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# Characterization and cDNA Cloning of Monomeric Lectins That Correspond to the B-Chain of a Type 2 Ribosome-Inactivating Protein from the Bark of Japanese Elderberry (*Sambucus sieboldiana*)

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**Two monomeric lectins, SSA-b-3 and SSA-b-4, were purified from the bark tissue of Japanese elderberry, *Sambucus sieboldiana*. SDS-PAGE of the purified lectins showed the presence of single bands of 35 and 33 kDa for SSA-b-3 and SSA-b-4, respectively, irrespective of the presence of reducing agent. MS analysis as well as gel filtration of these lectins indicated that they exist mostly as monomeric lectins. Analysis of the N-terminal amino acid sequences of SSA-b-3 and SSA-b-4 yielded an identical sequence, indicating their close structural relationship. Four cDNA clones with extensive homology were obtained from the bark cDNA library and indicated to encode SSA-b-3 or SSA-b-4 from the comparison with the N-terminal sequences of these lectins. These clones were classified into two groups, three for SSA-b-3 and one for SSA-b-4, based on the predicted isoelectric points. The amino acid sequences of the encoded polypeptides were almost identical with the B-chain of a type 2 ribosome-inactivating protein from the same bark tissue, sieboldin-b, except for the absence of a small peptide containing a cystein residue, which is critical for the heteromeric dimerization with an A-subunit. Carbohydrate binding specificity and biological activity of these lectins are also reported.**

**Key words: bark, cDNA, elderberry, lectin, ribosome-inactivating protein, *Sambucus sieboldiana*.**

Abbreviations: RIP, ribosome-inactivating protein; PVDF, polyvinylidenedifluoride; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ELISA, enzyme-linked immunosorbent assay; SSA, *Sambucus sieboldiana* agglutinin; SNA, *Sambucus nigra* agglutinin.

Ribosome-inactivating proteins (RIPs) from plants are RNA *N*-glycosidases that cleave a specific site of ribosomal RNA and stop protein synthesis (*1*, *2*). They are basically classified into two groups, *i.e.*, type 1 (single chain) and type 2 (two chain) RIPs. Typical type 2 RIPs such as ricin and abrin consist of a Gal/GalNAc-specific lectin subunit (B-chain), which serves to attach to the target cell surface by its ability to bind to Gal/GalNAc-containing cell surface glycoconjugates, and a ribosome-inactivating *N*-glycosidase subunit (A-chain), which kills the target cells by its ability to stop protein synthesis. Type 1 RIPs consist only of an A-chain. Because of the lack of the cell binding B-subunit, typical type 1 RIPs are much less cytotoxic than type 2 RIPs. Several type 1 RIPs, on the other hand, have been shown to carry antiviral or antifungal activity (*1–4*). Based on these findings, the possible involvement of RIPs in plant defense system has been discussed (*2–5*). The A-chains of these RIPs have also been used as a toxic moiety of immunotoxins (*1*, *3*).

Bark tissue of elderberry trees belonging to the genus *Sambucus* has been a rich source of novel RIPs and related proteins such as a group of type 2 RIPs that show potent *in vitro* ribosome-inactivating activity but little toxicity (*6*, *7*), a type 2 RIP with an inactive B-chain (*8*), sialylated oligosaccharide-specific lectins having a type 2 RIP-like structure (*9–11*), a truncated polypeptide corresponding to a part of the sialylated oligosaccharide-binding subunit (*12*) and Gal/GalNAc-specific lectins (*13*, *14*). The reason for such richness in RIPs and RIP-related proteins in the bark has not been well addressed, but a possible role of these proteins in the protection from potential predators has been indicated (*2*, *15*).

We here show that two new lectins from the bark of Japanese elderberry (*S. sieboldiana*) have almost identical primary structure with the B-chain of a type 2 RIP, sieboldin-b, from the same tissue, and exist abundantly as free, monomers. Biological activity and carbohydrate binding properties of these lectins are also discussed.

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## MATERIALS AND METHODS

*Purification of Lectins from Japanese Elderberry Bark*—Lectins were purified by affinity chromatography on acid-treated Sepharose 6B (Pharmacia, Uppsala, Sweden), gel filtration and cation exchange chromatography at 4°C, based on the method in ref. 10. Samples of 50 g of the bark tissue of Japanese elderberry collected in autumn and spring were each extracted with 400 ml of 0.28 M NaCl containing 5 mM sodium phosphate (pH 7.5). The extract was filtered and centrifuged at 15,000 rpm for 45 min at 0°C to remove solid materials. The supernatant was applied to a column of acid-treated Sepharose 6B (13 × 5 cm) (*16*), and the bound fraction was eluted with 0.2 M D-galactose. This fraction was concentrated with an Amicon YM-10 membrane filter and applied to a Superdex 75-HR column equipped with an FPLC system. The column was pre-equilibrated with 0.4 M NaCl containing 5 mM sodium phosphate (pH 7.5) and eluted with the same buffer. Elution was performed at a flow rate of 30 ml/h, the eluate was monitored for absorbance at 280 nm, and 0.5-ml aliquots were collected. Fractions corresponding to the second protein peak were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8). This fraction was applied to a Mono-Q column (Pharmacia) equilibrated with the same buffer used for the dialysis. After the elution of unadsorbed proteins with the same buffer containing 5 mM NaCl, adsorbed proteins were eluted with successive gradients of 5–75 mM NaCl and 75–300 mM NaCl. Fractions corresponding to each protein peak were collected, dialyzed against water and freeze-dried. The purified lectins obtained from the bark taken in autumn and spring were designated as SSA-b-3 and SSA-b-4, respectively.

