Bioinorganic Chemistry

Quaternary Ammonium Groups in Silica-Associated Proteins**

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The formation of inorganic minerals under the control of an organism (biomineralization) is a widespread phenomenon in nature. Silica biomineralization on earth is dominated by simple aquatic life-forms, which include unicellular organisms such as diatoms, radiolarian, and synurophytes as well as multicellular sponges.[1] These organisms produce silica based exo- and endoskeletons. In diatoms, the silica-based cell walls exhibit intricate patterns in the nano- to micrometer range. As these patterns are precisely reproduced in each generation of a diatom, genetic control of this process is evident. Cell-wall formation in diatoms has been regarded as an example for the controlled production of nanostructured silica. Therefore, understanding the underlying mechanism of silica fabrication may inspire synthetic routes to produce novel silica-based materials under mild reaction conditions.[2,3] Diatom biosilica is mainly composed of amorphous, hydrated SiO_2 (silica) that contains a small proportion of proteins, which have long been speculated to be involved in silica deposition.[4,5] Remarkably, more than three decades ago, Volcani's group recognized the existence of unusual amino acid derivatives such as ε-*N*,*N*,*N*-trimethyl-δ-hydroxylysine in diatom biosilica.[6,7] However, proteins associated with biosilica from *C. fusiformis* have only recently been purified to homogeneity and found to be the source of a number of unusual amino acid modifications. Typically, these proteins (denoted as silaffins) contain a high percentage of lysine and serine groups and all of these residues are modified.[8–10] The serine residues are phosphorylated, whereas the lysines are converted into three different derivatives: ε-*N*,*N*-dimethyllysine, ε-*N*,*N*,*N*-trimethyl-δ-hydroxylysine, and lysines that are covalently linked to long-chain polyamines (*N*-methylated polypropyleneimines). As well as silaffins, many cell walls in diatoms contain the same type of long-chain polyamines that are bound to putrescine.[11] Silaffins as well as long-chain polyamines have been shown to promote the formation of silica nanospheres from silicic acid in vitro.[8,11,12] A model which is based on phase-separation processes of polyamines explains the formation of patterns during silica formation.[13] Recently hexagonal silica structures were produced in vitro under the influence of polyamines.[14] Polyamines are known to affect silica formation in several ways: they catalyze the formation of siloxane bonds and act as flocculating agents.[15,16]

More than 40 years ago, quaternary ammonium ions were recognized as structure-directing agents in the synthesis of zeolites.[17] The tetramethylammonium cation favors the formation of symmetric oligosilicate anions, such as the cubic octamer $Si_8O_{20}^{8-}$, and this control of silicate speciation influences the nucleation phase of silica formation.[18] Furthermore, these organic cations exhibit a high affinity to silica surfaces. The existence of the ε-*N*,*N*,*N*-trimethyl-δ-hydroxylysine residue in silaffins from *C. fusiformis* indicates that nature exploits this very special interaction in the biomineralization of silica.

Herein we demonstrate that quaternary ammonium cations are indeed a characteristic feature of silica-associated proteins. Three novel lysine derivatives, which contain quaternary ammonium groups, were identified in silaffins that were purified from silica shells of the diatom *Eucampia zodiacus* (Figure 1 A). Silica was removed from the purified shells by treatment with anhydrous hydrogen fluoride, and the remaining silica-associated proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein extract from *Eucampia* shells exhibited one major protein band with an apparent molecular mass of 22 kDa and three or four additional minor components (Figure 1 B). These proteins were then analyzed for the presence of unusually modified amino acids. After acid hydrolysis, the resulting amino acid mixture was directly

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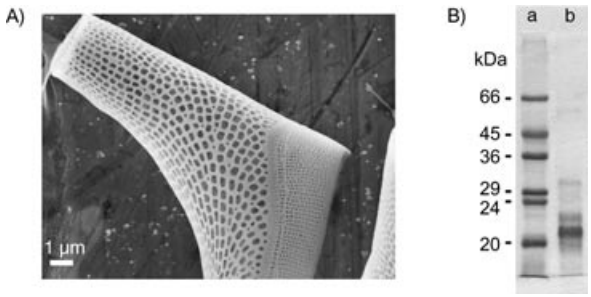


