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Aqueous Macromolecules with Silicon from Alcohol-insoluble Residues of Rice Seedlings

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Abstract

Rice (*Oryza sativa*) seedlings were grown in the presence of 5 mM orthosilicic acid (H_4SiO_4, IUPAC name: tetrahydroxyl silane). More than 80% of silicon absorbed in the rice was localized in the alcohol-insoluble residues (AIR) fractions. AIR refers to cell wall materials in the text. Driselase (commercial cell wall hydrolyzing enzyme preparation) released 3% of silicon present in the rice AIR into water-soluble fractions. Size-exclusion high performance liquid chromatography/inductively coupled plasma atomic emission spectroscopy (HPLC/ICP-AES) showed that an aqueous silicon-containing substance with high molecular weight was present in the water-soluble fractions. The compounds were stable at pH 6.5, while commercial silica sols which were stable at pH 9.0 were insoluble at the neutral pH. The stability in neutral pH may be due to the presence of a complex of polysaccharide and protein in the molecule. These results imply that silica-containing macromolecules exist in rice cell walls.

Discipline: Soil, fertilizer and plant nutrition
Additional key words: cell walls, HPLC/ICP-AES, *Oryza sativa*, silicon

Introduction

Silicon (Si) is the second most abundant mineral element in soil[3]. Plants accumulate quantitatively high levels of silicon; in particular, Poaceae plants contain 10–100 g Si kg^{-1} on a dry weight basis[3,4,22]. The beneficial effects of silicon on land plants have been demonstrated although its essentiality for land plants is not recognized. Silicon-deficient Poaceae plants are more susceptible to pests and pathogens[4,6,21], are less resistant to drought[6] and salt stress[12], are structurally weaker, and have abnormal growth and development, compared to silicon-replete plants[3,4,22]. Silicon modified the chemical properties of cell walls and regulated cell wall extensibility in oat leaves[9]. Some tropical woods contain silicon in ray parenchyma[26]. Silicon-containing woods are resistant to the attack of insects and microorganisms but sometimes high content of silicon in woods is an obstacle for the lumbering process[26].

Silicon in the soil solution is mainly present in the form of orthosilicic acid, H_4SiO_4. At circumneutral pH the equilibrium concentration of monomeric silicic acid is less than 2 mM and above this polycondensation produces initially oligomers and eventually colloidal particles of solid, hydrated silica (silicon dioxide)[1]. The silicic acid is readily absorbed by plants, especially in rice[3,4]. A silicon transporter Lsi1, which promotes the uptake of silicic acid, has recently been identified in rice[14]. The absorbed silicon is transported from root to stem. The monomeric silicic acid is finally deposited in the apoplast and forms a polymer of hydrated amorphous silica, making silica-cuticle double layers on the surface of leaves, stem and hulls[3,4,17]. The accumulated silica in the cell walls would contribute to structural strength of cell wall[3,4,17].

There have been a few reports on the chemical interactions between silica and cell wall constituents of organisms other than land plants[13,20,25]. In the cell walls of the diatom, *Cylindrotheca fusiformis*, a highly polyanionic phosphated protein, termed "silaffin", was associated with silica, and mediated precipitation of porous silica[13,20]. A protein, named "silicatein" that seems to control biosilicification was identified from the sponge, *Tethya aurantia*[25]. The horsetail, *Equisetum telmateia* and the grass, *Phalaris*

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canariensis had silica complex with protein[7]. The complex is considered to be involved in biosilica formation. It was suggested that silicon might be combined with a phenolic acid or aromatic ring moiety of the lignin-carbohydrate complexes in rice cell walls[10,11]. To date, however, there has been no direct evidence that silica binds to organic molecules such as polysaccharides in the cell walls of land plants. In this report we show that a water-soluble silicon-containing macromolecule was present in the Driselase hydrolysates of rice alcohol-insoluble residues (AIR) using size-exclusion high performance liquid chromatography/inductively coupled plasma atomic emission spectrometry (SE-HPLC/ICP-AES)[2]. We characterized the partially isolated molecules by amino acid and sugar composition analyses.

