Structural differences between two lectins from *Cytisus scoparius*, both specific for D-galactose and N-acetyl-D-galactosamine

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Three lectin fractions were obtained from seeds of the leguminous plant *Cytisus scoparius* (Scotch broom) by means of affinity chromatography on a N-acetyl-D-galactosamine medium. The first fraction, termed CSIa, was equally well inhibited in haemagglutination experiments by D-galactose and by N-acetyl-D-galactosamine and consisted of a group of isolectins formed from closely related polypeptide chains of approx. M_r 30000. The second fraction, CSIb, was closely related to CSIa in specificity, c.d. and other properties. The third fraction contained a homogeneous lectin, CSII, formed from subunits again of approx. M_r 30000. CSII was 100 times more readily inhibited by N-acetyl-D-galactosamine than by D-galactose. Despite the similarity in specificity, comparative studies of their amino acid composition, c.d. and N-terminal amino acid sequence showed that the CSIa and CSII lectins diverged considerably in structure. The lectin from *Cytisus sessilifolius*, specific for chitobiose, was also examined and resembled CSIa in composition and c.d. properties.

Though lectins occur in the seeds of many leguminous plants, only a few species are known to have more than one lectin. A well-known example is *Ulex europaeus* (Matsumoto & Osawa, 1969) whose seeds contain two anti-(blood group H) haemagglutinins, one specific for L-fucose and another usually described as chitobiose-specific, but actually recognizing a structure on erythrocytes comprised of other sugars (Pereira *et al.*, 1979). The most diverse case is that of *Griffonia* (*Bandeiraea*) *simplicifolia*, where extensive studies by Goldstein and co-workers have shown that the seeds contain four types of lectins, two of which occur in isolectin forms (Iyer *et al.*, 1976; Murphy & Goldstein, 1977; Murphy, 1978; Shibata *et al.*, 1982). So far, relatively little work has been reported on the structural relationship between multiple lectins from a single species. It is not known whether they are generally more closely related to each other than to lectins from other species. N-Terminal amino acid sequences have been determined for two lectins of different specificity from *Vicia cracca* (Baumann *et al.*, 1979), and these were not closely related.

An interesting case of multiple lectins is that of *Cytisus scoparius* (Scotch broom), which was reported in an abstract several years ago (Brossmer *et al.*, 1975) to contain two lectins. Both bound N-acetyl-D-galactosamine and D-galactose, but had

quite different relative affinities for the two ligands. We further investigated these two lectins to determine if they were distinct molecular species or isolectin forms analogous to those of the GSI lectin from *G. simplicifolia* (Murphy & Goldstein, 1977), which they resemble in specificity. In the work reported here, it is shown that the two lectins are distinct proteins, one of which has several isolectin forms. The lectins show much greater divergence in primary and secondary structure than their similarity in specificity would suggest. The *C. scoparius* lectins were also compared with a lectin from *C. sessilifolius* (Matsumoto & Osawa, 1969), which resembles the 'chitobiose-specific' *U. europaeus* lectin II in specificity.

Materials and methods

Purification of lectins

Milled seeds of *C. scoparius* (25 g; obtained from P-L Biochemicals) were extracted with 250 ml of phosphate-buffered saline (0.1 M-NaCl/0.01 M-phosphate, pH 7.3) for 4 h at 4°C. The extract was centrifuged and the supernatant was fractionated with $(\text{NH}_4)_2\text{SO}_4$ to obtain the material precipitating between 33% and 66% saturation. After dialysis against the above buffer, the extract was further fractionated by affinity chromatography. The column was prepared by coupling 50 mg of *p*-

aminophenyl 2-acetamido-2-deoxy- α -D-galactopyranoside (Calbiochem-Behring Corp.) to 12 ml of Affi-Gel 10 (Bio-Rad Laboratories Canada Ltd.), which is an *N*-hydroxysuccinimide-activated agarose gel of the type described by Cuatrecasas & Parikh (1972). The coupling was carried out in 0.1 M-NaHCO₃ at 4°C overnight. The dialysed extract was applied to a column (0.9 cm $\times$ 10 cm) of the affinity medium, which was then washed with the buffer. The lectins were obtained by elution of the column sequentially with (a) 10 mM-D-galactose, (b) 200 mM-D-galactose and (c) 50 mM-*N*-acetyl-D-galactosamine, each in the above buffer. The eluted lectin fractions are termed CS Ia, CS Ib and CS II respectively.

