Leguminous wood has a unique vacuolar lectin in bark - Lectin-carbohydrate interaction modulates solubility-insolubility transition of vacuolar glycoproteins -

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Abstract

Mannose/glucose-specific sophoragrin, a *Sophora japonica* bark lectin, shows unique multimerization and dissociation accompanied by transitions between insolubility and solubility, which are modulated by both a specific sugar and Ca^{2+} concentration.

Sophoragrin is composed of four subunits, a-1, a-2, b-1, and b-2, and all except for b-2 are reported to be glycosylated. Biotinylated sophoragrin bound sugar-specifically to subunit 'b' but not to subunit 'a' on the membrane. Sophoragrin was labeled by only one of the photoaffinity-probes derived from sophoragrin-glycans, and the labeling was inhibited by a specific sugar. In contrast, another probe-mixture, which contains major oligosaccharide of sophoragrin ($Man_3Xyl_1Fuc_1GlcNAc_2$), did not label sophoragrin. These results reveal that the multimerization of sophoragrin is caused by a particular signal oligosaccharide on subunit 'b'.

It was shown that sophoragrin can form a soluble complex with other endogenous glycoproteins, and may play a specific role through its transition between soluble and insoluble forms.

Introduction

Lectin is a specific carbohydrate-binding protein or glycoprotein. Since the first lectin was found in Castor bean (*Ricinus communis*) seed in 1888, a large number of lectins have been found in microorganisms, plants, and animals. In particular, leguminous plants are rich sources of lectins in seed, bark, stem, leaf, flower, and root. Several galactose/*N*-acetylgalactosamine-specific lectins werereported for *Sophora japonica* (Japanese pagoda tree), which are present inbark (B-SJA-I) [1], seed [2], and leaf [3].

Previously, we found a novel mannose (Man)/glucose-specific lectin, sophoragrin, from a bark of *S. japonica* other than B-SJA-I [4]. Sophoragrin is composed of four subunits, namely a-1 (19.4 kDa), a-2 (18.2 kDa), b-1 (15.0 kDa), and b-2 (13.2 kDa), which are classified in two groups, 'a' (a-1+a-2), and 'b' (b-1+b-2), based on their sequence homology. Subunit a-1 contains thee potential *N*-glycosylation sites and other subunits contain only one site [5]. Sophoragrin shows the unique property to multimerize and form precipitates under low concentrations of a specific sugar and this property is not found in other *S. japonica* lectins and most of other leguminous plant lectins.

To elucidate the function of sophoragrin, the mechanism of this unique multimerization property and endogenous receptor for sophoragrin were analyzed.

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Materials and Methods

Materials

S. japonica bark was stripped from branches harvested in the city of Tokyo, chopped into pieces, and stored at -20 °C. Sophoragrin and B-SJA-I were prepared from frozen bark as described previously [4][6]. Sophoragrin was biotinylated using *N*-hydroxysuccinimide biotin (Pierce, Rockford, IL, USA) in the presence of 90 mM methyl α-mannoside (Me α-Man) by employing reaction time of 2 h as described in the instructions. Glycopeptidase A (from almond) was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Horseradish peroxidase (HP)-streptavidin complex was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). Endoglycosidase H was obtained from Boehinger Mannheim (Mannheim, Germany). Pepsin was purchased from Sigma Fine Chemical Inc. (St. Louis, MO, USA). Dithiobis(succinimidylpropionate) (DSP) was obtained from Pierce. Standard pyridylamino (PA)-glucose oligomer were purchased from Takara Shuzo Co. (Kyoto, Japan).

