Solubility–insolubility interconversion of sophoragrin, a mannose/glucose-specific lectin in *Sophora japonica* (Japanese pagoda tree) bark, regulated by the sugar-specific interaction

Haruko UEDA^{*1}, Hisako FUKUSHIMA^{*}, Yasumaru HATANAKA[†] and Haruko OGAWA^{*2}

^{*}Department of Advanced Biosciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan, and [†]Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama, 930-0194, Japan

Sophoragrin, a mannose/glucose-specific lectin in *Sophora japonica* (Japanese pagoda tree) bark, was the first lectin found to show self-aggregation that is dependent on the sugar concentration accompanying the interconversion between solubility and insolubility [Ueno, Ogawa, Matsumoto and Seno (1991) *J. Biol. Chem.* **266**, 3146–3153]. The interconversion is regulated by the concentrations of Ca²⁺ and specific sugars: mannose, glucose or sucrose. The specific glycotopes for sophoragrin were found in the sophoragrin subunit and an endogenous galactose-specific lectin, B-SJA-I (bark *S. japonica* agglutinin I), and the lectin subunit that binds to the glycotop was identified by photoaffinity glycan probes. Remarkably, the insoluble polymer of sophoragrin is dissociated by interaction with B-SJA-I into various soluble

complexes. Based on these results, self-aggregation of sophoragrin was shown to be a unique homopolymerization due to the sugar-specific interaction. An immunostaining study indicated that sophoragrin localizes mainly in vacuoles of parenchymal cells coincidentally with B-SJA-I. These results indicate that sophoragrin can sequester endogenous glycoprotein ligands via sugar-specific interactions, thus providing new insights into the occurrence and significance of the intravacuolar interaction shown by a legume lectin.

Key words: glycoprotein, legume lectin, photoaffinity labeling, *Sophora japonica* (Japanese pagoda tree) bark, self-aggregation, vacuole.

INTRODUCTION

A large number of lectins have been found in plants and animals and, in particular, leguminous plants are known to contain lectins abundantly in various organs, such as seed, bark, stem, leaf, flower and root. The detailed sugar specificities and structures of these lectins have been vigorously studied for a long time, and a number of them are used as tools for studies in glycobiology, but their functions are little understood [1–5]. One well-understood function of plant lectins is the involvement in *Rhizobium* legume symbiosis [6]. Specific interactions between host cells and rhizobia to form nodulations in the leguminous root hair are mediated by leguminous lectins, indicating that lectins may play an active role in specificity by transmitting a signal to one or both symbiotic partners. Other examples are defensive functions against predators, such as the cytotoxicity represented by Type II ribosome-inactivating proteins such as ricin [7,8] and sieboldin-b [9], and the anti-nutrient effect represented by *Phaseolus vulgaris* lectin [10]. Plant lectins are considered to bind exogenous carbohydrate ligands to play symbiotic or defensive roles, which is in contrast with numerous animal lectins that exhibit functions in connection with their endogenous ligands. This is partly because endogenous ligands have not been discovered for plant lectins, and cellular plant lectins are mostly localized in vacuoles, suggesting *in vivo* roles limited to sink or scavenger.

Sophoragrin, a mannose/glucose-specific lectin found in the bark of the leguminous *Sophora japonica* (Japanese pagoda tree), is the first lectin reported that exhibits a unique self-aggregation

based on its sugar-binding specificity and the carbohydrate concentration; that is, isolated sophoragrin precipitates to an insoluble complex by itself under the depletion of specific sugars, and becomes soluble in their presence. It was discovered as B-SJA-II (bark *S. japonica* agglutinin II) [13], while B-SJA-I is a galactose/*N*-acetylgalactosamine (Gal/GalNAc)-specific isolectin in *S. japonica* bark [14]. Sophoragrin is found exclusively in bark [14], and its homologue has not been detected in seeds, either by affinity chromatography at the protein level [14] or by Northern blotting at the mRNA level [15], whereas Gal/GalNAc-specific lectins are abundant in both seed (SJA) and bark (B-SJA-I), exhibiting 89% identity in amino acid sequences [15]. The amino acid sequence of sophoragrin possesses a sequence identity of less than 50% with B-SJA-I [15], whereas legume lectins usually exhibit high sequence identities between isolectins in the same plant. Sophoragrin consists of four approx. 13–19 kDa glycopeptides [13], which are uncommon sizes among the leguminous lectin family, generated by proteolysis of two kinds of precursor peptides [15], whereas B-SJA-I has a subunit size of approx. 30 kDa [13].

Interconversion between a small soluble molecule and an osmotically inactive polymer is a known mechanism for storage of solutes in cells, such as sugar starch in plants and phosphoric acid polyphosphates in microbes. However, no such controlled mechanism of sequestering and retrieving glycoproteins has been elucidated. Immunomicroscopy revealed that electron-dense clumps consisting of most bark glycoproteins, including B-SJA-I and probably sophoragrin, are present in the vacuoles of *S. japonica* bark from autumn to winter, and diminish in the spring

Abbreviations used: AAL, *Aleuria aurantia* lectin; B-SJA-(I/II), bark *S. japonica* agglutinins I and II respectively; DSP, dithiobis(succinimidylpropionate); Con A, concanavalin A; FAB MS, fast-atom-bombardment MS; HRP, horseradish peroxidase; LCA, *Lens culinaris* agglutinin; Me α -Man, methyl α -D-mannoside; SEC-MALLS, size-exclusion chromatography–multi-angle laser-light scattering; TBS, Tris-buffered saline; TFMS, trifluoromethanesulphonic acid.