The *C. sessilifolius* lectin was obtained from E-Y Laboratories, San Mateo, CA, U.S.A. (lot no. 0613B).

Characterization of the lectins

Electrophoresis in polyacrylamide slab gels in the presence of sodium dodecyl sulphate was carried out by the method of Laemmli (1970). The standard proteins used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, β -lactoglobulin and lysozyme. Haemagglutination tests were performed with microtitre equipment (Cooke Engineering Co., Alexandria, VA, U.S.A.) and 2% suspensions of sheep erythrocytes which had been treated with *Vibrio cholerae* neuraminidase (EC 3.2.1.18; Calbiochem-Behring Corp.) by incubating 20 ml of a 2% suspension with 1 EC unit of enzyme at 37°C for 2 h. Amino acid compositions of duplicate lectin samples, hydrolysed at 110°C in 6 M-HCl for 22 h, were determined on a Durrum D500 analyser. Approximate M_r values were obtained by size-exclusion high-performance liquid chromatography on a TSK 3000SW column (0.75 cm $\times$ 60 cm) run at 0.5 ml/min in 0.2 M-phosphate buffer, pH 7.0, and by gel filtration on a column (1.6 cm $\times$ 85 cm) of Sephadex G-200, run in phosphate-buffered saline, each column being calibrated with suitable standard proteins.

The c.d. spectra of the lectins were measured on a Cary model 61 instrument. The protein samples were dialysed into 0.1 M-NaCl/0.01 M-acetate buffer, pH 5.4. The results are presented as mean residue ellipticities $[\theta]_{m.r.w.}$ in degrees $\cdot$ cm² $\cdot$ dmol⁻¹, calculated on the basis of a mean residue weight of 110. The absorption coefficients, $A_{1\%}^{1\text{cm}}$, used were 1.37 for CS Ia and CS Ib, 1.22 for CS II, and 1.38 for *C. sessilifolius* lectin.

N-Terminal sequence determination

Purified lectin samples containing 50–100 nmol of material were dissolved in 400 μ l of distilled water and transferred to the reaction cup of

Beckman 890C sequencer. The sample was dried as a film on the wall of the spinning reaction cup by using a Beckman sample application subroutine program (no. 101081). Sequential Edman degradation was performed by using a 1.0 M-Quadrol program (Beckman program no. 345562). The 2-anilinothiazolin-5-one derivatives of the amino acids were converted into corresponding phenylthiohydantoin derivatives by treatment with 20% trifluoroacetic acid at 65°C for 20 min. These derivatives were identified by reverse-phase high-performance liquid chromatography on a Varian Vista 54 chromatograph using an Altex PTH-Ultra-sphere ODS column (4.1 mm $\times$ 250 mm) maintained at 45°C in 15 mM-sodium acetate, pH 4.75, with gradient elution with acetonitrile. Confirmation of identity was achieved by back-hydrolysis of the derivatives in 6 M-HCl containing 0.1% SnCl₂ at 130°C for 20 h (Mendez & Lai, 1975), followed by amino acid analysis on a Durrum D-500 analyser. The amino acid placed at any given position in the sequence was not necessarily that present in the greatest amount, but was taken to be that amino acid which was shown to be increasing over the amount present in the previous step. It was also dependent on all other amino acids decreasing over the amount present in the previous step.