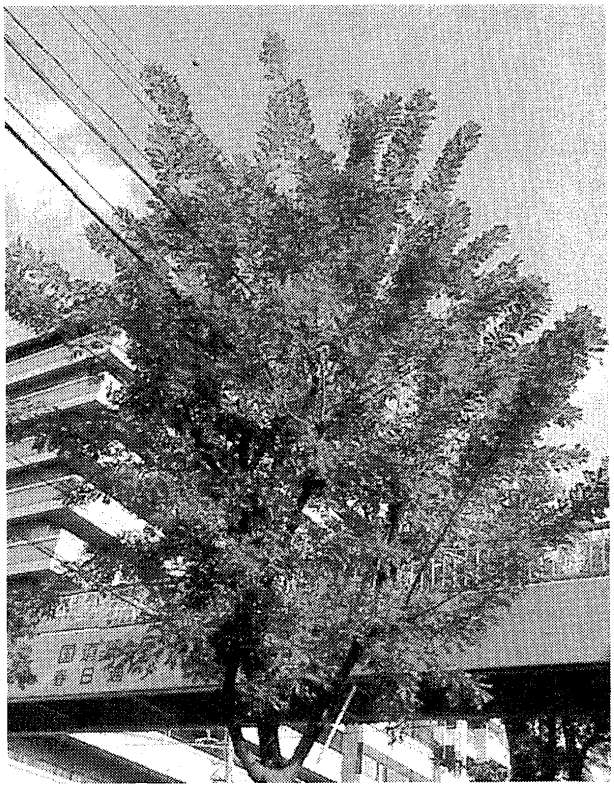

Figure 1 ***Sophora japonica*** **tree.**

Cross-linking analysis

Sophoragrin was cross-linked with cleavable cross-linker, dithiobis(succinimidylpropionate) (DSP). Sophoragrin was dissolved at a concentration of 150 μg/3 ml in 20 mM carbonate buffered saline (pH 8.0) containing 3 mM $CaCl_2$ and incubated with 9.2 mg of DSP for 27 h at room temperature. The cross-linking reaction was stopped by the addition of Tris to 50 mM followed by a 15 min incubation. The excess reagent was removed using microcon (exclusion MW 10,000, Millipore). The cross-linked products were examined by two-dimensional SDS-PAGE as follows; 30 μg of sample was loaded on 16% gel and run for first SDS-PAGE, then the gel was cut by lane and treated with 40 mM dithiotheitol in 0.1 M Tris-HCl (pH 6.8) containing 0.1% SDS for 20 min at room tempareture to cleave the cross linker. The treated gel or untreated gel as a control experiment, was laid on the top of other 16% gel and second SDS-PAGE was performed.

Molecular mass of sophoragrin analyzed by size exclusion chromatography-multiangle laser light scattering (SEC-MALLS)

Sophoragrin was dissolved at a concentration of 2 mg/ml in TBS at pH 7 or 10 mM acetate buffered saline at pH 5, containing various concentration of specific sugar and Ca^{2+}. The samples were filtrated through ultrafree (0.45 μm filter unit, Millipore) and 100 μl aliquots were subjected to SEC-MALLS (Figure 2). Molecular mass was calculated by analytical software ASTRA™ (Wyatt Technology Co.) assuming the dn/dc of sophoragrin as 0.18 ml/g, which value corresponds to general proteins. Pullulan (Shodex, Mw 47,300) was used as standard to characterize the performance of the SEC column.

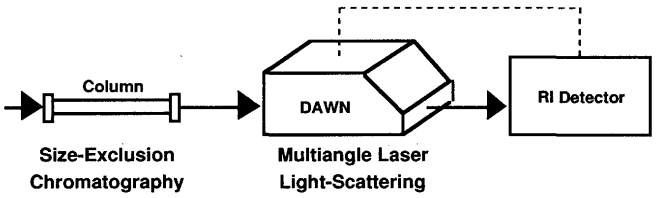

Figure 2 System of size exclusion chromatography-multiangle laser light scattering (SEC-MALLS). MALLS measurements were performed with a DAWN DSP systems (Wyatt Technology Co.) and a refraction index meter, Shodex RI-71, directly on-line with SEC using a KW803 Shodex HPLC-column (0.8 × 30 cm).

