Soluble Organic Matrices of the Calcitic Prismatic Shell Layers of Two Pteriomorphid Bivalves

PINNA NOBILIS AND PINCTADA MARGARITIFERA*

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The calcitic prisms of the shells of two bivalves, Pinna and Pinctada, are considered simple prisms according to some morphological and mineralogical characteristics. Scanning electron microscopic and atomic force microscopic studies show that the microstructures and nanostructures of these two shells are different. Pinna prisms are monocristalline, whereas Pinctada prisms are not. Moreover, intraprismatic membranes are present only in the Pinctada prisms. The soluble organic matrices extracted from these prisms are acidic, but their bulk compositions differ. Ultraviolet and infrared spectrometries, fluorescence, high pressure liquid chromatography, and electrophoresis show that the sugar-protein ratios and the molecular weights are different. Sulfur is mainly associated with acidic sulfated sugars, not with amino acids, and the role of acidic sulfated sugars is still underestimated. Thus, the simple prism concept is not a relevant model for the biominalization processes in the calcitic prismatic layer of mollusk shells.

Among more than 60 known different biominerals, calcium minerals are the most abundant. These exoskeletons (also called hard tissues or shells) have morphological, chemical, and physical properties that are never shown by the corresponding abiotic crystals. They are biocomposites (bioceramics), and organic macromolecules are their key components. Much of what is known about biominerals is deduced from the nacreous layers of mollusks, and it is believed that the organic matrix directs the organization of biocrystals. For example, they are large units that globally exhibit a single crystal-like organization. These prisms are built by a series of growth steps and surrounded by thick organic walls (6–10). According to Taylor et al. (11) the outer layers of Pinna and Pinctada are composed of calcite “simple prisms.” Their crystallographic c axes are normal to the layer surfaces. Illustrations of a transverse section of the prisms of Pinctada by Wise (12) are similar to those of Taylor et al. (11) and follow the simple prism concept. These calcitic layers have high S and magnesium contents (13–16). Acidic amino studies of the soluble intraprismatic matrices of both genera have shown high contents of aspartic acid, glycine, alanine, and glutamic acid (17, 18). However, repeated observations have shown some differences between the two shells. In Pinctada martensi, thin sections observed in polarized light show that each prism extinguishes in smaller smaller blocks (8, 19). Wada (9) observed uniaxial crystals and partial irregular extinctions in horizontal sections under crossed nicols. The individual prism of Pinna exhibits a monocristalline extinction (20, 21).

Dissolution of the crystal units releases various macromolecules into solution, and it is well known that organic components are key participants in the control processes of shape and structure in mineralized tissues (2, 3, 17, 22). Only some proteins have been isolated from the aragonitic nacreous layers of Pinctada (23) since the first evidence of the presence of organic matrices (24). Thus, before the cloning of “pure” proteins of mollusk shell layers, a better knowledge of the bulk composition of their SOM is necessary as a first step in understanding the mechanisms of layer formation. From preliminary studies of the molecular weights of the SOM extracted from the prisms, Pinna and Pinctada are different (15, 25, 26). While pursuing a long term study of calcified biominerals, the present study was undertaken to extract and compare the SOM of the calcitic prisms of Pinna and Pinctada and to relate these SOM to the simple prism concept. Unlike many studies, this work is not focused on only the protein content. The location of the organic matrices and the presence of sugars are also considered.

EXPERIMENTAL PROCEDURES

Materials—Specimens of P. margaritifera (L.) (Pteriomorpha, Pterioiida, Pteriacea, Pteriidae) were collected in French Polynesia. P. nobilis L. (Pteriomorpha, Mytiolda, Pinneacea, Pinnidae) samples came from the Mediterranean Sea.

