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A lectin from elder (*Sambucus nigra* L.) bark

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A lectin was isolated from elder (*Sambucus nigra*) bark by affinity chromatography on fetuin–agarose. It is a tetrameric molecule (M_r 140000) composed of two different subunits of M_r 34500 and 37500 respectively, held together by intramolecular disulphide bridges. The lectin is a glycoprotein and is especially rich in asparagine/aspartic acid, glutamine/glutamic acid, valine and leucine. It is also the first lectin isolated from a species belonging to the plant family Caprifoliaceae.

Plant lectins are a heterogeneous class of proteins or glycoproteins that have in common their unique ability to recognize specific sugars or sugar-containing macromolecules (for reviews, see Liener, 1976; Goldstein & Hayes, 1978). In the past, phytohaemagglutinins have been mostly studied in dry seeds, and especially in seeds of legume species. However, at present, evidence is accumulating that they occur also in vegetative tissue of a number of plant species. Well-known examples of typical non-seed lectins are the tuber, leaf and fruit lectins from solanaceous species such as potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) (Allen *et al.*, 1978; Kilpatrick, 1980) and phloem-exudate lectins of fruits and stems of *Cucurbita* (vegetable marrow) and *Cucumis* (cucumber) species (Sabnis & Hart, 1978; Allen, 1979). In addition, root lectins from *Trichosanthes kirilowii* (tiahuafen) (Yeung *et al.*, 1980), *Bryonia dioica* (bryony) (Peumans *et al.*, 1984) and *Phytolacca americana* (pokeweed) (Waxdal, 1974) typically occur in vegetative tissue as do mistletoe (*Viscum album*) leaf lectin (Olsnes *et al.*, 1982) and black-locust-tree (*Robinia pseudoacacia*) bark agglutinin (Horejsi *et al.*, 1978). Moreover, several legume species contain leaf and stem lectins that are related to, but not identical with, the respective seed lectins (Talbot & Etzler, 1978; Etzler & Borrebaeck, 1980; Lamb *et al.*, 1983).

In the present paper we describe the isolation and characterization of a lactose-specific lectin from elder (*Sambucus nigra L.*) bast (the living part of the bark). This lectin, which occurs in very high concentrations in living bark tissue, is the first lectin purified from a species of the family Caprifoliaceae.

Abbreviations used: SNA, *Sambucus nigra* agglutinin; SDS, sodium dodecyl sulphate; phosphate-buffered saline, 1.5mM-KH_2PO_4/10mM-Na_2HPO_4 (pH 7.4)/3mM-KCl/140mM-NaCl.

Materials and methods

Materials

Bark was stripped from elder (*Sambucus nigra* L.) stems and branches. After removal of the outer suberized (dead) tissue, it was cut into small pieces and either used immediately or stored at −20°C.

Reagents

All reagents used were analytical grade. Fetuin–agarose was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Extraction and lectin purification

All operations were carried out in a cold-room at 0–4°C. Bast tissue was homogenized with a mixer in 10vol. of ice-cold 10mM-Tris/HCl buffer, pH 7.8. After being left on ice for 1h, the homogenate was filtered through cheesecloth and the filtrate cleared by centrifugation at 3000*g* for 5min, followed by a high-speed centrifugation (100000*g*, 30min). The resulting supernatant was filtered through filter paper (Whatman 3MM) (in order to remove lipid material, which floats on top of the centrifuge tube) and applied to a column (2.5cm × 10cm; 10ml bed volume) of fetuin–agarose (equilibrated with phosphate-buffered saline). Unbound protein was eluted by washing the column with phosphate-buffered saline until the A_{280} fell below 0.01, and the lectin desorbed with 0.1M-lactose (in water). As shown in Fig. 1,