Results

Affinity chromatography of the *C. scoparius* extract on a *N*-acetyl-D-galactosamine column, using sequential elution with different sugar solutions, gave three lectin fractions (Fig. 1), which were termed CS Ia, CS Ib and CS II in view of their properties (described below). The purification is summarized in Table 1. The average yields of the lectins from four preparations were approx. 360 mg/kg of seed for CS Ia, 90 mg/kg for CS Ib and 430 mg/kg for CS II. In haemagglutination tests with neuraminidase-treated sheep erythrocytes, the total recovery of activity in the three lectin fractions (Table 1) corresponded to the initial activity of the extract, within the limitations of the haemagglutination assay. The specific activities of the lectins with the sheep erythrocytes were below those reported previously with neuraminidase-treated human erythrocytes (Brossmer *et al.*, 1975). One haemagglutination unit (i.e. the last dilution giving a 2+ agglutination) for CS Ia was approx. 60 μ g/ml, for CS Ib, 30 μ g/ml, and for CS II, 16 μ g/ml. In haemagglutination-inhibition experiments, D-galactose and *N*-acetyl-D-galactosamine were equally effective inhibitors of CS Ia, and *N*-acetyl-D-galactosamine was 128-fold better an inhibitor of CS II than was D-galactose. These values are similar to those reported by Brossmer *et al.* (1975). For CS Ib, *N*-acetyl-D-galactosamine

Table 1. Purification of *C. scoparius* lectins

	Volume (ml)	Total absorbance ($A_{280} \times \text{vol.}$)	Reciprocal haemagglutination titre	Total haemagglutination titre	Specific activity (haemagglutination units/ 1000 A_{280} units)
Extract	160	11 776	4	640	54
(NH_4) ₂ SO ₄ fraction	56	858	8	464	1850
CSIa lectin	15	9.2	8	120	13 000
CSIb lectin	2.5	1.7	16	40	23 500
CSII lectin	20	10.8	32	640	59 300

Fig. 1. Affinity chromatography of *C. scoparius* extract. After application of the sample and a wash with phosphate-buffered saline, the column was eluted with 10mM-D-galactose (at A), 200mM-D-galactose (at B) and 50mM-N-acetyl-D-galactosamine (at C), each in phosphate-buffered saline.

was 8-fold better an inhibitor than was D-galactose. These haemagglutination results are in accordance with the elution behaviour of the lectins on affinity chromatography.

Chemical properties

Polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate (Laemmli, 1970) showed that CSIa and CSIb each contained

Table 2. Amino acid compositions of *C. scoparius* and *C. sessilifolius* lectins

The values given are means of duplicate determinations, with 22h hydrolysis time. Tryptophan was determined spectrophotometrically (Edelhoch, 1967). Cysteine was not detected; a trace of methionine was found in CSIa only.

Amino acid	Composition (residues/30000 M_r)			
	<i>C. scoparius</i>			<i>C. sessilifolius</i>
	CSIa	CSIb	CSII	
Asx	35.5	34.3	33.4	37.7
Thr	19.7	19.2	25.3	24.5
Ser	25.6	26.6	26.6	27.1
Glx	16.6	17.3	20.7	17.1
Pro	15.3	13.8	14.5	9.4
Gly	16.2	16.6	17.0	18.6
Ala	26.7	25.5	23.6	21.5
Val	22.7	23.1	17.2	22.2
Ile	16.9	16.7	17.1	15.7
Leu	20.6	20.0	21.5	20.9
Tyr	9.6	9.2	8.5	11.4
Phe	8.7	19.6	16.8	20.1
His	3.5	5.2	3.9	4.3
Lys	13.4	14.1	17.6	12.2
Arg	6.7	7.6	4.5	5.4
Trp	6.1	5.1	6.3	5.5

two major peptide species, and CSII only one, all three chains having M_r values of about 30000 (Fig. 2). Densitometry of the gels gave the ratio of the two components in CSIa as 3:1, whereas for CSIb the ratio was 1.25:1, there being an increased content of the higher- M_r component. The CSII main band migrated to a distance intermediate between those of the CSI bands and, at high load, slight traces of these two bands could be seen in the CSII pattern. The *C. sessilifolius* lectin also showed two bands.

Size-exclusion liquid chromatography on a TSK 3000SW column gave apparent M_r values of 70000 for CSIa and 75000 for CSII, whereas gel filtration on Sephadex G-200 gave values of 90000 and 75000 respectively.