Materials and methods

1. Materials and growth conditions

Rice (*Oryza sativa* L. cv. Koshihikari) caryopses were purchased from a local seed store (Ibaraki, Japan). Silica sol, Cataloid SI-45P (diameter; 45 nm), was purchased from Catalysts & Chemicals Ind. Co., Ltd. Other chemicals and reagents used were obtained from Wako Pure Chemicals (Osaka, Japan). Caryopses of rice were sterilized for 1 day at 25ºC with aqueous 0.5% benomyl (Sumitomo Chemicals, Osaka, Japan), rinsed with water and then kept in water for 2 days at 25ºC. The caryopses were germinated and grown on agar containing 0 or 5 mM concentration of silicic acid in the dark for 7 days at 25ºC. Sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$) was used as the source of silicon. To avoid the effects of strong alkalization and excess of sodium ions on rice growth, the sodium metasilicate nonahydrate solution was passed through a cation-exchange column (Dowex 50W-X12, H^+ form). The resultant silicic acid solution was diluted to final silicon concentration. The concentration of silicon was determined with ICP-AES SII SPS4000 (Seiko Instruments Inc., Chiba, Japan) at a wavelength of 251.611 nm.

2. Preparation of alcohol-insoluble residues

The rice seedlings were suspended in aqueous 80% (V/V) ethanol and homogenized for 0.5 min at 1,500 rpm using a Polytron blender (Physco-Troller NS-610, Nichion Rikakikai Co., Tokyo). The suspensions were centrifuged at 2,500 *g* for 2 min. The AIR was then washed with 80% (V/V) ethanol, 95% (V/V) ethanol, 99.5% (V/V) ethanol, chloroform:methanol (1:1, V/V), and acetone, and then air-dried. The intercellular proteins and polyphenols are soluble in aqueous-alcohol and lipids and membrane fractions are extracted with chloroform:methanol treatment[24]. AIR refers to cell wall materials and usually the starting materials[24]. A part of the AIR (~10 mg) was digested with Driselase® (Kyowa Hakko Kogyo Co., Ltd.) as described elsewhere[15] and analyzed by SE-HPLC/ICP-AES. Driselase is a mixture of endo- and exo- enzymes from *Polyporus tulipiferae*. It is highly active on plant cell walls. The activity includes the following exo-hydrolases: α-D-galactopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, α-D-mannopyranosidase, β-D-mannopyranosidase, α-L-arabinofuranosidase, β-D-xylopyranosidase, α-L-fucopyranosidase, cellulose-cellobiohydrolase; and endo-hydrolases: cellulase [β-(1→4)-D-glucanase], β-D-galactanase, α-L-arabinanase, pectinase, β-D-mannase, xylanase[5].

3. Purification of silicon containing macromolecules

The AIR (10 g) was treated for 4 h at 4ºC with 0.1 M sodium hydroxide (500 mL) to saponify ester groups such as the methyl, acetyl and phenolic acids. The suspensions were adjusted to pH 5.0 with 10% V/V (~1.7 M) acetic acid and then treated for 16 h at 30ºC with Driselase. The suspensions were centrifuged and the insoluble residues washed with water. The solubilized material was dialyzed against deionized H_2O with 10,000 mol wt cut dialysis membrane (Amicon Hollow fiber HIP 10-20). The retentate was freeze-dried, and then loaded onto a column (1.6 × 90 cm) of Sephadex G-75, equilibrated with 50 mM ammonium formate buffer, pH 5.3, and eluted with the same buffer. Fractions (2 ml) were collected and the void volume fractions were pooled. The pooled fractions were loaded onto a column (1.6 × 30 cm) of DEAE-Sepharose Fast Flow that had been equilibrated with 50 mM ammonium formate buffer, pH 5.3. The column was eluted with a step gradient of 50, 100, 200, and 500 mM ammonium formate, pH 5.3 (200 mL each). Each fraction was dialyzed against deionized water with 12,000 mol cut dialysis tube and freeze-dried, and then analyzed by SE-HPLC/reflective index (RI) detector (JASCO PU1580i pump and RI-1530 detector) and SE-HPLC/ICP-AES.

