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Purification and characterization of *Robinia pseudoacacia* seed lectins

A re-investigation

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Two lectins, RPA 1 and RPA 3, were purified from *Robinia pseudoacacia* seeds. These two lectins differ in their physicochemical and biological properties. By analytical ultracentrifugation the M_r values of RPA 1 and RPA 3 were estimated to be 59000 and 105000 respectively. From SDS/polyacrylamide-gel-electrophoresis data it was estimated that RPA 1 consisted of two subunits of M_r 34000, and RPA 3 of two types of subunits (M_r 30500 and 29000). RPA 1 and RPA 3 were found to be glycoproteins of comparable amino acid composition. RPA 1 was the more highly glycosylated molecule (11.6% versus 4.3%). The carbohydrate-specificity of RPA 1 appears to be complex. RPA 3 was inhibited by *N*-acetyl-D-galactosamine and human α-glycoproteins. Both lectins exerted a mitogenic effect on human peripheral-blood lymphocytes. Concentrations between 0.5 and 1 μg of RPA 3/ml gave optimal proliferative responses, whereas for RPA 1 concentrations higher than 10 μg/ml were needed for these responses.

INTRODUCTION

Various procedures have been used to purify black-locust (*Robinia pseudoacacia*) lectin (Bourrillon & Font, 1968; Horějši *et al.*, 1978; McPherson & Hoover, 1979). In all cases mitogenic preparations were obtained. Mitogenicity was observed on human peripheral-blood (Sharif & Bourrillon, 1975), rabbit lymph-node (Hořejši *et al.*, 1978) and mouse spleen lymphocytes (McPherson & Hoover, 1979). Furthermore, *Robinia pseudoacacia* lectin has been described as a potent human T-cell mitogen (Sharif *et al.*, 1977).

Data concerning the saccharide-binding site of the *Robinia pseudoacacia* lectin are limited. Leseney *et al.* (1972) showed that this lectin could be inhibited by a variety of glycopeptides with galactose residues in the external branches and mannose residues in the core, but monosaccharides appeared to be devoid of inhibitory activity.

However, the diversity of the techniques used as well as the fragmentary characterization of these different lectin preparations make their biochemical comparison difficult. By ion-exchange chromatography Bourrillon & Font (1968) purified a slightly acidic (pI 5.9) 4.4 S protein, containing 17% carbohydrates. By specific adsorption on formaldehyde-fixed human erythrocytes Hořejši *et al.* (1978) isolated from black-locust bark a lectin of M_r 110000 composed of two types of subunits (M_r 29000 and 31500) containing about 8% carbohydrates. McPherson & Hoover (1979) used affinity chromatography on desialylated fetuin–Sepharose and reported the existence of two non-identical polypeptide chains of M_r 30000 and 31000.

From these data the status of the *Robinia pseudoacacia* lectin seemed rather unclear. We have undertaken the purification of *Robinia pseudoacacia* seed lectin by the usual chromatographic techniques. Close examination of the haemagglutinins thus obtained reveals the existence of at least two lectins. Their subunit composition, M_r values and biological properties are characterized and compared with those of the lectins previously described.

MATERIALS AND METHODS

Purification of *Robinia pseudoacacia* lectin

Robinia pseudoacacia seeds originating from Romania were purchased from Vilmorin Andrieux (Paris, France). Seed extraction and $(NH_4)_2SO_4$ precipitation were performed as described by Bourrillon & Font (1968). Briefly, seeds were ground and soluble proteins were extracted with 20 mM-sodium phosphate buffer, pH 6.8, containing 150 mM-NaCl at 4 °C. The insoluble material was pelleted by centrifugation and subjected to a second extraction under the same conditions. The supernatants were fractionated by two successive $(NH_4)_2SO_4$ precipitations at 30–80% and 30–70% saturation respectively. This 'crude lectin fraction' was dialysed against 10 mM-Tris/phosphate buffer, pH 8.2, and further purified by chromatographic steps on DE-52 DEAE-cellulose (microgranular preswollen anion-exchanger; Whatman, Maidstone, Kent, U.K.), Ultrogel hydroxyapatite (IBF, Villeneuve la Garenne, France) and Sephacryl S-200 (Pharmacia, Uppsala, Sweden) at 4 °C. The DE-52 DEAE-cellulose column (4.1 cm × 40 cm) was equilibrated in 10 mM-Tris/phosphate buffer, pH 8.2. Fractions (12 ml) were collected at a flow rate of 100 ml/h. The Sephacryl S-200 column (2.5 cm × 90 cm) was equilibrated in 20 mM-sodium phosphate buffer, pH 6.8, containing 150 mM-NaCl. Fractions (6 ml) were collected at a flow rate of 50 ml/h. Details of the chromatographic separation on Ultrogel hydroxyapatite are given in the legend to Fig. 1.

