Purification and partial characterization of a novel lectin from elder (*Sambucus nigra* L.) fruit

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A previously unknown haemagglutinin, named *Sambucus nigra* agglutinin-III (SNA-III), has been purified from the fruit of the elder (*Sambucus nigra*). Whereas elder bark agglutinin I (SNA-I) is highly specific for terminal α 2,6-linked sialic acid residues, SNA-III displays a high affinity for oligosaccharides containing exposed *N*-acetylgalactosamine and galactose residues. Different *N*-terminal sequences and the amino acid composition distinguish the fruit lectin from elder bark agglutinin II (SNA-II), which shows a similar carbohydrate specificity. The 40-fold higher affinity of SNA-III for asialofetuin than for human asialo- α ₁-acid glycoprotein and human asialotransferrin respectively suggests a preference for *O*-linked glycans. SNA-III occurs mainly as a monomeric glycoprotein, but tends to form di- and oligo-meric aggregates. This aggregation seems to mediate the multivalent interaction, leading to agglutination. SDS/PAGE revealed two major polypeptides with apparent molecular masses of 32 and 33 kDa respectively. This heterogeneity is probably a result of proteolysis in the C-terminal region. Binding to concanavalin A and susceptibility to peptide:*N*-glycosidase F indicated the presence of *N*-glycosidically linked oligosaccharides.

INTRODUCTION

Plant lectins have been most commonly isolated from dried seeds [for a review, see Goldstein & Poretz (1986)]. Nevertheless, several other plant tissues contain carbohydrate-binding proteins, for example leaves (Etzler & Borrebaeck, 1980), roots (Waxdal, 1974), tubers (Allen *et al.*, 1978) and bark (Horejsi *et al.*, 1978). Differences in saccharide specificity and in physicochemical properties have been related to the location within the plant (Talbot & Etzler, 1978; Gatehouse & Boulter, 1980; Wantyghem *et al.*, 1986), as established, for example, for two lectins from the roots and seeds respectively of the legume *Lotononis bainesii* (Law & Strijdom, 1984). In contrast, a lectin-like protein isolated from soybean (*Glycine max*) roots was demonstrated to be identical with soybean seed agglutinin (Malek-Hedaya *et al.*, 1987).

Elder (*Sambucus nigra* L.) bark contains the haemagglutinins *Sambucus nigra* agglutinin-I (SNA-I) (Broekaert *et al.*, 1984) and SNA-II (Kaku *et al.*, 1990). SNA-I exhibits a unique specificity for α 2,6-linked sialic acid residues (Shibuya *et al.*, 1987a). This remarkable property has made it a useful tool for the histochemical localization of sialic acid-containing glycoconjugates (Taates *et al.*, 1988) and for the characterization and separation of glycoprotein-derived oligosaccharides (Shibuya *et al.*, 1987b). However, its biological function in elder bark remains unclear, since plant glycoproteins do not acquire sialic acid residues (Faye *et al.*, 1989). The specificity of SNA-II for carbohydrates containing terminal *N*-acetylgalactosamine and galactose residues is rather common for plant lectins (Bhattacharyya *et al.*, 1988).

In the present paper we report on another lectin from *Sambucus nigra*, SNA-III, which occurs in the fruits, and demonstrate that it is not related to SNA-I and also that it is distinct from SNA-II.

MATERIALS AND METHODS

Materials

Mono- and di-saccharides, *p*-nitrophenyl glycosides, raffinose,

fetuin, asialofetuin, human α ₁-acid glycoprotein, human transferrin, ovalbumin (Grade V), BSA (Type V) and horseradish peroxidase (HRP) (Type II), as well as standard proteins for gel filtration and SDS/PAGE, were obtained from Sigma (St. Louis, MO, U.S.A.). Desialylation of human α ₁-acid glycoprotein and human transferrin was carried out by acid treatment (Spiro, 1966). Asialofetuin and fetuin were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) to a final concentration of 1.5 and 2.0 mg/ml of gel respectively, as outlined in the manufacturer's protocol. DEAE-Trisacryl M was purchased from LKB (Bromma, Sweden), and Bio-Gel P-100 was from Bio-Rad (Richmond, CA, U.S.A.). Peptide:*N*-glycosidase F (PNGase F; '*N*-Glycanase') was supplied by Genzyme (Boston, MA, U.S.A.). SNA-I was prepared from elder bark as described by Broekaert *et al.* (1984). Concanavalin A (Con A) was obtained from Pharmacia. All other reagents were of the highest purity available.