The similarity of CSIa and CSIb is also evident

Fig. 2. Gel electrophoresis of *C. scoparius* lectins, in the presence of sodium dodecyl sulphate
(a) Gel electrophoresis of CSIb, CSIa and CSII lectins and a standard protein mixture (see the Materials and methods section). The standard protein migrating the same distance as the lectins is carbonic anhydrase (M_r 30000).
(b) Photometric scans of the lectin electrophoretic bands.

Fig. 3. C.d. spectra of *C. scoparius* and *C. sessilifolius* lectins
The spectra are from CSIa (—), CSIb (---), CSII (....) and *C. sessilifolius* lectin (-.-.-) and are presented as mean residue ellipticities, assuming a mean residue weight of 110.

in their amino acid compositions (Table 2), that of CSII again being quite different. The *C. sessilifolius* lectin composition generally resembled the *C. scoparius* lectin compositions.

C.d. spectra

The spectra of the three *C. scoparius* lectins and the *C. sessilifolius* lectin were measured in the

Table 3. *N*-Terminal sequence analysis of CSII

Approx. 50 nmol of protein was analysed. The phenylthiohydantoin (PTH) derivatives were identified by high-performance liquid chromatography; in the back-hydrolysis to amino acids, threonine was identified as α -aminobutyric acid.

Residue no.	Identified amino acid	PTH derivative	Back-hydrolysis	
			Residue	Yield
1	Ser	Ser	Ala	28.2
2	Glu	Glu	Glx	42.1
3	Glu	Glu	Glx	43.8
4	Leu	Leu	Leu	31.9
5	Ser	Ser	Ala	21.6
6	Phe	Phe	Phe	15.7
7	Ser	Ser	Ala	16.8
8	Phe	Phe	Phe	12.9
9	Thr	Thr	Thr	15.1
10	Lys	Lys	Lys	23.2
11	Phe	Phe	Phe	12.4
12	Lys	Lys	Lys	22.5
13	Thr	Thr	Thr	13.8
14	Asp	Asp	Asx	19.2
15	Gln	Gln	Glx	22.7
16	Lys	Lys	Lys	15.1
17	Asn	Asn	Asx	17.4
18	Leu	Leu	Leu	15.8
19	Ile	Ile	Ile	8.3
20	Leu	Leu	Leu	20.4
21	Gln	Gln	Glx	18.6
22	Arg	Arg	Arg	10.6
23	Asp	Asp	Asx	13.5
24	Ala	Ala	Ala	15.8
25	Leu	Leu	Leu	11.5
26	Ile	Ile	Ile	6.3
27	Thr	Thr	Thr	6.7
28	Pro	Pro	Pro	9.3
29	Thr	Thr	Thr	6.8
30	Gly	Gly	Gly	11.6
31	Lys	Lys	Lys	8.5
32	Leu	Leu	Leu	7.8

'aromatic' (250–310 nm) and far-u.v. (195–250 nm) regions (Fig. 3). In the far-u.v. region the spectra are very similar to each other, and analysis by the procedure of Provencher & Glöckner (1981) showed the proteins had 51–61% β -sheet structure, 14–20% β -bend and no α -helix. In the aromatic region, CSIa and CSIb gave similar, but not identical, spectra, which also resembled that of the *C. sessilifolius* lectin. The CSII lectin had a very different spectrum in this region, including a band of negative ellipticity unusual in lectin c.d. spectra. Addition of the ligand *N*-acetyl-D-galactosamine caused an increase in ellipticity in the tyrosine region of the CSII spectrum (Fig. 4), whereas CSIa showed a decrease in its spectrum in both the tyrosine and tryptophan regions. CSIb gave a difference c.d. spectrum intermediate between those of the other two.

Fig. 4. *C.d. difference spectra of C. scoparius lectins*. The difference spectra caused by addition of *N*-acetyl-D-galactosamine are presented as $[\theta]_{\text{m.r.w.}}^{\text{protein + ligand}} - [\theta]_{\text{m.r.w.}}^{\text{protein}}$ for CSIa (—), CSIb (---) and CSII (....).

