Sialylated Oligosaccharide-specific Plant Lectin from J
apanese Elderberry (Sambucus sieboldiana) Bark Tissue
Has a Homologous Structure to Type II Ribosome-inactivating
Proteins, Ricin and Abrin

cDNA CLONING AND MOLECULAR MODELING STUDY*

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Bark lectins from the elderberry species belonging to the genus Sambucus have a unique carbohydrate bind-
ing specificity for sialylated glycoconjugates containing NeuAc(n-2-6)Gal/GalNAc sequence. To elucidate the
structure of the elderberry lectin, a cDNA library was constructed from the mRNA isolated from the bark tis-
sue of J\apanese elderberry (Sambucus sieboldiana) with lgt11 phage and screened with anti-S. sieboldiana
agglutinin (SSA) antibody. The nucleotide sequence of a cDNA clone encoding full-length SSA (LecSSA1) showed
the presence of an open reading frame with 1902 base pairs, which corresponded to 570 amino acid residues.
This open reading frame encoded a signal peptide and a linker region (19 amino acid residues) between the two
subunits of SSA, the hydrophobic (A-chain) and hydro-
philic (B-chain) subunits. This indicates that SSA is syn-
thesized as a preproprotein and post-translationally cleaved into two mature subunits. Homology searching
as well as molecular modeling studies unexpectedly re-
vealed that each subunit of SSA has a highly homologous
structure to the galactose-specific lectin subunit and ribosome-inactivating subunit of plant toxic pro-
teins such as ricin and abrin, indicating a close evolu-
tionary relationship between these carbohydrate-bind-
ing proteins.

Plant lectins with defined carbohydrate binding specificities
have been isolated from various origins and used as invaluable
tools for the detection, fractionation, and isolation of glycocon-
jugates. The biological roles of these plant lectins, however, are
still not clear compared with some animal or microbial lectins
that have been shown to play important roles in biological
recognition systems. Structural studies on these molecules may
provide useful information not only on the molecular basis for
the binding specificity, but also on their biological function
through the comparison of their structure with other functional
proteins.

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to the GenBank™/EMBL Data Bank with accession number(s) D25317.
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† The abbreviations used are: SSA, Sambucus sieboldiana agglutinin;
PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid
chromatography; MSSA, monomeric monovalent SSA; RCA, Ridi
nus communis agglutinin; RIP, ribosome-inactivating protein.
affinity-purified anti-SSA antibody until individual plaques could be identified and isolated (at least three times). The nucleotide sequence of subcloned cDNA in the BamHI site of a Bluescript II K5+ plasmid vector was analyzed using an automatic DNA sequencing system (Model 373A, Applied Biosystems, Inc., Foster City, CA). The deleted cDNA mutant clones in the Bluescript vector on both strands were prepared using a deletion kit (Takara Shuzo, Kyoto, J. apam) according to the manufacturer's instructions.

Northern and Southern Blot Analyses—Northern blot analysis was carried out with total RNA (10 μg) from the bark as described (9). Southern blot analysis was performed as described previously (10). The [α-32P]dCTP-labeled cDNA probe was prepared by random primer labeling (11). The Southern blot analysis was performed using an automatic DNA sequencing system (DSQ-300, Daini, Kyom, Tokyo, J. apam).

Preparation of SSA and Anti-SSA Antibody—A bark lectin (SSA) was purified from the extract of the twigs of J. japonense elderberry by affinity chromatography on fetuin-agarose as described previously (12). The SSA A-chain was isolated from reduced and alkylated SSA by reversed-phase HPLC using conditions similar to those reported (13). Carboxypeptidase Y treatment of the A-subunit tetramer was very effective in removing antibodies against glycan chains and increased the specificity of the antibody.

Cyanogen Bromide Cleavage of SSA—SSA (10 mg) was reduced and alkylated by 4-vinylpyridine as described previously (3). Cleavage of the methionine residues of alkylated SSA was carried out as previously reported (12). The resulting peptides were analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE) (13) and transferred to a nitrocellulose membrane. The nitrocellulose membrane was stained with Coomassie Brilliant Blue, and the peptide spots were cut and sequenced by a gas-phase protein sequencer (PPSQ-10, Shimadzu, Kyoto, J. apam).

Determination of C-terminal Amino Acid Residues of SSA A-chain—The SSA A-chain was isolated from reduced and alkylated SSA by reversed-phase HPLC using an Inertsil 300-C18 column (4.6 × 100 mm; GL Science Inc., Tokyo, J. apam) as previously reported (3). After the lyophilized SSA A-chain was dissolved with 95% SDS in 0.5 M citrate buffer (pH 5.6) and kept at 60°C for 20 min, the solution was diluted 10 times with distilled water and incubated with 1:200 (w/w) carboxypeptidase Y (Boehringer Mannheim GmbH) at 25°C. Aliquots of the reaction mixture were taken at appropriate time intervals, added to a sulfosalicylic acid solution (final concentration of 1%), kept for 15 min at 25°C, and centrifuged. The released amino acid in the supernatant was identified using a Hitachi Model LS-8500A amino acid analyzer.