Purification of SNA-III

All procedures were carried out at 4 °C. A 1 kg portion of elderberries (collected in the garden of the University then frozen) was thawed overnight and homogenized (10 × 30 s) in 2 litres of PBSA [10 mM-NaH₂PO₄/Na₂HPO₄/150 mM-NaCl (pH 7.4)/0.02% (w/v) NaN₃] using an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). After being stirred for 1 h, insoluble material was removed by centrifugation (3000 *g*, 30 min), and the supernatant was fractionated by successive addition of solid (NH₄)₂SO₄ to 20 and 60% saturation respectively. Each precipitate was collected after stirring for 1 h by centrifugation as described above. The final pellet was dissolved in a minimal volume (200 ml) of 20 mM-Tris/HCl, pH 7.4, and dialysed exhaustively against the same buffer (3 × 5 litres) over a period of 48 h. The resulting suspension was centrifuged at 27000 *g* for 1 h. The supernatant was filtered through glass wool and applied to a DEAE-Trisacryl M column (2.5 cm × 20 cm) equilibrated with 20 mM-Tris/HCl, pH 7.4

Abbreviations used: Con A, concanavalin A; DTE, dithioerythritol; HRP, horseradish peroxidase; PNGase F, peptide:*N*-glycosidase F (peptide:*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase; EC 3.5.1.52); PBSA, phosphate-buffered saline containing azide (composition and pH are given in the text); SNA, *Sambucus nigra* L. agglutinin.

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(equilibration buffer) at a flow rate of 8 ml/h. After washing with 500 ml of equilibration buffer, bound material was eluted with a linear gradient (total volume 500 ml) up to 1.0 M-NaCl in equilibration buffer. Fractions (10 ml) containing haemagglutinating activity were combined, dialysed against three changes of 2 litres of 0.1 M-ammonium acetate buffer, pH 6.0, and freeze-dried. This material was then dissolved in 38 ml of PBSA.

Aliquots (3 ml) were used for each affinity-chromatography run on an asialofetuin-Sepharose column (1 cm × 10 cm). After sample application the column was washed with 15 ml of PBSA containing 1.0 M-NaCl, and the lectin was then specifically desorbed with 0.1 M-galactose in PBSA. The A_{280} was monitored throughout the run. Haemagglutinin-containing fractions (1 ml each) were combined and concentrated as outlined above.

Gel-permeation chromatography

Separation of the protein monomer and its various oligomeric aggregates was achieved by gel filtration on a Bio-Gel P-100 column (1.5 cm × 100 cm) performed in 0.1 M-ammonium acetate buffer, pH 6.0. Fractions (2 ml each) were monitored for A_{280} and analysed for haemagglutinating activity.

Gel electrophoresis

SDS/PAGE, using a total monomer concentration of 12.5 % (w/v), was performed under reducing and non-reducing conditions as described by Laemmli (1970), with the minor modifications described by Hasilik & Neufeld (1980). Silver staining was done as outlined by Merrill *et al.* (1982).

Enzymic deglycosylation

The protein content of the samples was determined by the method of Lowry *et al.* (1951), with BSA as standard. A 5 µg portion of protein, treated with SDS and mercaptoethanol, was incubated for 16 h at 37 °C in the presence or absence of 5 munits of peptide:N-glycosidase F, essentially as described by Hanewinkel *et al.* (1987), before analysis by SDS/PAGE.