¹ Present address: Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan.

² To whom correspondence should be addressed (email hogawa@cc.ocha.ac.jp).

[11,12]. To elucidate the biological significance of the self-aggregation of sophoragrin, the ability of sophoragrin to sequester glycoproteins and dissociate them in response to sugar signalling was examined, with a view of providing an insight into a possible active mode of glycoprotein administration.

In the present study, the mechanisms regulating interconversion of sophoragrin were elucidated. Further adding to the self-aggregation, sophoragrin was found to bind with endogenous glycoproteins to form various complexes that are controlled by the specific sugar concentrations. The localization of sophoragrin in bark was elucidated using a specific antibody prepared with deglycosylated sophoragrin. The functional significance of sugar-specific interconversions of lectins in glycoprotein administration in vacuoles will be discussed.

EXPERIMENTAL

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 [13,14]. Sophoragrin, LCA (*Lens culinaris* agglutinin) and AAL (*Aleuria aurantia* lectin) were biotinylated using *N*-hydroxysuccinimide biotin (Pierce, Rockford, IL, U.S.A.) in the presence of 90 mM Me α -Man (methyl α -mannoside; specific sugar) by employing a reaction time of 2 h, according to the manufacturer's instructions. Horseradish peroxidase (HRP)—streptavidin complex was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, U.S.A.). Dithiobis(succinimidylpropionate) (DSP) was obtained from Pierce. Almond glycoamidase A was obtained from Seikagaku Corp. (Tokyo, Japan). Trifluoromethanesulphonic acid (TFMS; special grade) was purchased from Wako Pure Chemicals (Osaka, Japan). HRP-conjugated anti-rabbit IgG antibody (goat) was obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD, U.S.A.).

Measurement of the soluble–insoluble transition of sophoragrin by turbidity

Sophoragrin (2 mg) was dissolved in 2 ml of 10 mM TBS (Tris-buffered saline), pH 7.5, and aliquots (0.8 or 2 μl) of 0.1–1.0 M CaCl_2 in TBS were added to the solution and mixed. After incubation for 15 min at room temperature, the mixture was vortex-mixed, and the attenuation (D_{635}) was measured immediately with a spectrophotometer. The procedure was repeated several times. Subsequently, aliquots (1–7 μl) of EDTA (0.3 M), Me α -Man, glucose, sucrose or lactose (0.5 or 1.5 M) in TBS were added to the mixture in a stepwise fashion, and the turbidity was measured at each time in the same manner.

Size-exclusion chromatography–multi-angle laser-light scattering (SEC-MALLS)

MALLS measurements were performed with a DAWN DSP system (Wyatt Technology Co., Santa Barbara, CA, U.S.A.) and a Shodex RI-71 refractive index meter, directly online with SEC using a KW803 Shodex HPLC column (0.8 cm $\times$ 30 cm). Sophoragrin was dissolved at a concentration of 2 mg/ml in either TBS at pH 7.0 or 10 mM acetate-buffered saline at pH 5.0, containing various concentrations of specific sugars and Ca^{2+} . The samples were filtered through Ultrafree (0.45 μm filter unit; Millipore, Bedford, MA, U.S.A.), and 100 μl aliquots were injected. The molecular mass was calculated using analytical software (ASTRATM; Wyatt Technology Co.), assuming a specific refractive-index increment (dn/dc) of sophoragrin of 0.18 ml/g.

Pullulan (Shodex; M_r 47 300) was used as the standard to characterize the performance of the SEC column.

For the analysis of formation of the sophoragrin complex with B-SJA-I, 100 μl of sophoragrin solution (3.3 mg/ml in TBS/1 mM Ca^{2+}) was mixed with an equal volume of B-SJA-I solution (2.5 mg/ml in TBS/1 mM Ca^{2+}), and incubated for 1.5 h in the presence or absence of 0.2 M Me α -Man. After the mixture had been filtered through an Ultrafree 0.45- μm -pore-size filter unit, 100 μl aliquots were injected and analysed as described above.

Preparation of biotinyl photoprobes

Total oligosaccharides enzymically released from sophoragrin according to the method described previously [16] and maltotriose were separately derivatized to biotinyl photoprobes. Biotinyl photoprobes were prepared by coupling the oligosaccharides to the amino-oxy group of biotinylated photoreactive reagent 1 [17]. Reagent 1 (2 μmol) and oligosaccharides (1 μmol) were dissolved in aq. 80% acetonitrile (200 μl). After adjusting the pH to 5–6 with di-isopropylethylamine, 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 mm $\times$ 250 mm, Sensu Kagaku Co. Ltd, Japan) with aq. 80% acetonitrile at a flow rate of 1 ml/min. Products were monitored at 210 and 300 nm, and the peaks eluted at 10 min (fraction 1) and 15 min (fraction 2) respectively were pooled. The amount of photoprobes was determined from the UV adsorption of the photoreactive moiety ($\epsilon_{282} = 2400$ in methanol). The masses of probes were analysed by FAB MS (fast-atom-bombardment MS) using a JEOL JMS700T mass spectrometer.