4. Analytical methods

SE-HPLC/ICP-AES was performed using a HPLC pump (JASCO PU980) equipped with a TSKgel $G3000PW_{XL}$ (exclusion limit; 2×10^5) or $G5000PW_{XL}$ (exclusion limit; 2.5×10^6) column (TOSOH, Japan) coupled to the ICP-AES (SII SPS4000) through poly ether ether ketone (PEEK) tubing. The column was eluted at 1.0 mL/min with 200 mM ammonium formate, pH 6.5. The neutral and acidic glycosyl compositions of the samples were determined by GC-MS analysis of alditol acetate derivatives and trimethylsilyl (TMS) methyl glycoside derivatives, respectively[28]. The neutral sugars were

determined as follows. The samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121ºC for 1 h, and TFA was removed with a stream of air. The resulting hydrolyzates were reduced with sodium borohydride and then acetylated with acetic anhydride and pyridine. Per-*O*-acetylated alditol acetates were analyzed by GC and GC-MS. The glycosyluronic acids and neutral sugars were determined as follows. The samples were treated with 1 M HCl in methanol at 80ºC for 16 h. The resulting methyl glycosides and methyl ester methylglycosides were trimethyl silylated. TMS derivatives were analyzed by GC and GC-MS. Amino acid composition analysis was performed at Toray Research Center (Kamakura, Kanagawa, Japan).

Results and discussion

Rice seedlings were grown on agar containing 0 (control) or 5 mM concentration of silicic acid, because 5 mM concentration of silicic acid was the most effective for rice growth[8]. Cell wall materials were prepared from the rice seedlings containing coleoptiles, first, second, and third leaves as AIR. The silicon content in rice seedlings and AIR was determined by ICP-AES. The rice seedlings grown in the presence of 5 mM silicon contained higher content of silicon (12 mg/g) than the control (2 mg/g). The silicon contained in the control originates from the seed which was reported to contain a significant amount of silicon[16,23]. More than 80% of silicon present in rice seedlings are localized in AIR fractions. When the AIR was digested with Driselase, only ca. 3% of silicon present in the AIR was released into a water-soluble fraction. The solubilized silicon may have some interaction with polysaccharides in the cell walls because Driselase has cellulase, hemicellulase, and pectinase activities[5]. The soluble fraction with Driselase was analyzed with SE-HPLC/ICP-AES. A small peak of silicon at void volume was detected together with a large peak of silicic acid, indicating that a silicon-containing high molecular weight substance was present in the rice seedling AIR (Fig. 1). Similar silicon-containing high molecular weight compounds were detected in the void volume by molecular sieve open-column chromatography of cellulase digests of rice cell walls[10]. It should be noted that the samples grown without silicic acid also gave the peak at void volume. The result implies that the silicon-containing macromolecule may be essential and physiologically important in the silicon metabolism in rice plants.

To isolate the silicon-containing high molecular weight substances, the soluble fractions with Driselase obtained from rice seedlings grown on 5 mM silicon were separated with 10,000 molecular weight cut membrane

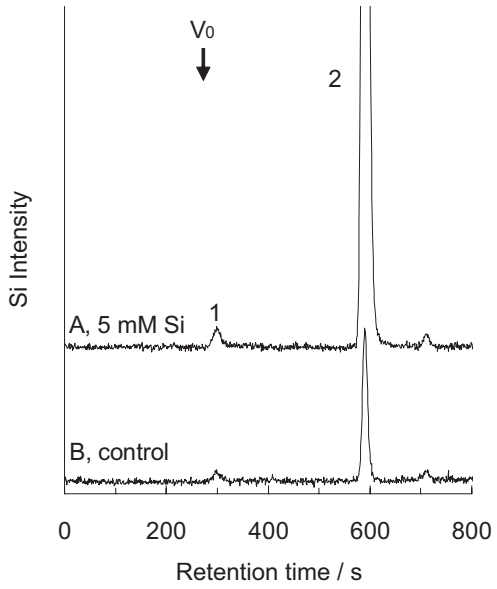

Fig. 1. SE-HPLC/ICP-AES Si profiles of the Driselase digests of rice cell walls

The rice was grown in the presence of 5 mM silicon (A) or without silicon (B). 1: high molecular weight Si compounds, 2: silicic acid. HPLC conditions: column, TSKgel G3000PW$_{XL}$; injection volume: 100 µL.

