Isolation and Characterization of an N-Acetylgalactosamine Specific Lectin from *Salvia sclarea* Seeds*

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Crude extracts from *Salvia sclarea* seeds were known to contain a lectin which specifically agglutinates Tn erythrocytes (Bird, G. W. G., and Wingham, G. (1974) Vox Sang. 26, 69–166). We have purified the lectin to homogeneity by ion-exchange chromatography and affinity chromatography. The agglutinin was found to be a glycoprotein of *M* = 50,000, composed of two identical subunits of *M* = 35,000 linked together by disulfide bonds. The purified lectin agglutinates specifically Tn erythrocytes and, at higher concentrations, also Cad erythrocytes. Native A, B, or O red blood cells are not agglutinated by the lectin and, even after treatment with sialidase or papain, these cells are not recognized. Tn red cells present 1.45 × 10⁵ accessible sites to the lectin which binds to these erythrocytes with an association constant of 1.8 × 10⁹ M⁻¹. On Cad red cells, 1.73 × 10⁵ sites are accessible to the lectin which binds with an association constant of 1.0 × 10⁹ M⁻¹. The carbohydrate binding specificities of the *S. sclarea* lectin have been determined in detail, using well defined monosaccharides, oligosaccharides, and glycopeptide structures. The lectin was found to be specific for terminal N-acetylgalactosamine (GalNAc) residues. It binds preferentially αGalNAc determinants either linked to Ser or Thr (as in Tn structures) or linked to a BGalNAc or to an unsubstituted BGal residue. Although more weakly, the lectin binds βGalNAc residues linked in 1–4 to a βGal (as in Cad structures). It does not recognize βGalNAc determinants linked in 1–3 to a Gal (as in globoside) or the αGalNAc residues of blood group A structures.

Lectins are carbohydrate-binding proteins which agglutinate erythrocytes and other normal or transformed cells (1). They show a wide diversity in their sugar specificity and they are often used to investigate the structures of carbohydrate components present on cell surfaces. They are also applied to the purification and the structural assessment of glycoproteins. Among the lectins already well characterized, several N-acetyl-D-galactosamine (GalNAc) specific lectins have been described (for reviews see Refs. 2–5). Since they exhibit different, well defined specificities toward GalNAc residues, they are valuable tools used to discriminate between cells bearing various GalNAc-containing oligosaccharides on their surfaces (4, 5).

Lectins specific for GalNAc residues have been shown to agglutinate Tn erythrocytes (6). The Tn transformation is an acquired disorder characterized by the exposure, at the red cell surface, of normally cryptic GalNAc residues α-linked to Ser or Thr on the membrane sialoglycoproteins (7). This is the result of a selective deficiency of the 3-β-p-galactosyltransferase involved in the biosynthesis of the T structure: Galβ1–3GalNAca-Ser/Thr (8). In patients presenting the Tn syndrome, this antigen has been detected at the cell surface of erythrocytes, granulocytes, platelets, and B and T lymphocytes (9). The Tn antigen has also been proposed as a marker of cancerous tissues (10, 11).

Since some time, crude extracts from *Salvia sclarea* seeds are known to agglutinate specifically Tn red cells (12) and are used in routine blood typing. However, nothing has been reported about the properties of the lectin and its precise carbohydrate binding specificity. In the present paper, we describe the purification and the characterization of an αGalNAc specific lectin from the seeds of *S. sclarea*.