Identification of oligosaccharides from sophoragrin

Oligosaccharides were released from sophoragrin by glycopeptidase A or hydrazynolysis and pyridylaminated. PA-oligosaccharides were fractionated by reverse-phase HPLC on a CLC-ODS column and the elution time of each peak was recorded in glucose units (GU) and plotted on the X-axis. Then, each of the separated *N*-glycans was applied to the Amide-80 column to separate on the basis of size. Each peak's elution time was recorded in GU and plotted on the Y-axis. The GU of sophoragrin glycans were compared with those of the reference glycans on the two-dimensional map [7][8]. The structures were confirmed by ^{1}H-NMR and ESI-MS.

Preparation of biotinyl photoprobes

Total oligosaccharides enzymatically released from sophoragrin and maltotriose were separately derivatized to biotinyl photoprobes. Biotinyl photoprobes were prepared by coupling the oligosaccharides to the aminooxy group of biotinylated photoreactive reagent **1** (Figure 3) [9]. The reagent **1** (2 μmol) and oligosaccharides (1 μmol) were dissolved in 80% aqueous acetonitrile (200 μl). After adjusting the pH to 5-6 with diisopropylethylamine, the mixture was incubated at 37 °C for 40 h in the dark. Photoprobes were purified by HPLC on a silica gel column (Aquasil SS-1251, 4.6 × 250 mm, Sensyu Kagaku Co. Ltd., Japan) with 80% aqueous acetonitrile at a flow rate of 1 ml/min. Products were monitored at 210 and 300 nm and the peaks eluting at 10 min (fraction 1) and 15 min (fraction 2) were pooled, respectively. The amount of photoprobes was determined from the UV adsorption of photoreactive moiety (ε^{282}=2400, in CH_3OH). The mass of probes were analyzed by FAB-MS using JEOL mass spectrometer JMS700T.

Other procedures are described in the legends to figures.

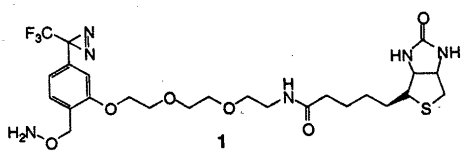

Figure 3 The structure of biotinylated photoreactive reagents 1 developed by Hatanaka *et al.* [9].

Results

i) Multimerization mechanism of sophoragrin

The amount of insoluble sophoragrin under various conditions was analyzed by turbidity which was measured with absorbance at 635 nm in the photometric cell. Turbidity of the sophoragrin solution increased by increasing Ca^{2+} concentration up to 1 mM and decreased by the addition of either EDTA or specific sugar (Figure 4), indicating that soluble-insoluble transition of sophoragrin was reversible and caused by Ca^{2+} and specific sugar.

The conformational change of sophoragrin was not caused by binding of Ca^{2+} and specific sugar by CD spectra (data not shown). Chemical cross-linking and multiangle laser light scattering connected with size-exclusion chromatography (SEC-MALLS) showed that the minimum unit of soluble sophoragrin is mainly heterotetramer $(a,b)_2$ corresponding to the molecular mass of about 55 kDa (data not shown).

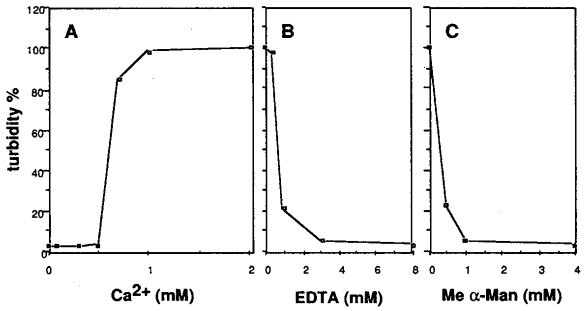

Figure 4 Effects of Ca^{2+} and specific sugar on the turbidity of sophoragrin. The concentration of Ca^{2+} in the sophoragrin solution in TBS was increased gradually (A). After the turbidity reached a maximum at 2 mM Ca^{2+}, the mixture was equally separated into two tube, and EDTA (B) or Me α-Man (C) was step-wisely added to the solution. The absorbance of the mixture at 635 nm was measured with spectrophotometer at each step. The turbidity (%) is represented by the proportion of the absorbance at 635 nm to that in the presence of 2 mM Ca^{2+}.

ii) Identification of signal oligosaccharide for soluble-insoluble transition

By western blot analysis, biotin-sophoragrin sugar-specifically bound to subunit 'b' but not to subunit 'a' (Figure 5B and C), suggesting that the receptor oligosaccharide exists on subunit 'b'.

