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Biomineralization

Silacidins: Highly Acidic Phosphopeptides from Diatom Shells Assist in Silica Precipitation In Vitro**

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Biomineralization is the formation of inorganic materials under the control of a living cell. Silica biomineralization occurs, for example, in unicellular diatoms with cell walls composed of silica. Although amorphous, diatom silica displays intricate structures of amazing beauty even on the nanometer scale.[1] Therefore, it is commonly assumed that the cellular processes that govern the biogenesis of this mineral may include structure-directing templates.[2–4] Single molecules, however, are much too small to act as templates for the observed shapes and patterns. Thus, only supramolecular assemblies are candidates as templates. Indeed, diatom silica is a composite material that contains organic substances with the potential to promote assembly. Highly zwitterionic proteins known as silaffins and long-chain polyamines have been identified as constituents of diatom biosilica, and have been shown to promote silica formation from monosilicic acid in vitro.[5,6]

To date, silaffins from *Cylindrotheca fusiformis* and *Thalassiosira pseudonana* have been characterized in detail. Silaffin-1 from *C. fusiformis* contains modified lysine and serine residues. Certain lysine residues are linked through their ε-amino groups to long-chain polyamines (four to eight propyleneimine repeated units), and all serine residues are phosphorylated.[7] Similar modification strategies are associated with silaffins extracted from the cell walls of *T. pseudonana*;[8,9] however, selected lysine residues are linked with only two propyleneimine units, which are further modified by methylation. Permanent positive charges are introduced by quaternary ammonium groups. Again, negative charges are introduced by the phosphorylation of serine residues. As a result of this zwitterionic nature, individual silaffins or silaffin mixtures are able to form supramolecular aggregates. In vitro, silaffins guide silica formation only in this aggregated state. Biosilica from all diatom species investigated so far also contains long-chain polyamines that are not attached covalently to a polypeptide backbone.[10] Their chemical structures display a remarkable degree of species specificity.[11] Interestingly, long-chain polyamines were also detected recently as constituents of biosilica produced by sponges.[12]

In vitro, polyamines display silica-precipitation activity if polyanions or silaffins with an acidic domain are present to allow their assembly by electrostatic interactions.[13,14] Evidence for the presence of phosphate and/or phosphorylated compounds in the shells of *Coscinodiscus* diatoms was obtained by ^{31}P NMR spectroscopy.[15] However, until now, no purely polyanionic substances that may serve as cross-linking agents for the assembly of long-chain polyamines could be identified in diatom biosilica. Herein, we describe a new class of aspartate/glutamate-rich and serine phosphate rich peptides as constituents of biosilica produced by the diatom *Thalassiosira pseudonana*. Owing to their presence in silica and their acidic nature, we refer to these peptides as silacidins.

It has been shown previously that organic constituents can be extracted from cell walls in a native state if an aqueous solution of ammonium fluoride is used to dissolve the silica.[7] By applying this procedure to the diatom *T. pseudonana*, several silaffins and polyamines could be extracted and separated by size-exclusion chromatography. The silaffins were denoted sil1/2L, sil1/2H, and sil3, respectively.[8]

If silaffin-1/2L is treated after purification by size-exclusion chromatography (Figure 1a) with a concentrated salt solution (2 M NaCl) and again subjected to size-exclusion chromatography under high-salt conditions, a previously undetected low-molecular-weight component dissociates and can be separated readily from silaffin-1/2L (Figure 1b). Amino acid sequencing of this material was only possible after treatment with anhydrous hydrogen fluoride, which indicates that this material is a peptide that contains many HF-labile posttranslational modifications.[16] After treatment with HF and purification by reversed-phase chromatography, this material can be separated further into three related substances (Figure 2). Edman sequencing of each of these substances identified in the N-terminal amino acid sequences given in Figure 2. These very unusual sequences consist mainly of serine residues and the acidic amino acids aspartic and glutamic acid. We named these related but slightly different peptides silacidins A, B, and C. These peptides were absent in fractions containing the more anionic silaffins sil1/2H and sil3.