**EXPERIMENTAL PROCEDURES**

**Materials**—*S. sclarea* seeds were a gift from Dr. J. Touche (Avignon, France). Oligosaccharides coupled to Synsorb were obtained from Chemibiotic Ltd, Edmonton, Alberta, Canada. Prepacked DEAE TSK 545 column was from LKB, Bromma, Sweden. Na₅¹⁵Ο⁺ (555 MBq of ¹⁵Ο⁻/µg of iodine) was supplied by Amersham. IODOGEN was purchased from Pierce. Dibutyl phthalate and bis(2-ethylhexyl)phthalate were obtained from Faikes, Buchs, Switzerland. Pepsin was from Prolabo, Paris, France. *Vibrio cholerae* neuraminidase was supplied by Behringwerke A.G., Marburg/Lahn, Federal Republic of Germany. BSM, BSA, Gal, GalNH₂, and GalNAc were purchased from Sigma. p-NO₂Ph-α-DGalNAc and p-NO₂Ph-β-DGalNAc were from Koch-Light Laboratories, Suffolk, United Kingdom. The blood group P-active trisaccharide (*GalNAcβ1–3Galα1–4Glc) was a generous gift from Dr. H. Paulsen (Hamburg, Federal Republic of Germany). The asialo-Cad trisaccharide (*GalNAcβ1–3Galβ1–4Glc) was synthesized and donated by Dr. J. C. Jacquetin and Dr. F. Sinay (Orléans, France). All the synthetic glycopeptides carrying α*-N-acetylgalactosamine residues were synthesized according to published methods (13) and donated by Dr. B. Ferrari and Dr. A. Pavia (Avignon, France). Asialo-BSM was prepared as described (14). The sialic acid and the hexosamine contents of native and desialylated BSM were estimated according to Refs. 15 and 16.

**Buffers**—PBS (phosphate-buffered saline): 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.137 mM NaCl, pH 7.35. TBS (Tris-buffered saline): 50 mM Tris-HCl, pH 7.2, 100 mM NaCl. TBS-BSA (Tris-buffered saline with BSA): TBS, pH 7.3, containing 1% (w/v) BSA.

**Cells**—Red cells from Tn (Mrs. Ba.) and Cad (Mr. Cad.) donors were supplied by Dr. D. Lee (Lancaster, United Kingdom) and M. Monis (Montpellier, France), respectively. Erythrocytes from blood group A₁, A₂, O, and B were supplied by the Centre National de...
Transfusion Sanguine, Paris, France. Protease treatment (1.35 mg of papain/ml of packed red cells, 7 min at 37 °C) and neuraminidase treatment (0.1 unit of neuraminidase/ml of packed red cells, 30 min at 37 °C) of intact erythrocytes were performed as previously described (17, 18).

Preparation of S. salarea Lectin—30 g of dry S. salarea seeds were ground in a Waring blender and the meal was stirred overnight in 400 ml of PBS, 5 mM EDTA at 4 °C. The solution was centrifuged at 20,000 × g for 30 min, and the pellet was re-extracted with another 400 ml of PBS, 5 mM EDTA, overnight at 4 °C. The two supernatants were combined and frozen at −20 °C. After thawing, the insoluble material was spun down (50,000 × g for 30 min), and the clear supernatant (crude extract) was precipitated with 50% (v/v) cold ethanol at 4 °C. After centrifugation (20,000 × g for 30 min), the pellet was discarded, and the supernatant was further precipitated with cold ethanol up to 80% (v/v) at 4 °C. After one night in the cold room, the solution was centrifuged (20,000 × g for 30 min), the supernatant was discarded, and the pellet was resuspended in water, dialyzed for 3 days at 4 °C against water with several changes, and lyophilized. A 40% fraction of the freeze-dried product was redisolved in 15 mM Tris- HCl buffer, pH 7.5, centrifuged 10 min at 3,500 × g, and the supernatant was filtered through a 0.2-μm nitrocellulose filter (Millipore). It was then applied to a DEAE-TSK 545 preparative column (2.15 × 15 cm) equilibrated in the same buffer. The chromatography was performed at room temperature, at a flow rate of 2 ml/min. The unbound material was pooled and concentrated at 4 °C by ultrafiltration using a PM-10 Diaflo membrane (Amicon). Standards were mixed with unlabeled material before being applied onto an affinity column containing GalNAc a-linked to Synsorb. The column of viscous and readily precipitable material present in the extract was saturated for 3 h in TBS-BSA, with several changes, in order to avoid nonspecific interaction. Incubation assays consisted of 0.5 mg of Synsorb (=20 μl) and 20 μl of the radioliodinated lectin solution (specific activity = 100,000 cpm/μg) in TBS-BSA. The mixtures were left at room temperature under gentle shaking. After 1 h, the supernatants were removed, and the Synsorb was rinsed three times with TBS-BSA and counted in a LKB γ counter model 1274.