Fractions were analysed for absorbance and agglutination on human and sheep erythrocytes.

Abbreviation used: RPA, *Robinia pseudoacacia* lectin.

Gel electrophoresis

Disc-gel electrophoresis was performed at pH 4.3 as described by Reisfeld *et al.* (1962) and at pH 10.2 as described by Rodbard & Chrambach (1971).

SDS/polyacrylamide-gel electrophoresis was performed by the procedure of Laemmli (1970) on a 0.75 mm slab gel containing 12.5% acrylamide and 0.27% bisacrylamide with Bromophenol Blue as the tracking dye. M_r standards were obtained from Pharmacia, and included phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and soya-bean trypsin inhibitor (M_r 20100).

Gels were stained with Coomassie Brilliant Blue G-250 as described by Blakesley & Boezi (1977).

Isoelectric focusing

Isoelectric focusing was performed according to the technique of Doerr & Chrambach (1971) with a 2% Ampholine pH 5–8 gradient (LKB, Stockholm, Sweden) at 4 °C for 16 h at 200 V.

Analytical ultracentrifugation

Analytical ultracentrifugation was carried out with a Beckman model E centrifuge equipped with Schlieren and interference optics. Sedimentation-velocity experiments were performed at 59780 rev./min at 20 °C, and the concentrations of haemagglutinins were between 1.01 and 3.33 mg/ml in 20 mM-sodium phosphate buffer, pH 7.2. Equilibrium experiments were performed in presence of 20 mM-sodium phosphate buffer, pH 7.2, containing 150 mM-NaCl. The temperature was 20 °C and the rotor speed was 23150 rev./min for RPA 1 (loading concentration 0.44 mg/ml) and 16160 rev./min for RPA 3 (loading concentration 0.43 mg/ml). Equilibrium measurements were made until the data did not change with time (72 h). Data were plotted as $\log c$ versus r^2 and M_r values calculated according to the equation:

$$M_r = 2.303[\mathrm{d}(\log c)/\mathrm{d}r^2] \times 2RT/(1-\bar{v}\rho)\omega^2$$

Partial specific volumes, $\bar{v}$, of 0.705 (RPA 1) and 0.708 (RPA 3) were calculated from the amino acid and carbohydrate compositions of the haemagglutinins in accordance with the procedures of Cohn & Edsall (1943) and Gibbons (1971) respectively.

Chemical analyses

The amino acid composition was determined after hydrolysis of the samples in 6 M-HCl under vacuum at 110 °C for 20 h. Analyses were performed by ion-exchange chromatography on a JEOL JLC 6AH amino acid analyser.

Carbohydrate composition was determined as described by Chambers & Clamp (1971) by g.l.c. on a Hewlett–Packard model 5710 A chromatograph.

Immunochemical methods

The anti-(*Robinia* lectin) serum raised in rabbits against a crude lectin extract was kindly provided by Dr. J. Font. Immunoelectrophoresis was performed as described by Hirschfeld (1960). Immunodiffusion was performed according to the method of Ouchterlony (1949).

Biological activities

Haemagglutination assays were carried out in Cooke microplates as previously described (Turpin *et al.*, 1984) with a 4% suspension of human erythrocytes (C.N.T.S., Orsay, France) or sheep erythrocytes (Flow Laboratories, Irvine, Ayrshire, U.K.).