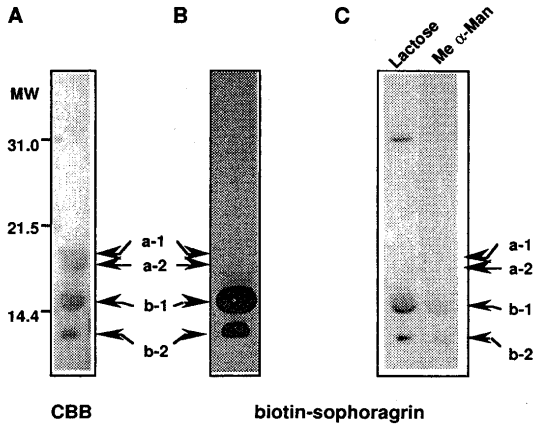

Figure 5 Binding study of biotin-sophoragrin to electroblotted sophoragrin on PVDF membrane. Intact sophoragrin was electrophoresed using 16% polyacrylamide gel, electroblotted onto PVDF membrane, and stained with CBB (**A**) or allowed to react with biotin-sophoragrin and the color was developed with avidin-peroxidase complex and 4-chloro-1-naphthol/H_2O_2 (**B**). The inhibition study of the binding of biotin-sophoragrin was performed in the presence of 0.2 M lactose or Me α-Man (**C**).

Oligosaccharides of subunit 'b' were released from sophoragrin by hydrazinolysis after treatment with glycopeptidase A, because subunit 'b' was found to be resistant to glycopeptidase A. Oligosaccharides were pyridylaminated and subjected to the structural analysis by two dimensional sugar mapping technique on HPLC. The major oligosaccharide structure of subunit 'b' was determined to be M2.1FX, while that of total sophoragrin was 000.1FX (Figure 6).

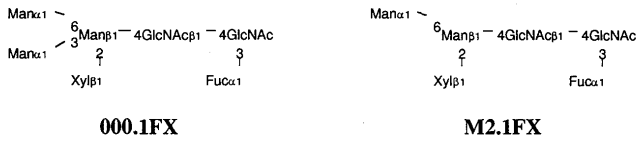

Figure 6 The proposed structure of major oligosaccharide of total sophoragrin (000.1FX) and that of subunit 'b' (M2.1FX).

To identify the signal oligosaccharide for multimerization process, sugar-photoprobes were prepared by conjugating the oligosaccharide mixtures, which were enzymatically released from Sophoragrin to biotinylated photoreactive reagents **1** (Figure 3) [9]. Two major photoreactive-probes prepared from total sophoragrin oligosaccharides had the mass corresponding to photoreactive-derivatives of 000.1FX or M2.1FX. Sophoragrin was labeled in solution only by M2.1FX-photoprobe (Figure 7A), and its labeling was inhibited with maltotriose but not with lactose (Figure 7C). In contrast, another 000.1FX-photoprobe could not label Sophoragrin (Figure 7B), demonstrating that the multimerization receptor of sophoragrin is M2.1FX. On the other hand, only subunit 'b' was labeled by maltotriose-photoprobe, indicating that the lectin site of sophoragrin exsists on subunit 'b'.

These results indicate that multimerization of sophoragrin is a sugar-specific process caused by a particular signal oligosaccharide and the lectin sites in subunit 'b'. Based on these observation, a schematic representation of multimerization process of sophoragrin is shown in Figure 8.