Affinoblotting

Transfer of proteins to nitrocellulose sheets was done as described by Towbin *et al.* (1979). Subsequent detection of glycoproteins was performed using the Con A/HRP technique, essentially as described by Faye & Chrispeels (1985).

Compositional analysis

Amino acid analysis after hydrolysis of 50 µg of SNA-III in 6 M-HCl at 110 °C for 20 h was performed on a Dionex LC amino acid analyser as outlined by Roughley & White (1980). The cysteine content was determined after acid hydrolysis in the presence of 0.2 M-dimethyl sulphoxide (Spencer & Wold, 1969). Tryptophan values were obtained by using the spectrophotometric approach described by Edelhoch (1967).

N-Terminal sequence analysis

SNA-III polypeptides (30 µg from pool 3 after Bio-Gel P-100 chromatography) were separated by SDS/PAGE under reducing conditions and transferred to a poly(vinylidene difluoride) membrane (Matsudaira, 1987). Individual protein bands were directly analysed on an Applied Biosystems protein sequencer, model 470 A, and an on-line operating phenylthiohydantoin-derivative analyser, model 120 A (Nguyen *et al.*, 1989).

Haemagglutination and haemagglutination-inhibition assays

Haemagglutination assays were performed essentially as described by Ahmed & Gabius (1989), using untreated rabbit erythrocytes isolated after blood collection from the main ear artery. Briefly, 2-fold serial dilutions of column fractions or protein solutions in PBSA (50 µl) were mixed in 96-well microtitre plates (U-bottom; Falcon) with 50 µl of 2 % (w/v) erythrocytes in PBSA. Agglutination was recorded visually after incubation for 1 h at room temperature. The reciprocal of the highest dilution resulting in visible agglutination was defined as the titre. Specific activities are expressed in terms of titre/ A_{280} .

For inhibition studies, 2-fold serial dilutions of the inhibitor in PBSA were incubated with the lectin (titre 8) in a 50 µl volume for 1 h at room temperature before addition of the erythrocyte suspension. The lowest concentration of the inhibitor that reduced the lectin titre to 4 (equivalent to 50 % inhibition) was determined.

Immunological methods

Antibodies against SNA-I were raised in a New Zealand White rabbit by initial immunization with 300 µg of purified SNA-I in 0.5 ml of PBSA containing 0.1 M-lactose after mixing with an equal volume of complete Freund's adjuvant (Difco). A booster injection of 200 µg of SNA-I was given after 2 weeks using incomplete Freund's adjuvant (Difco). Serum was collected on week 4 after the initial injection.

Radial double immunodiffusion was performed by the Ouchterlony (1948) method.

RESULTS

Carbohydrate-binding specificity of SNA-III

Crude extracts of elderberries were found to agglutinate rabbit and human, but not sheep, erythrocytes. This activity did not depend upon the presence of bivalent-metal ions such as Ca^{2+} and Mg^{2+} , and was destroyed by boiling (results not shown). The agglutinin, partially purified by anion-exchange chromatography, was effectively inhibited by the monosaccharides *N*-

Table 1. Inhibition of SNA-III mediated haemagglutination by carbohydrates and glycoproteins

The following substances did not inhibit: D-glucose, *N*-acetyl-D-glucosamine, L-fucose, D-mannose, D-rhamnose, D-arabinose and D-xylose (at 50 mM each); fetuin, α_1 -acid glycoprotein, transferrin, ovalbumin, thyroglobulin and BSA at 1 mg/ml each.