RESULTS
Purification of S. salarea Lectin—Crude extracts of S. salarea seeds are known to agglutinate specifically Tn erythrocytes (12), which bear unmasked GalNAcα-Ser/Thr structures on their membrane glycoproteins (7). Purification strategy was therefore based on affinity chromatography using GalNAc-derivatized Synsorb. Because of large amounts of viscous and readily precipitable material present in the extract, a preliminary purification was required before the affinity chromatography step. As shown in Table I, the first purification stages were achieved by ethanol fractionation, followed by anion-exchange chromatography, which together resulted in a 50-fold increase in specific activity of the lectin. Most of the hemagglutinating activity was recovered in the 50–80% ethanol fraction and applied onto an ion-exchange column. A typical elution profile after DEAE-chromatography is shown in Fig. 1A. Almost all the loaded material was retained by the anion exchanger, while the hemagglutinating activity passed through unretarded.

The lectin which was immediately eluted from the DEAE-column proved to be still contaminated by another protein as seen by SDS-electrophoresis and gel filtration experiments (data not shown). A further step was then necessary to obtain the lectin in pure form. This was achieved by affinity chromatography as shown in Fig. 1B. For an easier monitoring of the elution, an aliquot of the protein mixture was radiolabeled as described under "Experimental Procedures." It was then applied onto an αGalNAc-Synsorb column. The protein which eluted with the starting buffer had no agglutinating activity, while the protein which eluted with 0.1 M GalNAc in the buffer corresponded to 85% of the total hemagglutinating activity loaded onto the column. The two radioactive peaks eluted from the Synsorb column represented together 98% of the applied radioactivity. After this last step, the purification
Affinity chromatography
Anion-exchange chromatography (flow-through)
Ethanol precipitate
ishes Tn agglutinability by the lectin.

trofocusing (Fig. 2).

using different kinds of human red cells, as shown in Table

of the red cells with papain leads to a slight agglutinability of
dase are more strongly agglutinated by the lectin. Treatment

A1, AO, 0,
sclarea seeds agglutinates specifically Tn erythrocytes, and, to

although at a lower level. This is consistent with the fact that the lectin is more homo-
genic and isoelectrically homogeneous. The molecular mass of the lectin, determined
g the globular structure of the lectin by its precipitation with cold ethanol, was found to


gain activity for Tn erythrocytes is higher than for Cad red cells. The reason for this

The homogeneity of the purified lectin has also been checked by isoelectrofocusing (Fig. 2, lane c). Two sharp bands could be detected by Coomassie Blue staining: one major one migrating at pH 8.8 and a minor one migrating at pH 9.0.

The amino acid and carbohydrate compositions of the lectin are given in Table II. The lectin is rich in acidic amino acids, aspartic and glutamic acids, and in hydroxyproline, serine and threonine, but it contains few cysteine and methionine. Moreover, it shows a relatively high content of hydrophobic amino acids. Sugar analysis revealed the presence of Man, GlcNAc, Fuc, and Xyl as the major constituents. Gal, Glc, and Ara could also be detected, however, in lower amounts.

Since their ratios varied with the lectin samples analyzed, it is likely that these last three sugars are contaminants. Total carbohydrate content accounts for about 15% by weight of the glycoprotein.

Agglutination Properties of the Lectin—Some agglutination properties of the pure lectin toward Tn erythrocytes have been investigated. The lectin has no apparent requirement for divalent cations since it is still capable of hemagglutination after incubation with up to 40 mM EDTA. Moreover, the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+} in the incubation medium does not alter the hemagglutination titer. The lectin agglutinates better at basic pH (near the PI of the lectin) than in an acidic environment.

Agglutination specificity of the lectin has been determined using different kinds of human red cells, as shown in Table III. Among the native cells tested, the isolated lectin from S. sclarea seeds agglutinates specifically Tn erythrocytes, and, to a lesser extent, Cad erythrocytes. It does not agglutinate native A_{1}, A_{0}, O, or B cells. Treatment of the red cells with sialidase leads to a slight agglutinability of A_{1}, A_{0}, O, or B cells, while there is only a small increase of hemagglutination of Cad cells. In contrast, Tn erythrocytes treated with sialidase are more strongly agglutinated by the lectin. Treatment of the red cells with papain leads to a slight agglutinability of A_{1}, A_{0}, O, or B cells and to a strong increase of hemagglutination of Cad cells. On the contrary, papain treatment abolishes Tn agglutinability by the lectin.

