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EVIDENCE FOR INTRAMINERAL MACROMOLECULES CONTAINING PROTEIN FROM PLANT SILICAS

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Key Word Index—*Equisetum telmateia*; Pteridophyta; *Phalaris canariensis*; Gramineae; Canary Grass; silica; silicification; biomineralisation; protein; intramineral.

Abstract—Macromolecular assemblages intimately associated with biogenic silica in plants are released on solubilization of the siliceous phase by treatment with buffered aqueous solutions of HF following treatment of plant material with concentrated oxidizing acids (perchloric, nitric and sulphuric) to remove cytoplasmic contents and the largely polysaccharidic cell wall. The non-dialysable material, which may form 0.015–0.030% dry weight of the silica, has an amino acid composition rich in Pro–Glu, Pro–Lys, or Ser–Asp–Gly depending on the extent of treatment with oxidizing acids. The materials are suggested to have an intrasilica location with materials of similar composition being extracted from the branches of *Equisetum telmateia* (a primitive plant) and from hairs from the lemma of the grass *Phalaris canariensis*. The role of such material in regulating nucleation, particle growth and aggregate structure development in silicas is discussed.

INTRODUCTION

Biominerals are produced by a variety of single cell organisms, higher plants and animals [1]. Minerals produced by biological organisms include oxides of silicon and iron, and carbonates, sulphates, oxalates and phosphates of the Group IIA elements (calcium, strontium and barium). Organisms regulate the chemical composition (including trace element composition), the crystal phase, the point of deposition and the morphology of the biomineral. Additionally, all biominerals are formed in confined spaces in the presence of membrane structures and/or cell wall matrices. Acidic proteins/glycoproteins are often found in association with mineral phases as components of the organic matrix and are considered to be key components in the mineralization process. It is proposed that their use in controlling mineralization evolved long before the advent of skeletal formation some 540 million years ago [2]. The organic phase is largely found external to the mineral phase but low levels (*ca* 0.03% by weight) of these proteinaceous biopolymers can also be found intercalated within the mineral phase, perhaps at crystal domain boundaries. The intercalated biopolymers may have fundamental roles to play in nucleation and/or crystal growth and their presence has also been shown to have dramatic effects on the mechanical properties of crystalline biominerals [3].

In comparison with studies on the formation of crystalline biominerals such as calcium carbonate very little is known concerning the organic phase associated with hydrated amorphous silica deposits. The existence of an organic matrix which interacts with diatom silica during mineralization was proposed by Hecky *et al.* [4], based on the discovery that cell wall protein, when compared with cellular extracts, was enriched in serine, threonine and glycine and depleted of acidic, sulphur-containing and aromatic amino acids. They proposed that in diatoms, condensation of silicic acid to produce covalently bonded organic/inorganic composites is mediated by a protein template enriched in serine and threonine. Other workers have also shown that in these organisms there is enzymatic activity associated with protein synthesis immediately prior to silica deposition [5]. More recently, Swift and Wheeler [6] have extracted what they believe to be a proteinaceous matrix material from diatoms which is enriched in serine and glycine and is relatively more hydrophilic (results based on the method of Kyte and Doolittle [7]) than the general proteins of the cell. Glycoproteins of a very different composition have been extracted from the diatom *Cylindrotheca fusiformis* [8]. The main feature of these acidic, *ca* 75 kDa glycoproteins is the presence of repeated acidic cysteine-rich hexapeptides within domains rich in acidic residues. The glycoproteins also contain rhamnose, galactose, xylose, glucose, mannose and an unidentified sugar in the ratio 5:4:4:2:1:4. The accumulation of these glycoproteins in the cell wall corresponds to the formation of new valves, and is therefore thought to play some role in mineral formation. For plant material where silica deposition occurs in the presence of an extensive carbohydrate

matrix (the secondary cell wall) no such information on the role of protein containing macromolecules is currently available.

In the formation of amorphous silica there are three distinct processes which occur. The processes are oligomerization from monomer units to yield stable nuclei, particle growth, and aggregate development. For silicas produced by aqueous industrial processes these stages can be identified but are poorly understood due to the inherent difficulty of regulating the various stages of oligomer formation in an aqueous environment. The general product from such routes is an ill-defined material with a wide range of particle sizes and pore texture. In direct contrast are the materials produced by biological organisms where the result of a well-regulated polymerization process is the formation of silicified structures built up from particles of well defined size, morphology, surface chemistry and texture [9]. In our search to understand how such materials are produced (and ultimately to extend our knowledge to biomimetic routes for the preparation of novel silicas) we operate a two pronged approach to our research. We investigate the biological systems in order to learn how they operate and at the same time perform model oligomerization experiments in order to investigate the potential regulatory ability of individual components present in the biological system.