Photoaffinity labelling

Photoaffinity labelling of sophoragrin and Con A (concanavalin A) with biotinyl-sugar photoprobes was performed as described by Hatanaka et al. [17]. Briefly, 10 nmol of biotinyl-sugar photoprobe was added to 2.5 μg of sophoragrin or Con A dissolved in 15 μl of 0.1 M phosphate buffer, pH 7.0, containing 1 mM Ca^{2+} and incubated for 30 min at room temperature in the dark. For inhibition assays, the incubation was performed in the presence of 0.2 M inhibitor sugar. After irradiation with UV light (365 nm, 10 W $\times$ 2) for 100 min on ice, these samples were electrophoresed using a 15% separation gel, blotted on to a PVDF membrane (Nihon Millipore Co., Tokyo, Japan) and detected by chemiluminescence with luminol [BM Chemiluminescence Blotting Substrate (POD); Boehringer Mannheim, Mannheim, Germany] as the substrate, according to the manufacturer's instructions.

SDS/PAGE and binding studies of sophoragrin with biotin–lectin on the membrane

SDS/PAGE was performed as described by Laemmli [18] under reducing conditions with 5% (v/v) 2-mercaptoethanol. For the binding study, the electrophoresed proteins were transferred to a PVDF membrane, and reactivity with biotin–lectins was studied as described previously [16].

Cross-linking analysis

Sophoragrin was dissolved at a concentration of 100 $\mu\text{g}/100 \mu\text{l}$ in 0.1 M *N*-ethylmorpholine acetic acid/2 mM CaCl_2 buffer, pH 8.0, containing 200, 50 or 5 mM Me α -Man. An aliquot (40 μl) of 25% (v/v) glutaraldehyde as a cross-linker and 10 mg of sodium cyanoborohydride were added to the solution. After incubation at room temperature for 15 h, the products of the cross-linking reaction were examined by SDS/PAGE on 14% (w/v) gels.

To study chemical cleavage of the cross-linked product, sophoragrin was cross-linked with another cleavable cross-linker, DSP. Sophoragrin (150 μ g) was dissolved in 3 ml of 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 a final concentration of 50 mM, followed by a 15 min incubation. The excess reagent was removed by using a Microcon centrifugal filter unit (exclusion molecular mass 10 000 Da; Millipore). The cross-linked products were examined by two-dimensional SDS/PAGE as follows: 30 μ g of sample was loaded on to a 16% gel for the first SDS/PAGE run; then the gel was cut by lane and treated with 40 mM dithiothreitol in 0.1 M Tris/HCl, pH 6.8, containing 0.1% (w/v) SDS for 20 min at room temperature to cleave the cross-linker. The treated gel, or untreated gel as a control, was laid on top of another 16% gel, and a second SDS/PAGE was performed.

Preparation of anti-TFMS-treated sophoragrin antibody and immunohistochemistry of sophoragrin

The localization of sophoragrin in autumn bark was studied with specific antibodies against deglycosylated sophoragrin, because the major glycan of sophoragrin is so immunogenic that anti-sophoragrin antibodies were cross-reactive with the glycan moieties of other plant glycoproteins, including B-SJA-I [19]. Chemical deglycosylation of sophoragrin was performed with TFMS according to the procedures published previously [20,21], but with modifications. In short, 1 ml of anisole and 2 ml of TFMS were mixed in a glass tube with a Teflon-lined screw cap, and cooled to 0 °C. Dry sophoragrin (3 mg) was dissolved in 0.3 ml of this mixture in a glass tube, and nitrogen was bubbled through the solution for 30 s, followed by magnetic stirring at 0 °C (ice-water bath) for 3 h. The reaction mixture was then added to 5 ml of pyridine/diethyl ether (1:9, v/v) in a solid CO_2 /acetone bath for 1 h. The co-precipitated protein and pyridinium salt of the acid were collected by centrifugation at 1800 g (3000 rev./min) for 10 min at 4 °C. The pellets were suspended in 0.1 M NH_4HCO_3 , and dialysed extensively against this solution to remove salts. The deglycosylated sophoragrin, a flocculent precipitate, was recovered by centrifugation. TFMS-treated sophoragrin (200 μ g) in complete Freund's adjuvant was injected subcutaneously into a rabbit. The same quantity of TFMS-treated sophoragrin in incomplete Freund's adjuvant was injected again 4 weeks later. Blood was collected on days 10–14 after the final boost.

S. japonica twigs were harvested in autumn and cut into pieces. Fixation was carried out for 4 h at 4 °C in 20 mM piperazine-1,4-bis-(2-ethanesulphonic acid) buffer, pH 7.2/0.5 mM CaCl_2 containing 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde. After washing in 20 mM Tris/HCl, pH 7.4, the samples were dehydrated in ethanol at 4 °C, embedded in paraffin and cut into 7 μ m sections. For immunostaining, sections were deparaffinized with xylene and rehydrated in ethanol. To inhibit the activity of internal peroxidase, sections were treated with 3% H_2O_2 . After blocking in PBS containing 0.5% (w/v) skimmed milk, the sections were incubated with anti-(TFMS-treated sophoragrin) antibodies, blocked with 0.5% skimmed milk in PBS again, and then incubated with goat anti-rabbit IgG conjugated with HRP. Sections were stained with 3,3'-diaminobenzidine and H_2O_2 , and analysed by bright-field microscopy.