Standards—Isoelectric focusing calibration kit (pH 2.5–6.5) was from Amersham Biosciences. Low range SDS-PAGE standards (19–107 kDa), kaleidoscope-prestained standards (6.9–202 kDa), and gel filtration standards were from Bio-Rad. Chondroitin sulfate A was purchased from Sigma. Rapid Decalcifier was from Apex Engineering Prod-
Scanning Electron Microscopy (SEM)—Atomic Force Microscope (AFM)—The traditional method for obtaining information about the shell microstructures is scanning electron microscopy. Fractures and polished etched sections have been observed with Philips 505 and XL30 SEM. Acidic and enzymatic etchings were used to reveal the details of the microstructures of the polished sections. Samples were also studied using a Nanoscope IIIa (Digital Veeco) multi-mode scanning probe microscope operating in tapping mode. The tapping mode AFM utilizes an oscillating tip at a tip amplitude of approximately several tens of nanometers when the tip is not in contact with the surface. Because the tip is no longer in permanent contact with the sample surface during the scan, sample alteration can be avoided. The resolution of tapping mode AFM is on the order of a few nanometers. Details of the etchings for SEM and AFM observations are given in the figure legends.

Extraction and Purification of the Soluble Organic Matrix—Samples were immersed in 3% NaClO for 1 h to remove organic contaminants, rinsed with Milli-Q water, dried, and ground into powder. Powdered samples were immersed in 5 ml of Milli-Q water and then decalcified by progressive addition of 50% acetic acid so that the pH (automatically controlled with a titrimer) is above 4. The entire extract was directly centrifuged at 21,000 × g for 15 min, which separated the supernatant (soluble) and precipitated (insoluble) fractions. The soluble fraction was desalted by exchange with Milli-Q water on a Microconcentrator (Filtration). Other analyses were done using the same TSK columns connected in series with the Bio-Rad Model 111 Mini IEF cell, the gel was run without electrode buffers (non-denaturing dry IEF). The lyophilized SOM were dissolved in Milli-Q water. Focusing was carried out in a horizontal plane perpendicular to the beam collects the fluorescence emission photons. This geometry minimizes the contribution of elastic scattering. Provided the sample is thin enough, a Si photodiode can be mounted downstream from the sample to exploit the transmission signal as well. An energy range between 2 and 7 keV is available, which gives access to the K-edge of sulfur at 2472 eV. The low pI calibration kit (APB) with pH among 2.5 and 6.5, BSA, and ribonucleic acid were used. They were stained with Coomassie Blue R-250.

Two-dimensional Gel Electrophoresis—The molecular groups were separated in the first dimension by isoelectric focusing in IEF strips (Prefest Immobilon DryStrip, 11 cm, pH 3–10, APB) on a Multiphor II system. The low pI calibration kit (APB) with pH among 2.5 and 6.5, BSA, and ribonucleic acid were used. They were stained with Coo
portion, only the outer thick organic walls are present. However, the polished and etched transverse sections are different from those of Pinna. Enzymatic hydrolyses reveal intraprismatic sinuous lacunae (Fig. 2C). The nonspecific enzymes used and the basic pH suggested that these lacunae are the remains of intraprismatic organic membranes. The orientations of crystallites on both sides of these intraprismatic membranes within a prism are not similar (Fig. 2D). These sinuous intraprismatic lacunae are also present in fixed and etched transverse sections (Fig. 2E). In such preparations, the interprismatic walls are partially destroyed. Transverse thin sections observed with cross-nicols confirm that the prisms are composite crystals (Fig. 2F). AFM observations show that the elongated crystallites are irregularly aligned (Fig. 2, G and H). Their width is ~110 nm, whereas their length varies from 250 to 400 nm. Round units at 50–70 nm in diameter are sometimes present. As with Pinna, the crystallites seem surrounded by a thin organic layer according to the phase images (Fig. 2F). The long polygonal calcitic prisms in both Pinna and Pinctada are surrounded by a thick organic wall and exhibit growth lines in longitudinal sections. However, prismatic units of Pinna are monocristalline, whereas those of Pinctada are not, as shown by thin sections and complex preparative processes. The enzymatic hydrolyses were done at pH >7 so that...
the mineral part is not strongly etched and only a small dissolution can occur. Thus, the intraprismatic sinuous lacunae in *Pinctada* indicate the presence of sinuous organic membranes. Besides, there is a clear difference between these sinuous intraprismatic organic membranes and the outer walls. The sinuous intraprismatic organic membranes are destroyed by acid and enzymes, whereas the outer walls are not. The ultrastructure of *Pinctada* is then more complex than that of *Pinna*, which suggests that the intraprismatic organic matrix of *Pinctada* is also more complex. Hence, the simple prism concept appears not to be appropriate.