In Vitro Translation Experiment—In vitro protein synthesis was carried out with total RNA (2.9 μg) using rabbit reticulocyte lysate (Amersham, J. apam, Tokyo, J. apam) according to the manufacturer's protocol. The radioactive bands were detected by autoradiography.

RESULTS

Cloning of cDNA Encoding J japonense Elderberry Bark Lectin (SSA)—Total RNA (4.4 mg from 10 g of fresh tissue) was prepared from the bark tissue of Japanese elderberry (S. sieboldiana) at the beginning of October, when the tissue was actively synthesizing SSA (data not shown). Poly(A+) RNA was obtained by oligo(dT)-cellulose chromatography. In vitro translation using rabbit reticulocyte lysate followed by coprecipitation with anti-SSA antibody showed the synthesis of one major band corresponding to M, 58,000, along with several weaker bands (Fig. 1).

A bark cDNA library was constructed using the EcoRI site of the expression vector λgt11 phage with the double-stranded cDNA and was screened three times with an affinity-purified anti-SSA antibody. Four positive clones were obtained and subcloned into the BamHI site of the Bluescript II K5+ plasmid vector. Sequence as well as Southern blot analyses of these four cDNA clones showed that these had significant overlapping regions, indicating that they were derived from the same gene. Arrangement of the sequence of these four clones yielded a 834-base pair sequence, which corresponded to 278 amino acid residues. Portions of the deduced sequence coincided with those of two internal peptides isolated by CNBr deavage of SSA, suggesting that these clones encoded the cDNA of SSA. However, none of these four clones contained the region corresponding to the N-terminal sequences of the two subunits of SSA, indicating that they were not full-length clones. To isolate a full-length clone, the cDNA library was rescreened by plaque hybridization using a probe of the 243-base pair nucleotide corresponding to the 5′-terminal region of the 834-base pair sequence. Seven positive clones, each 2000 base pairs in size, were isolated. Southern blot analyses indicated that these clones belonged to the same group. Analysis of a clone (Lec-SSA1) with the longest insertion showed a sequence of 1902 nucleotide base pairs with an open reading frame encoding a polypeptide with 570 amino acid residues (Fig. 2).

Alignment of the known sequences of the N-terminal regions as well as the internal peptides of SSA revealed that both subunits of SSA were encoded in this open reading frame (Fig. 2). This indicated that a precursor polypeptide synthesized from the mRNA corresponding to this open reading frame was post-translationally cleaved into two subunits. From the N-terminal sequences of the two subunits of SSA (3), it was shown that the hydrophobic subunit (SSA A-chain) was encoded at the 5′-terminal side of the cDNA and that the hydrophilic subunit (SSA B-chain) was encoded at the 3′-terminal side. To determine the coding region for the first subunit, the SSA A-chain, the C-terminal sequence of the mature A-subunit was analyzed. Reduced and alkylated A-subunit was isolated by reversed-phase HPLC using conditions similar to those reported previously (3). Carboxypeptidase Y treatment of the A-subunit liberated serine, threonine, and valine successively, which corresponded to the sequence of Val287-7 Ser289 in the structure of the precursor polypeptide (Fig. 2). Combining this information
with the known N-terminal sequences of both subunits, coding regions for the A- and B-subunits were determined as Val29–Ser289 and Gly309–Ala570, respectively. These results also showed the presence of the linker peptide portion, Ser290–Arg308, between the A- and B-subunits.

Hydropathy plot analysis (Fig. 3) as well as the N-terminal sequence of the hydrophobic subunit indicated the presence of a signal peptide consisting of 28 amino acid residues (Met1–Arg28; Fig. 2). Thus, SSA is synthesized as a single preproprotein and processed into two mature subunits by post-translational removal of the signal peptide and the internal linker peptide between the two subunits. The hydropathy plot also supported the identification of the region for the hydrophobic and hydrophilic subunits as described above.

The calculated molecular weights of the A- and B-subunits were 28,774 and 29,055, respectively. The discrepancy between these values and those previously obtained for the two subunits by SDS-PAGE, 31,000 and 35,000, may be explained by the presence of sugar chains in each subunit (2).