RESULTS

Regulators of sophoragrin solubility

Measurement of the turbidity (attenuance) of sophoragrin solutions demonstrated that the solubility–insolubility transition of

Figure 1 Effects of Ca^{2+} and specific sugars on the turbidity of self-aggregated sophoragrin

The concentration of Ca^{2+} in the sophoragrin solution in TBS was increased gradually, as described in the Experimental section (A). After the turbidity reached a maximum at 2 mM Ca^{2+} , the mixture was divided equally into two tubes, and EDTA (B) or Me α -Man (C) respectively was added in a stepwise manner to the solution. The absorbance of the mixture at 635 nm was measured with a spectrophotometer at each step. The turbidity (%) is represented by the proportion of absorbance to that in the presence of 2 mM Ca^{2+} .

sophoragrin is regulated by the concentration of Ca^{2+} and specific sugars. As shown in Figure 1(A), turbidity increased as sophoragrin in TBS (1 mg/ml) was precipitated by the addition of Ca^{2+} , and reached a plateau at concentrations higher than 1 mM Ca^{2+} . The precipitated sophoragrin was dissociated by the addition of EDTA, and it dissolved completely at 3 mM EDTA (Figure 1B), indicating that the solubility–insolubility transition is Ca^{2+} -dependent. Alternatively, the precipitated sophoragrin was differentially dissociated by various specific sugars in the presence of Ca^{2+} ; complete solution was achieved with Me α -Man at 1 mM, glucose at 5 mM or sucrose at 9 mM (Figure 1C), but not with lactose, even at 0.1 M (results not shown). In the absence of Ca^{2+} , sophoragrin remained soluble irrespective of the sugar concentration. The CD spectra of sophoragrin solutions did not change in 1 mM Ca^{2+} containing 0.1 M Me α -Man and 5 mM EDTA, demonstrating that the secondary structure was not changed between the sugar-bound form and the inactive form, and suggesting that a serious conformational change was not caused by sugar binding (results not shown).

Molecular form of soluble sophoragrin

Sophoragrin is composed of four subunits, namely a-1 (19 kDa), a-2 (18 kDa), b-1 (15 kDa) and b-2 (13 kDa) (see Figure 3A, lane 1) [13], which are encoded by two homologous genes coding for proteins with 72% similarity in amino acid sequences: one gene encodes the a-1 and b-1 subunits, and the other encodes the a-2 and b-2 subunits in each polypeptide sequence [15]. Cross-linking studies indicated that soluble sophoragrin is composed of a heterodimer irrespective of the concentration of Me α -Man (Figure 2A), and cleavage of the cross-linked product indicated that it consists of one subunit a (a-1 or a-2) and one b (b-1 or b-2) (Figure 2B). In the absence of a cross-linker, the subunit assembly was dissociated with 1% (w/v) SDS (Figure 2A, 'intact'), but not with a reducing agent (results not shown), indicating that the subunits are not linked with a disulphide bond. Since the precursor polypeptides of sophoragrin are supposed to be proteolytically processed into two polypeptides each, the results suggest that the initially formed polypeptide structure is retained after proteolytic processing.

The molecular mass of soluble sophoragrin was measured by SEC–MALLS, in which SEC was used as a pre-filter for MALLS

Figure 2 Cross-linking analysis (A and B) and molecular-mass measurement (C) of sophoragrin

(A) Cross-linking of sophoragrin with glutaraldehyde and sodium cyanoborohydride was performed in the presence of 5, 50 or 200 mM Me α -Man, as described in the Experimental section. The products were examined by SDS/PAGE on 14 % gels, and stained with Coomassie Brilliant Blue. The values on the left indicate the migration positions of molecular-mass markers. (B) Sophoragrin was cross-linked by DSP, and two-dimensional SDS/PAGE was performed using 16 % polyacrylamide gels before and after reductive cleavage, as described in the Experimental section. (C) Sophoragrin solution in TBS, pH 7.0/1 mM Ca^{2+} containing 2 or 100 mM Me α -Man was fractionated by size-exclusion HPLC on a KW803 column connected to a DAWN-DSP system, as described in the Experimental section. Chromatograms indicate elution volume (ml) versus refractive index (voltage, shown on the left of the y-axis), and dots indicate elution volume (ml) compared with molecular mass (shown on the right of the y-axis).

to estimate the concentration of each molecular form, whereas the mass was calculated absolutely from MALLS. As shown in Figure 2(C), a symmetrical major peak [V_e (elution volume) = 9.4 ml] and a minor peak (less than 10%; V_e = 8.5 ml) were detected in 2–100 mM Me α -Man and 1 mM Ca^{2+} at pH 7.0 by refractive index on SEC. The peaks were smaller in 2 mM Me α -Man than in 100 mM Me α -Man, whereas the calculated masses were constantly 5.7×10^4 (± 3 %) Da for the major peak, corresponding to a heterotetramer (ab)₂, and $(13\text{--}14) \times 10^4$ (± 7 %) Da for the minor peak, corresponding to an octamer (ab)₄, under both conditions. In the absence of the specific sugar, the signal on the refractive index disappeared due to the precipitation

of sophoragrin, indicating that the solubility of sophoragrin depends extensively on the Me α -Man concentration. Changing conditions to pH 5.0, 200 mM glucose, or eliminating Ca^{2+} did not change the chromatogram from that in 100 mM Me α -Man (results not shown).

Combining the results of SEC–MALLS and cross-linking, the solubility depends on the concentration of sugar in the presence of Ca^{2+} , but the molecular size of soluble sophoragrin is unchanged.