**Bulk Composition**

The emission scan of CS exhibits a small peak at 287 nm and a main peak at 353 nm for a 257-nm excitation. Peaks are at 290 and 342 nm for BSA (Fig. 3A). Only a small peak is visible at 287 nm in *Pinctada* and a shoulder is visible in *Pinna*. The emission scan of CS shows a shoulder at 310 nm and a main peak at 353 nm for a 257-nm excitation. A peak at 342 nm is visible in the BSA spectrum. A small peak at 303 nm is present in *Pinna* and *Pinctada* SOM (Fig. 3B). CS shows a peak at 353 nm for a 287-nm excitation, whereas that of BSA is at 342 nm. Only shoulders are present in *Pinna* and *Pinctada* spectra (Fig. 3C). From UV spectra, it appears that *Pinna* and *Pinctada* SOM are not pure proteins and that these proteins have a low content of aromatic amino acids (data not shown). From fluorescence and UV spectra (data not shown), it may be inferred (1) that the SOM of *Pinna* and *Pinctada* are different (2), that these matrices are not pure proteins (3), and that their aromatic amino acid contents are very low.

In infrared spectra, the amide I band is the most intense absorption band for proteins. The presence of bands arising from amino acid side chains in the region between 1800 and 1400 cm\(^{-1}\) (amides I and II) has been thoroughly investigated (29). It has been established that among the 20 proteinogenic amino acids, only residues arginine, asparagine, glutamine, aspartic and glutamic acids, lysine, tyrosine, histidine, and phenylalanine have intense absorption in this region. *Pinna* has nine bands, and *Pinctada* has eight bands in this part of the spectrum. The prominent band in the two matrices is the amide I band near 1653 cm\(^{-1}\); thus, it may be supposed that components have adopted the \(\alpha\)-helical conformation (Figs. 4 and 5). However, precise interpretations of bands in the amide I region are difficult, because there is an overlap of the \(\alpha\)-helical with random coil structures. The 1647 cm\(^{-1}\) band (amide I) may be assigned to unordered structures, this band being absent from *Pinctada*. Other bands show the presence of \(\beta\)-turn and \(\beta\)-sheet structures. The prominent amide II bands are near 1560 cm\(^{-1}\) (*Pinna*) and 1540 cm\(^{-1}\) (*Pinctada*). Strong carboxylate absorption bands are present at 1419–1420 and 1717 cm\(^{-1}\) in both SOM. Bands at 1717 and 1575 cm\(^{-1}\) are usually assigned to aspartic acid, whereas bands at 1712 and 1558 cm\(^{-1}\) are assigned to glutamic acid. These four bands are present in *Pinctada*, whereas bands corresponding to aspartate (1622 and 1678 cm\(^{-1}\)) and glutamate (1610 and 1670 cm\(^{-1}\)) are absent. In *Pinna*, bands assigned to aspartic acid (1574 and 1717 cm\(^{-1}\)) are present, but bands related to aspartate are absent. One band is present for glutamic acid (1560 cm\(^{-1}\)), and two bands are present for glutamate. The small band near 1245 cm\(^{-1}\) may imply that some molecules are sulfated (Fig. 5). Amide A bands are similar in both samples and are not specific for secondary structures.