*SDS Polyacrylamide Gel Electrophoresis and Isoelectric Focusing*—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (*17*) using a precast acrylamide gel (15%, ATTO, Tokyo). Standard proteins were purchased from Pharmacia. Electrophoresis was carried out at 20 mA for 2 h, and the gels were stained with Comassie Brilliant Blue. Isoelectric focusing of proteins was performed using a Phast Gel IEF 3-9 (Pharmacia, Uppsala) on the Phast electrophoresis system.

The gels were blotted onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Tokyo) and analyzed for glycosylation using a Glycan Detection Kit (Boehringer Mannheim. Germany).

*Enzymatic Deglycosylation*—Before the enzymatic digestion, 6 μg of each lectin was denatured with 0.5% SDS and 2% mercaptoethanol for 10 min at 100°C. The denatured lectin was incubated first with PNGase-F at 37°C and pH 7.5 for 15 h, then with a mixture of glycopeptidase A and endoglycosidase F at 37°C and pH 5.5 for 2 h. Deglycosylated products were analyzed by SDS-PAGE and stained with Commasie Brilliant Blue.

*Ribosome-Inactivating Activity*—Ribosome-inactivating activity of the purified lectins was analyzed using rabbit reticulocyte lysate or a cell-free system of wheat germ as described previously (*7*).

*Antiserum*—Rabbit polyclonal antibody to sieboldin-b was raised and purified as described in the previous paper (*7*). The antiserum was partially purified using an affinity column containing immobilized SSA-b-3 and SSA-b-4 on CNBr-activated Sepharose 4B. The bound fraction eluted with Glycine/HCl buffer was used for immunological analyses.

*Analysis of N-Terminal Amino Acid Sequence*—The lectins were separated by SDS-PAGE and electroblotted onto a PVDF membrane. Protein bands were detected with amidoblack 10B, and the corresponding bands were cut out and used for N-terminal amino acid sequence analysis with a Shimadzu model PPSQ-10 protein sequencer.

*Estimation of Molecular Weight by Mass Spectrometry*—The purified proteins were dissolved in PBS and analyzed with a time-of-flight (TOF) mass spectrometer, Voyager RP (PerSeptive Biosystems, MA). Mass spectra were obtained in a positive mode using 3,5-dimethoxycinnamic acid as a matrix.

*Preparation of cDNA Library, Cloning and Nucleotide Sequencing*—Total RNA was isolated from the bark tissue by the guanidinium isothiocyanate/cesium chloride method, and poly(A)-rich RNA was isolated by oligo(dT) column chromatography. A cDNA library was constructed by the method described by Gubler and Hoffmann (*18*) with λgt 10 as vector. The blotted nitrocellulose membranes were hybridized with the cDNA for sieboldin-b (*7*) in 25% (v/v) formamide, 5× SSPE, 0.5% (w/v) SDS, 2.5× Denhart's solution, 100 μg/ml denatured salmon sperm DNA at 42°C followed by washing three times with 2× SSC, 0.1% (w/v) SDS at 62°C. Positive clones were further selected by PCR with 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by incubation at 72°C for 5 min using the forward and reverse primers of λgt10 (5′-GCTGGGTAGTCCCCACCTTT-3′ and 5′-CTTATGAGTATTTCTTCCAGGGTA-3′, respectively), and a degenerated primer (5′-AT(T/C/A)ACNGGNAA(T/C)AT(T/C/A)AT(A/T/C)GG-3′), which is complementary to the deduced sequence from the partial amino acid sequence of SSA-b-3 and SSA-b-4 (ITGNIIG). From among the clones from which two distinct PCR products were detected, clone Lec SSA-b-3a, Lec SSA-b-3b, Lec SSA-b-3c, Lec SSA-b-4 were subjected to the determination of nucleotide sequence by the dideoxy chain terminating method (*19*) using model 373A automatic DNA sequencer (Applied Biosystems, USA).

*Genomic Southern Blot Analysis*—Total DNA was extracted from 5 g of leaves of *Sambucus sieboldiana* by the SDS/phenol method of Dellaporte *et al*. (*20*). Genomic southern hybridization was carried out in the usual manner (*21*). The cDNA of monomeric lectin (clone Lec SSA-b-3a) was used as a hybridization probe.