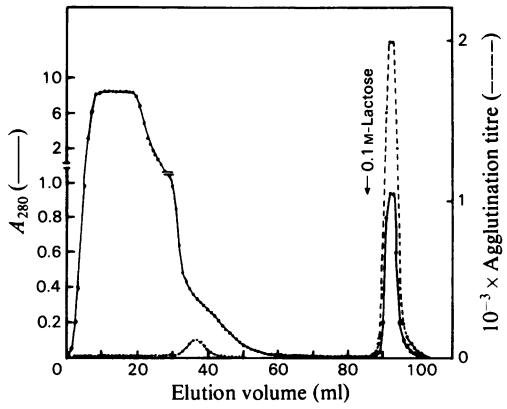

Fig. 1. *Affinity chromatography of SNA on fetuin–agarose*
A portion (20ml) of a crude extract from elder bast equivalent to 2g of tissue was applied to the column (10ml bed volume). Unbound protein was eluted with phosphate-buffered saline until the A_{280} fell below 0.01. The lectin was desorbed with 0.1M-lactose (in water). About 95% of the total agglutination activity and 3.6% of the total protein was recovered in the fraction desorbed with lactose.

virtually all agglutinating activity bound to the column and was eluted as a sharp peak upon elution with lactose. At complete saturation, the binding capacity of the fetuin–agarose column was about 1.5mg/ml of bed volume. It is worthwhile to mention here that elder lectin can hardly be eluted from freshly used fetuin–agarose with lactose (even at a concentration of 0.3M). A convenient way to collect the lectin is desorption with unbuffered 20mM-ethylenediamine. The reason for the poor desorption from a fresh column is not known. After three to five rounds of affinity chromatography (followed by desorption with ethylenediamine), however, the lectin is readily eluted with 0.1M-lactose.

After affinity chromatography, the lectin was further purified by hydroxyapatite chromatography, ion-exchange chromatography and gel filtration. The fractions containing the affinity-purified lectin were applied to a small column (10ml bed volume) of hydroxyapatite [granular; 80–200 mesh ASTM (American Society for Testing and Materials); from Merck, Darmstadt, Germany] equilibrated with water. The lectin bound to the column, and lactose (together with other impurities) was washed off with water and 0.3M-NaCl successively. Then the lectin was eluted in a sharp peak with 0.1M-phosphate buffer, pH7.2 (Fig. 2*a*). Subsequently, the fractions containing the lectin were pooled, dialysed against 10mM-Tris, pH8.7, and applied to a column (1.4cm × 25cm) of DEAE-Bio-Gel (from Bio-Rad,