The recently completed genome sequence of *T. pseudonana* has opened the door for genomic and proteomic approaches to compounds synthesized by this diatom.[17,18] A search in the corresponding data base uncovered a gene model that indeed encoded all three silacidin sequences. The terminal part of the corresponding open reading frame

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

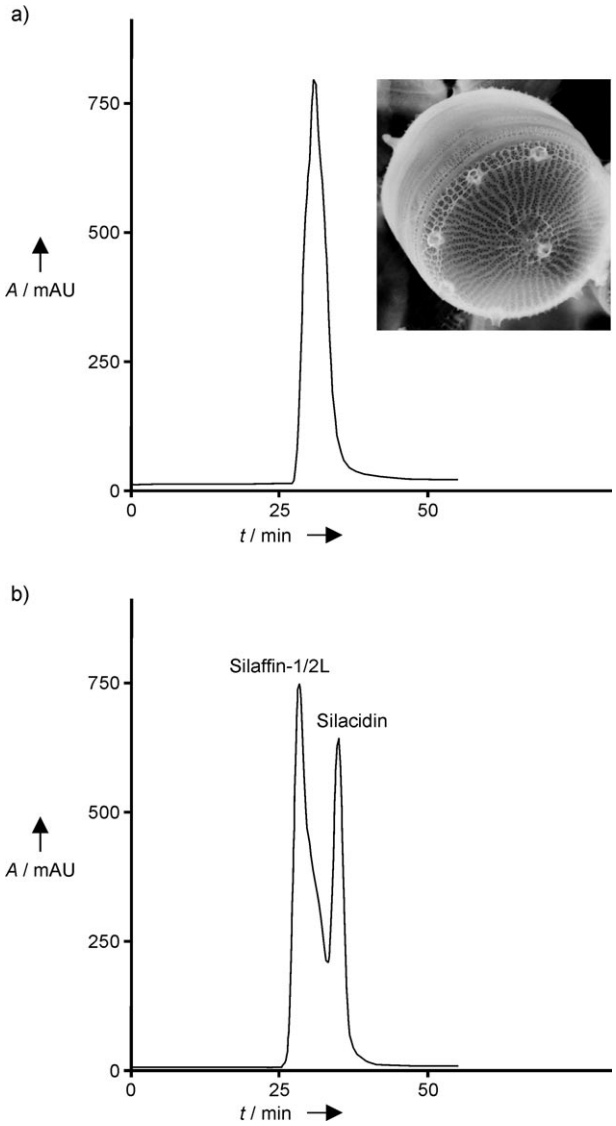

Figure 1. Size-exclusion chromatography of purified silaffin-1/2L under different ionic-strength conditions. a) Chromatography under low-salt conditions (50 mM NaCl) of a silaffin-1/2L preparation enriched from *T. pseudonana* cell walls (see inset); b) chromatography of the same preparation in the presence of 1 M NaCl. The procedure for the purification of silaffin-1/2L is described in the Supporting Information.

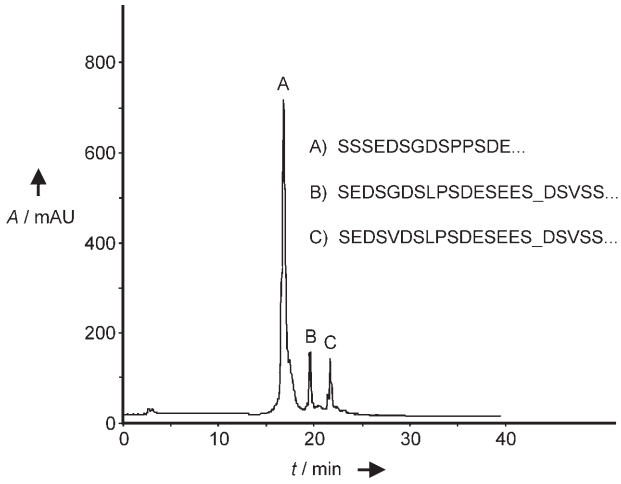

Figure 2. Reversed-phase chromatography of silacidin after treatment with HF. After treatment with HF, purified silacidin can be fractionated further into three peptide species. The corresponding N-terminal amino acid sequences are shown.