*Analysis of Carbohydrate Binding Properties by ELISA*—Enzyme-linked immunosorbent assay (ELISA) for the analysis of lectin-carbohydrate interaction was carried out as follows. A 96-well flat-bottom microtiter plate was coated with glycoprotein solution (1 μg/50 μl/well) in 0.1 M carbonate buffer (pH 8.5) and kept overnight at 4°C. The glycoprotein solution was then discarded, and the plate was incubated with 1% BSA in the same buffer (100 μl) at 37°C for 1 h to prevent non-specific binding. The wells were rinsed three times with phosphate-buffered saline (PBS, pH 7.2)/0.05% Tween 20 (this rinsing was repeated after all subsequent steps). Then, 50 μl of PBS containing varying amounts of each

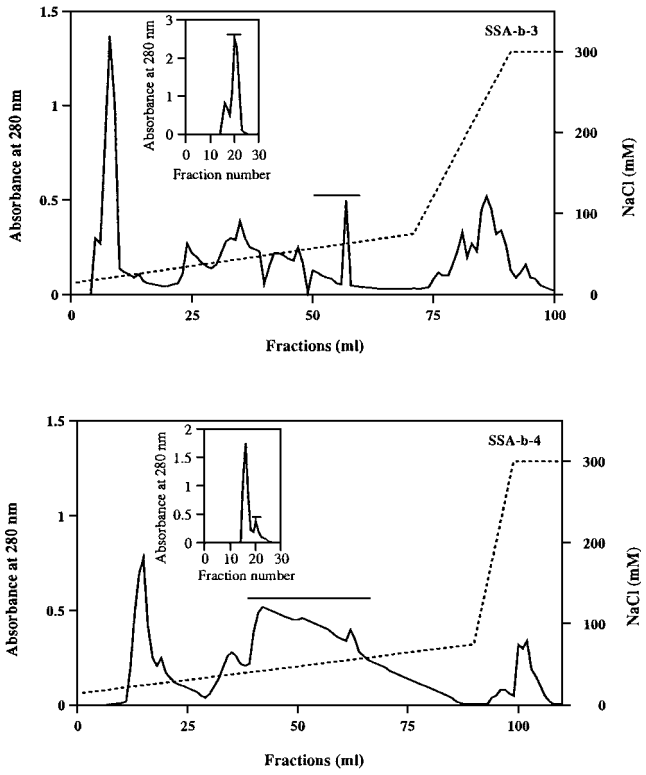


Fig. 1. **Purification of SSA-b-3 and SSA-b-4 by gel filtration and ion-exchange chromatography on a Mono-Q column.** These chromatograms show the purification of SSA-b-3 (upper) and SSA-b-4 (lower) from the bark taken in autumn and spring, respectively. SSA-b-3 and SS-b-4 fractions obtained from the gel filtration step (chromatograms inserted) were applied to a Mono-Q column and eluted with the gradient of NaCl. The solid line and dotted line indicate $A_{280}$ and NaCl concentration, respectively. Fractions indicated by the bars, which showed hemagglutination activity with rabbit erythrocytes, were collected and used for further analyses.

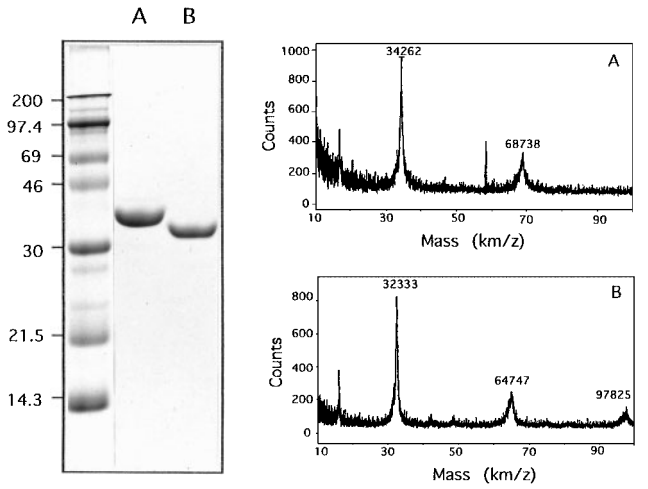


Fig. 2. **Analysis of purified SSA-b-3 and SSA-b-4 by SDS-PAGE and MALDI-TOF-MS.** The panel at left shows the SDS-PAGE pattern. SDS-PAGE was conducted using 15% polyacrylamide gels in the presence of β-mercaptoethanol. Portions of 5 μg of purified protein were applied to each well. The gels were stained with Comassie Brilliant Blue. Lane A, SSA-b-3; lane B, SSA-b-4. The sizes of standard proteins are shown in kDa. The panel at right shows the results obtained by MALDI-TOF-MS analyses. Panel A, SSA-b-3; Panel B, SSA-b-4.