All of these bands are present in both proteins and sugars. Thus, it is possible that the strong amide A, I and II bands, are also due to sugars as shown by the spectra of CS (30, 31). On the other hand, bands between 1000 and 1150 cm\(^{-1}\), which are absent from protein spectra, are usually considered characteristic of only the sugars. Thus, in complex components, it is
difficult to infer the low or high contents of sugars from the intensity of bands of only the 1000–1150-cm$^{-1}$ region. In both SOM, these specific bands are weak, but this is not necessarily evidence of a low sugar content. Moreover, some molecules seem sulfated.

**Molecular Masses**

The HPLC chromatogram of *Pinna* at 226 nm in non-dissociative buffer (Tris) shows a small peak of excluded large molecules (>1 × 10$^6$ Da), a main peak at 277 kDa, and a minor peak estimated at 12 kDa (Fig. 6A). At 254 and 278 nm, the overall peaks are similar to those detected at 226 nm, but the excluded molecules are the main peak. Only the largest and smallest molecular masses are present in the refractometric chromatogram, the main peak being at 277 kDa (Fig. 7). There is a strong decrease in the intensity of the peaks from 226 to 278 nm, showing a low protein content. Refractive index detectors (or refractometers) are sensitive to a wide range of organic compounds, but they are typically used to analyze compounds that do not have strong absorbance in the UV range such as sugars. In the experimental conditions used, low quality commercial BSA shows one or two small peaks, whereas high purity globular proteins do not show peaks. CS shows a strong peak. These data may imply that the main components of the *Pinna* SOM are sugars or highly glycosylated proteins.

The 226-nm profile of *Pinctada* shows three distinct peaks of increasing intensities: one of excluded molecules, a second one at 377 kDa, and the last one at 110 kDa (Fig. 6B). As for *Pinna*, the 278- and 254-nm profiles are similar to each other and different from the profile at 226 nm. The main peak is at 110 kDa, the middle peak is a shoulder, and the excluded molecules are present. These three peaks are present in the refractometric profiles (Fig. 7), the excluded molecules being the most intense. It should be noted that in Figs. 6 and 7, elution times are different for the equivalent peaks in each elution profile because of the serial connection of the two detectors.

The comparison of the UV and refractometric HPLC profiles confirms the UV-fluorescence data. The SOM of *Pinna* and

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**Fig. 5.** Detailed FTIR spectra of the SOM of *Pinna* and *Pinctada* showing the different bands in the amide I, amide II, and the sugar region (A) and in the amide I and II domains (B).

**Fig. 6.** HPLC UV profiles of *Pinna* (A) and *Pinctada* (B) showing the composite nature and the different molecular weights of the SOM. Non-dissociative buffer (Tris).
Superose 12 chromatography in the dissociative buffer (1,000–300,000 Da) is narrower than that of the TSK columns. The UV and refractometric profiles of a sample were acquired during the same elution, so that their ratios are not dependent of the quantity of SOM. According to the comparison of these profiles and the 226/278 nm ratios, Pinna seems to contain a higher proportion of sugars than Pinctada.

Components with molecular masses greater than 10^6 Da are present in Pinna (Fig. 8A), the main peak being at ~120 kDa. The Pinctada profile shows more peaks. A faint shoulder is indicative of excluded components (>10^6 Da), other peaks being at ~98 and 23 kDa (Fig. 8A). The separation range of Superose 12 chromatography in the dissociative buffer (1,000–300,000 Da) is narrower than that of the TSK columns. The Pinna profile shows an excluded peak (>300 kDa) and a broad peak at 34 kDa (Fig. 8B). The Pinctada profile is more complex with a small excluded peak, several shoulders, a main peak of apparent molecular mass of 20 kDa, and several small peaks (Fig. 8B). Clearly, there is a discrepancy in the molecular mass estimated for the molecules in the major peak of the Pinna SOM on the two different columns (120 and 34 kDa). The basis for this discrepancy is not readily apparent but may indicate some peculiarity regarding the molecules contained in this peak.