Glycotope and lectin site on sophoragrin

The receptor oligosaccharide (glycotope)-bearing subunit responsible for self-aggregation was addressed by a membrane-binding

Figure 3 Localization of glycotope (A) and identification of lectin subunit (B and C)

(A) Intact sophoragrin was electrophoresed, electroblotted and stained with Coomassie Brilliant Blue (lane 1) or allowed to react with biotin–sophoragrin in the absence (lane 2) or presence (lanes 3 and 4) of 0.2 M inhibitor or biotin–LCA (lane 5). The colour was developed as described in the Experimental section. (B and C) Oligosaccharides prepared from sophoragrin or maltotriose was separately derivatized to biotinyl photoprobes (B), and photoaffinity labelling of sophoragrin and Con A with the biotinyl–sugar photoprobe was performed in the presence or absence of inhibitor sugar (C). The samples were electrophoresed and electroblotted, and the labelling was detected by chemiluminescence. Labelling of sophoragrin with a maltotriose probe in the presence of 0.2 M maltotriose, lactose, or no inhibitor (lanes 1–3), labelling of sophoragrin with probe 1 (lanes 4 and 5) or probe 2 (lanes 6 and 7), and labelling of Con A with probe 1 (lanes 8 and 9) or probe 2 (lanes 10 and 11) in the presence of 0.2 M Me α -Man or Gal is shown.

assay with biotin–sophoragrin. Each subunit of sophoragrin contains one or three potential N-glycosylation sites [15]. As shown in Figure 3(A) (lane 2), biotin–sophoragrin bound to subunits b-1 and b-2, but not to subunits a-1 and a-2, and the binding was inhibited with 0.2 M Me α -Man, but not with 0.2 M lactose (lanes 3 and 4). The same subunit reactivity was observed for biotin–LCA (lane 5), whereas biotin–AAL bound only to a-1 and a-2 subunits (results not shown). The results indicate that subunits b and a are differentially glycosylated, and that the glycotope is located on subunits b-1 and b-2. Although a potential N-glycosylation site in subunit b-2 had previously been considered to be unglycosylated [13], a glycotope was shown to exist on b-2 as well as b-1 because the glycan was clearly detected in Figure 3(A).

The lectin subunit was identified by biotinylated photoreactive reagents [17] derivatized with maltotriose or sophoragrin oligosaccharides (Figure 3B). Using the maltotriose photoprobe (mass = 685 Da), only subunit b-1 was labelled, and the binding was completely inhibited by maltotriose (Figure 3C, lanes 1–3). The ability of maltotriose to dissociate self-aggregated sophoragrin (results not shown) indicates that the sugar-combining site of subunit b-1 is involved in self-aggregation of sophoragrin. From sophoragrin oligosaccharides released by almond glycoamidase A, two types of probe, probes 1 and 2, were obtained on silica-gel HPLC. FAB MS measurement of each probe showed that probe 1 contained a major peak (mass = 1635 Da) that corresponds to the photoreactive derivative of M2.1FX, whereas probe 2 contained

Figure 4 Binding of biotinyl-sophoragrin to B-SJA-I on membrane (A) and complex formation of sophoragrin with B-SJA-I in solution (B-G)

(A) B-SJA-I was electrophoresed, electroblotted and stained with Coomassie Brilliant Blue (lane 1) or allowed to react with biotinyl-sophoragrin (lanes 2-4). The binding was inhibited in the presence of 0.2 M Gal (lane 3) or Me α -Man (lane 4). (B-E) Sophoragrin and B-SJA-I mixed in TBS in the presence or absence of 0.1 M Me α -Man was analysed by SEC-MALLS. x- and y-axes represent the same parameters as described in the legend to Figure 2(C). (B) Sophoragrin in the presence of Me α -Man; (C) B-SJA-I in the presence or absence of Me α -Man; (D) mixed sophoragrin and B-SJA-I in the presence of Me α -Man; and (E) mixed sophoragrin and B-SJA-I in the absence of Me α -Man. (F) Peaks labelled 1-3 in (E) were collected, and the components were analysed by SDS/PAGE. (G) A plot of the molecular masses of peaks 1-3 in (E) from MALLS measured against the sophoragrin content, which was estimated by quantification of the band intensity using Lane Analyser Ver.3 (ATTO Corporation, Bunkyo-ku, Tokyo, Japan).

a major peak (mass = 1797 Da) that corresponds to 000.1FX, the most major oligosaccharide of sophoragrin (Figure 3B), with several other oligosaccharides in both probes. As shown in Figure 3(C), only probe 1 bound to sophoragrin; binding was significantly weakened in the presence of 0.2 M Me α -Man (lane 4), but not with 0.2 M Gal (lane 5). Probe 2 did not bind to sophoragrin

under either condition (lanes 6 and 7), but, in contrast, both probes equally bound with Con A (lanes 8-11). The results indicate that sophoragrin exhibits marked specificity towards the endogenous glycotope that is contained in probe 1, but not the major oligosaccharides in probe 2. Taking the mass of the probe into consideration, four bands labelled with probe 1 (lane 5) were

Figure 5 Immunolocalization of sophoragrin in the *S. japonica* bark

Sections of *S. japonica* twigs harvested in autumn were prepared and Coomassie Brilliant Blue-stained (**A**) or immunostained with specific antibodies against TFMS deglycosylated sophoragrin (**B–E**). The nuclei were stained with haematoxylin. (**A**, **C** and **D**) phloem (P); (**B**) phloem and xylem (X); **E**, xylem. CZ, cambial zone; s, sieve tube; bf, bast fibre; rp, ray parenchyma; ap, axial parenchyma. (**A** and **C**) Magnification $\times 160$; (**B**) $\times 100$; (**D** and **E**) $\times 500$; scale bars represent 10 μm .

attributable to conjugates in the 1:1 stoichiometry of probe 1 and subunits a-1, a-2, b-1 and b-2 from the top to the bottom of the gel. The labelling of subunit 'a' with probe 1 suggests that the oligosaccharide-binding site stretches between subunits 'b' and 'a'. In support, seven residues forming the carbohydrate-binding site, which are common to legume lectins with some conservative replacements, are present in sophoragrin polypeptides; two of those residues are located in subunit 'a', and the other residues are in subunit 'b', surrounding the monosaccharide-binding clefts between the two subunits in the superimposed three-dimensional model [15]. The topological relationship of the determinant sugar residue and the diazirino-reactive group (Figure 3B) would have caused the discrepancy between the labelling of the maltotriose probe and probe 1.