Sialidase-treated erythrocytes were obtained by incubating a 1 ml erythrocyte pellet with 0.5 unit of *Vibrio cholerae* neuraminidase (Behring, Marburg, West Germany) in sodium acetate buffer, pH 5.5, at 37 °C for 50 min. Cells were extensively washed and resuspended at 4% in 0.9% NaCl. Haemagglutination was inhibited by preincubating equal volumes of serial dilutions of inhibitor solutions and four minimal haemagglutinating doses of lectin for 1 h, and then adding the erythrocyte suspension. Results are expressed as the minimal concentrations (mM) required to inhibit one haemagglutinating dose completely. The inhibitors tested were glucosamine, *N*-acetyl-D-glucosamine, galactosamine, *N*-acetyl-D-galactosamine, L-fucose, D-fucose, D-mannose, D-galactose and α_1-acid glycoprotein, all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Mitogenic activity was tested on lymphocytes isolated from human peripheral blood by Ficoll–Hypaque-density-gradient centrifugation (Pharmacia) (Boyum, 1968). Assays were carried out in quadruplicate in Linbro flat-bottomed micro-titre plates. Cells (2×10^5) in 0.2 ml were cultured with increasing concentrations of lectin in RPMI 1640 medium (Flow Laboratories) supplemented with 5% (v/v) heat-inactivated human AB serum and antibiotics (50 units of penicillin/ml and 50 μg of streptomycin sulphate/ml; Gibco, Grand Island, NY, U.S.A.). Cell cultures were incubated at 37 °C in CO_2/air (1:19) and 95% humidity.

Cells were labelled with 1 μCi of [*Me*-^{3}H]thymidine (5 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) for the final 6 h of a 4-day incubation period. Cells were harvested by precipitation on glass-fibre filter paper with a Titertek multiple cell harvester and processed as described by Sharif *et al.* (1980).

RESULTS

Purification of *Robinia pseudoacacia* lectins

The crude lectin extract obtained by $(NH_4)_2SO_4$ precipitation was fractionated on a DE-52 DEAE-cellulose column by stepwise elution with Tris/phosphate buffer of increasing molarities (results not shown). Four peaks were obtained. Peak I, which passed through the column with the equilibration buffer, had no haemagglutinating activity and was discarded. Elution with 50 mM-Tris/phosphate buffer gave one peak (II) with a shoulder (III). A fourth peak, weakly active, was eluted with 50 mM-Tris/phosphate buffer containing 100 mM-NaCl. Peak II, which contained the bulk of the haemagglutinating activity (12 μg/ml for human erythrocytes, 24 μg/ml for sheep erythrocytes), was subsequently studied.

After dialysis and concentration, this material was applied to an Ultrogel hydroxyapatite column and eluted with sodium phosphate buffer. As shown in Fig. 1, three active fractions were obtained. They exhibited decreasing haemagglutinating activities when tested on human erythrocytes. Only fraction 3 was found to agglutinate sheep erythrocytes. These fractions were further purified on a Sephacryl S-200 column (results not shown). Each fraction gave a symmetrical peak, and minor contaminants were eliminated. Elution volumes were identical for fractions 1 and 2, which were slightly retarded compared

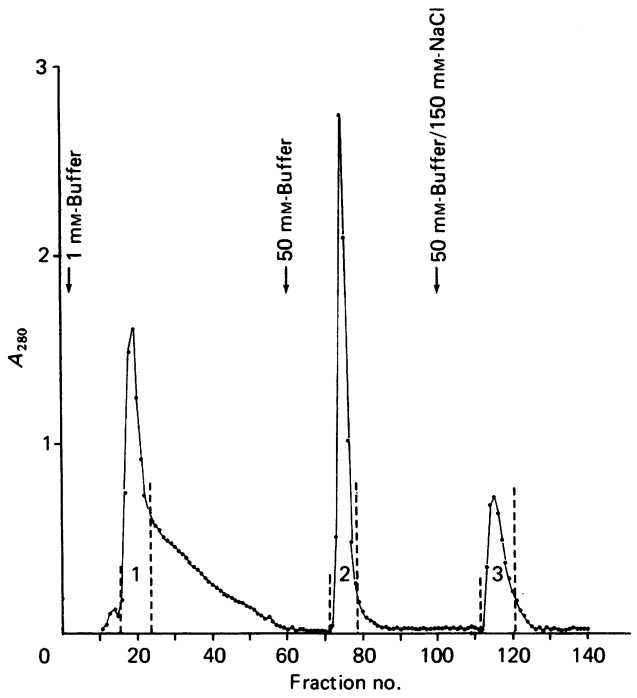

Fig. 1. Ultrogel hydroxyapatite chromatography of *Robinia pseudoacacia* lectins

The haemagglutinating material obtained by ion-exchange chromatography (peak II, DE-52 DEAE-cellulose column chromatography; see the Results section) was concentrated and applied to a hydroxyapatite column (2.5 cm × 23 cm) equilibrated in 1 mM-sodium phosphate buffer, pH 6.8, at 4 °C. Fractions (6 ml) were collected at a flow rate of 50 ml/h. One peak (fraction 1) was eluted with the equilibration buffer. A second peak (fraction 2) was eluted with 50 mM-sodium phosphate buffer, pH 6.8, and another (fraction 3), with 50 mM-sodium phosphate buffer, pH 6.8, containing 150 mM-NaCl. Haemagglutinating titres with human erythrocytes and sheep erythrocytes respectively were: fraction 1, 3.6 and 0 μg/ml; fraction 2, 8.6 and 0 μg/ml; fraction 3, 21.0 and 5.3 μg/ml.

with fraction 3. These three fractions are referred to below as RPA 1, RPA 2 and RPA 3.