N-Terminal sequence analysis

To compare further the structures of the *C. scoparius* lectins, the *N*-terminal amino-acid sequences of CSIa and CSII were investigated. The CSII lectin showed a single sequence, but the CSIa lectin gave multiple phenylthiohydantoin derivatives from several sequence positions, particularly in the initial ten residues. The CSII sequence up to residue 32 was established by identification of the phenylthiohydantoin derivatives and of the amino acids given by back-hydrolysis of the derivatives (Table 3). Data for the CSIa sequence are included in the sequence comparison in Fig. 5. A second sample of CSIa was prepared by elution of the affinity column with a linear gradient of D-galactose, and the protein emerged as a single symmetric peak at approx. 2 mM-D-galactose. This sample also gave two bands on gel electrophoresis and the same multiple derivatives in the sequence analysis.

Discussion

The experiments reported here show that *C. scoparius* produces at least three lectin polypeptide chains. One forms the homogeneous protein, CSII, more specific for *N*-acetyl-D-galactosamine, whereas the others form the mixed species present in the CSIa and CSIb fractions. The differences between the latter chains may extend to carbohydrate specificity, on the basis of the small differences in behaviour of CSIa and CSIb in the affinity-chromatography and haemagglutination experiments. CSIa had the same affinity for D-galactose as it had for *N*-acetyl-D-galactosamine. The CSIa lectin is probably the same as the CSA₁ lectin described by Brossmer *et al.* (1975), though

Fig. 5. *N*-Terminal amino acid sequences of lectins

The sequence of CSII is compared in order of homology with those of the two *Phaseolus vulgaris* subunits (PHA-R and PHA-L; Miller *et al.*, 1973); *Glycine max* agglutinin (SBA; Vodkin *et al.*, 1983); the *V. cracca* lectin specific for *N*-acetyl-D-galactosamine (VC-GN; Baumann *et al.*, 1979); *Dolichos biflorus* agglutinin (DBA; Etzler *et al.*, 1977); *G. simplicifolia* lectin II subunits (GSIa and GSIb; Lamb & Goldstein, 1984) and *A. hypogaea* agglutinin (PNA; Foriers *et al.*, 1979). The sequence of the prototypic D-glucose-specific lectin, concanavalin A (CON A), is shown from residue 122 (Cunningham *et al.*, 1975). Boxed residues are identical with the sequence of CSII. The results of the CS Ia sequence analysis are shown separately at the bottom; and at positions where multiple derivatives were found, the residues are listed in order of abundance. The one-letter notation for amino acids is described in *Biochem. J.* (1969) 113, 1-4.

details of its preparation were not reported. Brossmer *et al.* (1975) found that their CSA_{II} lectin was mitogenic towards human lymphocytes treated with neuraminidase, and their CSA_I lectin agglutinated platelets without causing release of serotonin (Patscheke *et al.*, 1977).

The subunits of the lectins had *M_r* values of approx. 30000. The size of the oligomeric species that they form could not be determined accurately by chromatographic methods. The molecular masses obtained suggest trimers, but other lectins known to be tetrameric, such as peanut (*Arachis hypogaea*) agglutinin, also gave molecular masses below 100 kDa by these methods. Since nearly all lectins of the D-galactose and *N*-acetyl-D-galactosamine type are tetrameric, it is probable that the *C. scoparius* lectins are also. Both CS Ia and CSII contain Mn²⁺, Mg²⁺ and Ca²⁺ (Young, 1983).

The c.d. and sequence data suggest the CS Ia and CSII lectins are significantly different in structure, despite their common affinity for *N*-acetyl-D-galactosamine. The c.d. spectra in the aromatic region are obviously dissimilar, and binding of the sugar produced opposite changes in ellipticity. The presence of multiple isomeric chains in CS Ia precludes exact comparison of its sequence with that of CSII, but some differences are evident in the region from residue 13 onwards, where the

heterogeneity was much less. Of the fourteen residues in CS Ia, at least seven differ from those of CSII, and several of these occur in the region of residues 19-24, which usually shows high homology between lectins (Fig. 5).