Binding Specificity of the S. sclarea Lectin—The binding of the purified lectin to native and papain- and sialidase-treated Tn red cells, as well as to native and papain-treated Cad red cells, was studied as described under "Experimental Procedures." The results are presented in Fig. 3, A and B. They show that S. sclarea lectin does not bind to papain-treated Tn erythrocytes. For all other cells studied, the binding of the lectin is saturable. The Scatchard plots obtained from these data are linear. The binding parameters derived from the plots show that the lectin affinity is highest for desialylated Tn red cells ($K_u = 3.8 \times 10^9$ M^{-1}) which display also the greatest number of sites per cell ($2.1 \times 10^9$). Native Tn cells and papain-treated Cad cells carry about the same amount of sites for the lectin ($1.45 \times 10^8$ and $1.07 \times 10^8$, respectively), which shows the same affinity for these two cell types ($K_u = 1.8 \times 10^9$ M^{-1}); however, their association constant is two times lower than for desialylated Tn erythrocytes. The native Cad red cell presents also $1.73 \times 10^9$ sites accessible to the lectin, but the affinity for these erythrocytes ($K_u = 1.0 \times 10^6$ M^{-1}) is lower by about one-half than for papain-treated Cad cells. The binding to all cells is inhibited in the presence of free GalNAc in the medium.

Inhibition of Tn Red Cell Hemagglutination by the S. sclarea Lectin—Hemagglutination assays were performed, on native Tn erythrocytes, in the presence of a variety of carbohydrate inhibitors (Table IV). N-Acetylgalactosamine proved to be the best monosaccharide inhibitor, 50 times more potent than galactosamine and 750 times more potent than galactose. p-Nitrophenyl-N-acetyl-$\alpha$- or $\beta$-D-galactosaminide were as good inhibitors as N-acetylgalactosamine, suggesting that the presence of the $\beta$-nitrophenyl group does not alter the binding specificity of the lectin. Moreover, with these substrates, no strong preferences for either the $\alpha$ or the $\beta$ anomers could be observed. Oligosaccharides containing $\beta$GalNAc in the terminal position were also inhibitors. The structure in which the $\beta$GalNAc is linked in 1-4 to a $\beta$Gal (as in the asialo-Cad structure) inhibited Tn agglutination in the same range as free N-acetylgalactosamine, but was 10 times more inhibitory than the $\beta$ structure in which the GalNAc is linked in 1-3 to an $\alpha$Gal (as in globoside).

Structures consisting of $\alpha$GalNAc directly linked to the amino acid chain (as in Tn structures) were also tested as inhibitors. All the synthetic glycopeptides listed in Table IV, consisting of one to three GalNAc units-$\alpha$-linked to Ser or Thr, gave almost identical inhibition scores, irrespective of their amino acid sequence or of their GalNAc content. However, they are slightly better inhibitors than N-acetylgalactosamine. Bovine submaxillary mucin (BSM) which contains many GalNAc-$\alpha$-Ser/Thr structures, most of them sialylated, also has a good inhibitory effect; if the amount of free terminal GalNAc is taken into account, the inhibitory activity of BSM is in the same range as for the glycopeptides tested. When the mucin was desialylated, it was an even more potent inhibitor of the Tn erythrocyte agglutination, about 50 times better than free N-acetylgalactosaminide.
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Fig. 1. Anion exchange chromatography of the 50–80% ethanol fraction (A) followed by affinity chromatography on GalNAc-Synsorb (B). The precipitate, recovered after fractionation between 50% and 80% ethanol, was dissolved in 15 mM Tris-HCl buffer, pH 7.3 (8 mg/ml). 5 ml of this solution was applied onto a DEAE-TSK 545 column (2.15 x 15 cm) equilibrated in the same buffer. A typical elution profile is shown in A. The column was washed at a flow rate of 120 ml/h with the starting buffer until the absorbance at 280 nm (M) returned to <0.02. The column was then eluted with a gradient of NaCl up to 0.3 M in order to desorb the retained material from the gel. Aliquots of the DEAE-eluate, after concentration and radiolabeling, were applied onto a column of GalNAc-Synsorb (0.5 x 5 cm) equilibrated with TBS, at 4 °C. A typical elution profile is shown in B. The gel was washed with TBS, at 4 °C, until the radioactivity (—-—) of the effluent was <10^4 cpm. The column was then transferred to room temperature, and the lectin was specifically eluted with TBS containing 0.1 M GalNAc. All the fractions eluted were monitored for their hemagglutinating activity (○) either directly or after exhaustive dialysis against TBS.