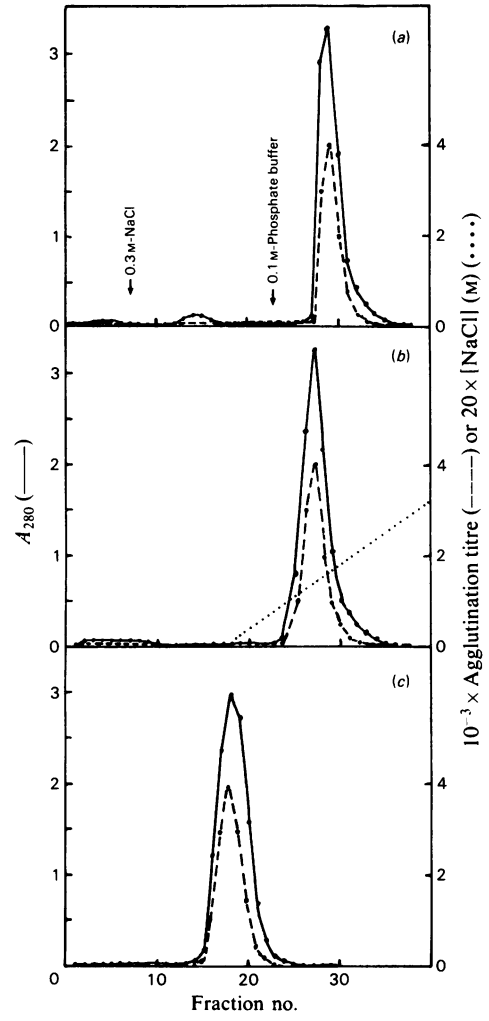

Fig. 2. *Hydroxyapatite (a), DEAE-Bio-Gel ion-exchange (b) and Sephadex G-100 gel-filtration (c) chromatography of affinity-purified SNA*
(*a*) After the column had been loaded (10ml bed volume), it was washed with water and 0.3M-NaCl before eluting the lectin with 0.1M-phosphate buffer, pH7.2). The fraction size was 2ml. (*b*) After the column (1.4cm × 25cm) had been loaded with SNA, purified on hydroxyapatite, it was washed with 25ml of 10mM-Tris/HCl, pH8.7, before eluting the lectin with a salt gradient (in Tris buffer). The fraction size was 2ml. (*c*) Peak fractions of SNA purified on DEAE-Bio-Gel were concentrated by $(NH_4)_2SO_4$ precipitation and chromatographed on a column (1.4cm × 28cm) of Sephadex G-100. Phosphate-buffered saline was the running buffer. The fraction size was 1ml.

Richmond, CA, U.S.A.) equilibrated with the same Tris buffer. After the column had been washed with Tris buffer, the lectin was eluted with a linear gradient (100ml) of NaCl (0–0.4M) in

10mM-Tris/HCl, pH8.7 (Fig. 2*b*). The peak fractions of the lectin were pooled and precipitated with $(NH_4)_2SO_4$ (60% relative saturation). Then the precipitated lectin was collected by centrifugation (20000*g*; 10min), dissolved in a minimal volume of phosphate-buffered saline and passed through a column (1.4cm × 2.8cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) equilibrated with phosphate-buffered saline (Fig. 2*c*). Finally, peak fractions were collected and used.

Agglutination assays

Agglutination assays were carried out in small glass tubes in a final volume of 0.1ml containing 80μl of a 1% suspension of trypsin-treated red blood cells and 20μl of crude extracts or lectin solutions (each serially diluted with 2-fold increments). Agglutination was monitored visually after the tubes had been left for 1h at room temperature.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

SDS/polyacrylamide-gel electrophoresis

Lectin preparations were analysed by SDS/polyacrylamide-gel electrophoresis by using a discontinuous system (Laemmli, 1971) on 12.5–25% (w/v)-acrylamide gradient gels.

Sugar determination

Total neutral sugar was determined by the phenol/H_2SO_4 method (Dubois *et al.*, 1956), with D-glucose as a standard. Amino sugars in a hydrolysed sample were determined in an amino acid analyser.

Amino acid analysis

Lectin samples were hydrolysed (in sealed glass tubes under an N_2 atmosphere) for 24h at 110°C in 6M-HCl containing 0.02% β-mercaptoethanol. After hydrolysis, HCl was removed by evaporation, and the residue was dissolved in sodium citrate buffer, pH1.9. Amino acid analysis was done with a Biotronik LC 2000 amino acid analyser. This apparatus allows a separation of all 20 amino acids on a single column of Durrum resin, by using an elution program consisting of four different buffers. Amino acids were directly quantified by using an Infotronics integrator. Tryptophan was determined after hydrolysis with 3M-mercaptoethanesulphonic acid. Cysteine was determined as cysteic acid after hydrolysis in performic acid.

Cell-free protein synthesis in a wheat embryo extract

Tobacco-mosaic-virus RNA translation activity of a wheat embryo cell-free protein-synthesizing system was measured by determining the incorporation of [^{3}H]leucine into hot (80°C)-trichloroacetic acid-insoluble material (Peumans *et al.*, 1980).

Results

Occurrence, agglutination properties and carbohydrate specificity of elder bark agglutinin

Crude extracts from elder bark exhibit high agglutination titres (up to 10000 and 100 with trypsin-treated and untreated human blood-group-A erythrocytes respectively). Although the agglutination is not really blood-group-specific, titres obtained with group-A red blood cells were 1.5 and 2 times higher than those obtained with group-B and -O erythrocytes respectively. The carbohydrate-specificity of the agglutinin in crude extracts was determined with a series of simple sugars (*N*-acetylgalactosamine, lactose, melibiose, fucose, galactose, galactosamine, raffinose, stachyose, glucose, galacturonic acid, sucrose, mannose, trehalose, arabinose, cellobiose, glucosamine, *N*-acetylglucosamine, ribose, maltose, mannosamine and rhamnose) all at a final concentration of 100mM. As shown in Table 1, lactose and *N*-acetylgalactosamine were the best saccharide inhibitors, being respectively 1.5 and 2 times as potent as galactose and raffinose. Melibiose, fucose and stachyose were 3 times, and galactosamine 8 times, less active than lactose. Galacturonic acid and ribose also inhibited the agglutination, but only at higher concentrations.