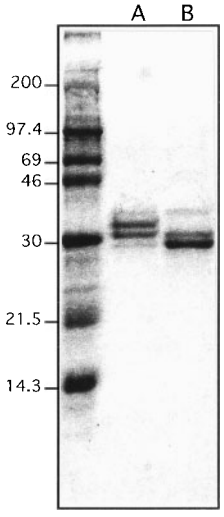


Fig. 3. **Enzymatic deglycosylation of SSA-b-3 and SSA-b-4. SDS-PAGE was conducted using 15% polyacrylamide gels.** Portions of 6 μg of the enzymatically deglycosylated proteins, obtained as described under Materials and Methods, were applied to each well. The gels were stained with Comassie Brilliant Blue. Lane A, SSA-b-3; lane B, SSA-b-4. The sizes of standard proteins are shown in kDa.

lectin (0.1% BSA/0.05% Tween 20) was added to each well and incubated at 37°C for 1 h. The lectin that reacted with the glycoprotein was detected with the partially purified anti-SSA-b-3/4 antibody (1/100) and HRP-labeled goat anti rabbit IgG (1/2,000, Sigma Chemical Co., St. Louis, MO, USA). After incubation with the substrate (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), the color intensity at 415 nm was determined by a microplate reader (MTP-32, Corona Electric).

Hapten inhibition experiments were carried out as follows. A solution of PBS-Tween-BSA containing varying amount of test sugars (25 μl) was added to each well of a glycoprotein-coated plate and incubated at 37°C for 30 min. The glycoproteins used for coating were asialoglycophorin for SSA-b-3 and Gal-BSA for SSA-b-4. Then, each lectin solution (100 ng/25 μl) was added and incubated for 1 h. Determination of the bound lectin with the anti-SSA-b-3/4 antibody was carried out as described above.

## RESULTS

*Isolation of Two Monomeric Lectins from the Bark Tissue of Sambucus sieboldiana*—Two new lectins were isolated from the bark tissue of *Sambucus sieboldiana* by affinity chromatography on acid treated-Sepharose 6B, gel filtration and high-performance cation exchange chromatography (Fig. 1). These lectins were named SSA-b-3 and SSA-b-4, for the reasons discussed later. SSA-b-3 was a minor component among the extractable proteins, but SSA-b-4 was one of the major components. Although these lectins were first isolated from the bark tissue taken in autumn (SSA-b-3) and spring (SSA-b-4), respectively, later analyses confirmed the presence of both

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LecSSA-b-3a:           DGEPITGNIIGRNGLCVDVRNGYSTDGTPIQLWPC--GRQRNQQWTFYEDGTIRSMGKCMTA       60
LecSSA-b-3b:           ************************************--************************       60
LecSSA-b-3c:           ************************************--************************       60
LecSSA-b-4a:           ************************************--************************       60
LecSNAIVf:             ***********D**********D*****L******--*T*********T*D**********       60
Sieboldin-b(B): DGETCSLPASF*RR*V**D*******D**************--*S*********T*D**********       64
SSA(B):       GGEYEKVCSVVEV*RR*S*WD*******G*HYI**N*V**R**---*NEC**L***RT*****WL***LT-       67
Ricin(B):        ADVCM*P***VR*V-**********D*RFHN*NA******KSNTDA**L**LKR*N****N**CL*T       66

LecSSA-b-3a:           NGLNSGSNIMIFNCSTAVENAIKWEVTIDGSIINLSSGLVMTAPSAASRTILLLENNIYAAS      122
LecSSA-b-3b:           ************************************************************      122
LecSSA-b-3c:           ************************************************************      122
LecSSA-b-4a:           *******D**********G****G*****R*********V****G***************      122
LecSNAIVf:             ***SN*****************************P************F*****Q*******      122
Sieboldin-b(B)         ****T************Q*******P*******T***R********T******Q*******      124
SSA(B):                ----TS*SV**YD*N*VPPE*T**V*S***T*T*PR****L***Q**EG*A*S**K**H**R      125
Ricin(B):              Y*YSP*VYV**YD*N**ATD*TR*QIWDN*T***PR*S**LA*T*GN*G*T*TVQT****V*      128

LecSSA-b-3a:           QGWTVSNDVKPIVASIVGYKEMCLQANGENNGVWMEDCEVTSLQQQWALFGDRTIRVNSSRG      184
LecSSA-b-3b:           *************************V**********************************      184
LecSSA-b-3c:           ************************************************************      184
LecSSA-b-4a:           ************************************************************      184
LecSNAIVf:             *********Q****L****N*****S*************A******************D**      184
Sieboldin-b(B):        ***S*T*N*Q*********N************************F***********A**      186
SSA(B):                *****G-**E*L*TF*****Q***RE*****F**L***VLNRVE*E***Y**G******NHS      186
Ricin(B):              ****PT*NTQ*F*TT***LYGL*****--SGQ**I***SSEKAE*****YA*GS**PQQN*D      188