In this paper we present data which for the first time suggests that protein containing material is trapped within biologically precipitated silica, and which, by analogy to intracrystalline proteins found in calcium carbonate minerals, may have an important role to play in structure control [3, 10]. The presence of biomolecules intimately associated with the siliceous phase may also have profound effects on the physical and mechanical properties of the inorganic/organic composites.

The plant materials chosen for study are the heavily silicified hairs from the lemma of the grass *Phalaris canariensis* L. and branches from the primitive plant *Equisetum telmateia*. Both have well characterized silica structures [11, 12]. For contrast, leaves from a poorly silicified grass (*Phragmites* sp.) were also examined.

RESULTS

The three plant materials differ greatly in their % weight of silica. The *Phalaris* hairs are most heavily silicified and contain *ca* 40% dry weight silica. The branches of *Equisetum* contain *ca* 20% dry weight silica and the leaves of *Phragmites*, *ca* 2% dry weight silica. In addition, the samples are different at the microscopic level. Figure 1 shows the representative types of silica structures obtained from the three plant materials. For *Phalaris canariensis* L., three different microstructural forms of silica are observed within a single hair cell from the lemma of the plant. Fibrous, globular and sheet-like structures can be distinguished by differences in morphology, size and organization of the silica at the microscopic scale. The fibrous material seen in Figure 1(a) exhibits clear linear direction and particles are *ca* 5 nm in length and 2–3 nm in diameter and joined end-on to give

Fig. 1. Transmission electron micrographs of silica structures from plant materials. Scale bars represent 200 nm. (a) Fibrillar material from *Phalaris* hairs, (b) sheet-like material from *Phalaris* hairs, (c) globular material from *Phalaris* hairs, (d) globular material from *Equisetum* branches, (e) disordered material from *Phragmites* leaves.

Table 1. Amino acid composition by functional type for the three plant materials investigated (Numbers given are as mol% of the recovered amino acids)

Amino acid side chain	*Phalaris canariensis* hairs (mol% of amino acids recovered)				*Equisetum telmateia* branches (mol% of amino acids recovered)				*Phragmites* sp. leaves (mol% of amino acids recovered)	
	Intact hairs	After treatment (1)	After treatment (1 + 2)	Insoluble residue after treament (1 + 2)	Intact branches	After treatment (1)	After treatment (1 + 2)	Insoluble residue after treatment (1 + 2)	Intact leaves	After treatment (1)
Aliphatic	39.8	35.6	30.4	29.2	41.0	38.4	26.9	12.8	33.3	17.9
Hydroxyl	9.9	23.5	4.6	6.1	11.5	23.4	3.7	2.9	8.0	2.6
Aromatic	3.0	1.8	0.1	0.8	4.3	1.4	0.0	0.0	1.0	0.0
Basic	7.9	6.5	10.6	26.0	12.7	5.5	15.8	40.7	10.8	15.7
Acidic	20.2	25.0	17.8	16.4	23.8	25.2	22.4	12.7	27.3	26.0
Sulphydryl	0.7	0.4	0.1	0.3	1.3	0.7	0.0	0.0	0.4	0.0
Proline	18.6	7.2	36.3	21.2	5.5	5.5	24.0	31.2	19.2	37.8

continuous strands. Globular and sheet-like structural types are composed of silica spheres 5–8 nm in diameter. In the sheet-like materials, Fig. 1(b), thin layers of particles are observed. In the globular material, Fig. 1(c), irregular spheres of varying diameter are packed together in a random manner. For more detail see Perry *et al.* [11]. Silica deposits extracted from the branches of *Equisetum telmateia* are built up from large areas of globular material (Fig. 1(d)). Individual particles are 7–8 nm in diameter. In addition, small areas of fibrillar material, normally no larger than 300 nm by 150 nm are sometimes observed interspersed with the globular matrix with particles being arranged in short parallel rows [12]. Silica extracted from *Phragmites* leaves shows no recognizable structural motif, Fig. 1(e), with a range of particle sizes *ca* 4–15 nm in diameter. The microscopic appearance of silica obtained after the second acid treatment showed no appreciable difference to those samples prepared after the first acid treatment which is not perhaps surprising as the difference in the amount of organic material associated with the silica was very small, see below.