Comparison of SSA Sequence with Other Protein Sequences—The deduced amino acid sequence of the SSA precursor showed extensive homology to the sequences of the precursor proteins of the well known plant toxic proteins abrin and ricin, which are composed of a galactose-specific lectin subunit and a toxin subunit with RNA N-glycosidase activity (Fig. 4). The hydrophilic subunit of SSA (SSA B-chain) showed 46.3 and 44.7% identity to the lectin subunits of ricin and abrin, respectively. The hydrophilic subunit (SSA A-chain) also showed 34.8 and 40.1% identity to the toxin subunits of ricin and abrin, respectively. Moreover, the amino acid residues that have been reported to be highly conserved among these ribosome-inactivating proteins were completely conserved within the SSA sequence, except Gin1, which replaced the conserved arginine residue in the other ribosome-inactivating proteins (Fig. 4).

Comparison of the SSA sequence with that of the castor bean lectin (Ricinus communis agglutinin (RCA)) precursor (19) also showed the presence of high homology (45.2% identity). These results indicate that SSA belongs, at least structurally, to the family of these toxin/lectins and probably originated from the same ancestral gene(s), although the carbohydrate binding specificity is significantly different compared with these proteins.

Ricin is a glycoprotein and contains two asparagine-linked oligosaccharides at Asn374 and Asn414 in the B-chain and another at Asn10 in the A-chain (20). Both SSA subunits were also shown to be glycosylated by periodic acid-Schiff staining of the SDS-polyacrylamide gel as well as by lectin blotting using several horseradish peroxidase-labeled lectins (data not shown). The sequence of SSA indicated the presence of six potential N-glycosylation sites in the A-chain and two sites in the B-chain, although none of them coincided with the glycosylation sites of ricin, nor were the real glycosylation sites identified.

Molecular Modeling—A computer-assisted, three-dimensional model of SSA was constructed based on the crystal structure of ricin (14–17). The amino acid residues of both subunits of SSA were aligned with those of the corresponding subunits of ricin to optimize identities. The amino acid substitutions, insertions, and deletions were made on this model, which was further stabilized using an energy minimization program. Fig. 5 shows the structure of SSA thus obtained, indicating the clear similarity in overall structure of these proteins, even though ricin has 15 additional residues in the whole molecule.

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The disulfide linkages in the SSA molecule were generated based on their corresponding position in ricin as the positions of the cysteine residues in the primary structure of SSA coincided well with those in ricin, except for Cys351 in the SSA B-chain.
Structural Similarity of Elderberry Bark Lectin to Type II RIPs

Fig. 4. Comparison of the amino acid sequences of the prepro-protein of SSA, abrin (23), ricin (20), and RCA (19). Identical amino acid residues are indicated (*), as are conserved amino acid residues among ribosome-inactivating proteins (31). The amino acid residues are indicated (+), as are conserved amino acid residues among ribosome-inactivating proteins (31). The amino acid residues are indicated (+) (16).

One of these disulfide linkages (Cys284-Cys316) connects the A- and B-subunits, and the other is present within the B-subunit. The free cysteine residue in the B-subunit (Cys316) that was not found in the ricin B-chain was located on the surface of the SSA molecule (Fig. 5A, indicated by the arrow).

Biological Activity of SSA—The effect of intact SSA and also disulfide linked dimeric SSA on in vitro protein synthesis was analyzed using rabbit reticulocyte lysate. SSA and MSSA showed only a very weak inhibitory potency of SSA and MSSA on protein synthesis were 2700–5000-fold weaker than those of ricin and 100–200-fold weaker than that of RCA. The property of SSA is significantly different from that of these toxic proteins. Ricin/abrin-type toxic proteins or RCA is basically specific to D-galactose residues, but the elderberry bark lectins including SSA are specific to the NeuAc(α2–6)Gal/GalNAc sequence (1, 23), other legume lectins (24, 25), and plant storage proteins such as soybean glycinn (26) and pea legumin (27). A polypeptide corresponding to the approximate size of unprocessed A-B chain, although associated with some other minor bands, was detected by in vitro translation experiments using rabbit reticulocyte lysate, which may lack such a specific endopeptidase.

Although there is no direct evidence for the identification of a SSA subunit that carries the carbohydrate-binding site, the extensive homology of the hydrophilic subunit (SSA B-chain) to the lectin subunit of abrin/ricin suggests the presence of the binding site in this subunit. Structural similarity of the B-subunit of SSA to that of ricin in the three-dimensional model (Fig. 5) also supports this. We recently found, by a chemical modification study, that histidine and tyrosine residues in SSA play an important role in the binding to sialylated oligosaccharides. The absence of a histidine residue in the coding region for the hydrophilic subunit (SSA A-chain) further supports that the carbohydrate-binding site is located in the SSA B-chain.