LecSSA-b-3a:           LCVTSNGYNSKDLIITLKC-QGLPSQRWFFNSNGAIVNPNSTLVMDVRASNVSLREIIISPA      245
LecSSA-b-3b:           ******************-****************************************Y**      245
LecSSA-b-3c:           ***************I***-****************************************Y**      245
LecSSA-b-4a:           ******************-*****************T**********************Y**      245
LecSNAIVf:             ****T**********I*Q*-*************************KE*D********F*Y      245
Sieboldin-b(B):        ***************I***-************D******S******************F*P      247
SSA(B):                *****EDHEPS***VI***-E*SGN***V**T**T*S***AKL****AQHDI***K**LYRP      247
Ricin(B):              N*L**DSNIRETVVKILS*GPASSG***M*KND*T*L*LY*G**L***R*DP**KQ**LY*L      250

LecSSA-b-3a:           TGNPNQQWVTQVLPS                                                     260
LecSSA-b-3b:           ***************                                                     260
LecSSA-b-3c:           ***************                                                     260
LecSSA-b-4a:           ***************                                                     260
LecSNAIVf:             H*D************                                                     260
Sieboldin-b(B):        ***************                                                     266
SSA(B):                ***S****I*TTQ*A                                                     262
Ricin(B):              H*D***I*LPLF                                                        262
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Fig. 4. **Comparation of the amino acid sequences of the proteins encoded by SSA-b-3a-c, SSA-b-4, Sieboldin-b (SSA-b-2) (7) and related proteins.** Identical amino acid residues are indicated by asterisks. The peptide sequence obtained from the analysis of purified SSA-b-3 (identical to that of SSA-b-4) is underlined.

lectins in the bark sampled at different seasons (data not shown).

SSA-b-3 and SSA-b-4 lectins each showed a single protein band of 35 kDa and 33 kDa, respectively, on SDS-PAGE, irrespective of the presence or absence of reducing agent. Gel filtration of these proteins indicated the *M*r of 33 k for SSA-b-3 and 32 k for SSA-b-4 (data not shown). Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS showed the presence of major ions with $m/z$ = 34,262 for SSA-b-3 and 32,333 for SSA-b-4 (Fig. 2). These results indicated that both lectins exist mainly as a monomeric protein.

Both lectin bands were stained with the Glycan Detection Kit on the PAGE gels, showing that they are glycoproteins. Enzymatic deglycosylation of these lectins resulted in the appearance of additional bands. In the case of SSA-b-3, three new bands of 33, 31 and 29 kDa appeared instead of the original 35 kDa band. Similarly, two new bands of 31 and 29 kDa were obtained from SSA-b-4 (Fig. 3). These results indicated that SSA-b-3 and SSA-b-4 contain two or three N-linked glycan chains in the original molecules, and the size of the deglycosylated polypeptides are very similar. In other words, the difference in the molecular weight observed for SSA-b-3 and SSA-b-4 seems to derive from the difference in the number/structure of their glycan chains.

Analysis of the N-terminal amino acid sequences of the two lectins showed a completely identical structure up to 30 residues, indicating a very close structural relationship between them. Their isoelectric points were determined to be 5.0 (SSA-b-3) and 5.5 (SSA-b-4) by isoelectric focusing.

*Isolation and Characterization of cDNA Clones for SSA-b-3 and SSA-b-4*—A very close structural relationship between SSA-b-3 and SSA-b-4 was expected from the fact that their N-terminal amino acid sequences were identical. Additional information on the structural relationship between the bark lectins came from the fact that the antibody against sieboldin-b (SSA-b-2) cross-reacted with both SSA-b-3 and SSA-b-4. Based on this knowledge, a cDNA library from the bark of *S. sieboldiana* was screened with the cDNA clone for sieboldin-b (7) under less stringent conditions in combination with PCR.

Four clones (LecSSA-b-3a, LecSSA-b-3b, LecSSA-b-3c, LecSSA-b-4) were obtained as candidates for the cDNAs encoding SSA-b-3 or SSA-b-4. These clones were composed of 1,132–1,213 bp and encoded open reading frames which were identified as the primary amino acid

sequences of SSA-b-3 or SSA-b-4 because the N-terminal amino acid sequence of these lectins was found in the reading frames (Fig. 4, underlined). All the reading frames of these clones encoded polypeptides in which a signal peptide sequence of 13 amino acids is followed by the mature polypeptides consisting of 260 amino acids. The *M*r of the mature polypeptides, 28,292–28,478, depending on the amino acid composition, corresponded well with that of the deglycosylated polypeptides derived from SSA-b-3 and SSA-b-4.

Because the structures of the polypeptides encoded by these clones were similar (96.9 to 98.8% identity) and the N-terminal amino acid sequences of both SSA-b-3 and SSA-b-4 were identical, it was impossible to identify unambiguously which cDNA clone corresponds to each lectin. However, based on the difference in the isoelectric points of SSA-b-3 and SSA-b-4 (5.0 and 5.5, respectively), we were able to classify the clones into two groups that may correspond to each of these lectins. Predicted pI values for the polypeptides encoded by these cDNAs were 5.1 for LecSSA-b-3a, LecSSA-b-3b and LecSSA-b-3c, and 5.4 for LecSSA-b-4. Thus, we tentatively assigned the former three clones to SSA-b-3 or very closely related lectins and the latter one to SSA-b-4. The deduced amino