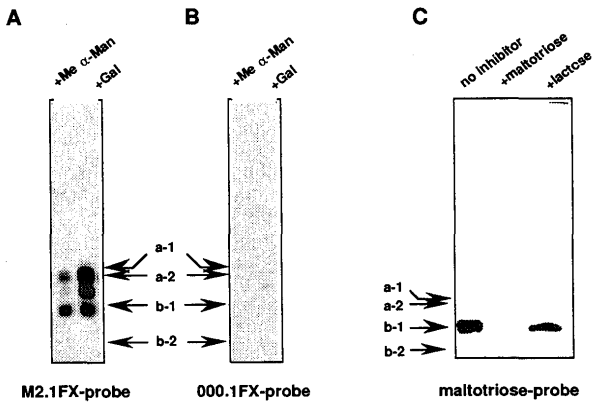

Figure 7 Photoaffinity labeling of sophoragrin with the photoprobes derivatized with sophoragrin glycan (A and B) or maltotriose (C). Sophoragrin was incubated with biotinyl glycan-photoprobes and labeled by irradiation of ultraviolet light (365 nm), electrophoresed and blotted on PVDF membrane. The membrane was incubated with HP-streptavidin and detected with chemiluminescence. **A** and **B**, labeling with M2.1FX-probe (**A**) or 000.1FX-probe (**B**) in the presence of 0.2 M Me α-Man or Gal; and C, labeling with maltotriose-probe in the presence of 0.2 M maltotriose, lactose, or no inhibitor.

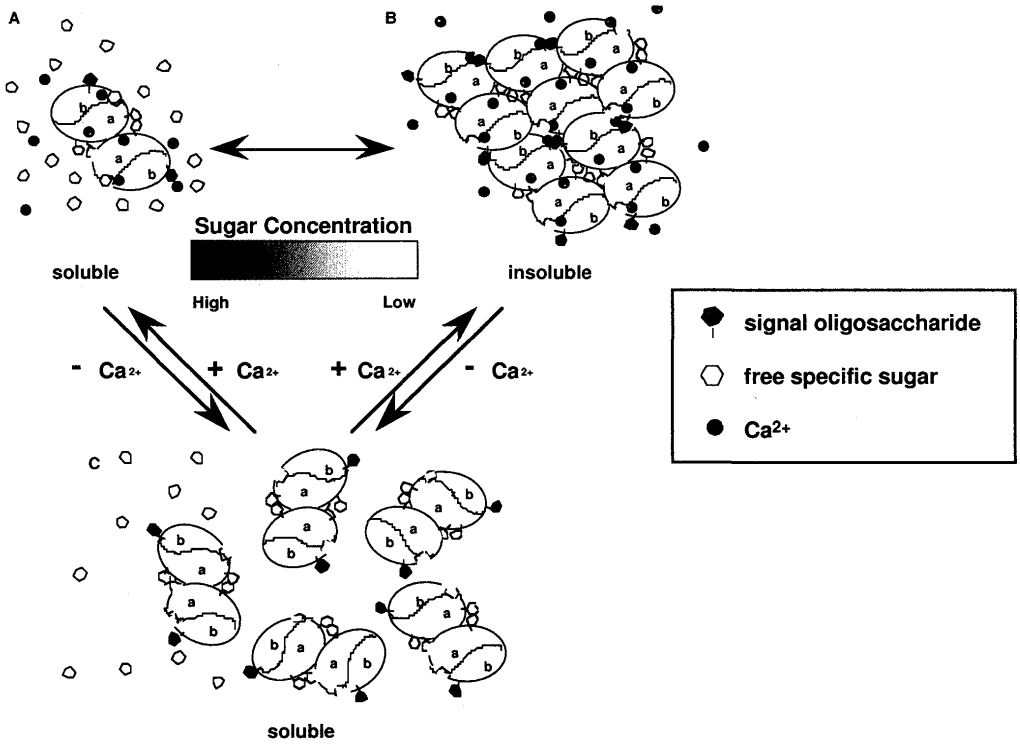

Figure 8 Hypothetical mechanism for soluble-insoluble transition of sophoragrin. Multimerization of sophoragrin is caused by a sugar-specific interaction between its signal oligosaccharides on subunit 'b' and lectin site. In the presence of Ca^{2+}, sophoragrin exhibits the carbohydrate binding activity and multimerise to form insoluble precipitate in the absence of specific sugar (**A**), and the amount of soluble sophoragrin increases with the increase of specific-sugar concentration (**B**). In the absence of Ca^{2+}, sophoragrin is soluble regardless of the absence (**C**) or presence (**D**) of the specific-sugar.