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     MVKYNVLAFIAVLGVSLINTSSAKTSL
RGHRQLAKPEKLGNANTSYALGSSINKVRRL
SSSEDSGDSPPSDESEESEDSVSSEDEDRRL  Silacidin A
SSSEDSGDSPPSDESEESEDSVSSEDEDRRL  Silacidin A
  SEDSVDSLPSDESEESEDSVSSEDEDRRL  Silacidin C
  SEDSGDSLPSDESEESEDSVSSEDEDRRL  Silacidin B
SSSEDSGDSPPSDESEESEDSVSSEDEDRRL  Silacidin A
SSSEDSGDSPPSDESEESEDSVSSEDEDRRL  Silacidin A
SSSEDSGDSPPSDESEESEDSVSSED-
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Figure 3. The silacidin A, B, and C peptides are encoded in a single open reading frame derived from a proposed gene model located on chromosome 2 of *T. pseudonana*. The spacer sequence between individual silacidin repeats is shown in red. Diagnostic amino acid residues for the identification of silacidins A, B, and C are shown in blue.

encodes a polypeptide with a striking repetitive structure (Figure 3). In this stretch of six highly homologous repeated units, four repeats correspond to silacidin A, and the remaining two repeats encode silacidins B and C. This arrangement suggests that silacidins originate from the endoproteolytic processing of a precursor polypeptide. The spacer sequence RRL that separates each repeated unit is not found in the mature silacidins, as has been proved for silacidin A by mass spectrometry. The calculated mass for the silacidin A sequence SSSEDSGDSPPSDESEESEDSVSSEDED (without posttranslational modifications) is 2920.0. MALDI-TOF analysis showed silacidin A to have exactly this mass (2920.2) after treatment with HF, thus confirming the absence of the RRL spacer sequence. Remarkably, the same gene organization was found previously for the silica-precipitating silaffin-1 peptides from *C. fusiformis*. The spacer sequences encoded in this gene were RRNL and RRIL.[5] This similarity suggests the operation of analogous processing pathways for the two silica-associated protein families.

To elucidate the chemical nature of the HF-sensitive modifications in silacidins, the native silacidin preparation (containing silacidins A, B, and C) was subjected to 15 cycles of Edman degradation. With the exception of serine, all expected amino acids were detected in the corresponding cycles. This result indicates a posttranslational modification of the serine residues. A single signal was observed at $\delta =$ 4.8 ppm in the ^{31}P NMR spectrum of a native silacidin preparation (pH 7, 500 mM NaCl). This signal indicates the presence of serine phosphates. To explore this hypothesis, native silacidins were hydrolyzed in 6 N HCl at 110 °C for varying periods of between 2 and 5 h, and the resulting hydrolysates were analyzed for the presence of serine phosphate by HPLC anion-exchange chromatography. Sila-

cidin-derived material with the same retention time as authentic serine phosphate was collected and identified as serine phosphate by mass spectrometry (negative-ion mode). After acidic hydrolysis for 2 h, the ratio of serine phosphate to free serine was 1.35 (see Table 1 in the Supporting Information), which indicates that at least 60% of the serine moieties present in the silacidins are phosphorylated. Thus, silacidins are extremely acidic peptides.

Silica precipitation in vitro guided by long-chain polyamines has been shown to depend on multivalent anions, such as phosphate or pyrophosphate. These anions induce the assembly of polyamines, which is a prerequisite for silica precipitation.[13] Phosphate anions exert a striking degree of control over the size of precipitating silica nanoparticles.[19] In the light of these findings, the silacidins in diatom cell walls may serve as the biologically relevant agent that guides the assembly of polyamines. Therefore, we tested the potential of a polyamine/silacidin binary system with respect to its ability to precipitate silica from a silicic acid solution. Polyamines isolated from *T. pseudonana*[20] were combined with increasing amounts of native silacidins. Following the addition of silicic acid, we determined the amount of silica that precipitated within 12 min (Figure 4). In the presence of polyamines and acetate anions only (the buffer system), no precipitate was formed. The addition of silacidins induced silica formation in a concentration-dependent manner, even in the micromolar range. The efficiency of the silacidins is remarkable: For phosphate anions to induce comparable amounts of silica precipitation, they would need to be present in concentrations at least two to three orders of magnitude higher. As shown previously for phosphate anions, the diameters of the silica nanospheres produced increase as the concentration of the silacidins increases. These results strongly support the hypothesis that silacidins serve as the polyanion required in vivo for silica formation directed by polyamines (and/or silaffins).