RPA 1 and RPA 2 were recovered in smaller quantities than RPA 3 (60 mg/kg of seeds versus 300 mg/kg of seeds).

The homogeneity of these lectins could be deduced from the following criteria. One symmetrical peak was observed by chromatography on Sephacryl S-200. The lectins sedimented as a single peak through the sedimentation-velocity runs. Disc-gel electrophoresis at pH 4.3 and 10.2 yielded one band (Fig. 2*a*). Minor components in RPA 1 observed upon electrophoresis at pH 10.2 represented less than 5% of the material applied to the gel.

Immunoelectrophoresis (Fig. 3*a*) showed that RPA 1 and RPA 2 had the same migration pattern, whereas RPA 3 exhibited increased cathodic migration. Immunodiffusion revealed cross-reactivity between RPA 1, RPA 2 and RPA 3. In addition, RPA 3 gave a diffuse precipitation line, which was also observed when the lectin was tested against human serum (Fig. 3*b*, wells 3 and 4). This additional precipitation line could be attributed to a non-immune interaction between RPA 3 and human or rabbit serum glycoproteins. Migration of the human glycoproteins was in the α_1- and α_2-globulin zones. Among them, α_1-acid glycoprotein, transferrin and haptoglobin were tested and formed precipitation lines with RPA 3.

From these analytical data, RPA 1 and RPA 2 seemed quite comparable with each other, whereas RPA 3 appeared to be different. Furthermore, RPA 1 and RPA 2 exhibited the same erythrocyte-haemagglutinating specificities. Consequently, comparative studies focused on RPA 1 and RPA 3.

Physicochemical characterization of RPA 1 and RPA 3

The sedimentation coefficients of *Robinia pseudoacacia* lectins showed only slight dependence on the protein concentration between 1.01 and 3.33 mg/ml at 20 °C. $s^0_{20,w}$ values of 4.22 and 6.47 S were found for RPA 1 and RPA 3 respectively.

Sedimentation-equilibrium analyses of the lectins gave linear plots of log$c$ versus r^2 (results not shown), and no evidence of association–dissociation equilibria could be observed for either RPA 1 or RPA 3. The M_r values were 59000 ± 4000 for RPA 1 and 105000 ± 6000 for RPA 3. From these values, frictional ratios (f/f_0) of 1.41 and 1.33 were calculated for RPA 1 and RPA 3, indicating that *Robinia pseudoacacia* lectins are rather extended molecules.

The subunit M_r was estimated by SDS/polyacrylamide-gel electrophoresis (Fig. 2*b*). The same electrophoretic patterns were obtained in the presence and in the absence of 2-mercaptoethanol, suggesting the absence of inter-chain disulphide bonds. One major band of M_r 34000 and a faint band of M_r 32000 were found for RPA 1, RPA 3 was constituted of two polypeptide chains of M_r 30500 and 29000. It could be deduced from their staining intensities that these two polypeptides were present in approximately equal ratios.

Isoelectric focusing with an Ampholine pH 5–8 gradient revealed one major band of pI 5.9 and minor ones of pI 5.5 and 5.3 for RPA 1. RPA 3 gave two main bands of pI 8.3 and 6.9 and an intermediate one of pI 7.5 (Fig. 2*c*).

Chemical composition

The chemical compositions of RPA 1 and RPA 3 are reported in Table 1. Very little difference was observed between the amino acid compositions of the two lectins. The major disparity was in the proline content, which was lower in RPA 3.

The carbohydrate determination by g.l.c. revealed a high mannose content. The different carbohydrates were present in approximately the same proportions for the two lectins, but RPA 1 was more highly glycosylated (11.6% versus 4.3%).