iii) Complex formation of sophoragrin with endogenous ligand

Biotin-sophoragrin bound sugar-specifically to a galactose-specific *S. japonica* bark lectin (B-SJA-I) on membrane (Figure 9). By SEC-MALLS, sophoragrin and B-SJA-I exist indepentdently in solution in the presence of Me α-Man (Figure 10C). On the other hand, sophoragrin and B-SJA-I were found to form high molecular weight complex in the absence of specific sugar (Figure 10D and E). These results indicate that sophoragrin can form soluble complex with endogenous glycoproteins in solution.

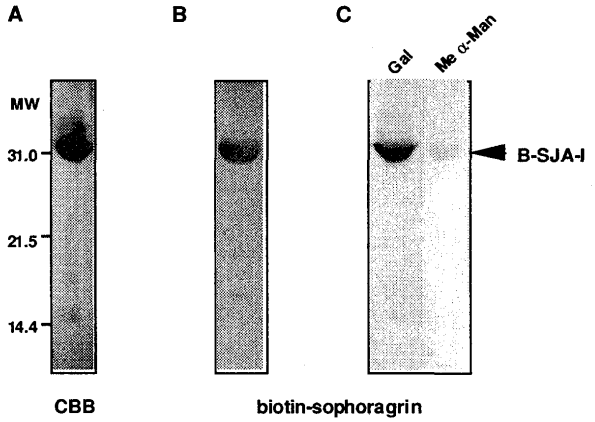

Figure 9 Binding study of biotinyl sophoragrin to electroblotted B-SJA-I on PVDF membrane. B-SJA-I was subjected to SDS-PAGE and electroblotted on PVDF membrane and stained with CBB (**A**), or allowed to react with biotinyl sophoragrin (**B** and **C**). The inhibition study of the binding was performed in the presence of 0.2 M galactose or Me α-Man (**C**).