Figure 1. The silica cell wall of the diatom *Eucampia zodiacus* and its protein components: A) SEM image of the cell wall. B) Silaffin proteins extracted from purified cell walls with anhydrous HF—the extracted proteins were subjected to 12-% SDS-PAGE and were stained with Coomassie blue (lane a); molecular-weight standards were applied in lane b.

subjected to high performance anion-exchange chromatography (Figure 2 A). Remarkably, a significant fraction of the amino acid mixture (Figure 2 A, peak a) did not bind to the anion-exchange matrix even under the extremely alkaline conditions (NaOH, 60 mM) which indicates the existence of amino acid derivatives with a permanently cationic modification that compensates the charge of the carboxylate anion. Electrospray ionization mass spectrometry (ESI-MS) of this fraction revealed the presence of two main components at *m*/*z* 246 and 317 as well as a further component at *m*/*z* 260 (Figure 2 B). The chemical structures of these derivatives were analyzed by MS/MS analysis. The resulting fragmentations of all three species are documented in Figure 3 A, Figure 4 A, and Figure 5 A. Each

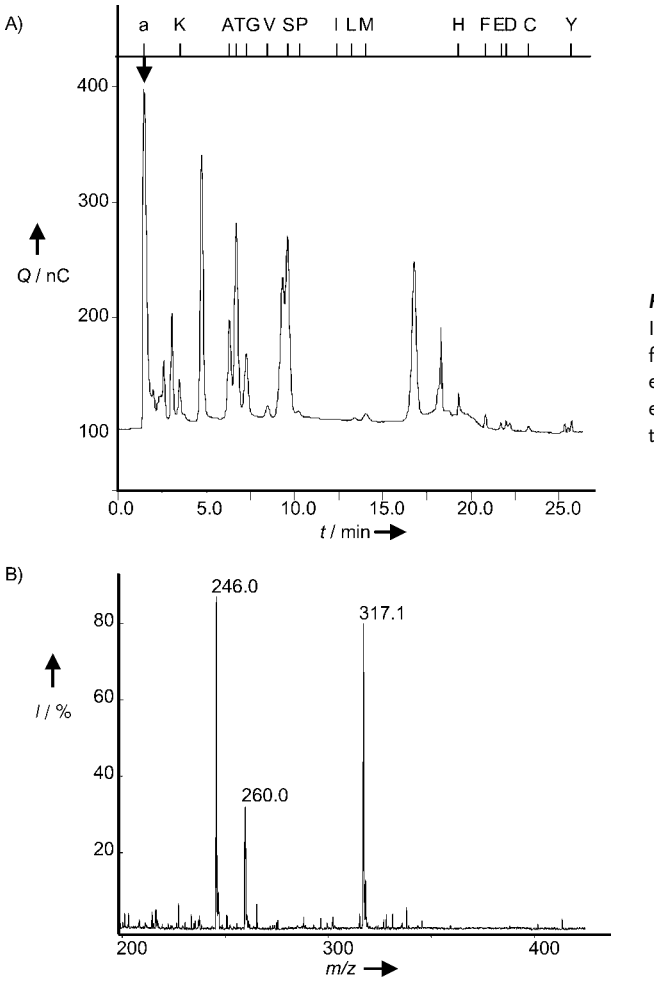


Figure 2. Amino acid analysis of silaffins: A) High performance anion-exchange chromatography of amino acids obtained by acidic hydrolysis of silaffins; the elution times of amino acid standards are indicated on the upper bar; peak a represents amino acids that contain a cationic modification. B) ESI-MS analysis (*I* = relative intensity) of the fraction that contains cationic amino acids (part A, peak a).