Despite the observed differences in the dissociative buffer, Pinna and Pinctada SOM are different. The comparison of the 280-nm (non-dissociative buffer) and 275-nm (dissociative buffer) profiles shows that the excluded peaks are less prominent in the dissociative buffer. There is a good correspondence of the apparent molecular masses among the three profiles of Pinctada but not in Pinna. The different behavior of the two SOM in the two buffers is consistent with the previous results. Some of the macromolecules of Pinna are poorly dissociated by guanidinium chloride, a behavior characteristic of highly glycosylated molecules. In contrast, Pinctada SOM is better dissociated and the sugar component is probably of lesser proportion.

Acidity

CS (an acidic and sulfated sugar) can be stained with Acridine Orange, but no discrete bands are present in this non-denaturing electrophoresis (Fig. 9A, center lane). Standard proteins are not stained with Acridine Orange but are stained with Coomassie Blue-Crocein Scarlet (Fig. 9A, left lane). The Pinna SOM is successfully stained with Acridine Orange, showing that the main part of the organic components consists of sulfated sugars, the pI of which is lower than 5.8 (Fig. 9A). A faint Coomassie Blue-Crocein Scarlet stain indicates that the protein components are slightly less acidic (Fig. 9A). Acidic sulfated sugars are also stained by Acridine Orange in Pinctada, but the proteins stained with Coomassie Blue-Crocein Scarlet are more acidic than those of Pinna (Fig. 9A). The two dyes used show that the SOM of Pinna and Pinctada are composed of proteins and acidic sulfated sugars. However, Pinna and Pinctada SOM are different. The protein part of Pinctada seems more acidic than that of Pinna.

Two-dimensional Electrophoretic Separation

Pinna—The Alcian Blue stain of the IPG strip shows a gradient from a light color (basic part) to an intense blue color at the acidic end (Fig. 9B). Thus, it may be suggested that large molecular mass components (>300 kDa) do not penetrate into the two-dimensional gel. These acidic components are strongly stained with Alcian Blue but not with silver; thus, it may be inferred that they are sulfated sugars. A very weak silver stain is visible only in the basic part of the IPG strip (Fig. 9B). The main part of the two-dimensional gel is not stained, and only the low molecular masses-acidic pI are stained with Alcian Blue (Fig. 9B). There is a contrast between the acidic parts of the IPG strip and the two-dimensional gel. The strip is heavily Alcian Blue-stained, whereas the two-dimensional gel is faintly stained. The silver stain is faint and visible only in the acidic part of the two-dimensional gel. Despite the use of dissociative denaturant buffers containing urea, SDS, dithiothreitol, 2-mercaptoethanol, and Triton X-100, there is no discrete band or

![Fig. 7. HPLC refractometric profiles of Pinna and Pinctada showing the high content of sugars in the SOM of Pinna.](Image 71x569 to 293x738)

![Fig. 8. HPLC UV profiles at 275 nm of Pinna and Pinctada in a dissociative buffer (guanidinium chloride). A, profiles showing the composite nature and the different molecular masses of the SOM. Same columns as in Fig. 6 (TSK columns). B, profiles in a Superose 12 column.](Image 338x424 to 542x737)
spot, and according to the stain, the main part seems composed of acidic sulfated sugars.

**Pinctada**—The entire length of the IPG strip is heavily stained: the acidic part with Alcian Blue, indicative of sulfated sugars, and the basic part with silver, indicative of proteins (Fig. 9B). The main part of the two-dimensional gel is silver-stained with a good separation according to pI but with no distinct band according to molecular masses (Fig. 9B). The acidic-low molecular mass part of the gel shows some unusual patterns and is also Alcian Blue-stained. As for **Pinna**, it may be suggested that a part of the acidic portion of the SOM is composed of molecular masses higher than 300 kDa, and these components do not penetrate into the two-dimensional gel. The Alcian Blue-stained two-dimensional gel prior to the silver stain does not show a strong blue color (data not shown).