Biological activities

The haemagglutinating activities of the two lectins are presented in Table 2. The minimal concentrations needed to agglutinate human erythrocytes were different for RPA 1 (5 μg/ml) and RPA 3 (20 μg/ml). No blood-group-specificity was observed. Only RPA 3 was shown to agglutinate sheep erythrocytes, at a minimal dose of 8 μg/ml. Sialidase treatment of human O Rh$^-$ and sheep erythrocytes enhanced the haemagglutinating titre of RPA 3. *N*-Acetyl-D-galactosamine inhibited haemagglutination of human erythrocytes by both lectins, and RPA

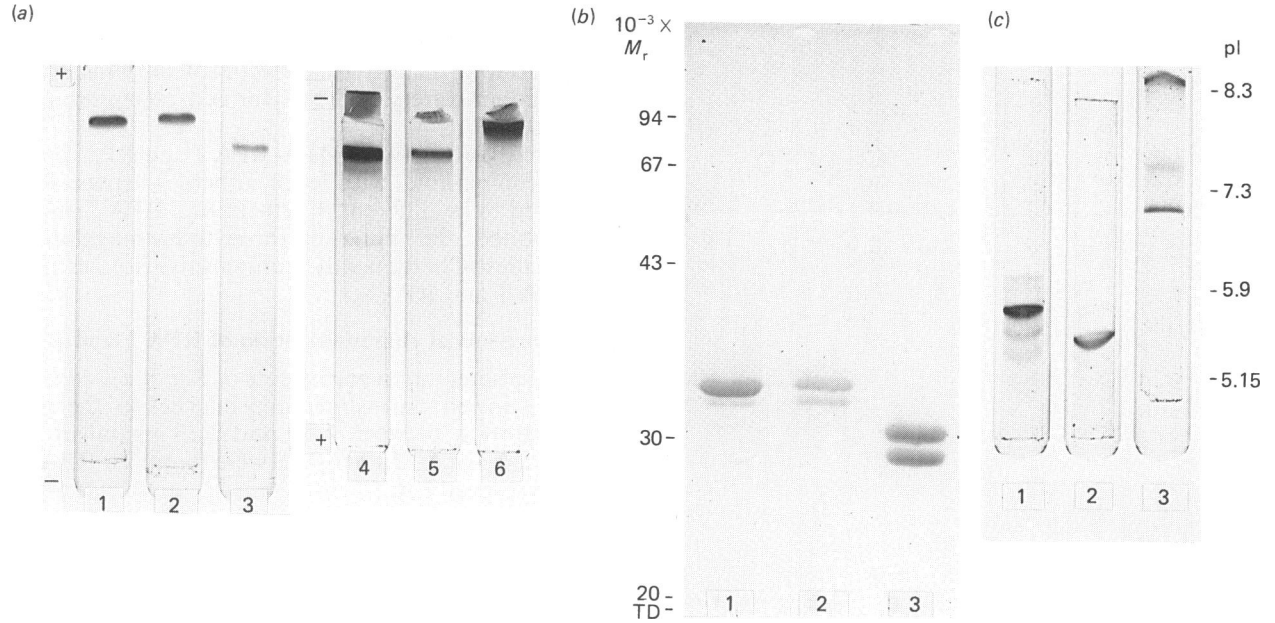

Fig. 2. Electrophoresis of *Robinia pseudoacacia* lectins

(*a*) Disc-gel electrophoresis was performed in glass tubes (6 mm × 120 mm) with a 5% acrylamide concentration. Protein samples (50–100 μg) were subjected to electrophoresis at pH 4.3 (tubes 1–3) and pH 10.2 (tubes 4–6). Tubes 1 and 4, RPA 1; tubes 2 and 5, RPA 2; tubes 3 and 6, RPA 3. (*b*) SDS/polyacrylamide-slab-gel electrophoresis. Samples were treated with SDS and 2-mercaptoethanol. A 30 μg portion of protein was applied to each lane. Lane 1, RPA 1; lane 2, RPA 2; lane 3, RPA 3. Approximate M_r values are indicated on the left-hand side. TD indicates the position of the tracking dye (Bromophenol Blue). The same electrophoretic patterns were obtained when samples were treated with SDS only. (*c*) Analytical disc-gel isoelectric focusing. A 50–100 μg portion of protein was applied to each gel. Tube 1, RPA 1; tube 2, RPA 2; tube 3, RPA 3. pI values of standard proteins are indicated on the right-hand side.