The amino acid analyses of plant materials and acid-treated samples are given in Table 1 and Figs 2 (*Phalaris*), 3 (*Equisetum*) and 4 (*Phragmites*). Table 1 gives the % amino acid composition by functional group type after the various treatments and Figs 2–4 give information on the individual mol% recoverable amino acid compositions of the extracts obtained after the treatments described below. Acid treatment has a dramatic effect on the absolute amount (wt %) of proteinaceous material associated with the silica phase. After the first treatment the % protein is reduced from *ca* 3% for *Phalaris canariensis* hairs to 0.06% and after the second acid treatment to *ca* 0.02% by weight. Similar levels are found in the intact *Phragmites* leaves and considerably more in the intact *Equisetum* sample although after the acid extraction the levels of protein remaining in association with the silica were very similar. For all three plant materials investigated there was a decrease in the levels of the aliphatic, aromatic and sulphur-containing amino acids in the protein extracts obtained after the sequence

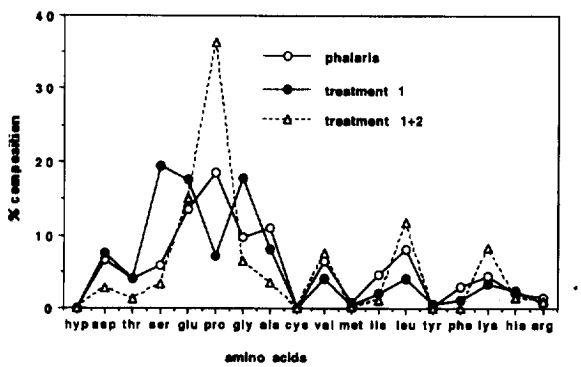

Fig. 2. The amino acid composition of protein-containing material extracted from hairs taken from the lemma of the grass *Phalaris canariensis*, (○) intact hairs, (●) after first acid treatment, (△) soluble material after both acid treatments.

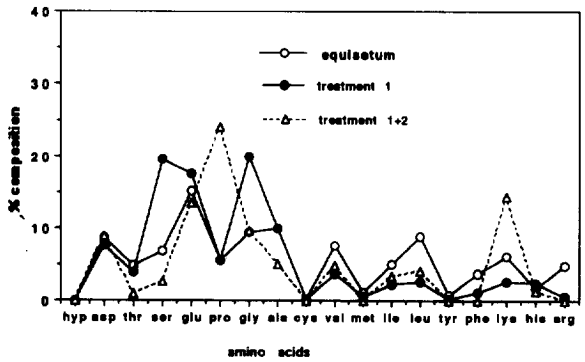

Fig. 3. The amino acid composition of protein-containing material extracted from the branches of *Equisetum telmateia*, (○) unextracted samples, (●) after first acid treatment, (△) soluble material after both acid treatments.

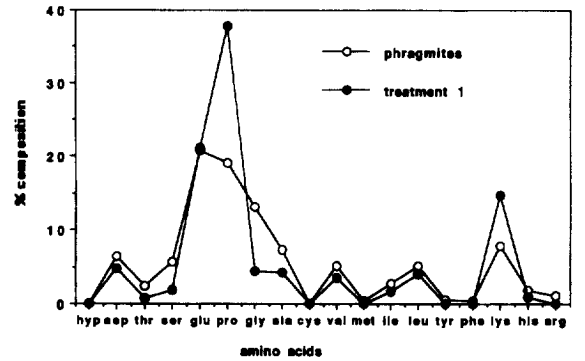

Fig. 4. The amino acid composition of protein-containing material extracted from *Phragmites* leaves, (○) intact leaves, (●) after first acid treatment.

of acid treatments performed prior to silica dissolution to yield the soluble intrasilica phase. After both acid treatments had been performed on the *Phalaris* and *Equisetum* samples the soluble extracts contained lower levels of amino acids with hydroxyl groups, higher levels of basic amino acids and considerably higher levels of proline (levels of hydroxyproline present in intact samples was minimal (< 1%) and was not detected for any of the other samples analysed using ion chromatography). The levels of acidic amino acids were little changed from the original levels in the intact plant materials. For the *Phragmites* samples, the alterations observed in the composition of the amino acid hydrolysates as described above were observed after treatment of samples with mixtures of concentrated nitric and sulphuric acids only. In contrast, the amino acid compositions of hydrolysates obtained after this acid treatment from the *Equisetum* and *Phalaris* samples had increased levels of hydroxylated amino acids. There were smaller decreases in the levels of the basic amino acids and a small increase in the relative concentration of the acidic residues present. For the *Phalaris* sample only, there was a small increase in the amount of proline present in the extract. A feature of the insoluble residues obtained after acid treatments (1 + 2) and subsequent HF solubilization of the silica phase are the high levels of basic amino acids, proline and aliphatic amino acids.