The striking feature of the two-dimensional gels is the absence of the spotty pattern usually seen for soft tissue proteins, despite the use of dissociative buffers. Smears are usually indicative of a high degree of glycosylation. **Pinna** SOM seems to be composed of acidic sulfated sugars, a part of which has molecular masses higher than 300 kDa. **Pinctada** SOM also shows components with molecular masses higher than 300 kDa, but this SOM contains proteins and sulfated sugars with a large pI range as shown by the IPG strip. The lower molecular weight components of the SOM also seem to be composed of proteins with a larger range of molecular weights and pI.

**Sulfur Contents**—Reference spectra are in accordance with published data (32, 33). The S–K edge spectra of methionine and cysteine show a main peak at 2.473 keV (Fig. 10A). Cystine with a disulfide bond shows a double peak in the same region (Fig. 10B). A similar double peak is also present in phenyl disulffides (data not shown). The sulfated sugar, CS, shows a main peak at 2.482 keV and no peak in the S amino acid region (Fig. 10B). The spectra of the SOM extracted from **Pinna** and **Pinctada** also show a main peak at 2.482 keV, indicative of a high sulfate content based on the CS spectrum (Fig. 11A). The profile of **Pinctada** shows a double peak similar to that observed with CS. **Pinctada** shows small peaks at 2.47 keV corresponding to S amino acids according to the reference spectra, whereas the **Pinna** profile is flat for S amino acids. These results are consistent with the HPLC and electrophoretic data. **Pinctada** SOM contains more proteins than that of **Pinna**.

SEM micrographs show sinuous intraprismatic membranes in **Pinctada** prisms (Fig. 2, C–F, arrows) but not in **Pinna** (Fig. 1, D–F). In situ spectra of these sinuous intraprismatic membranes of **Pinctada** confirm that they are organic. A main peak at 2.48 keV and a very faint shoulder at 2.47 keV may be indicative of some S amino acids (Fig. 11B). The S amino acid region is weaker in this spectrum than that of the extracted SOM. The detailed structures of the intraprismatic membranes are unknown, but as for membranes in other microstructures in mollusk shells (**i.e.** the nacreous layer), they are probably composed of soluble and insoluble parts, the compositions of which are different. It is conceivable that the difference between the SOM spectrum and the in situ spectrum is due to the ratio of soluble and insoluble matrices involved in the intraprismatic membranes.

In both samples, XANES spectra show that the main sulfur species is sulfate, not S amino acids. However, the **Pinctada** SOM also contains some sulfur corresponding to amino acids with a small double peak, which may suggest the presence of cystine. However, the ratios of the three small peaks in the amino acid region as well as the energy of the third peak are not the same in the spectra of cystine (Fig. 10B) and **Pinctada** SOM (Fig. 11A).

**DISCUSSION**

From the above results, three topics can be discussed: 1) the methods used, 2) a comparison with other calcitic prisms, and 3) the relevance of the simple prism concept.
**Methods**—Because EDTA tends to form non-removable complexes with organic materials extracted from bones or shells (34–36), a moderate acidic decalcification was used (pH 4). It is sometimes said that acetic acid decalcification is a harsh process denaturing the soluble organic matrix, but the accuracy of this assessment is questionable. If the acetic acid decalcification hydrolyzes and cleaves the SOM into smaller molecules, low molecular weight peaks and bands should be present in HPLC and electrophoresis. The presence of large molecular weight species shows that the hydrolysis of the SOM during the decalcification is moderate if not absent. These results are consistent with the usual hypothesis saying that when the decalcifying solution and organic components have similar pH, the organic matrices are not altered. Previous amino acid analyses of the SOM of mollusk shells have shown that the average pI is near 4 (17, 18). Thus, it may be inferred that the decalcification used does not substantially alter the SOM. In addition, concentrated HCl has been used to extract the intraskeletal macromolecules of ascidians and no major effect was observed on the extracted macromolecules (37).