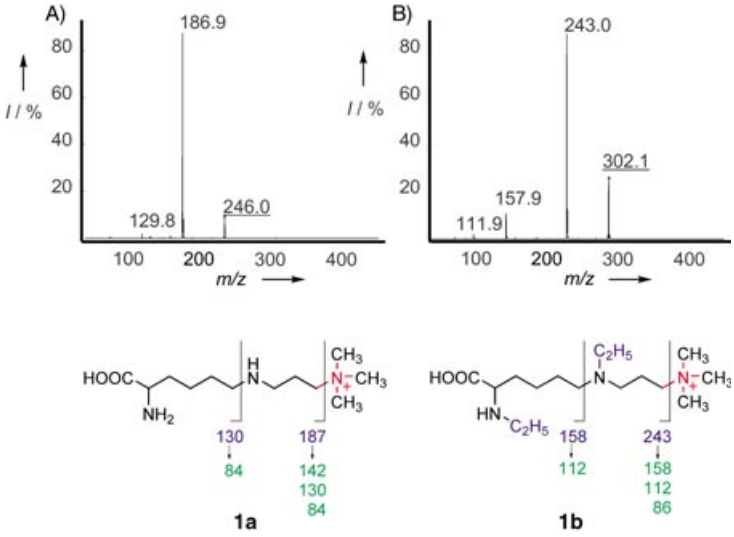


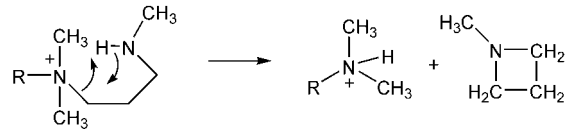
Figure 3. Structural analysis by mass spectrometry of the fragment with *m*/*z* 246. Ions were isolated by the ion-trap mode and were subjected to collision-induced fragmentation. Fragmentation patterns obtained A) before and B) after reductive ethylation (*m*/*z* after ethylation was 302 which indicates the introduction of two ethyl groups). *m*/*z* values shown in green indicate the results of $(MS)^3$ fragmentations of selected ions.

of the spectra displays a fragment at *m*/*z* 130 that subsequently (MS/MS/MS) decays into a species of *m*/*z* 84 (elimination of 46 mass units, which corresponds to the loss of H_2O and CO). This indicates the presence of a lysine moiety. Aminopropylations as well as methylations of the ε-amino groups of lysine residues were previously found to take place in silaffins isolated from the diatom *C. fusiformis*.[8] Aminopropylation followed by exhaustive methylation of the terminal amino group would create a lysine derivative of *m*/*z* 246 that contains a quaternary ammonium group as depicted (**1a**; Figure 3). Further methylation of the ε-amino group of the lysine moiety would create a derivative with *m*/*z* 260 (**2a**; Figure 4). These proposed structures are able to account for all of the remaining mass fragments observed in MS/MS analysis (Figure 3 A and Figure 4 A).

To prove the correctness of this interpretation, both of the components at *m*/*z* 246 and *m*/*z* 260 were converted into their fully methylated derivatives by reductive alkylation. As expected, reductive methylation converted both species into a derivative with *m*/*z* 288, which corresponds to the introduction of three and two methyl groups, respectively (data not

shown). Reductive ethylation is known to be less efficient and converted the α-amino group of the lysine moiety only into the secondary amine.[19] Under the conditions employed, only two ethyl residues were introduced into the component at *m*/*z* 246 and only one ethyl residue was introduced into the component at *m*/*z* 260. The resulting fragmentation patterns of the partially ethylated derivatives (Figure 3B and Figure 4B) confirm the proposed structures (**1b**; **2b**).