When the composition profile for individual amino acids is considered for the various samples it is clear that there are marked changes in the composition of the proteinaceous phase extracted. Figure 2 shows the amino acid composition of the *Phalaris* samples. Acid treatment (1) followed by HF extraction results in soluble macromolecules which show dramatic increases in serine and glycine relative to the intact hairs. There are also specific, but smaller increases in the levels of the threonine and acidic residues with the relative proportions of the different aliphatic amino acids being also modified. The second acid treatment followed by HF extraction produces a material dramatically reduced in functionality, with much lower levels of glycine and considerably higher levels of proline and lysine. The insoluble extracts produced at this stage are highly charged and have extremely high levels of lysine (*ca* 22%), histidine (12%), glutamic acid (14%) and proline (25%). For extracts produced from *Equisetum* branches a similar amino acid profile is observed for proteinaceous material extracted after acid treatment (1) (related by the Cornish–Bowden method [13]) but the material extracted after treatment (2) contains even higher levels of basic amino acids, specifically lysine and marginally lower levels of proline. The amino acid profile obtained from *Phragmites* is more closely related to that obtained from *Phalaris* hairs than from *Equisetum* branches possibly because both *Phalaris* and *Phragmites* are members of the Gramineae. After the first acid treatment the proteinaceous material extracted is very rich in proline (*ca* 38% molar composition), glutamic acid (*ca* 20% molar composition) and lysine (15% molar composition). The composition of this extract is more like that obtained after *both* acid treatments for the other two plants.

Preliminary evidence has been obtained for the presence of carbohydrate material in the intrasilica extract from *Phalaris*. After both acid treatments the soluble extract contains approximately equimolar concentrations of protein (as measured by amino acid concentrations) and carbohydrates (as measured by monosaccharide concentrations). Polymers built up from glucose and xylose make up more than 90% of the total weight of the saccharidic component of the material extracted. The molar ratio of glucose to xylose is *ca* 2:1. Insufficient material has been available to investigate the possibility of carbohydrate being present in the other intrasilica extracts.

DISCUSSION

The experimental data described above provides evidence for the presence of protein containing material which is intimately associated with the biologically produced silica. The extensive oxidizing treatment methods used to remove the cell wall debris have a dramatic effect on the amount of organic material which remains associated with the silica. It is clear that whatever organic material remains at the various stages is well protected

by the siliceous phase and is largely unaffected by the acid treatments used. At present it is not possible to say whether we are dealing with glycosylated proteins and/or mixtures of peptides and carbohydrates although preliminary data tends to suggest that silica extracts comprise several distinct peptide containing materials (Harrison, C. C. and Harthill, J. E., unpublished information).

The amino acid composition of the organic material extracted after various levels of acid treatment has little in common with material extracted from diatoms [6, 8]. The close similarity in composition of the material extracted from both *Phalaris* hairs and *Equisetum* branches is perhaps not surprising if the proteinaceous phase has a similar role to play in the different plant systems. It is likely that the materials obtained after the different treatments used may have different roles to play in the regulation of silica polymerization and precipitation.

In order for silica to form polymerization of orthosilicic acid molecules must occur to produce stable nuclei and these nuclei grow to produce recognizable particles which are generally arranged into aggregate structures which may or may not have definite structural patterns. The general oligomerization and aggregation process will occur in solution provided that the concentration of orthosilicic acid exceeds 100 ppm. The pH of the solution and the presence of ions can have profound effects on the sizes of the particles produced [14]. Our recent studies have also shown that organic molecules in solution and present as solid surfaces can have an effect on the nature of the silica aggregate structures formed [15]. Carbohydrate polymers such as cellulose and oligomers as small as trisaccharides, can regulate particle growth to produce particles with a very narrow ($\pm$ 0.5 nm) size distribution [15, 16]. However, we found that the presence of a wide range of neutral organic molecules have little effect on the kinetics and rate of oligomerization of the system (Harrison, C. C. and Loton, N., unpublished data). It is well established that charged species have a major effect on oligomerization kinetics [14]. The effect is dependent on charge density with high valent ions having more of an effect than unicharged ions. However, both inorganic and organic unicharged ions do affect the rate of oligomerization reactions to produce stable nuclei, but in general do not have the ability to produce ordered structures as are found in *Phalaris* hairs.

It is possible that *in vivo* a range of molecules, organic and inorganic with different chemical characteristics and structure may provide the means by which well regulated siliceous structures are formed.