Each subunit of SSA is connected to the other subunits by disulfide linkage to form the tetrameric glycoprotein molecule. As the SSA A-chain has only one cysteine residue (Cys284) near the C terminus, it must form a disulfide linkage with a SSA B-chain. Otherwise, SSA cannot form the tetrameric molecule connected through disulfide linkage. On the other hand, the sequence of the SSA B-chain contains 10 cysteine residues. For the same reason, one of them should form a disulfide linkage with the SSA A-chain, and at least another one should participate in the cross-linkage between another SSA B-chain. The previous findings that the selective reduction and alkylation of the disulfide linkage between the SSA subunits yielded 1.4 pyridylethylated cysteines per subunit (7) indicate that the A- and B-subunits are connected through one disulfide linkage to form a tetrameric molecule in the form of A-B-B-A. Two cysteine residues, Cys284 and Cys316, which connect the A- and B-subunits, could be assigned because of their conserved position in the corresponding subunits of ricin/abrin. Concerning the cysteine residue responsible for the connection between two B-subunits, the SSA B-chain was shown to contain one additional cysteine residue (Cys316) near the C terminus (23), which also supports this. The additional cysteine residue in the SSA B-chain might be responsible for the connection of two B-subunits as these connections are not present in dimeric molecules such as ricin and abrin. The presence of this cysteine residue on the surface of the three-dimensional model of SSA further supports the possible involvement of this residue in the cross-linkage between the subunits (Fig. 5A).

The striking structural similarity of SSA to ricin/abrin-type ribosome-inactivating proteins as well as to RCA revealed the close evolutionary relationship of SSA to these toxic proteins (28, 29), although elderberry is taxonomically very far from those plants that produce these toxins (for example, elderberry and R. communis, which produces ricin, belong to different subclasses). However, there are significant differences between the properties of SSA and these proteins. First, despite the fact that the three-dimensional model of the SSA B-chain suggested the presence of two domains corresponding to two carbohydrate-binding domains of the ricin B-chain (Fig. 5B), SSA has only one carbohydrate-binding site, probably in this subunit. Also, the carbohydrate binding specificity of SSA is significantly different from that of these toxic proteins. Ricin/abrin-type toxic proteins or RCA is basically specific to D-galactose residues, but the elderberry bark lectins including SSA are specific to the NeuAc(α2–6)Gal/GalNAc sequence (1).
Although it is difficult to indicate the amino acid residues responsible for such differences in the binding specificity at present, further comparison of their structure coupled with site-directed mutagenesis and chemical modification and crystallographic/NMR studies will eventually clarify why the elderberry lectins recognize 2,6-linkedsialylated oligosaccharides so specifically.

The structural similarity of the SSA A-chain to the A-chain of ricin and abrin raised the question about the biological function of this subunit. The A-chain of ricin and abrin is a N-glycosidase that hydrolyses a very specific site of rRNA, resulting in the inhibition of protein synthesis at the ribosome (30–32). The invariant amino acid residues known for most of the ribosome-inactivating proteins including those of bacterial origin (Shiga-like toxin) were conserved in the SSA A-chain, except for Gin (33). However, SSA showed only a very weak (several thousandfold weaker than ricin) inhibitory activity against the in vitro protein synthesis of rabbit reticulocyte lysate (Table I). MSSA (free and stabilized subunits of SSA) also showed only a very limited activity, suggesting that the inability of SSA to terminate protein synthesis does not relate to its tetrameric structure and reflects its intrinsic property. SSA is also quite different compared with the structurally related tetrameric lectin RCA-120, which was reported to inhibit strongly the in vitro protein synthesis of rabbit reticulocyte lysate (34). In this context, it is noteworthy to point out the recent report of the presence of a new group of ribosome-inactivating proteins (RIPs), ebulin I and nigrin b, that have been isolated from the bark tissue of elderberry species (Table I) (21, 22). These proteins are composed of two different subunits and have a molecular size corresponding to that of ricin/abrin, although their structure has not yet been elucidated. Interestingly, they could inactivate mammalian ribosomes in vitro, but were inactive on the cell itself. Actually, we recently discovered the presence of a similar RIP in bark extract from S. sieboldiana, and this makes it difficult to determine whether the very weak inhibitory activity detected in the SSA preparation reflects the property of SSA itself or the contamination of a trace amount of such a RIP. Thus, it can be said that, despite the structural similarity to ricin/abrin-type toxins, SSA has only a very weak activity as a RIP or actually does not have such activity. Structural comparison of these proteins with

### Table I

| Protein      | IC₅₀a (ng/ml) |
|--------------|--------------|
| SSA          | 985          |
| MSSAb        | 540          |
| Ricin        | 0.2          |
| RCA          | 5.5c         |
| Nigrin b     | 3d           |
| Ebulin I     | 8.5e         |

aIC₅₀ is the concentration required for 50% inhibition of protein synthesis.

bMSSA was prepared as previously described (7).
cData are taken from Citores et al. (34).
dData are taken from Girbes et al. (22).
eData are taken from Girbes et al. (21).
proteins. as well as the structure/function relationships of these activities will give more insight into their evolutionary relationship as well as the structure/function relationships of these proteins.

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