UV spectrometry and fluorescence show that the SOM studied have very low aromatic amino acid contents. Although these methods are relatively simple, they are informative and not time-consuming. Moreover, they show that the main methods used to estimate the protein contents based on the tryptophan, tyrosine, and phenylalanine absorptions are not suitable for such SOM.

There is good agreement between the results of HPLC and electrophoreses. Despite the use of dissociative buffers in both techniques, large molecular size species are observed (>10^3 kDa).

**Comparison with Other Calcitic Prisms**—Addadi et al. (38) and Albeck et al. (36) have studied the SOM extracted from the outer calcitic prisms of *Atrina* (*Pteriomorpha, Pinnidae*). *Atrina* and *Pinna* are taxonomically related. Infrared spectra of *Atrina* show a main peak at 1653 cm⁻¹, strong bands at 1575 and 1417 cm⁻¹, and weak specific sugar bands. The band at 1160–1630 cm⁻¹ is assigned to protein amide I but also "possibly to the N-acetyl groups of polysaccharide" (36). Bands at 1255 and 1230 cm⁻¹ may be attributed to amide III and/or sulfate (36). However, an acidic part of the matrix shows a large specific sugar band. Thus, the similarities between the infrared spectra of *P. nobilis* and *Atrina* are high despite the different decalcification processes. The prisms of *Atrina* also show high contents of acidic amino acids (36). S amino acids (Cys and Met) are low, but the special hydrolysis necessary to avoid destruction of these amino acids was not done by Albeck et al. (36).

**The Simple Prism Concept**—Despite some similar aspects, the microstructures and nanostructures of the prisms of *Pinna* and *Pinctada* are clearly different as previously shown by optical microscope studies. *Pinna* prismatic units are monocrystalline, whereas those of *Pinctada* are not. *Pinctada* SEM observations showed small globular crystals (200–600 nm in diameter) surrounded by a thin granular envelope 3–5-nm thick (17). This size is similar to that of the elongated crystallites observed with AFM but is larger than that of the rounded granules. The ultrastructure of *Pinna* was not illustrated previously (17). *Pinna* and *Pinctada* prisms were also called megaprism (39). They are said to be built up of small crystallites
components.2

result in any improvement in resolution of the SOM

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position of the bands, whereas the high S contents and the

sensitive compared with silver staining.

Non-denaturing IEF also shows strong acidity based on the

position of the bands, whereas the high S contents and the
differences in sugar contents are demonstrated by the stains. Both Pinna and Pinctada SOM contain up to 79% aspartic acid

(17, 18), but the amino acid composition is dependent on the

species. For example, aspartic acid varies from 23 to 76% in two

Pinctada species, whereas glutamic acid varies from 3.6 to 18%
in two Pinna species (17). XANES spectra confirm that the

main sulfur species is sulfate in both SOM, but S amino acids are also present in Pinctada. Previous microprobe analyses have shown that these calcitic prisms have high S contents, typically 5650 ppm in Pinna and 9100 ppm in Pinctada (13, 14).

Disulfide bonds, if present, are rare as confirmed by liquid

chromatography (HPLC) and electrophoresis data. The use of
dissociative denaturant-containing detergent buffers does not result in any improvement in resolution of the SOM components.2

CONCLUSION

The techniques used do not allow us to identify the glycoproteins precisely and/or their core proteins or sugar moieties. CS is one of the most widespread sulfated sugars and is frequently observed in extracellular matrices involved in calcification processes (41–43) and in shell formation (44, 45), but the data available for Pinna and Pinctada are not sufficient to ascertain its presence in these species. The high molecular weights and sugars in Pinna suggest the presence of mucins. Mucins are high molecular weight, heavily glycosylated, and sometimes sulfated proteins and chromatograph in the void volume of most commercially available gels. They do not penetrate well into electrophoresis gels. They are stained in situ or in gels with Alcian Blue. The subunits are joined by disulfide bonds. However, the amino acid contents of mucins are different from those of Pinna and Pinctada SOM. Mucins have high threo-
nine, serine (20%), and proline (10%) contents and low aspartic acid (4%) and glutamic acid (8%) contents.