Interestingly, polypeptides with similar structures were found in quite different biomineralization processes involving the amorphous inorganic phases calcium phosphate[21,22] and calcium carbonate.[23] Thus, this first highly acidic peptide from diatom biosilica puts the concepts of acidic proteins, phosphates, and stable/transient amorphous phases in biomineralization[24] and the hierarchical assembly of composite materials[25,26] into a broader evolutionary context.

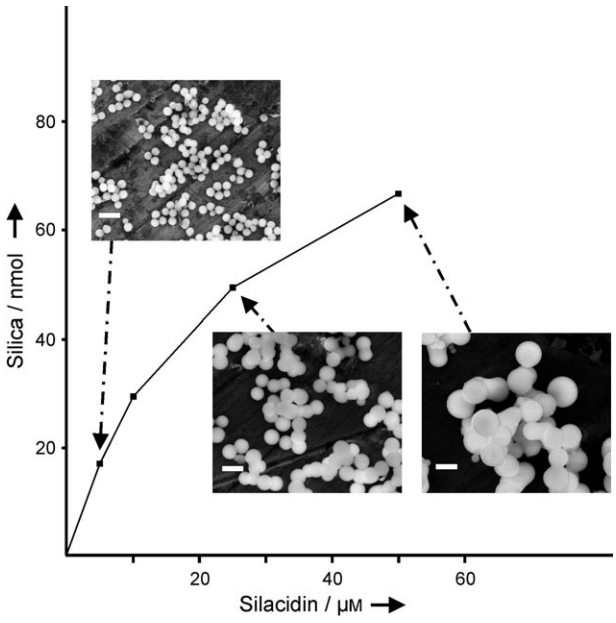

Figure 4. Polyamine-induced silica formation from silicic acid depends on silacidin polyanions. Silica precipitation was performed in the presence of *T. pseudonana* polyamines (1 mM), silicic acid (100 mM), and increasing amounts of silacidin at pH 5.5. The insets show the size distribution of the precipitated nanospheres (SEM images) at a given silacidin concentration. Scale bar: 1 μm.

Experimental Section

Silacidin was purified as described in the Supporting Information. Polyamines from *T. pseudonana* were purified as described previously.[20] The concentrations of solutions of silacidin were determined by ^{1}H NMR spectroscopy on the basis of the methyl signals of leucine and valine at $\delta = 1.0$ ppm; valine (1 mM) served as a reference. The concentrations of polyamines were determined on the basis of their methylene signal at $\delta = 1.7$ ppm; spermine served as a reference.

A detailed description of the analysis of serine phosphate and N-terminal sequencing is provided in the Supporting Information.

Mass spectrometry: The molecular mass and fragmentation pattern of silacidin after treatment with HF was determined by MALDI-TOF/TOF MS (Applied Biosystems 4700 Proteomics Analyzer).

The search tool used for the database search is described in the Supporting Information.

Silica formation in vitro: Silica-formation assays were performed in water (10 μL) containing polyamines (1 mM) from *T. pseudonana*, sodium acetate (pH 5.5, 25 mM), silacidin as indicated, and silicic acid (100 mM; tetramethoxysilane (1M) was subjected to hydrolysis for exactly 15 min in 1 mM HCl at room temperature and used immediately). After 12 min at room temperature, precipitated silica was collected by centrifugation, washed with water, dissolved in 2M NaOH at 95°C, and quantified by the molybdenum blue method.[27]

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