Carbohydrate or glycoprotein	Concentration required for 50 % inhibition
D-Galactose	12.5 mM
D-Galactosamine	12.5 mM
Lactose	12.5 mM
Raffinose	12.5 mM
<i>N</i> -Acetyl-D-galactosamine	1.56 mM
<i>p</i> -Nitrophenyl α - <i>N</i> -acetyl-D-galactosaminide	0.31 mM
<i>p</i> -Nitrophenyl α -D-galactoside	5.0 mM
<i>p</i> -Nitrophenyl β -D-galactoside	2.5 mM
Asialofetuin	0.003 mg/ml
Asialo- α_1 -acid glycoprotein	0.125 mg/ml
Asialotransferrin	0.125 mg/ml

Table 2. Purification scheme for SNA-III

Purification step	Total volume (ml)	Total protein (mg)	Total agglutinating activity (units)	Purification (-fold)
(NH ₄) ₂ SO ₄ precipitation	295	708	94 400	1.0
DEAE-Trisacryl M	38	195	37 500	1.5
Asialofetuin-Sephacrose 4B	4.0	15.0	12 800	6.4

Fig. 1. Gel-permeation chromatography of purified SNA-III on Bio-Gel P-100

Affinity-purified SNA-III was chromatographed on Bio-Gel P-100 as outlined in the Materials and methods section. Three A_{280} (Δ) peaks were pooled as indicated and the specific activity was determined. The column was calibrated with Blue Dextran 2000 (V_0), BSA (molecular mass 66 kDa), bovine β -lactoglobulin (36 kDa) and sperm-whale myoglobin (18 kDa).

Fig. 2. SDS/PAGE of SNA-III

Each pool (1, 2 and 3) obtained after gel filtration (Fig. 1) was analysed by SDS/PAGE (5 μ g of protein/lane), with (+) or without (–) reduction with dithioerythritol (DTE), and subsequent silver staining as outlined in the Materials and methods section. The standard proteins used were: BSA (molecular mass 66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20.1 kDa).

Fig. 3. Enzymic deglycosylation of SNA-III

A 5 μ g portion of protein from each pool (1, 2 and 3) after gel filtration was treated with (+) or without (–) PNGase F. Portions (2.5 μ g) were subjected to SDS/PAGE, subsequently stained with AgNO₃ (left panel) or subjected to affino blotting using Con A (right panel) as described in the Materials and methods section.

acetylgalactosamine and, to a lesser extent, by galactose and galactosamine. The corresponding *p*-nitrophenyl glycosides showed higher inhibitory activity, whereas lactose and raffinose reacted like galactose (Table 1). A more extensive characterization of the elder fruit lectin, which we propose to call SNA-III, was achieved by further inhibition experiments using glycoproteins with a well characterized glycan moiety (Montreuil, 1984). Agglutination of rabbit erythrocytes by SNA-III was not inhibited by fetuin, human α_1 -acid glycoprotein and human transferrin. Desialylation of these glycoproteins, however, led to strong inhibitory capacity (Table 1). Indeed, the finding that the fruit agglutinin binds to immobilized asialofetuin provided a useful means by which it could be purified.

Purification of SNA-III

Crude elderberry extract, concentrated by (NH₄)₂SO₄ precipitation, was fractionated by anion-exchange chromatography on DEAE-Trisacryl M to remove coloured contaminants. This step resulted in a slight enrichment of the lectin, which was eluted as one peak at approx. 0.15 M-NaCl (results not shown). Subsequent affinity chromatography on asialofetuin-Sephacrose with desorption by 0.1 M-galactose (results not shown) led to a 6.4-fold purification (Table 2). Since no retardation of the lectin on either fetuin-Sephacrose or Sepharose 4B was observed, non-specific binding to the matrix was excluded (results not shown).

Physicochemical and immunological characterization of SNA-III

Gel filtration of the affinity-purified material resolved various protein peaks (Fig. 1). Analysis by SDS/PAGE revealed two major polypeptides, with apparent molecular masses of 32 and 33 kDa, to be present in all protein-containing fractions (Fig. 2, left panel). The different ratios of both polypeptides in the three pools are in part due to differences in *N*-glycosylation (Fig. 3, left panel).

These results suggest the existence of different aggregated forms of SNA-III that interact in general with gel-filtration media, resulting in aberrant retardation (Fig. 1, and results not shown). A specific haemagglutinating activity of 1160, 230 and 5 units per mg of protein for pools 1, 2 and 3 respectively showed that agglutination is dependent on oligomerization of the lectin. However, in spite of its negligible haemagglutinating activity, pool 3 also retained the ability to bind to asialofetuin-Sephacrose (results not shown). The interaction between the subunits is not

Main sequence (75 mol%): Asp-Gly-Glu-Pro-Ile-Thr-Gly-Asn-Ile-Ile-Gly-Arg-...