Binding of S. sclarea Lectin to Immobilized Oligosaccharides—Another approach was used to further investigate the specificity of the lectin. It consisted of binding experiments of the radiolabeled lectin to well-defined oligosaccharide

Fig. 2. SDS-gel electrophoresis and isoelectrofocusing of the purified S. sclarea lectin. Samples containing about 1 µg of lectin (lanes a and d) were submitted to SDS-gel electrophoresis. Coomassie Blue-stained proteins are compared with standards (lanes b and c) of known molecular weight. Lanes a and b, reduced samples. Lanes c and d, nonreduced samples. Molecular weight standards: phosphorylase B (M_r = 92,500); bovine serum albumin (M_r = 66,200); ovalbumin (M_r = 45,000); carbonic anhydrase (M_r = 31,000); and soybean trypsin inhibitor (M_r = 21,500). A sample containing about 10 µg of lectin was submitted to isoelectrofocusing (lane e). The migration of the Coomassie Blue-stained band is compared with those of standards of known pI, indicated by the arrows. Isoelectrofocusing markers: lysozyme (pI = 10); horse heart myoglobin (pI = 6.7–7.1); and human carbonic anhydrase (pI = 6.5).

Table II
Amino acid and carbohydrate composition of S. sclarea lectin

The purified lectin was analyzed as described under "Experimental Procedures." The composition of the lectin was calculated assuming a M_r = 50,000.

<table>
<thead>
<tr>
<th>Residues/molecule</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO_3H</td>
<td>7.5^a</td>
</tr>
<tr>
<td>Asx</td>
<td>36.8</td>
</tr>
<tr>
<td>MeSO_3</td>
<td>2.0^b</td>
</tr>
<tr>
<td>Thr</td>
<td>35.4^b</td>
</tr>
<tr>
<td>Ser</td>
<td>34.4^b</td>
</tr>
<tr>
<td>Glx</td>
<td>22.0</td>
</tr>
<tr>
<td>Pro</td>
<td>14.1</td>
</tr>
<tr>
<td>Gly</td>
<td>39.6</td>
</tr>
<tr>
<td>Ala</td>
<td>34.4</td>
</tr>
<tr>
<td>Val</td>
<td>31.3</td>
</tr>
<tr>
<td>Ile</td>
<td>18.9</td>
</tr>
<tr>
<td>Leu</td>
<td>20.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>13.8^b</td>
</tr>
<tr>
<td>Phe</td>
<td>31.7</td>
</tr>
<tr>
<td>His</td>
<td>10.3</td>
</tr>
<tr>
<td>Lys</td>
<td>8.6</td>
</tr>
<tr>
<td>Arg</td>
<td>12.7</td>
</tr>
<tr>
<td>Trp</td>
<td>N.D.*</td>
</tr>
<tr>
<td>Man</td>
<td>14.4</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>8.4</td>
</tr>
<tr>
<td>Xyl</td>
<td>8.2</td>
</tr>
<tr>
<td>Fuc</td>
<td>6.6</td>
</tr>
<tr>
<td>Gal</td>
<td>1.4</td>
</tr>
<tr>
<td>Glc</td>
<td>1.2</td>
</tr>
<tr>
<td>Ara</td>
<td>1.8</td>
</tr>
</tbody>
</table>

^a Determined after performic oxidation.
^b Determined by extrapolation to zero time hydrolysis.
*N.D., not determined.
Salvia sclarea Lectin