Table 1. *Carbohydrate-binding specificity of SNA*
Agglutination was assayed with trypsin-treated human blood-group-A red blood cells.

	Minimal concentration (mM) required for 50% inhibition of agglutination with	
	Crude bark extract (titre = 320)	Purified bark lectin (10μg/ml)
Lactose	0.8	0.8
N-Acetylgalactosamine	0.8	0.8
Galactose	1.2	1.2
Raffinose	1.8	2.4
Melibiose	2.4	3.6
Fucose	2.4	3.6
Stachyose	2.4	3.6
Galactosamine	6.25	6.25
Galacturonic acid	12.5	18.7
Ribose	25	25

Purification of Sambucus nigra agglutinin (hereafter called 'SNA')

SNA could be purified from crude extracts in one step by affinity chromatography on fetuin–agarose (Fig. 1). The lectin content of bark is relatively high, as SNA represents up to 5% of the total soluble protein content. When prepared as described in the Materials and methods section, crude extracts contain about 20mg of protein/ml. The overall yield was usually about 1mg of SNA/g fresh weight of bark tissue.

Stability of SNA under different conditions

The stability of purified SNA was investigated under different conditions of pH, temperature and salt concentration. Thereby the lectin was found to be stable over the pH range 6–12 (Fig. 3*b*). It was heat-stable (in phosphate-buffered saline) up to 55°C, but was completely inactivated at 70°C (Fig. 3*a*). The lectin was also irreversibly inactivated by NaCl at concentrations above 2M (Fig. 3*c*). Finally, reduction of SNA with 1% β-mercaptoethanol (for 30min at 20°C) resulted in an almost

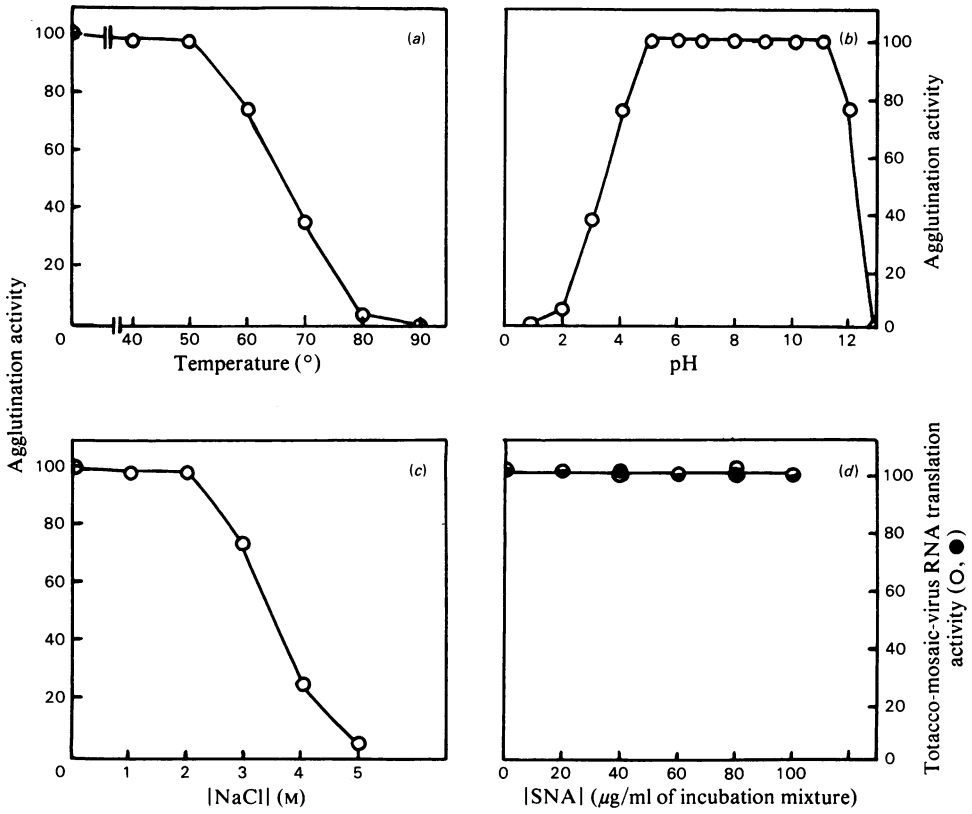

Fig. 3. *Heat (a)-, pH (b)- and NaCl (c)-stability of SNA, and effect of SNA on tobacco-mosaic-virus RNA translation in a wheat embryo cell-free system (d)*