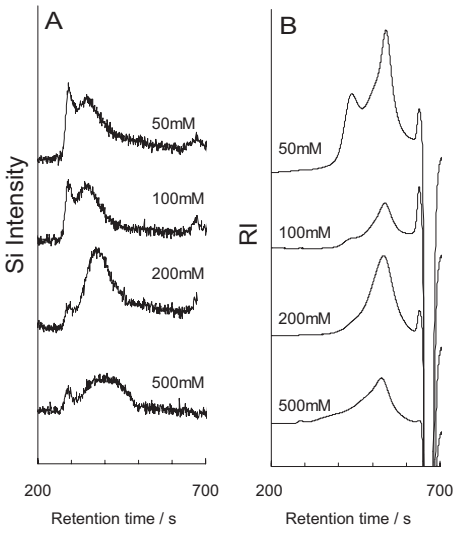

Fig. 2. SE-HPLC/ICP-AES Si profiles (A) and SE-HPLC/refractive index (RI) profiles (B) of the Driselase-soluble materials separated by ion-exchange chromatography

The samples (A: 3.14 mg, B: 1.50 mg, C: 1.98 mg, D: 1.30 mg) dissolved in 500 µL of deionized water were injected. HPLC conditions: column, TSKgel G5000PW$_{XL}$; injection volume: 100 µL (A), 20 µL (B).

filter, size-exclusion chromatography and ion-exchange chromatography. As the Si-containing substances were expected to have high molecular weight, the void volume fraction of size-exclusion chromatography with a Sephadex G-75 column was collected and then applied to weak-anion exchange chromatography, and separated by a step gradient of 50, 100, 200, and 500 mM buffer solutions. Each fraction was analyzed by HPLC/RI and HPLC/ICP-AES. A relatively sharp peak of silicon at the void volume and a broad peak at the inclusion volume were observed in the four fractions (Fig. 2). The results show that the silicon-containing macromolecules solubilized from rice AIR have wide molecular weight distribution. The purification of the Si-containing macromolecules seems to be not enough because the chromatographic profiles of Si (Fig. 2A) are different from those of RI (Fig. 2B). During the purification, it was frequently observed that the freeze-dried materials were not easily dissolved in buffer solutions and the silica precipitation occurred.

As the amount of available silicon-containing substances was limited, the 500 mM buffer eluted fraction was subjected to sugar composition analysis and protein content analysis without further purification. As shown in Table 1, arabinose and xylose are the main glycosyl residues, indicating that arabinoxylan[18] is one of components of the Si-containing AIR. The presence of galacturonic acid, rhamnosyl, and galactosyl residues indicated that it contained rhamnogalacturonan I and homogalacturonan[18]. The Si-containing substance contained 3% protein. Amino acid analysis of the substance showed that glycine, alanine, glutamic acid, aspartic acid, threonine, leucine, and serine were the main amino acids (Table 2). The amino acid composition of the Si-containing substance of rice had some similarity with that of silaffin[20]. The silaffin was identified as a silica-protein complex from a diatom, *Cylindrotheca fusiformis* and is considered to be involved in controlling silicic acid polymerization[19]. The possibility of the protein being a contaminant from Driselase can be excluded because characteristic glycosyl residues of glycoproteins such as GalNAc and GlcNAc were not detected in the glycosyl sugar analysis.