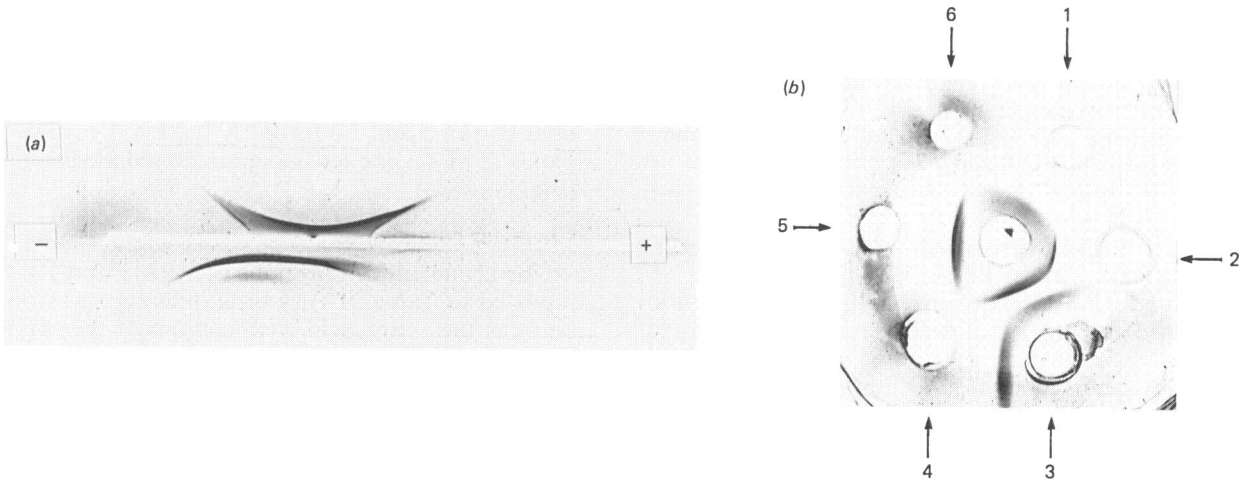

Fig. 3. Immunochemical analyses of *Robinia pseudoacacia* lectins

(*a*) Immunoelectrophoresis. The top well contains RPA 1, the bottom well RPA 3. (*b*) Ouchterlony double immunodiffusion. The central well contains anti-(*Robinia pseudoacacia* lectin) serum. Well 1, RPA 1; well 2, RPA 2; well 3, RPA 3; well 4, human AB serum. In each case the protein concentration was 1–2 mg/ml in saline.

3 was more efficiently inhibited than RPA 1. Furthermore, *N*-acetyl-D-glucosamine inhibited haemagglutination of human erythrocytes by RPA 3. These results led to the examination of the haemagglutination of erythrocytes with terminal *N*-acetyl-D-galactosamine residues (T_n and Cad erythrocytes). The haemagglutinating titre of RPA 3 on T_n erythrocytes was greatly enhanced and comparable with its titre on sialidase-treated normal erythrocytes. α_1-Acid glycoprotein was a potent inhibitor of RPA 3.

Both lectins were mitogenic (Fig. 4). RPA 1 increased [^{3}H]thymidine incorporation, which plateaued at 10 μg/ml, and its cytotoxicity was less than 10% at the different concentrations tested. RPA 3 yielded an optimal

Table 1. Chemical composition of *Robinia pseudoacacia* lectins

	Amino acid and carbohydrate composition			
	RPA 1		RPA 3	
	(g/100 g)	(mol/100 mol)	(g/100 g)	(mol/100 mol)
Asx	15.24	16.00	15.94	15.38
Thr	7.29	8.72	8.51	9.77
Ser	8.50	11.80	10.06	12.83
Glx	6.61	6.18	7.99	6.87
Pro	5.99	7.46	3.46	3.96
Gly	5.12	10.84	5.50	10.71
Ala	4.94	8.40	5.57	8.71
Cys	Trace		Trace	
Val	3.81	4.65	4.84	5.43
Met	0.53	0.48	0.83	0.70
Ile	2.64	2.83	2.28	2.24
Leu	6.08	6.50	7.57	7.44
Tyr	3.52	2.61	4.15	2.83
Phe	9.33	7.67	10.10	7.62
His	1.06	0.94	1.14	0.93
Lys	2.69	2.53	3.01	2.61
Arg	3.01	2.39	2.77	1.98
Fuc	1.54		0.56	
Man	5.20		1.97	
Xyl	1.76		0.67	
GlcNAc	3.13		1.08	