Introduction of a second aminopropyl residue into the species at *m*/*z* 260 would create a homologous structure with *m*/*z* 317 (**3a**; Figure 5). The fragmentation pattern of the component at *m*/*z* 317 supported this interpretation (Figure 5A). As expected, reductive methylation introduced three methyl groups into the compound and reductive ethylation introduced two ethyl groups. The fragmentation spectra of the partially ethylated derivative again confirmed the proposed structure (**3b**). Remarkably, the compound with *m*/*z* 317 produced an unexpected fragment (*m*/*z* 246) that appears to result from N–C bond cleavage at the quaternary ammonium site. In the partially ethylated derivative, the corresponding fragment (calculated *m*/*z*: 274) was no longer observed in the fragmentation pattern. This behavior is likely to be caused by a favored internal proton transfer from the terminal secondary amino group as depicted in Scheme 1. This proton transfer would no longer be possible after ethylation, and this observation strongly supports the proposed structure. Furthermore, ^{1}H NMR analysis of the compound with *m*/*z* 317 at pH 11.8 gave rise to a signal at $\delta = 3.14$ ppm (methyl) whose position did not shift upon lowering the pH (pH 5). This is characteristic of methyl groups attached to a quaternary ammonium group (data not shown) and confirms the conclusion drawn from mass spectrometry.



Scheme 1. Proposed mechanism of internal proton transfer to explain the production of a fragment with *m*/*z* 246 (see also Figure 5A) in the collision-induced fragmentation of **3a**.

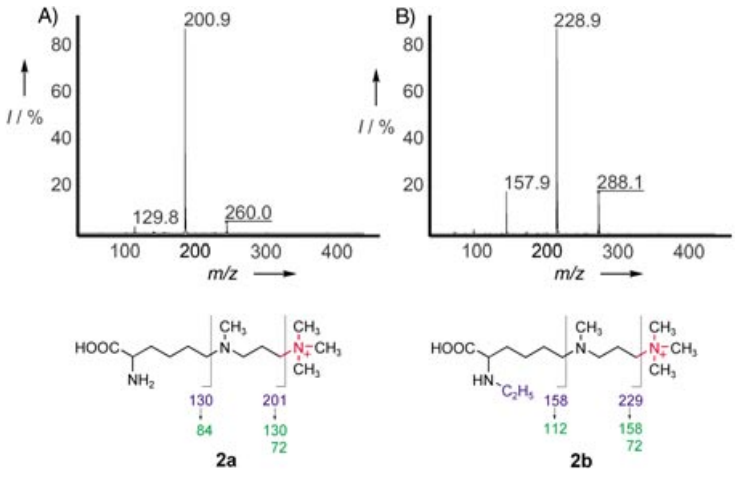


Figure 4. Structural analysis by mass spectrometry of the fragment with *m*/*z* 260. Ions were isolated by the ion-trap mode and subjected to collision-induced fragmentation. Fragmentation patterns obtained A) before and B) after reductive ethylation (*m*/*z* after ethylation was 288 which indicates the introduction of one ethyl group). *m*/*z* values shown in green indicate the results of $(MS)^3$ fragmentations of selected ions.

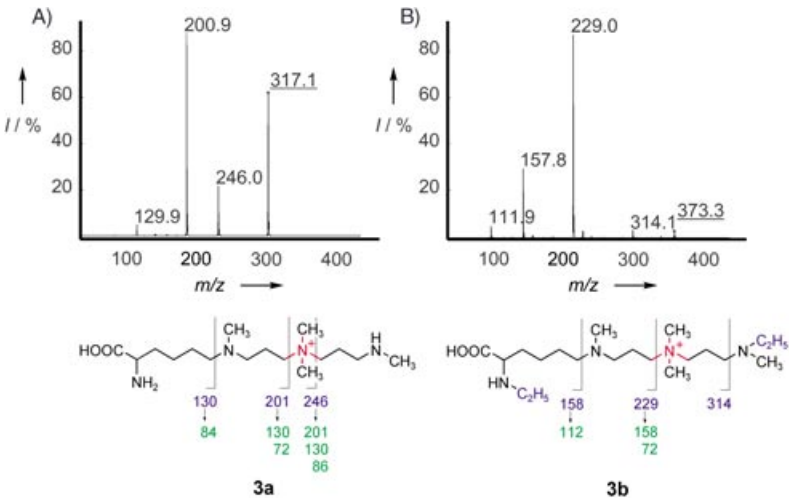


Figure 5. Structural analysis by mass spectrometry of the fragment with *m*/*z* 317. Ions were isolated by the ion-trap mode and subjected to collision-induced fragmentation. Fragmentation patterns obtained A) before and B) after reductive ethylation (*m*/*z* after ethylation was 373 which indicates the introduction of two ethyl groups). *m*/*z* values shown in green indicate the results of $(MS)^3$ fragmentations of selected ions.