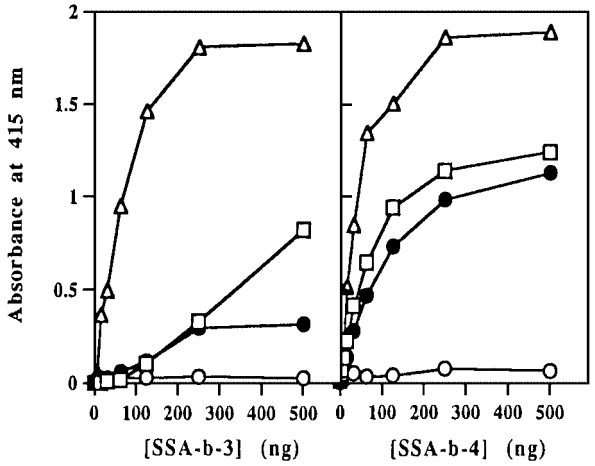


Fig. 5. **Reactivity of glycoproteins to SSA-b-3 and SSA-b-4.** Glycoproteins were coated on a 96-well microtiter plate and reacted with purified SSA-b-3 and SSA-b-4. The amounts of bound SSA-b-3 and SSA-b-4 were determined using partially purified rabbit anti-SSA-b-3/4 antibody and peroxidase-labeled goat anti-rabbit IgG. Triangles, asialoglycophorine; squares, Gal-β-BSA; open circles, fetuin; closed circles, asialofetuin.

Table 1. **Inhibitory potency of various sugars for the binding of SSA-b-3 and SSA-b-4 to glycoproteins[a].**

| Sugar | Concentration required for 50% inhibition (mM)[b] | |
|---|---|---|
| | SSA-b-3 | SSA-b-4 |
| D-Galactose | 4.2 (1.0)[c] | 9.7 (1.0)[c] |
| *N*-Acetyl-D-galactosamine | 2.5 (1.7) | 0.7 (14) |
| D-Galactosamine | 16.0 (0.26) | 22.0 (0.44) |
| D-Talose (C-2 epimer) | 5.5 (0.76) | 30.0 (0.32) |
| D-Fucose (6-deoxy-D-galactose) | 5.7 (0.74) | 6.4 (1.5) |
| L-Rhamnose | 8.1 (0.52) | 7.5 (1.3) |
| L-Arabinose | 47.5 (0.09) | 37.0 (0.26) |
| D-Ribose | 18.0 (0.23) | 34.0 (0.29) |
| D-Allose | 20.0 (0.21) | 49.0 (0.20) |
| D-Gulose (C-3 epimer) | No inhibition at 100 mM | 40% inhibition at 100 mM |
| D-Glucose (C-4 epimer) | 92.0 (0.046) | 40% inhibition at 100 mM |
| D-Mannose | 74% inhibition at 100 mM | 45% inhibition at 100 mM |
| D-Lyxose | 65% inhibition at 100 mM | 44% inhibition at 100 mM |
| D-Idose | No inhibition at 100 mM | 41% inhibition at 100 mM |
| Lactose | 0.97 (4.3) | 0.85 (11.4) |
| Melibiose | 7.5 (0.56) | 6.2 (1.6) |
| Raffinose | 5.4 (0.78) | 7.5 (1.3) |
| *N*-Acetyllactosamine | 1.6 (2.6) | 1.3 (7.5) |
| Methyl-α-D-galactopyranoside | 8.4 (0.5) | 3.1 (3.1) |
| Methyl-β-D-galactopyranoside | 4.5 (0.93) | 2.0 (4.9) |
| Methyl-α-D-mannopyranoside | 88% inhibition at 100mM | 22% inhibition at 100 mM |
| Gal(β1-6)GalNAc | 0.5 (8.4) | 0.6 (16.2) |
| GalNAc(α1-3)Gal(α1-2)Fuc | 0.5 (8.4) | 0.2 (49) |
| D-Altrose | 69% inhibition at 100 mM | No inhibition at 100 mM |
| D-Xylose | 66% inhibition at 100 mM | No inhibition at 100 mM |
| *N*-Acetyl-D-mannosamine, *N*-Acetyl-D-glucosamine | No inhibition at 100 mM | No inhibition at 100 mM |
| *N*-Acetylneuramic acid (Neu5Ac) | No inhibition at 20 mM | No inhibition at 20 mM |
| Neu5Ac(α2-6)lactose | No inhibition at 1 mM | No inhibition at 1 mM |
| Neu5Ac(α2-3)lactose | 90.3% inhibition at 1 mM | No inhibition at 1 mM |

[a]Obtained by ELISA as described under "MATERIALS AND METHODS." [b]Asialoglycophorin was used as a reacting glycoprotein for SSA-b-3 and Gal-β-BSA was used for SSA-b-4. [c]Relative inhibitory potency compared to D-galactose.

acid sequences of these lectins contained five putative glycosylation sites.