Minor sequence (25 mol%): Glu-Pro-Ile-Thr-Gly-Asn-Xaa-Ile-Gly-Xaa-...

Fig. 4. N-Terminal sequence of SNA-III

SNA-III polypeptides were separated by SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane. The 32 and 33 kDa protein bands were subjected to automated N-terminal amino-acid sequencing as described in the Materials and methods section.

Table 3. Amino acid composition of SNA-III compared with SNA-I and SNA-II

Lectin...	Composition			
	SNA-III	SNA-I	SNA-II	
Amino acid	(residues/molecule)*	(mol%)+	(mol%)+	(mol%)+
Asx	36.75	16.1	11.6	15.2
Thr	15.75	6.9	8.1	6.6
Ser	20	8.6	7.9	7.0
Glx	22.5	9.8	9.2	8.4
Pro	8	3.5	5.3	5.3
Gly	20.75	9.1	6.8	9.8
Ala	11.5	5.0	5.4	6.7
Val	14.75	6.5	9.7	7.7
Met	11.75	4.7	1.3	2.5
Ile	14.75	6.5	4.9	6.7
Leu	16.5	7.2	9.3	7.8
Tyr	4.5	2.0	3.0	1.7
Phe	6	2.6	3.4	2.6
His	1	0.4	1.0	0.2
Lys	3	1.4	2.0	2.5
Arg	8	3.5	5.7	6.0
Cys	6	2.6	2.3	3.0
Trp	7.75	3.4	1.7	n.d.§

* Results are expressed as residues per monomer, assuming a molecular mass of 29.2 kDa (exclusive of carbohydrate).

† From Broekaert *et al.* (1984).

‡ From Kaku *et al.* (1990).

§ n.d., not determined.

Fig. 5. Immunological relationship of SNA-III with SNA-I

Double immunodiffusion in 1% (w/v) agarose was performed in PBSA containing 0.1 M-lactose. The concentration of each lectin (purified SNA-I and SNA-III after gel filtration respectively) in the well was 10 µg/ml of PBSA. To reveal the precipitin lines, the dried gel was stained with Coomassie Brilliant Blue R-250. Well 1, SNA-I; well 2, SNA-III, pool 1; well 3, SNA-I, pool 2; well 4, SNA-III, pool 3; well 5, anti-SNA-I antiserum.

only a consequence of the formation of intermolecular disulphide bridges, since dissociation of the aggregated forms occurs even without reduction. Under these conditions, the same subunit

pattern was obtained for the three pools, the monomers exhibiting apparent molecular masses of 28 and 30 kDa (Fig. 2, right panel).

The binding of both polypeptides to Con A demonstrated the glycoprotein nature of SNA-III. Treatment with PNGase F before analysis by SDS/PAGE resulted in increased electrophoretic mobility, but the weak reaction with Con A, even after exhaustive enzymic deglycosylation, indicates the existence of oligosaccharide chains resistant to PNGase F. However, the carbohydrate moiety cannot account solely for the electrophoretic heterogeneity observed (Fig. 3).

Protein sequencing yielded two closely related N-termini in both SNA-III polypeptides. The minor N-terminal sequence represents a truncated version of the main sequence missing the first two amino acids (Fig. 4). The fruit lectin is rich in aspartic acid/asparagine, glutamic acid/glutamine, glycine, serine and threonine, and has a low histidine content. In addition, the calculation of amino acid residues per molecule of monomeric SNA-III with respect to the heterogeneous N-terminal region suggests a partially truncated C-terminus (Table 3).

In double-immunodiffusion experiments, the fruit lectin showed no cross-reactivity with an antiserum against SNA-I, indicating two immunologically distinct proteins (Fig. 5).

