 M) vapor in vacuo for 24 h. Following evaporation of HCl, the hydrolysates were analyzed with an automatic amino acid analyzer (HP Aminoquant system).

**Crystal growth experiments and analyses**: Soluble proteins, either the total macromolecule assemblage or the fractions obtained after each purification step, were adsorbed on $\beta$-chitin and silk substrate and crystallization was induced as described elsewhere.[14, 16] The crystals were characterized by FTIR spectrometry (described in Levi et. al.)[16] and by Raman imaging spectroscopy.

Raman spectroscopy: Pieces of chitin (~0.5 $cm^2$) containing embedded $CaCO_3$ spherulites, were placed on glass slides directly after crystallization. The chitin was suspended in $CaCl_2$ solution (10 mM) to keep it wet and care was taken to keep the samples hydrated at all times. The spherulites inside the chitin were observed with a Leica microscope at a magnification of 50 × by using reflected white light. After focusing at the center of the spherulites, the light source of the microscope was transferred to a diode laser (780 nm). The sample was scanned for 10 s in the range 100 – 1200 $cm^{-1}$ with a Raman imaging microscope. The Raman laser beam was then focused on the same spherulites at the same location for two additional minutes and changes in the spherulite internal texture were observed with reflected white light, after which a second Raman spectrum was obtained under the same conditions as used for the initial measurement. In order to ensure that the organic matrix does not contribute to the spherulite spectra, spectra of $\beta$-chitin and silk were taken separately as controls.

**Polyacrylamide gel electrophoresis of proteins**: Tris-tricine ready-to-use Minigels of 10 – 20% polyacrylamide gradient were used. Usually 1 – 10 μg proteins were applied per gel lane. The gels ran at 100 V for 1.5 – 2 h and were developed by silver staining.

Silver staining procedure: Fresh solutions for staining were prepared daily (500 mL). The silver nitrate solution in particular must be prepared immediately before use. The silver nitrate procedure used here is very sensitive, in particular to nonacidic proteins. It is thus sufficient to load as little as 0.5 μL protein standard onto a gel. Immediately after running, the gel was immersed for 1 h in the first fixative solution, which contained methanol (50%), acetic acid (12%), and formaldehyde (18%). This step, as well as the entire staining procedure, was performed on a rocking table. The gel was then immersed for 30 min in a second fixative solution containing glutaraldehyde (10%), followed by washing in ethanol solution (50%; 3 × 20 min). The gel was transferred to $Na_2S_2O_3$ solution (5 ml of an 80-mM stock solution diluted to 0.5 L) for 1 min and washed 3 times with a small volume of water for 20 sec each time. The impregnation step was performed by treatment with freshly made solutions containing $AgNO_3$ (1 g) and formaldehyde (18%) for 20 min. The gel was rinsed in water twice (20 seconds) and developed in a solution containing $Na_2CO_3$ (30 g), formaldehyde

  

(18%), and $Na_2S_2O_3$ (100 μL of an 80-mM stock solution) until bands started to appear. This developing step was performed above a light table and followed by quick washing with water (2×) as soon as faint bands started to appear. The bands become more intense during washing. Staining was stopped by immersion in a solution containing methanol (50%) and acetic acid (12%) for 10 min. The gel was kept in a methanol solution (50%) and transferred to water for 1 h before drying between nitrocellulose sheets.

**FPLC separation of the macromolecular ensemble**: High-performance liquid chromatography was carried out with an FPLC system at 4 °C. All buffer solutions for the separations were filtered before use. The sample solutions were centrifuged (3500 rpm, 10 min, 4 °C) before injection. Monitoring was performed at 220 nm rather than 280 nm because of the low aromatic content of the proteins.

**Anion exchange**: The first purification step was performed on an anion-exchange Mono Q column HR 5/5. The solution of macromolecules was diluted 1:2 in Tris base (20 mM, pH 8.2), glycerol (5%), and dithiothreitol (0.1 mM). The column was equilibrated for 5 min with buffer (20 mM Tris base, pH 8.2), followed by elution with NaCl (0.2 M) for 1 min (first step), then with an NaCl gradient (0.2 – 0.5 M) for 25 min. The column was washed with NaCl (2 M) for 5 min followed by re-equilibration with Tris buffer for 10 min. The protein fractions were collected by hand at a flow rate of 1 mL $min^{-1}$.

**Gel filtration**: Fraction I was concentrated under vacuum (Speed-vac concentrator, Savant) to a final volume of 200 μL and injected directly into a gel-filtration Superdex 75 column. The proteins were eluted at a flow rate of 0.5 mL $min^{-1}$ by using an isocratic run with buffer (pH 8.2) containing Tris (50 mM) and NaCl (0.1 M). The fractions were extensively dialyzed against water.

In order to avoid protein adsorption to dialysis tubes or vials (which is typical of these acidic proteins), the fractions were desalted and concentrated by using a PAGE prep kit (Pierce) prior to characterization by gel electrophoresis. Low protein absorbance onto the resin in the kit was confirmed by comparing the yields of dialysis and Speed-vac concentration.

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