The characterization of novel lysine derivatives with quaternary ammonium groups indicates that this cationic modification represents a special structural feature of silica-associated proteins from diatoms. It is well documented that quaternary ammonium ions exhibit very unusual behavior in silica systems which is probably owed to the high affinity of the ions to silica surfaces and their inability to form coordinate bonds with the oxygen of SiOH groups as sodium does.[16] Nature probably uses these special properties to design proteins with high affinities for silica surfaces.

Experimental Section

Culture Conditions: *Eucampia zodiacus* was isolated from the North Sea and cultivated in an artificial seawater medium according to the protocol from the North East Pacific Culture Collection (www.ocgy.ubc.ca/projects/nepcc/media.htm).

Purification of Silaffins: A 20-L culture of silaffins was harvested by filtration through a nylon screen (40 μm). The diatom colonies were suspended in 10-mL lysis buffer (EDTA (100 mM), SDS (2%)), and the suspensions were incubated at 95°C for 10 min. The resulting silica shells were collected by

centrifugation (2500 × *g*; 10 min), washed extensively with water, and dried in vacuo. Silica shells (100 mg) were dissolved in anhydrous hydrogen fluoride and the solution was incubated at 0 °C for 30 min. Hydrogen fluoride was evaporated, and the remaining material was neutralized with Tris-HCl (250 mM, pH 7.5; 2 mL). HighS cation-exchange resin (NH_4^+ form; Bio-Rad; 100 μL) was added to this extract, and the mixture was incubated at 4 °C for 1 h. The resin was washed with H_2O, followed by NH_4OH (4 N), and again with H_2O. The silaffins were then eluted with NaCl (2 M)/Na_2CO_3 (20 mM). After dialysis against H_2O (Spectra/Por CE dialysis tubing; molecular-mass cutoff: 500 Da), the eluted proteins were size-fractioned on a Superdex-Peptide HR 10/30 column (Amersham Pharmacia; running buffer ammonium acetate (250 mM); flow rate: 0.3 mL min^{-1}). Fractions were analyzed for silaffins by 12-% SDS PAGE with subsequent staining with Coomassie blue. Silaffin-containing fractions were pooled and dried by lyophilization.

Purification of Basic Amino Acids: Purified silaffins were hydrolyzed in HCl (6 N; Pierce; Sequanal Grade; 1 mL) at 110 °C for 24 h. After evaporation, the material was dissolved in H_2O (300 μL), filtered (Millipore; Millex PV 0.22 μm), and loaded onto an AminoPac PA10 4/250 mm anion-exchange column (Dionex; elution gradient: NaOH/NaOAc). Amino acids were detected electrochemically (Dionex ED40). Basic amino acids modified with a quaternary ammonium group eluted with NaOH (60 mM). To eliminate Na^+ ions, this fraction was loaded onto a Biorex 70 cation-exchange column (H^+-form; Bio-Rad; 0.6 mL), washed with H_2O (1.0 mL) and NH_4OH (0.15 N; 3.4 mL), and eluted with NH_4OH (0.5 N; 0.7 mL). After filtration and drying, the residue was dissolved in H_2O (200 μL).

Alkylation of Modified Lysine Residues: A dried sample, which contained lysine residues modified with a quaternary ammonium group, was dissolved in sodium phosphate (50 mM, pH 7.0), and the solution was treated with sodium cyanoborohydride and acetaldehyde (reductive ethylation) according to a previously described protocol.[19] Reductive methylation was similarly carried out with sodium cyanoborohydride and formaldehyde.[19]

Mass Spectrometry of Basic Amino Acids: Electrospray ionization MS and fragmentation were performed by using an Ion Trap ESQUIRE LC instrument (Bruker, Billerica, MA). Samples were infused by a nanospray source in MeOH (50 %)/AcOH (0.5 %).

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