Table 2. Comparison of the haemagglutinating activities of RPA 1 and RPA 3 lectins from *Robinia pseudoacacia*

	RPA 1	RPA 3
(*a*) Haemagglutinating titre (μg/ml) on different erythrocytes		
Human O erythrocytes	5	20
T_n erythrocytes	2.5	4
Cad erythrocytes	10	20
Sheep erythrocytes	>1000	8
Human O erythrocytes (sialidase-treated)	5	4
T_n erythrocytes (sialidase-treated)	5	4
Cad erythrocytes (sialidase-treated)	10	10
Sheep erythrocytes (sialidase-treated)	>1000	0.8
(*b*) Inhibition of haemagglutination (minimum concentration in mM required to inhibit haemagglutination completely)		
N-Acetyl-D-glucosamine	>200	6
N-Acetyl-D-galactosamine	25	1.6*
		25†
L-fucose	>200	50*
		100†
α_1-Acid glycoprotein	>0.1	0.001*
		0.004†
Galactosamine, glucosamine, D-fucose, D-mannose, D-galactose did not inhibit haemagglutination		

* Human erythrocytes.
† Sheep erythrocytes.

response at 1 μg/ml, and its cytotoxic effect increased at high doses, where the cell count diminished.

The physicochemical and biological properties of the two lectins are summarized in Table 3.

DISCUSSION

Two lectins were isolated from *Robinia pseudoacacia* seeds and characterized. To the best of our knowledge, heterogeneity of *Robinia pseudoacacia* seed lectin has not been described, although several authors (Bourrillon & Font, 1968; McPherson & Hoover, 1979) have reported the purification and characterization of this lectin.

The lectin described by Bourrillon & Font (1968) and RPA 1 seem to be comparable on the basis of their physicochemical characteristics. Both have pI 5.9 and a sedimentation coefficient of 4.4 S. Although we used similar purification processes, no evidence is given

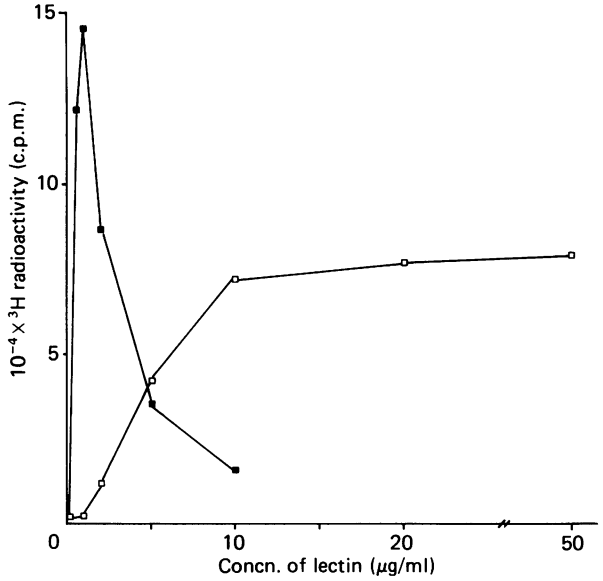

Fig. 4. Dose–response curves for mitogenic stimulation of human lymphocytes by *Robinia pseudoacacia* lectins

Human peripheral-blood lymphocytes were cultured at 37 °C for 4 days with various concentrations of lectins. The proliferative response was estimated by [^{3}H]thymidine incorporation. □, RPA 1; ■, RPA 3.

suggesting the presence of another lectin in their preparation. McPherson & Hoover (1979), who used affinity chromatography on fetuin–Sepharose, purified a lectin exhibiting two subunits of M_r comparable with those of RPA 3. In their case haemagglutination occurred only with trypsin-treated erythrocytes. Since only RPA 3 has a strong precipitation reaction with human or rabbit glycoproteins, it should be noted that this lectin would be preferentially selected by affinity chromatography. Hořejši *et al.* (1978) isolated a lectin nearly identical with RPA 3 (M_r, subunit composition) from black-locust bark. The major difference resides in its pI, which is approx. 5.9. These slight differences between the lectin extracted from seeds and that prepared from the bark may arise from their location in the plant. Differences in activity and specificity have been reported between *Dolichos biflorus* seed lectin and the lectin-like glycoprotein contained in stems and leaves (Talbot & Etzler, 1978).

Immunochemical comparison of RPA 1 and RPA 3 reveals a close relationship between these two lectins.