DISCUSSION

The unique specificity described for elder bark agglutinin I (SNA-I) for α2,6-linked sialic acid residues suggested not-yet-elucidated physiological roles for endogenous lectins in plants (Broekaert *et al.*, 1984; Shibuya *et al.*, 1987a). This led to extensive characterization and comparison of bark lectins from different *Sambucus* species (Nsimba-Lubaki *et al.*, 1986; Tazaki & Shibuya, 1989; Shibuya *et al.*, 1989) and finally yielded the identification of a second class of bark haemagglutinins (Kaku *et al.*, 1990; Harada *et al.*, 1990) with a rather widespread affinity towards terminal Gal/GalNAc-residue-containing oligosaccharides. Although specific physiological functions for these diverse bark lectins have been proposed (Greenwood *et al.*, 1986; Kaku *et al.*, 1990), no information is available about the occurrence of these lectins in other elder tissues.

Our hapten inhibition studies using various mono- and disaccharides revealed some similarities between a haemagglutinin identified in *Sambucus nigra* fruit extracts and the bark lectins, and the variability observed was in the same range as described for various bark lectins isolated from different *Sambucus* species (Broekaert *et al.*, 1984; Nsimba-Lubaki *et al.*, 1986; Shibuya *et al.*, 1989; Tazaki & Shibuya, 1989; Harada *et al.*, 1990; Kaku *et al.*, 1990). However, differential reactivity towards glycoprotein glycans made it possible to distinguish clearly the elder fruit lectin from SNA-I. While the α2,6-linkage of sialic acid to galactose enhances the binding to SNA-I (Shibuya *et al.*, 1987a), the fruit agglutinin requires unsubstituted terminal N-acetyl-galactosamine or galactose residues. Nevertheless, these results indicate some similarities of the fruit lectin, SNA-III, to the bark agglutinin, SNA-II, although differences in the fine carbohydrate specificity do exist. In contrast with SNA-II (Kaku *et al.*,

1990), the fruit lectin exhibits a high affinity towards asialofetuin. This exceptionally strong interaction compared with other desialylated glycoproteins could be due to the presence of *O*-linked glycans on asialofetuin based on a Gal β 1-3GalNAc disaccharide unit (Spiro & Bhoyroo, 1974).

SNA-III is a glycoprotein and forms aggregates of various complexities. It consists of two isoforms with apparent molecular masses of 32 and 33 kDa. The heterogeneity is not solely the result of differences in the number of attached *N*-linked oligosaccharide chains, as it was proposed for *Dolichos biflorus* (Etzler *et al.*, 1981) and *Robinia pseudoacacia* (Wantyghem *et al.*, 1986) agglutinins. Both polypeptides exhibit almost identical *N*-termini. However, the presence of a proteolytically truncated C-terminus could be, according to the amino acid composition, an alternative explanation for the results obtained.

The haemagglutinating activity of SNA-III depends, as it is commonly believed for plant lectins (Roberts *et al.*, 1982), on oligomerization. However, in contrast with SNA-I (Broekaert *et al.*, 1984), this aggregation is due to non-covalent interactions. As SNA-II exists as a homodimer (Kaku *et al.*, 1990), our data suggest a marked functional difference between the different elder lectins. The lack of immunological cross-reactivity between SNA-I and SNA-III confirms their unrelatedness. On the other hand, the unique *N*-terminal sequence obtained for SNA-III and its amino acid composition distinguish it from SNA-II. However, the possibility still exists that the fruit lectin represents a proteolytically processed form of this bark agglutinin, as the immunological relationship of the two proteins has not yet been addressed.

The biochemical and physicochemical characterization of SNA-III from *Sambucus nigra* fruit may contribute to the study of the role of different lectins in various plant tissues. The marked specificity for oligosaccharide structures resembling the basic unit of *O*-linked glycans emphasize potential applications in glycoconjugate research and biomedical procedures, but a refined analysis of its carbohydrate-binding properties will be essential for these purposes.

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