Amino acid compositions can also be used as a measure of the relationship between proteins. The composition data for CS Ia (Table 2) were compared with the data for the other *Cytisus* lectins and with a number of other lectins prepared in this laboratory and analysed in the same fashion. The composition index used was the *SΔn* index of Cornish-Bowden (1977):

$$S\Delta n = \frac{1}{2} \sum_{i=1} (n_{iA} - n_{iB})^2$$

where *n_{iA}* is the number of residues of the *i*th type in protein A, and *n_{iB}* in protein B, and the correction terms for polypeptide length (Cornish-Bowden, 1979) were used with concanavalin A. The results are shown in Table 4 for those lectins that gave values of *SΔn* below 115, the value for the 'strong' test for homology (Cornish-Bowden, 1979, 1980). Another nine lectins specific for D-galactose or *N*-acetyl-D-galactosamine, including peanut agglutinin and *Phaseolus vulgaris* agglutinin, gave values of *SΔn* above 115. The relationship of CS Ia to CSII by this criterion is not as close as that of

Table 4. Comparison of the amino acid compositions of CSIA and other lectins

$S\Delta n$ values compared with CSIA were calculated as described by Cornish-Bowden (1977, 1979). M_r values were assumed to be 30000, except for concanavalin A (26500; Cunningham *et al.*, 1975). Lectin compositions were obtained after hydrolysis at 110°C for 22h.

Lectin	$S\Delta n$
CSIB	7.1
CSII	61.8
<i>C. sessilifolius</i>	54.9
<i>G. simplicifolia</i> I	40.8
<i>G. max</i>	52.2
Concanavalin A	72.9
<i>Dolichos biflorus</i>	77.2
<i>Caragana arborescens</i>	112.1

CSIA to *C. sessilifolius* or GSI or soya-bean (*Glycine max*) agglutinin, but clearly is closer than that of many other lectins. By the various criteria of c.d. and amino acid sequence and composition, CSIA and CSII have clearly diverged considerably, possibly from before the emergence of *C. scoparius* as a distinct species. The same may also be true for the two lectins from *V. cracca* (Baumann *et al.*, 1979), which differ greatly in specificity and structural type.

The similarity of the *C. sessilifolius* lectin to CSIA is interesting, particularly since this lectin, being classified as chitobiose-specific, is not obviously related to the *N*-acetyl-D-galactosamine class of lectins. The c.d. and amino acid composition data show that it should be considered a member of this class. The *C. sessilifolius* lectin resembles the *Ulex* II agglutinin in specificity, both being termed *Cytisus*-type lectins by Matsumoto & Osawa (1970), and the two organisms are both classified botanically in the same tribe, the Genisteae. It has been shown that the *Ulex* II lectin recognizes a complex carbohydrate determinant that does contain *N*-acetyl-D-galactosamine (Pereira *et al.*, 1979).

It is probable that more species will be shown to have multiple lectins, since the erythrocytes commonly used to detect lectins by agglutination may lack constituents such as uronic acids that frequently occur in rhizobial polysaccharides. It is worth considering how the occurrence of multiple lectins may accord with a current theory for the biological role of lectins in leguminous plants, namely that they aid in the establishment of symbiosis between the plant and nitrogen-fixing bacteria (Bauer, 1981; Dazzo & Truchet, 1983). Dazzo & Hubbell (1975) proposed, on the basis of studies of clover (*Trifolium*) root interaction with

rhizobia, that lectins form cross-links between root hairs and bacteria by recognizing carbohydrate determinants common to both. The occurrence of multiple lectins and of isolectins arising from heterologous associations of subunits of slightly different specificities might be of value to a plant by permitting some variation in carbohydrate structure between the two organisms to occur without loss of the ability to cross-link them. Thus the presence in *C. scoparius* and other plant seeds of isolectins and structurally distinct lectins is not incompatible with the symbiosis theory, but does not, of course, provide proof of its validity.

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