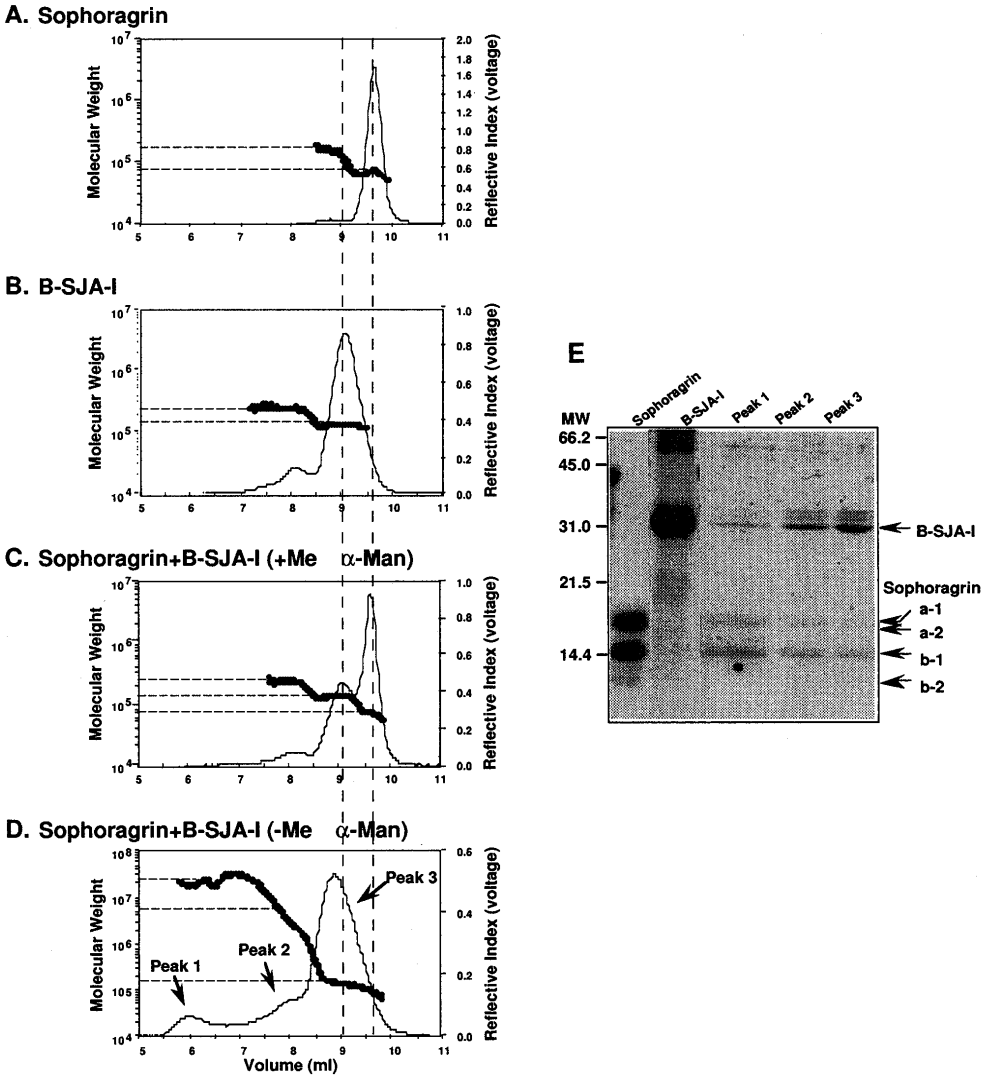

Figure 10 The behavior of sophoragrin and B-SJA-I in the presence or absence of Me α-Man measured by SEC-MALLS. Sophoragrin and B-SJA-I mixed in TBS in the presence or absence of 0.1 M Me α-Man was analyzed by SEC-MALLS. Chromatograms indicate elution volume (ml) versus reflective index (voltage) corresponding to the left of Y-axis, and dots indicate elution volume (ml) versus molecular mass corresponding to the right of Y-axis. **A**, sophoragrin in the presence of Me α-Man; **B**, B-SJA-I in the presence or absence of Me α-Man; **C**, the mixture of sophoragrin and B-SJA-I in the presence of Me α-Man; and **D**, the mixture of sophoragrin and B-SJA-I in the absence of Me α-Man. Peaks 1-3 in **D** were collected and the components were analyzed by SDS-PAGE (**E**).

Discussion

This study showed that the mechanism of self-aggregation of sophoragrin, which is a unique sugar-specific multimerization due to the intersubunit interaction between the lectin site and the specific oligosaccharide of subunit 'b'. In addition, we found that sophoragrin and B-SJAI, which were reported to exist in vacuole of a *S. japonica* bark cell, form a complex in solution. Based on these findings, we suggest a model of sophoragrin function in vacuole. Sophoragrin is soluble at high concentration of specific sugar. On the other hand, at very low concentration of specific sugar, sophoragrin aggregates to form an insoluble precipitate or forms a soluble complex with other glycoproteins, such as B-SJA-I, through signal oligosaccharides (Figure 11). Sophoragrin may play a specific role depending on Ca^{2+} and sugar concentrations; sophoragrin may regulate the turgor pressure of the vacuole involved in the growth of the plant, or it may function as a sink of storage or waste molecules in the bark cell, through the soluble-insoluble transition.