The amino acid sequence of the mature lectin encoded by LecSSA-b-3a was 98.8% identical with that of LecSSA-b-3b, 98.5% with LecSSA-b-3c and 96.9% with LecSSA-b-4. These cDNA clones also had a very high similarity in their base sequences (only 5–6 base differences were found between any combination of SSA-3-a, SSA-3-b and SSA-3-c). The structure of these lectins also showed a striking homology (86–89%) with that of the B-chains of sieboldin-b, a type 2 RIP from the same bark (*7*). However, a hexapeptide portion in the B-chain of sieboldin-b, which contains a cystein residue ($Cys^5$) conserved among type 2 RIPs and involved in the formation of disulfide linkage between A and B-chain, was lacking in SSA-b-3 and SSA-b-4. SSA-b-3 and SSA-b-4 also shared a high structural similarity with the B-chains of SSA (SSA-b-1, 56.6%) (*9*) and ricin (46.3%) (*22*).

Southern blot analysis using genomic DNA digested with *Eco*RI, *Hin*dIII or *Bam*HI and LecSSA-b-3a probe detected 2, 5, and 1 bands, respectively, indicating the presence of small multigene family related to SSA-b-3 and SSA-b-4 (data not shown).

*Biological Activities*—SSA-b-3 and SSA-b-4 agglutinated rabbit erythrocytes at a minimal concentration of 3.1 μg/ml and 6.2 μg/ml, respectively. As several lectins and related proteins isolated from elderberry trees were reported to show ribosome-inactivating activity, the effect of these two lectins on *in vitro* protein synthesis in rabbit reticulocyte lysate or cell-free extract of wheat germ was examined. Both lectins, however, showed the ribosome-inactivating activity only at unusually high concentrations (50% inhibition at 20–30 μg/ml for rabbit reticulocyte lysate and 200–400 μg/ml for wheat germ extract).

*Carbohydrate Binding Characteristics*—Reactivity of these lectins with several glycoconjugates was studied using ELISA (Fig. 5). Both lectins showed similar reactivity with asialoglycophorin, but SSA-b-4 showed higher reactivity with asialofetuin and Gal-BSA. Neither SSA-b-3 nor SSA-b-4 reacted with fetuin, suggesting that both lectins require terminal Gal/GalNAc residues for the interaction.

Inhibition studies with various sugars gave further information on the binding specificity of each lectin (Table 1). The results indicated that both lectins can be classified as Gal/GalNAc specific lectins, similarly to sieboldin-b, a previously purified type 2 RIP from the bark (*7*). However, the relative affinity of SSA-b-4 to GalNAc or GalNAcα1,3Galα1,2Fuc was 8–11 times higher than that of SSA-b-3, when Gal was used as a reference sugar. These results indicate that GalNAc fits much better than Gal to the binding site of SSA-b-4, similarly to sieboldin-b, but less well to that of SSA-b-3. Significant differences were also observed for the preference of Gal-containing oligosaccharides and glycosides between SSA-b-4 and SSA-b-3. Although the first group of lectins isolated from the bark of elderberry trees specifically bind to 2,6-linked sialylated oligosaccharides (*23*), neither SSA-b-3 nor SSA-b-4 showed such specificity.

## DISCUSSION

In this paper, we have described the isolation and characterization of two new lectins which have almost identical amino acid sequences to the B-chain of a type 2 RIP, sieboldin-b, from the bark of *Sambucus sieboldiana* (Japanese elderberry) (*7*). Because elderberry plants are a rich source of various lectins, Van Damme et al. proposed a system for naming these lectins based on the order in which they are found, such as SNA I-V from *S. nigra* (*24*). If we follow this system, the lectins reported here should be called SSA III and IV, because we have previously reported the isolation of two lectins from *S. sieboldiana*, namely, SSA and sieboldin-b (*25*, *7*). However, as these two new lectins correspond to neither SNA III nor IV (*26*, *27*), which were oligomeric lectins isolated from the seed and fruit of *S. nigra*, respectively, this terminology may cause some confusion. The two new lectins from *S. sieboldiana* also do not correspond to two other bark lectins from *S. nigra*, SNA II (*13*) and SNA V (nigrin-b) (*6*, *24*). We have already reported the isolation and cDNA cloning of the counterpart for SNA V from *S. sieboldiana*, sieboldin-b (*7*). SNA II is a homodimeric lectin and its N-terminal amino acid sequence is significantly different from these new lectins reported here (about 70% identity). To avoid such confusion, we have used Arabic numerals instead of the Roman numerals of SNA series and also indicated the origin of the tissue, bark, with the letter "b" after SSA. Thus, these two lectins have been called SSA-b-3 and SSA-b-4. The two previously isolated lectins can also be called SSA-b-1 (sialylated oligosaccharide-specific lectin) and SSA-b-2 (sieboldin-b).