From the data presented here we propose that the organic material which is most difficult to remove and is highly charged (very high levels of lysine and acidic groups) with a reasonably rigid backbone (high levels of proline) may be involved in regulating nucleation. Those materials which are a little easier to expose and contain correspondingly higher levels of amino acids (such as serine) which would bind to silica by a hydrogen bonding mechanism (in a similar way to the proposed interaction between saccharides and silica [15]) and would provide the means by which growth of particles is regulated. Other cell wall proteins and some of the carbohydrate polymers present in the secondary cell wall may also be involved in regulating particle growth and in the delineation of spaces with specific shapes in order for structural motifs of silica and macroscopic features to result. At present the nature of interactions between silicas and protein/proteoglycan materials are not known but it is likely that the interactions will include a significant contribution from hydrogen bonding given the nature of the functional groups present. It is important to note that specific effects are most likely to occur when relatively weak interactions are involved, since even slight differences in recognition will tend to be amplified. In support of this idea, current studies on the separation of protein assemblages from calcified sea urchin spines according to charge have shown that acidic and moderately acidic members exhibit different effects on *in vitro* crystallization when present at levels similar to those found in the biological situation [18]. It is clear that in the formation of biogenic silica a combination of biomolecules of different chemical composition and structure may be used to control all stages in the formation of silica structures from the fundamental building blocks through to the macroscopic structures. Further work is aimed at elucidating the structures of the macromolecules intimately associated with the silica and investigating their effects on silica polymerization in a model system. These topics will be the subject of future publications.

EXPERIMENTAL

Sample preparation. Samples for the study of intrasilica proteins were obtained as follows. *Phalaris canariensis* L. hairs were obtained from Darling Downs Grain Exporters Pty (Queensland, Australia) and were separated from other plant fragments by sieving. This was continued until no other plant fragments were visible in the sample when viewed with a light microscope operating at a magnification of 100 ×. *Equisetum telmateia* samples were collected from Wytham Woods (Oxford, U.K.) and the branches separated from the stems before drying. Samples of *Phragmites* sp. were collected from Hulda (Israel) the leaves separated and dried before further treatment. In all cases, samples of plant material were ground up using a pestle and mortar before being treated with concentrated acids. In general, three aliquots of each plant material were investigated. Treatments used were slight modifications of a method used to routinely extract phytoliths from plants (Method (1)) [19] a method used by environmental paleontologists for the preparation of siliceous samples for stable isotope work (Method (2) alone) [20]. Acid treatment (1) involved heating samples at 60–70° for 24 hr in a 4:1 mixture of concentrated nitric–sulphuric acids followed by centrifugation and washing up to eight times with deionized water before lyophilizing. Acid treatment (2) was performed on samples of *Phalaris* hairs and *Equisetum* branches with a 1:1 mixture of concentrated nitric acid and 60% perchloric acid at 60° for 6 periods of 30 min with the acid being renewed after each period of 30 min. Any remaining

material was then washed and lyophilized as above. Acid treated siliceous samples (after treatment (1) only or after the sequence of treatments ((1) < (2)) were then placed into teflon tubes in a mixture of 1–3% HF in 3% NH_4F (pH 5) and left stirring or shaking at 4° for a period of 48 hr, before extensive dialysis (Spectropor 3 tubing with a molecular weight cutoff of 3500) against a minimum of 6 changes of deionized H_2O, and lyophilized. Occasionally a soluble and insoluble fraction resulted after HF treatment and these were separated by centrifugation prior to lyophilization of both fractions. The HF treatment had no noticeable effect on the dialysis tubing nor on the molecular weight of test proteins [21]. Protein admixed with abiotic silica prior to the acid treatments described above did not result in any dialysable material after HF treatment to solubilize the siliceous phase.

Amino acid analysis. For amino acid analysis samples were hydrolysed under vacuum using 6M HCl at 112° for 24 hr after flushing twice with nitrogen. The hydrolysates were analysed on a Dionex BIOLC amino acid analyser.

Monosaccharide analysis. For monosaccharide analysis samples were treated with 2M TFA at 120° for one hour and analysed using a DIONEX 450 HPLC system with pulsed amperometric detection of the monosaccharides.

Electron microscopy. Electron microscopy was performed using a JEOL2000FX electron microscope operating at 200 keV. Samples were prepared by suspending the silica phase obtained after acid-treatment (1) in chloroform and placing a drop on a holey carbon, Formvar-coated electron microscope grid. Samples were also investigated after the second acid treatment but no significant differences in morphology were observed.

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