Complex with endogenous glycoprotein

Sophoragrin and B-SJA-I are the two major glycoproteins in *S. japonica* bark [14]; interaction with B-SJA-I was therefore investigated as being representative. As shown in Figure 4(A), biotin–sophoragrin was found to sugar-specifically bind to B-SJA-I on the membrane, indicating that it contains the glycotope for sophoragrin. Surprisingly, B-SJA-I in solution was found to completely dissolve sophoragrin, forming a striking contrast with the

fact that sophoragrin alone was precipitated in the absence of a specific sugar. On SEC–MALLS, a mixed solution of sophoragrin and B-SJA-I (2.7:1, mol/mol) in the presence of Me α -Man showed a simple additional curve on SEC and a molecular mass that was unchanged (Figure 4D) from those of single component solutions (Figures 4B and 4C) by MALLS, suggesting that sophoragrin and B-SJA-I do not interact in 0.1 M Me α -Man. In the absence of a specific sugar, the mixed solution showed a contrasting pattern (Figure 4E). Two new peaks, peaks 1 and 2, appeared, and their calculated masses from MALLS were 2×10^7 and 3×10^6 Da respectively, higher by two or one order(s) from those in the presence of a specific sugar, indicating that a large soluble complex is formed sugar-specifically. Peak 3 was eluted at a smaller volume than the major peak of B-SJA-I in Figure 4(C), and had a larger calculated mass (1.7×10^5 Da) compared with that of B-SJA-I alone (1.2×10^5 Da). Recovery of lectins was equal to that in 0.1 M Me α -Man on the refractive index.

As shown by SDS/PAGE of peaks 1–3 (Figure 4F), sophoragrin and B-SJA-I are partitioned in every peak at various molecular ratios. As shown in Figure 4(G), the molecular mass of the complex from MALLS correlates with the sophoragrin content. The high B-SJA-I content (80%) in peak 3 suggests that a 1:1 complex of sophoragrin and B-SJA-I is contained with free B-SJA-I in the peak.

Immunolocalization of sophoragrin

Localization of B-SJA-I was previously demonstrated with an anti-SJA polyclonal antibody by Baba et al. [11]. Although the anti-SJA polyclonal antibody exhibits good reactivity with B-SJA-I, it may react with sophoragrin too, due to the cross-reactivity with the glycan moiety. Therefore we prepared sophoragrin-specific antibody using TFMS-deglycosylated sophoragrin as an immunogen. As shown in Figures 5(B) and 5(C), sophoragrin was detected mainly in the ray and axial parenchymal cells in the phloem and the vessels in the xylem, but not in the cells of the cambial zone, the bast fibres, the sieve tubes or the xylem parenchyma. At higher magnification, sophoragrin was found to concentrate in the parenchymal vacuoles in the phloem (Figure 5D), whose localization coincides with B-SJA-I [11]. The clear detection of sophoragrin around the vessel walls (Figure 5E) was in contrast with the fact that B-SJA-I was not detected in the vessel walls [11]. The results showed co-existence of the two lectins in the vacuoles in the phloem, but existence of only sophoragrin in the vessels in the xylem.

DISCUSSION

The present study has revealed the mechanism of unique complex formation and localization of sophoragrin in the bark. Self-aggregation was shown to be a sugar-specific polymerization of sophoragrin due to its own glycotope and lectin activity. Although numerous studies have been performed on the interaction between lectins and other glycoproteins [22–25], this is the first that demonstrates the homopolymerization mechanism of one lectin that accompanies the solubility–insolubility transition. A remarkable finding is that B-SJA-I, a major endogenous glycoprotein, induced solubilization of sophoragrin precipitate by assembling homopolymers with sophoragrin, because it possesses a glycotope for sophoragrin. It is now clear that sophoragrin is present at the same time and place as B-SJA-I, as shown by immunostaining patterns in the parenchymal vacuoles in the phloem (Figure 5). Immunocytochemistry with anti-(deglycosylated sophoragrin) showed that sophoragrin localizes around the vessel walls in the xylem, in addition to the phloem (Figure 5E), suggesting that sophoragrin was released by vacuole explosion during vessel formation and/or that sophoragrin is transported via vessels. Both lectins are equally major glycoproteins in *S. japonica* bark in autumn [14], but the distribution suggests that sophoragrin may play a distinctive role in the bark.