TABLE III
Agglutination of human erythrocytes by S. sclarea lectin

<table>
<thead>
<tr>
<th>Human red cells</th>
<th>Minimum amount of lectin (μg/ml) for complete agglutination of red cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
</tr>
<tr>
<td>A</td>
<td>27</td>
</tr>
<tr>
<td>A&lt;sub&gt;s&lt;/sub&gt;</td>
<td>110</td>
</tr>
<tr>
<td>O</td>
<td>&gt;435</td>
</tr>
<tr>
<td>B</td>
<td>&gt;435</td>
</tr>
<tr>
<td>Cad (Cad.)</td>
<td>5</td>
</tr>
<tr>
<td>Tn (Ba.)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

FIG. 3. Binding of S. sclarea lectin to Tn (A) and Cad (B) erythrocytes. Binding studies were performed in triplicate. The saturation curves for native (●), desialylated (▼), and papain-treated (▲) erythrocytes are shown in the insets. Nonspecific binding was always <1% and was subtracted from the total binding. The data were analyzed according to the method of Scatchard (25) and represented with open symbols: native (O), desialylated (V), and papain-treated (0) erythrocytes. Binding parameters were calculated using 50,000 for the molecular weight of the lectin.

structures immobilized on an inert matrix. These derivatized Synsors are listed in the legend to Fig. 4. Under the conditions used ("Experimental Procedures"), binding curves were obtained which are given in Fig. 4. They show that the lectin binds preferentially, and to about the same extent to terminal αGalNAc residues, either directly linked to the matrix (as in Tn structure) or linked in 1-3 to a βGalNAc or to a βGal. In contrast, the lectin binds weakly to αGalNAc determinants

TABLE IV
Inhibition of hemagglutination

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration*</th>
<th>Relative inhibitory potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono- and oligosaccharides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GalNAc</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>β-NO₂Ph-α-bGalNAc</td>
<td>0.12</td>
<td>0.8</td>
</tr>
<tr>
<td>β-NO₂Ph-β-bGalNAc</td>
<td>0.06</td>
<td>1.7</td>
</tr>
<tr>
<td>GalNH₂ HCl</td>
<td>5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Gal</td>
<td>75</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GalNAcβ1-4Galβ1-3GalNAc</td>
<td>0.13</td>
<td>0.8</td>
</tr>
<tr>
<td>GalNAcβ1-3Galα1-4Galβ1-4Glc</td>
<td>1.15</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Glycopeptides and glycoproteins

Ser-Ser-Thr-Thr-Glu<sup>+</sup> 0.02  5
Ser-Ser-Thr-Thr-Gly<sup>+</sup> 0.05  2
Ser-Thr  0.01  10
Thr  0.06  1.7
Ser  0.03  3.3
Native BSM  0.04  2.5
Asialo-BSM  0.02  50

* Minimal concentration required for inhibition of agglutination of Tn (Ba) erythrocytes.

▼ = αGalNAc residue.

Expressed as the concentration of free terminal GalNAc.

FIG. 4. Binding of S. sclarea lectin to immobilized sugar structures. Binding studies were performed in triplicate. The symbols related to each structure tested are: ○, GalNAcβ1-3GalNAcβ1-R (Forssman); ▲, GalNAcβ1-3Galβ1-R; ▼, GalNAcβ1-R; △, GalNAcβ1-4Galβ1-R; ◯, GalNAcβ1-4Galβ1-R (asialo-Cad); □, GalNAcβ1-3 (Fucα1-2) Galβ1-R (T); ▽, Galβ1-4GlcNAcβ1-R (LacNAc); ▼, Galβ1-3Galα1-4Galα1-R (T); ◯, GalNAcβ1-R in presence of 100 mM GalNAc. R = Synsorb. The data have been corrected for the hapten content of each type of Synsorb.
linked in 1-4 to an \( \alpha \text{Gal} \), as well as to \( \beta \text{GalNAc} \) residues linked in 1-4 to a \( \text{aGal} \) (as in asialo-Cad structures). Blood group A structure is bound even more weakly although the same structure without substitution of the subterminal Gal by a fucose is an effective ligand. The lectin does not recognize either terminal Gal residues (as in T and LacNAc structures), or GalNAc residues in internal position (as in the T structure).