(*a*) Portions of an SNA solution (0.1 mg/ml in phosphate-buffered saline) were heated in a water bath for 5min at the temperatures indicated, quenched cold in ice and assayed for agglutination activity. Results are expressed as percentages of the control (which was kept at 20°C) value. (*b*) Portions of an SNA solution (0.1 mg/ml) in phosphate-buffered saline were adjusted to different pH values (by adding 0.1 M-HCl or 0.1 M-NaOH). After being left for 1 h at room temperature, samples were adjusted to 0.2M-Tris/HCl, pH 7.8, and assayed for agglutination activity. Results are expressed as percentages of the control (samples kept in phosphate-buffered saline) value. (*c*) Portions of SNA (0.1 mg/ml in 10mM-Tris/HCl, pH 7.8) were adjusted to different concentrations of NaCl and left for 1 h at room temperature. Then they were diluted 10-fold with 10mM-Tris/HCl, pH 7.8, and assayed for agglutination activity. Results are expressed as percentages of the control (sample kept in 10mM-Tris/HCl, pH 7.8) value. (*d*) A cell-free protein-synthesizing system, derived from wheat embryos, was supplemented with different concentrations of either native (○) or reduced (●) SNA (the latter was treated with 1% β-mercaptoethanol for 2h at 25°C before its addition to the cell-free system). Results are expressed as percentages of the control (mixture without SNA) value. Absolute control leucine incorporation was 1240pmol/25μl of reaction mixture.

complete loss (about 99%) of its agglutinating activity.

Characterization of purified SNA

Molecular structure. Highly purified SNA was analysed by SDS/polyacrylamide-gel electrophoresis with and without reduction with 2% β-mercaptoethanol. As shown in Fig. 4, reduced SNA yields two bands of M_r 34500 (A-subunit) and 37500 (B-subunit) respectively, indicating that the lectin molecules are build up of two different types of subunits. Unreduced SNA gives a pattern typified by the appearance of irregularly shaped faint bands of M_r around 200000. These high-M_r bands seem to be inherent to SNA. Indeed, they appear also on SDS/phosphate-buffered saline of crude extracts, but are absent when the lectin is removed by affinity chromatography (results not shown). The M_r of native SNA was also estimated by gel filtration (Fig. 5*a*) and sucrose-density-gradient centrifugation (Fig. 5*b*). Both methods

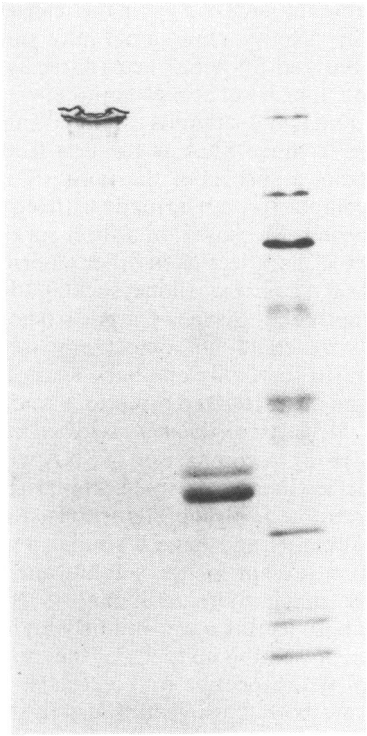

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of purified SNA (25 μg) in the presence (b) or absence (a) of 2% β-mercaptoethanol*

M_r reference proteins (lane *c*) were lysozyme (M_r 14300), soya-bean trypsin inhibitor (M_r 21000), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumin (M_r 67000), phosphorylase *b* (M_r 94000), galactosidase (M_r 130000) and myosin (M_r 200000). Gels were stained with Coomassie Brilliant Blue.