Table 1. Glycosyl composition analysis of 2 M TFA hydrolyzates of 500 mM buffer eluted fraction from rice cell walls

Residue	mole %
Ara	25
Rha	7
Fuc	1
Xyl	34
Man	1
Gal	14
Glc	1
GalA	17

Table 2. Amino acid analysis of 500 mM buffer eluted fraction from rice cell walls

Amino acid	mole %
Asp	9.5
Thr	7.8
Ser	8.8
Glu	9.9
Gly	13.7
Ala	13.3
Val	6.7
Cys	0
Met	0.7
Ile	4.4
Leu	7.5
Tyr	1.8
Phe	2.9
Lys	3.7
His	1.7
Arg	2.5
Try	0
Pro	5.1

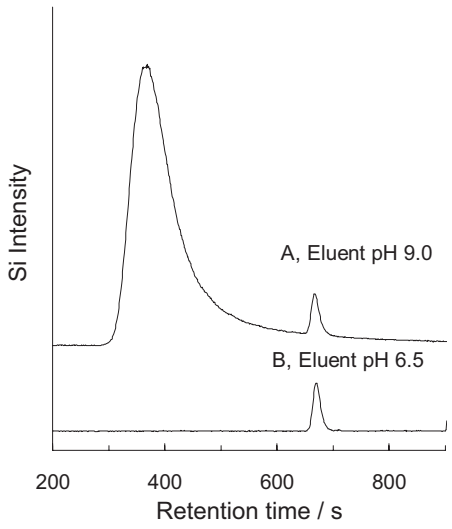

Fig. 3. SE-HPLC/ICP-AES Si profiles of commercial silica sol, "cataloid SI-45P"
A: eluent pH 9.0, B: eluent pH 6.5. The silica sol diluted 1,000 fold with 0.2 M HCO_2NH_4 (pH 9.0) was injected. HPLC conditions: column, TSKgel G5000PW$_{XL}$; injection volume, 5 µL.

Moreover, it is interesting that the high molecular weight fraction was not detected when commercial water-soluble silica, cataloid SI-45 was analyzed with HPLC/ICP-AES at pH 6.5 (Fig. 3). The silica sols which are soluble at pH 9.0 are insoluble at pH 6.5. In contrast, the Si-containing macromolecule from rice AIR is stable at pH 6.5, suggesting that the Si-containing macromolecule is not simple inorganic silica. These results imply that the silicon macromolecule, which originated from the rice AIR, might be an organic-silica like macromolecule that forms a stable silica sol at the neutral pH in the walls.

It was reported that the horsetail, *Equisetum telmateia* and the grass, *Phalaris canariensis* contained silica complex with protein, in which silica was biologically precipitated[7]. The protein-silica complex was rich in proline-glutamic acid, proline-lysine, or serine-aspartic acid-glycine[7]. The protein-silica complex is considered to have some biological functions to process and to organize soluble silicon into highly intricate biosilica structures with great control and reproducibility[19]. The Si-containing substance of rice might be concerned with aggregation, precipitation and structure-directing formation of silica. A schematic model for silica biomineralization in diatoms was proposed[27]. In the model, at the onset of silica polymerization, small silica sols and peptides such as silaffin aggregate to larger silica gel structure. The aqueous organic-silica like macromolecules proposed above might be involved in the first step of silica polymerization in rice.

The present results did not provide any direct evidence that Si binds carbohydrates and proteins in the rice cell walls. However, they imply that there is some unidentified association in the Si-containing substances between Si and cell wall polymers. It would be challenging to identify the linkage between Si and cell wall polymers because the yield of the complex is too small to analyze them by ^{29}Si NMR and chemical analysis. It is well known that in rice most of Si is localized in the apoplast as silica gel. During digestion of the AIR with Driselase, this silica gel was removed as insoluble fractions. In fact, the amount of Si in the Driselase digests was only 3% of the total Si present in the rice AIR as mentioned above. It may happen that some of the silica gel would be dissolved during the saponification of the AIR. Even so, Si-containing high molecular material was present in the Driselase hydrolyzates of the rice AIR.

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