In this context, Figure 6 illustrates the interconversion among three forms of sophoragrin in a vacuole. In the presence of Ca^{2+} , sophoragrin is active and multimerizes to form insoluble clumps in the presence of low sugar concentrations and in an absence of ligand glycoprotein (Figure 6A). Although sophoragrin is bivalent, containing two lectin sites per soluble tetramer (Figure 3C), it can bind to other sophoragrin molecules through its oligosaccharide moiety too, serving as both receptor and lectin simultaneously, which is an exception to the simple lattice hypothesis proposed by Brewer [26]. In this way, sophoragrin behaves multivalently, having heterogeneous sites that can form a unicomponent, insoluble macrocomplex (Figure 6A). When glycoprotein ligands possessing specific glycotopes such as B-SJA-I are present in the vacuole, sophoragrin binds to them to form soluble complexes of various sizes in the presence of low sugar concentrations (Figure 6B). At a high concentration of a specific sugar, which includes glucose, sucrose and mannose or their glycosides, sophoragrin and the ligand glycoproteins are dissociated into separate soluble forms (Figure 6C). Although the lectin activity of sophoragrin is regulated by the Ca^{2+} concentration too (see

Figure 6 Solubility–insolubility transition via three forms of sophoragrin

(A) The sophoragrin multimer in the presence of Ca^{2+} in the presence of low sugar concentrations and in the absence of ligand glycoprotein. (B) Soluble complexes of various sizes and ratios of sophoragrin to ligand glycoprotein, depicted as B-SJA-I, which are proposed by taking the molecular mass and component ratio in each peak from Figure 4(E) into consideration. (C) The soluble monomers of sophoragrin and the ligand glycoprotein dissociate at high concentrations of the specific sugar.

Figure 1), it is considered to be active under normal physiological conditions because the Ca^{2+} concentration in the vacuole is generally maintained by Ca^{2+} -ATPase and the $\text{H}^{+}/\text{Ca}^{2+}$ exchanger at more than 10^{-3} M, levels which are three to four orders of magnitude higher than that in the cytoplasm [27].

As the immunomicroscopic observations indicated that bark lectins are mainly localized in the intravacuolar electron-dense clumps from autumn to winter [11,12], sophoragrin would form a complex with B-SJA-I to reduce the osmotic pressure inside the vacuole. The glycoproteins in clumps are supposed to be utilized to produce phloem tissue or to dilate the outer parenchyma from spring to summer [11]. One possible mechanism by which clumps are thought to be dissociated is the up-regulation of the sugar concentration by sugar influx as a result of increased photosynthesis (Figure 6C); the other is digestion of lectins by glycoamidases or glycosidases that can remove the receptor glycans. Free N-glycans released by glycoamidase may simultaneously act as signal molecules, such as growth factors in flax plantlets [28]. In this manner, the hypothesis of glycoprotein sequestration and administration via a self-aggregatable lectin agrees with the localization of lectins and seasonal changes of the concentration in bark tissue of perennial legumes, leading to efficient mobilization of nitrogen sources.

Except for two self-aggregatable lectins, CLA-I and CLA-II, in the bark of a leguminous tree, *Cladrastis lutea* (American yellowwood) [29], a sophoragrin-like lectin has never been found in any other tissue of a leguminous plant, although enormous quantities of lectins were reported in seeds rather than in barks. The striking difference between the aggregation of legume seed conglutins and sophoragrin is a dependence on sugars that may be related to their functional disparity; the seed storage proteins, *Lupinus* conglutins, electrostatically self-aggregate depending on the $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration and are dissociated by charged compounds, but not by neutral sugars, even though some of the included proteins have lectin activity [30]. Recently, we found by preliminary screening of leguminous plants that the barks of *Robinia pseudoacacia* and *Wisteria floribunda* contain sophoragrin-like lectins and co-existing glycoproteins like B-SJA-I (H. Ueda, I. Ishizuka, K. Tadano-Aritomi, N. Iida-Tanaka, Y. Hatanaka, M. Yamamoto and H. Ogawa, unpublished work). The distribution and function of the lectins capable of soluble–insoluble interconversions that are dependent on sugars needs to be elucidated in relation to perenniality.

We thank Mr Takashi Takakuwa (Jasco Co.) for the CD spectrometric analysis, Mr Masahide Nakamura (Shoko Co., Ltd) for support in SEC–MALLS, and Katherine Ono for editing the English. This work was supported in part by Research Fellowships for Young Scientists and a Grant-in-Aid for JSPS (Japanese Society for the Promotion of Science) Fellows No. 07729 (H.U.), and Grants-in-Aid for Scientific Research (C) nos 12680607 and 14580622 (H.O.) from the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