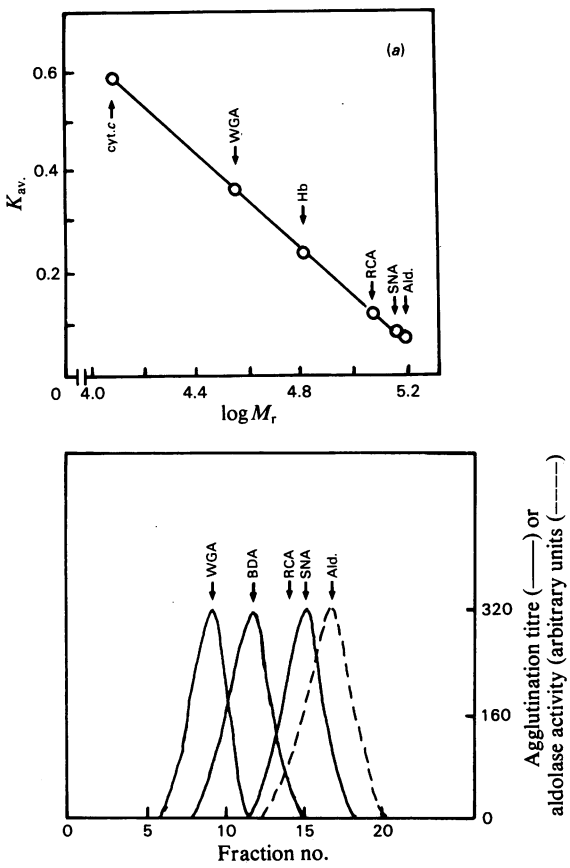

Fig. 5. *Sephadex G-100 gel filtration (a) and sucrose-gradient centrifugation (b) of SNA*

(*a*) Column dimensions were 1.4 × 28cm; the running buffer was phosphate-buffered saline. M_r reference proteins were cytochrome *c* (cyt.c, M_r 12400); wheat-germ agglutinin (WGA, M_r 36000); haemoglobin (Hb, M_r 65000), *Ricinus communis* agglutinin (RCA, M_r 120000) and aldolase (Ald., M_r 160000). The $K_{av.}$ of *Ricinus communis* agglutinin was determined in a separate experiment (with the same column and the same marker proteins). (*b*) SNA was centrifuged in a linear 12–38% (w/v)-sucrose gradient (in phosphate-buffered saline) at 2°C for 24h at 50000rev./min in a Beckman SW 50.1 rotor. Gradients were fractionated into 0.2ml portions with an ISCO density-gradient fractionator and assayed for agglutination activity. Wheat-germ agglutinin, *Ricinus communis* agglutinin, *Bryonia dioica* agglucinin (BDA, M_r 65000) and aldolase were used as M_r markers. The position of *Ricinus communis* agglutinin was determined on a parallel gradient with the same reference proteins.

indicated a M_r around 140000. It appears, therefore, that SNA is a tetrameric protein composed of two A (M_r 34500)- and two B (M_r 37500)-chains.

Amino acid composition

The amino acid composition of SNA (Table 2) is characterized by high contents of asparagine/aspartic acid, glutamine/glutamic acid, leucine and valine. SNA contains 16 methionine and 28 cysteine residues per molecule. It also contains 5.6% (w/w) total sugar and 1.5% (w/w) glucosamine.

Agglutination properties

Hapten inhibition assays with simple sugars indicated that both purified SNA and crude extracts exhibit the same sugar-specificity (Table 1). Like crude extracts, purified SNA agglutinated blood-group-A erythrocytes slightly better (1.5 and 2 times better than group-B and -O red blood cells respectively). The specific agglutination activity of SNA is comparable with that of other plant lectins. Minimal concentrations required for agglutination were 0.12 and 11 μg/ml with trypsin-treated and untreated group-A erythrocytes respectively.