Despite some common features, the Pinna and Pinctada SOM are not mucins. SOM extracted from the calcitic prismatic layer of Pinna is probably composed of a peptide core of repeating units decorated with carbohydrate chains. Pinctada SOM is also probably composed of a peptide core of repeating units, despite a low glycosylation level and low sulfate content.

Further studies are required to isolate and characterize the core proteins and the carbohydrate moieties of the SOM of mollusk shells and the relationships between the extrapallial fluid and the SOM. Hattan et al. (46) have shown that the major protein of extrapallial fluid of Mytilus is a glycoprotein “that is a homodimer composed of 14.3% carbohydrate.” Denaturing gel electrophoresis of this protein under reducing or non-reducing conditions indicates the lack of disulfide bonds. The estimated molecular mass of the subunit is 28,350 Da, and the pI is 4.43. The major secondary structures are α-helix and then β-sheet. The Mytilus shell is composed, as are Pinna and Pinctada, of an inner aragonitic layer and an outer calcitic one. However, a direct comparison between the extrapallial fluid and SOM of the calcitic layers remains difficult, because the extrapallial fluid is involved in the secretion of all of the layers of the shells and it is well known that in a single shell the SOM of the nacreous layer and of the prismatic layer are different.

High S contents, high sugar contents, and sulfate as main sulfur species are not exclusively known in calcitic minerals. Similar compositions have been described in the SOM extracted from the aragonitic skeletons of Scleractinia (28). Sulfated mucopolysaccharides are known to be closely associated with the mineralization process, and their involvement in the nucleation process of calcitic and aragonitic biominerals in vivo has been shown in various taxa (9, 34, 47–50). Moreover, from histochemical and microscopic studies, Wada (47) has shown that acidic sulfated mucopolysaccharides are not present in non-calcified organic matrices of mollusk shells.

The pioneer results of Wada (47) showing differences in the organic contents of the calcitic prisms of Pinna attenuata and P. martensii on the one hand and the presence of components similar to CS on the other hand are confirmed. Although they are present at or before the beginning of the calcification, few data are available on the structure and the composition of the thick organic walls or interprismatic sheaths. A detailed study of the organic matrices of the prismatic layer of Pinna shows that the interprismatic walls consist of soluble and insoluble fractions (31). Nevertheless, the role of each fraction in the biomineralization process is not explained. Similar data are not available for the interprismatic walls of Pinctada.

The simple prism concept is not corroborated by other microscopic observations despite a common mineralogy. In many ways, the prisms of Pinna and Pinctada are not identical. The SOM differ markedly in their structures and composition and in the molecular weights and acidities of their molecular constituents. The “similar” features of these prisms are less numerous than the “different” features. Both Pinna and Pinctada are Pteriomorpha bivalves, but they belong to distinct orders. The main microstructural differences (composite structure in Pinctada) and the major compositional differences (sugar contents) probably reflect fundamental evolutionary trends. Thus, the simple prism concept is not a relevant model for the biomineralization processes in the prismatic calcitic layer of mollusk shells.

From a methodological point of view, highly focused studies on the protein contents of biominerals are not sufficient to estimate the phylogenetic similarities of taxa on the one hand

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2 Y. Dauphin, unpublished data.
and to understand the role of the organic matrices in biomineralization processes on the other hand. The next step of this comparison is a separation of sugars and proteins followed by a separation of the constituents of each fraction. From a geological point of view, the observed distinct characteristics imply a different behavior during the fossilization processes despite a common size and mineralogy.

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