**DISCUSSION**

Extracts from *S. sclarea* seeds contain a lectin specific for GalNAc residues. Using ethanol precipitation, anion-exchange, and affinity chromatography on immobilized \( \alpha \text{GalNAc} \), it was purified about 90-fold to apparent homogeneity. From 100 g of seeds, 5 mg of pure lectin can be obtained which corresponds to a yield of 25%. The purified lectin gives only one band on SDS-PAGE and isoelectrofocusing shows one major protein (pI = 8.8) and one minor band of slightly lower pI (8.0).

The lectin is a glycoprotein containing 15% of carbohydrates. It sugar composition with Man, GlcNAc, Xyl, and Fuc in a molar ratio 3:2:2:1 greatly resembles those of other plant lectins as Tora bean lectin (26), *Vica graminea* lectin (27), *Erythrina cristagalli* lectin (28), *Griffonia simplicifolia* lectin (29), and also the B1 lectin from *Vicia villosa* (30). Its amino acid composition shows a high content of acidic and hydroxyamino acids, which is also common to many lectins. *S. sclarea* agglutinin gives upon SDS-PAGE a \( M_r = 50,000 \). The action of a reducing agent dissociates the molecule in two identical subunits linked by disulfide bonds. The discrepancies in \( M_r \) observed between gel filtration and SDS-PAGE experiments can be attributed to differences in the hydrodynamic volume of the protein, either in the absence (gel filtration) or in the presence (electrophoresis) of SDS, and to the glycoprotein nature of the lectin. Sedimentation equilibrium analysis of this glycoprotein will probably allow a determination of its molecular weight with higher accuracy.

One of the most interesting features of this lectin was that it had been described as Tn specific (12). Indeed, our first hemagglutination assays were in good agreement with this finding: the purified lectin agglutinated native Tn red cells at a minimum concentration of 0.3 \( \mu \text{g/ml} \). However, native Cad red cells were also agglutinated, but at a minimum concentration of 5.0 \( \mu \text{g/ml} \). Red cells with other blood group specificities were agglutinated only at much higher concentrations (>27.0 \( \mu \text{g/ml} \)).

Tn and Cad erythrocytes are well known to carry specific exposed GalNAc residues. Tn red cells present GalNAc-Ser/Thr structures on their membrane glycoproteins (7). Cad erythrocytes show a specific structure:

\[
\text{GalNAc}\beta_1 \rightarrow 3 \text{Gal}\beta_1 \rightarrow 3 \text{GalNAc}\alpha_2 \rightarrow 6 \text{GalNAc}\alpha_2 \rightarrow \text{Ser/Thr}
\]

On their glycoporphins (31). Moreover, Cad cells present among their glycolipids, a major specific ganglioside (32):

\[
\text{GalNAc}\beta_1 \rightarrow 3 \text{Gal}\beta_1 \rightarrow 4 \text{GlcNAc}\alpha_2 \rightarrow 1-3 \text{Gal}\beta_1 \rightarrow 4 \text{Glc}\beta_1 - \text{ceramide}
\]

The most striking observation in our hemagglutination experiments was that after papain treatment of the erythrocytes, under conditions which destroy the major membrane sialoglycoproteins, the reactivity of Tn cells with the lectin was abolished, whereas Cad cells, after the same treatment, were on the contrary much better agglutinated. On the other hand, none of the papain-treated A, B, or O red cells were recognized by the lectin. This indicates that *S. sclarea* lectin specifically binds \( \alpha \text{GalNAc} \) determinants directly linked to Ser and Thr as in Tn structures. It can also bind to \( \beta \text{GalNAc} \) determinants linked in 1-4 to Gal as in Cad structures, but the lectin does not recognize \( \beta \text{GalNAc} \) determinants linked in 1-3 to Gal as in the P structures accessible at the surface of all papain-treated red cells.