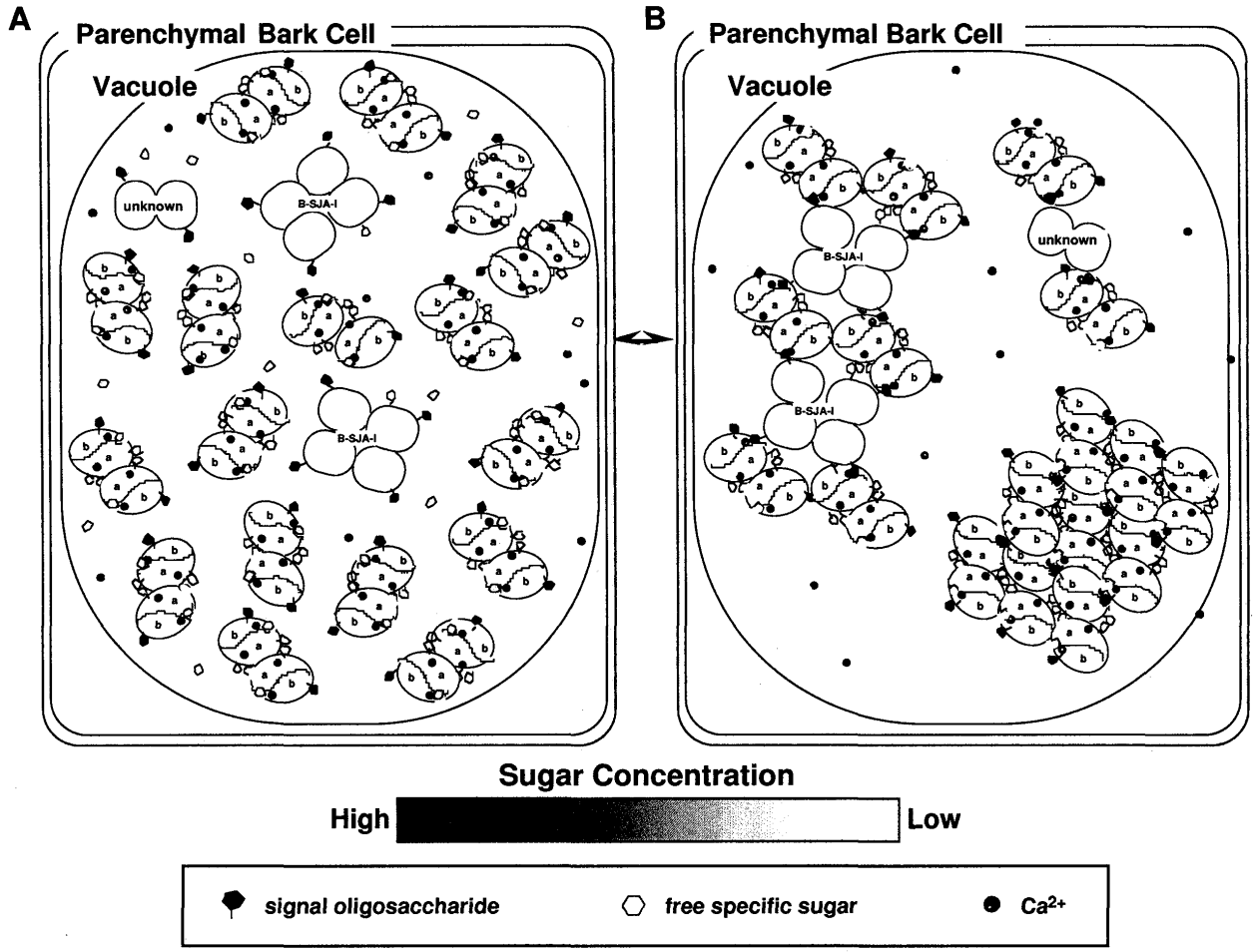

Figure 11 Model of complex formation between sophoragrin and B-SJA-I or other glycoproteins in parenchymal bark cell of *Sophora japonica* tree. **A**, in the presence of high concentration of specific sugar, sophoragrin and B-SJA-I or other glycoproteins are independently present in their soluble forms. **B**, in the presence of very low concentration or absence of specific sugar, sophoragrin forms insoluble multimer itself or form complex with B-SJA-I or other glycoproteins that posesses signal oligosaccharides.

Acknowledgments

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