- Van Damme, E. J. M., Peumans, W. J., Pusztai, A. and Bardocz, S. (1998) Handbook of plant lectins: properties and biomedical applications. John Wiley & Sons, Chichester
- Rüdiger, H. and Gabius, H. J. (2001) Plant lectins: occurrence, biochemistry, functions and applications. *Glycoconj. J.* **18**, 589–613
- Peumans, W. J., Van Damme, E. J., Barre, A. and Rouge, P. (2001) Classification of plant lectins in families of structurally and evolutionarily related proteins. *Adv. Exp. Med. Biol.* **491**, 27–54
- Goldstein, I. J. (2002) Lectin structure–activity: the story is never over. *J. Agric. Food Chem.* **50**, 6583–6585
- Sharon, N. and Lis, H. (2002) How proteins bind carbohydrates: lessons from legume lectins. *J. Agric. Food Chem.* **50**, 6586–6591
- Hirsch, A. M. (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr. Opin. Plant Biol.* **2**, 320–326
- Olsnes, S. and Kozlov, J. V. (2001) Ricin. *Toxicol.* **39**, 1723–1728
- Lord, M. J., Jolliffe, N. A., Marsden, C. J., Pateman, C. S., Smith, D. C., Spooner, R. A., Watson, P. D. and Roberts, L. M. (2003) Ricin. Mechanisms of cytotoxicity. *Toxicol. Rev.* **22**, 53–64
- Rojo, M. A., Yato, M., Ishii-Minami, N., Minami, E., Kaku, H., Citores, L., Girbes, T. and Shibuya, N. (1997) Isolation, cDNA cloning, biological properties, and carbohydrate binding specificity of sieboldin-b, a type II ribosome-inactivating protein from the bark of Japanese elderberry (*Sambucus sieboldiana*). *Arch. Biochem. Biophys.* **340**, 185–194
- Mirkov, T. E., Wahlstrom, J. M., Hagiwara, K., Finardi-Filho, F., Kjemtrup, S. and Chrispeels, M. J. (1994) Evolutionary relationships among proteins in the phytohemagglutinin-arcelin- α -amylase inhibitor family of the common bean and its relatives. *Plant Mol. Biol.* **26**, 1103–1113
- Baba, K., Ogawa, M., Nagano, A., Kuroda, H. and Sumiya, K. (1991) Developmental changes in the bark lectin of *Sophora japonica* L. *Planta* **183**, 462–470
- Baba, K., Ogawa, M. and Sumiya, K. (1990) Electron microscopical studies on the deposition route of the bark lectin of *Sophora japonica*. *Wood Res.* **77**, 1–7
- Ueno, M., Ogawa, H., Matsumoto, I. and Seno, N. (1991) A novel mannose-specific and sugar specifically aggregatable lectin from the bark of the Japanese pagoda tree (*Sophora japonica*). *J. Biol. Chem.* **266**, 3146–3153
- Ueno, M., Arai, T., Kojima, K., Ogawa, K. H., Matsumoto, I. and Seno, N. (1992) Improved affinity chromatographic purification of D-mannose-N-acetyl-D-glucosamine specific lectin from the bark of *Sophora japonica* eliminating the loss by sugar specific self-aggregation. *J. Chromatogr.* **597**, 197–200
- Van Damme, E. J. M., Barre, A., Rouge, P. and Peumans, W. (1997) Molecular cloning of the bark and seed lectins from the Japanese pagoda tree (*Sophora japonica*). *Plant Mol. Biol.* **33**, 523–536
- Kitagaki-Ogawa, H., Matsumoto, I., Seno, N., Takahashi, N., Endo, S. and Arata, Y. (1986) Characterization of the carbohydrate moiety of *Clerodendron trichotomum* lectins. Its structure and reactivity toward plant lectins. *Eur. J. Biochem.* **161**, 779–785
- Hatanaka, Y., Kempin, U. and Jong-Jip, P. (2000) One-step synthesis of biotinyl photoprobes from unprotected carbohydrates. *J. Org. Chem.* **65**, 5639–5643
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Ogawa, H., Hijikata, A., Amano, M., Kojima, K., Fukushima, H., Ishizuka, I., Kurihara, Y. and Matsumoto, I. (1996) Structures and contribution to the antigenicity of oligosaccharides of Japanese cedar (*Cryptomeria japonica*) pollen allergen Cry j I: relationship between the structures and antigenic epitopes of plant N-linked complex-type glycans. *Glycoconj. J.* **13**, 555–566
- Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, Jr, L. E. and Weber, P. (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* **118**, 131–137
- Karp, D. R., Atkinson, J. P. and Shreffler, D. C. (1982) Genetic variation in glycosylation of the fourth component of murine complement. Association with hemolytic activity. *J. Biol. Chem.* **257**, 7330–7335
- Mandal, D. K. and Brewer, C. F. (1992) Cross-linking activity of the 14-kilodalton β -galactoside-specific vertebrate lectin with asialofetuin: comparison with several galactose-specific plant lectins. *Biochemistry* **31**, 8465–8472
- Mandal, D. K. and Brewer, C. (1992) Interactions of concanavalin A with glycoproteins: formation of homogeneous glycoprotein–lectin cross-linked complexes in mixed precipitation systems. *Biochemistry* **31**, 12602–12609
- Brewer, C. F. (2001) Lectin cross-linking interactions with multivalent carbohydrates. *Adv. Exp. Med. Biol.* **491**, 17–25
- Ramachandrarajah, G., Chandra, N. R., Suroliya, A. and Vijayan, M. (2003) Computational analysis of multivalency in lectins: structures of garlic lectin–oligosaccharide complexes and their aggregates. *Glycobiology* **13**, 765–775
- Brewer, C. F. (1996) Multivalent lectin–carbohydrate cross-linking interactions. *Chemtracts: Biochem. Mol. Biol.* **6**, 165–179
- Bush, D. S. (1995) Calcium regulation in plant cells and its role in signals. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 95–122
- Berger, S., Menudier, A., Julien, R. and Karamanos, Y. (1995) Do de-N-glycosylation enzymes have an important role in plant cells? *Biochimie* **77**, 751–760
- Van Damme, E. J., Barre, A., Berner, V., Rouge, P., Van Leuven, F. and Peumans, W. J. (1995) A lectin and a lectin-related protein are the two most prominent proteins in the bark of yellow wood (*Cladrastis lutea*). *Plant Mol. Biol.* **29**, 579–598
- Ferreira, R. B., Freitas, R. L. and Teixeira, A. R. (2003) Self-aggregation of legume seed storage proteins inside the protein storage vacuoles is electrostatic in nature, rather than lectin-mediated. *FEBS Lett.* **534**, 106–110