Binding experiments with radiolabeled lectin on Tn and Cad red cells gave results fully consistent with those obtained from the hemagglutination data. Furthermore, the number of accessible binding sites calculated from our studies (1-2 x 10^6 sites per cell) is in rather good agreement with the number of Tn (33) and Cad (34) potential sites present on glycoporphin (12-15 x 10^6 sites/cell). Fewer sites are present among Cad glycolipids, since about 70% of sialosylparagloboside is transformed to Cad ganglioside (32); however, also about 10^6 sites per cell are accessible to the lectin.

Hemagglutination inhibition assays confirmed that the lectin recognizes much better terminal \( \beta \text{GalNAc} \) determinants linked in 1-4 than those linked in 1-3. Furthermore, all the substrates carrying \( \alpha \text{GalNAc} \) residues linked to Ser/Thr were more potent inhibitors than the other terminal GalNAc-containing structures tested. Thus, it may be concluded that the lectin is most specific for GalNAc-Ser/Thr structures. The strong agglutination inhibition shown by asialo-BSM may be explained by the cluster effect due to the high density of terminal \( \alpha \text{GalNAc} \) on the molecule. This can be compared to the high affinity of the lectin for desialylated Tn erythrocytes. However, it is not known to what extent the GalNAc-Ser/Thr structures present on the glycoporphins are sialylated. Desialylation of Tn erythrocytes did not only lead to a higher affinity of the lectin for these cells, but also to a higher number of accessible sites. This increased accessibility may be explained by an effect of charge due to an overall desialylation, as well as by a specific desialylation of the GalNAc residues possibly present on the Tn membrane glycoproteins. On the other hand, Cad red cells are well known to be sialidase-resistant (34). In good agreement with these findings, we observed that the agglutination score of the lectin was only slightly better after neuraminidase treatment of the Cad erythrocytes.

The sugar combining site of the lectin was further investigated by binding experiments to immobilized oligosaccharides. The data once again show that the lectin specifically recognizes terminal \( \alpha \text{GalNAc} \) residues and to a lesser extent, terminal \( \beta \text{GalNAc} \) residues. However, the linkage of the GalNAc to the subterminal sugar is important for the recognition: when the \( \alpha \text{GalNAc} \) is linked in 1-4 to Gal, the
binding of the lectin is diminished. On the other hand, hemagglutination and inhibition of hemagglutination experiments had already shown that when the terminal βGalNAc is linked in 1–3 to a Gal, the recognition is almost abolished. Important also is the substitution of the subterminal sugar, since the A structure, where the subterminal Gal residue is substituted by a fucose, is no longer recognized. Again, the same conclusion could be obtained by the hemagglutination experiments, since the lectin does not agglutinate A red cells. However, the presence of NeuAc linked in α2–3 to the subterminal Gal of Cad structures does not prevent the lectin binding.

All the data presented in this paper show that this newly purified lectin from S. sclarea seeds interacts preferentially with terminal αGalNAc residues, either directly linked to Ser or Thr, or linked in 1–3 to a βGalNAc or to a βGal. Other GalNAc specific lectins as Helix pomatia agglutinin, soybean agglutinin, Dolichos biflorus agglutinin, or lima bean agglutinin, reviewed recently by Sharon (37) are often used as tools in immunology, but do not show the same narrow specificity for αGalNAc-Ser/Thr residues, since, for instance, most of them recognize also blood group A structures. However, the B lectin isolated from Vicia villosa seeds has strong homologies in its binding specificity with the S. sclarea lectin. It was also found to recognize native Tn and Cad cells which present the same number of sites for this lectin (35, 36). Vicia villosa lectin was also described as highly specific for αGalNAc structures either linked to Ser and Thr (35) or linked in 1–3 to a Gal (57). However, the B, V. villosa lectin, as well as soybean agglutinin, do not recognize A structures. But are able to agglutinate normal O red blood cells after proteolytic treatment (38, 39). On the other hand, terminal αGalNAc linked in 1–3 to βGalNAc or to unsubstituted βGal, are not normally encountered in human tissues. Therefore, we think that S. sclarea lectin is a new specific tool which can be used as a marker for the initiation of the O-glycosylation process (40) and for the detection of Tn-like structures in cancerous tissues (11).

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