SNA does not affect tobacco-mosaic-virus RNA translation in a wheat embryo cell-free system

Several plant lectins are toxic and inhibit protein synthesis in cells or cell-free sytems (Olsnes, 1978*a*,*b*; Gasperi-Campari *et al.*, 1978). To find out whether SNA, too, inhibits protein synthesis, its effect on tobacco-mosaic-virus RNA translation was investigated in a wheat-embryo-derived cell-free system. Native SNA had no inhibitory effect, even at a final concentration of 0.1 mg/ml (Fig. 3*d*). Since some lectins [e.g. *Ricinus communis* (castor bean) and *Vicia cracca* (tufted vetch) agglutinins] inhibit cell-free protein synthesis only after reduction with β-mercaptoethanol (Barbieri *et al.*, 1979), SNA was reduced with 1% β-mercaptoethanol before its addition to the cell-free system. Again, however, no inhibition was observed, even at SNA concentrations up to 0.1 mg/ml (at which concentrations reduced *Ricinus communis* and *Vicia cracca* lectins are extremely inhibitory) (Fig. 3*d*).

Table 2. *Amino acid composition of lectins from elder (SNA) and black-locust-tree bark*

Amino acid	SNA (Residues/molecule)*	SNA (Mol. %)	Black-locust-tree agglutinin (Mol. %)†
Asx	144	11.6	13.4
Thr	102	8.1	8.3
Ser	98	7.9	9.08
Glx	114	9.2	6.43
Pro	66	5.3	5.66
Gly	84	6.8	7.72
Ala	68	5.4	8.11
Val	122	9.7	9.24
Met	16	1.3	0.34
Ile	62	4.9	5.55
Leu	116	9.3	7.76
Tyr	38	3.0	2.60
Phe	42	3.4	5.95
His	12	1.0	0.88
Lys	24	2.0	4.44
Arg	72	5.7	3.10
Trp	22	1.7	1.32
Cys	28	2.3	0

* Results are expressed as the number of residues per molecule, assuming an M_r of 136000 (exclusive of carbohydrate).

† Data from Horejsi *et al.* (1978).

Discussion

Living bark tissue of elder trees contains considerable amounts of a lectin that can readily be isolated by affinity chromatography on fetuin–agarose. Purified SNA has been partially characterized and appears to be a glycoprotein composed of two A- and two B-subunits (M_r 34000 and 37000 respectively). Since SNA is the first lectin to be isolated from a species of the family Caprifoliaceae, no comparison can be made with lectins from related species. Moreover, in a short survey of the occurrence of bark lectins in other Caprifoliaceae species [e.g. *Lonicera* (honeysuckle), *Viburnum* (wayfaring-tree), *Symphoricarpus* (snowberry), *Weigelia*], we could not detect any other bark agglutinin. In fact, only one bark lectin has been isolated and characterized hitherto, namely that of the black locust tree (*Robinia pseudoacacia*) (Horejsi *et al.*, 1978). A comparison of SNA with black-locust-tree lectin reveals some similarities. They are both tetrameric glycoproteins consisting of two types of subunits and have a similar amino acid composition (except for the sulphur amino acids, which are much more abundant in SNA). In addition, both lectins occur in fairly high concentrations, representing up to 5% of the total protein content of the respective bark extracts.

However, both bark lectins differ in several aspects. They are structurally different in that SNA subunits are covalently linked by disulphide bridges whereas black-locust-lectin subunits are not. In addition, both lectins exhibit striking differences in sugar-specificity. Indeed, whereas black-locust bark lectin is only inhibited by high concentrations (25 mM) of *N*-acetylgalactosamine (and no other simple sugars), SNA is inhibited not only by low concentrations (< 1 mM) of lactose and *N*-acetylgalactosamine, but also by relatively low concentrations of galactose, melibiose, fucose,

stachyose and galactosamine (Table 1). Moreover, unlike the black-locust lectin, which agglutinates blood-group-A, -B and -O erythrocytes equally well, SNA agglutinates blood cells of group A 1.5 and 2 times better than those of groups B and O respectively.

The presence of high concentrations of lectins in living bark tissues of some trees leaves intriguing questions about their possible physiological role(s). At present, only speculations are possible. However, the high concentration of bark lectins might suggest that they fulfil a rather specific role (either defensive or structural), or perhaps even serve as storage protein.

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