Although the structure of four cDNA clones obtained from the bark cDNA library showed extensive homology, they could be classified into two groups corresponding to SSA-b-3 and SSA-b-4 based on the differences in their predicted isoelectric points. Isolation of these cDNA clones indicates the presence of corresponding lectins in the bark tissue. However, we could not detect or distinguish related lectins other than SSA-b-3 and SSA-b-4. It seems possible that all three lectins encoded by LecSSA-b-3a to LecSSA-b-3c might be contained in our SSA-b-3 preparation. Because of their practically identical amino acid sequences (99% identity with each other) and identical isoelectric point, these three lectins would be impossible to separate or distinguish from each other, unless they had significantly different glycosylation patterns, as in the case of SSA-b-3 and SSA-b-4.

The extensive sequence similarity between the polypeptides encoded by the cDNAs and the B-chain of sieboldin-b (SSA-b-2, 86–89% identity) is particularly interesting. Actually, SSA-b-3 and SSA-b-4 seem to be the B-chain itself, with the exception of the differences in the small peptide portion near the N-terminal, which contains a conserved cystein residue responsible for the formation of the disulfide linkage between A and B-chains in type 2 RIPs. The rest of the sequence of these lectins is almost identical with that of the B-chain of sieboldin-b (Fig. 4). The lack of the cystein residue well explains why these lectins exist in monomeric form rather than a part of two-chain RIPs. The structure of SSA-b-3 and SSA-b-4 also explains why these monomeric lectins can agglutinate red blood cells, as it is well known that the B-chain

of typical type 2 RIPs such as ricin and abrin have two carbohydrate-binding sites in a single polypeptide (*1*, *28*).

The amino acid sequences of SSA-b-3 and 4 are very similar to that of SNAIVf (*29*) previously isolated from the fruit of *S. nigra* (Fig. 4 for comparison). SNAIVf also has a structure corresponding to the B-chain of a type 2 RIP from the fruit, SNAVf, showing similar correspondence to the relationship between SSA-b-3/4 and sieboldin-b. However, SNAIV behaved as a homodimer on SDS-PAGE, differently from the monomeric behavior of SSA-b-3. Interestingly, in the case of *S. nigra*, the presence of similar B-chain like lectins was not detected in the bark even after extensive survey (*29*). Carbohydrate-binding specificity of SNAIVf was also not well understood.

Both SSA-b-3 and SSA-b-4 are Gal/GalNAc-specific lectins like sieboldin-b (*7*). The fact that only 7–8 out of 260 amino acid residues are different between SSA-b-3 and SSA-b-4 makes the observed differences in their binding specificity quite interesting. Expression of these lectins in a suitable system such as *E. coli* and introduction of mutation for the amino acid residues differing between these lectins will clarify the amino acid residue(s) responsible for such differences in the binding specificity, though the contribution of the differences observed in their glycosylation also should be taken into consideration.

In plants, type 1 RIPs are more widely distributed than type 2 RIPs, which seem to be more developed in terms of their structure and biological activity (*1*). It has been suggested that type 2 RIPs might have evolved by the fusion of a type 1 RIP-like gene and a B-chain like lectin gene (*30*). In addition to the known presence of free A-chains, type 1 RIPs, the finding of free, monomeric lectins with similar structure to the B-chain of a type 2 RIP further supports the above hypothesis. If this is correct, the genes for SSA-b-3 and SSA-b-4 may reflect the structure of such an ancestral gene for the B-chain of type 2 RIPs. Of course, the reverse can also be true, that they represent a product of the mutation of the genes for the B-chain that resulted in the deletion of the part responsible for dimerization.

The bark of elderberry plants is especially rich in RIPs and lectins. Type 2 RIPs with *in vitro* activity but not toxicity (*6*, *7*), a type 2 RIP with inactive B-chain (*8*), a group of Neu5Acα2,6Gal/GalNAc-specific lectins (*9–11*, *25*, *31*), a truncated polypeptide corresponding to a part of the sialylated oligosaccharide-binding subunit (*12*), Gal/GalNAc-specific lectins (*13–14*), and SSA-b-3 and SSA-b-4 have all been isolated from the bark. In addition, we recently isolated two other Gal/GalNAc-specific dimeric lectins from the bark of *S. sieboldiana* (Rojo *et al.*, unpublished). Concerning the biological role of these RIPs and lectins in the bark tissue, it has been postulated that they function to prevent the attack of potential predators by their toxicity (*15*). In addition to the possible synergistic effect of these molecules, the presence of different types of RIPs/lectins with different binding specificities and biological activities may contribute to the effective defense against a wide array of potential predators. On the other hand, RIPs/lectins in the bark seem to be a good model system to examine the course of molecular evolution of these molecules. Available information on the structure of these proteins indicates that most of them are structurally related and obviously evolved from a small number of ancestral genes. On the other hand, the characteristics of some of these proteins, such as non-toxic type 2 RIPs, raise the question about their function, because of the apparent "incomplete" biological activity compared to traditional type 2 RIPs. The presence of free B-chain-like lectins also stimulates curiosity about whether they have definite function by themselves or they just reflect the course of molecular evolution to (or from) type 2 RIPs. Further characterization and structural elucidation of related proteins in the bark may give further insight into the evolution of structure and function of these groups of plant proteins.

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