RPA 1 appears to be a dimeric molecule and RPA 3 a tetramer. Ultracentrifugation data permit the exclusion of an equilibrium between a dimeric and a tetrameric form of lectin. Comparison of the subunit M_r values confirms that RPA 3 does not result from the dimerization of RPA 1. The variations of the subunit M_r values of the two lectins may be attributed to the percentage of carbohydrates, which is sufficient to explain the overall change in M_r value. Nevertheless this does not preclude variations in the amino acid sequences of *Robinia pseudoacacia* lectins.

Furthermore the heterogeneity of each lectin is shown by analytical isoelectric focusing. This is commonly observed and easily explained for isolectins by the combination of different subunits in non-covalently bound polymers (Leavitt *et al.*, 1977; Murphy & Goldstein, 1977), but has also been attributed to charge variations in the lectin subunit, as described for *Pisum sativum* (Entlicher & Kocourek, 1975) and *Phaseolus lunatus* (Roberts *et al.*, 1982) lectins.

Two-dimensional electrophoresis reveals that the different bands obtained by isoelectric focusing of RPA 1 and RPA 3 give the same electrophoretic patterns in the second dimension in the presence of SDS (results not shown). Thus RPA lectin heterogeneity probably results from the existence of subunit subtypes.

The data concerning the lectin-haptenic sugar are controversial. High concentrations of *N*-acetyl-D-galactosamine (Hořejši *et al.*, 1978) or α-D-galactose (McPherson & Hoover, 1979), *N*-linked oligosaccharide chains from erythrocyte or immunoglobulin glycopeptides (Leseney *et al.*, 1972) have been described as inhibitors of *Robinia pseudoacacia* lectin. Our results support the differences in the carbohydrate-binding specificities of RPA 1 and RPA 3. Whether or not they are linked to the conformation of the two molecules remains to be defined.

The carbohydrate specificity of RPA 1 seems to be complex, since simple monosaccharides are non-inhibitory. Inhibition of RPA 3 by *N*-acetyl-D-galactosamine is in agreement with the agglutination of T_n erythrocytes, in which α-*N*-acetyl-D-galactosamine residues are exposed on glycophorin molecules (Dahr *et al.*, 1974; Springer & Desai, 1974). Agglutination of Cad erythrocytes, the determinant of which is a pentasaccharide with a terminal β-linked *N*-acetyl-D-galactosamine residue (Blanchard *et al.*, 1983), is comparable with normal erythrocyte

Table 3. Physicochemical and biological properties of *Robinia pseudoacacia* lectins

	RPA 1	RPA 3
$s_{20,w}$ (S)	4.22±0.04	6.47±0.07
M_r		
Sedimentation equilibrium	59000±4000	105000±6000
SDS/polyacrylamide-gel electrophoresis	34000±1000	30500±1000
	32000±1000	29000±1000
pI	5.9±0.1	8.3±0.2
	5.5±0.1	7.5±0.2
	5.3±0.2	6.9±0.1
Haemagglutinating titre		
Human	5 μg/ml	20 μg/ml
Sheep	>1000 μg/ml	5 μg/ml
Optimal mitogenic concentration	10 μg/ml	0.5–1 μg/ml

agglutination. This might indicate that RPA 3 is either specific for α-*N*-acetyl-D-galactosamine residues, or that *N*-acetyl-D-galactosamine must be in close association with the protein counterpart. Nevertheless the carbohydrate-specificity of RPA 3 remains puzzling. Precipitation with α-glycoproteins prompted us to study the proteins responsible for this reaction. Among them, α_1-acid glycoprotein has been found to be a potent inhibitor of haemagglutination, but this glycoprotein is devoid of *N*-acetyl-D-galactosamine residues. One possible explanation would be the existence of two different carbohydrate-binding sites in RPA 3.

Although both RPA lectins are mitogenic, the responses observed are different. The mitogenic dose–response curve for RPA 1 is saturable, whereas high doses of RPA 3 lead to diminished thymidine incorporation, which can be correlated to the lectin-induced cytotoxicity.

The valence differences may contribute to the different mitogenic capacities of RPA 1 and RPA 3, as described for concanavalin A (Gunther *et al.*, 1973) or *Phaseolus lunatus* lectin (Munske *et al.*, 1981; Pandolfino *et al.*, 1983). Nevertheless a role for the binding specificities of each lectin in the mitogenic stimulation, as observed for *Griffonia simplicifolia* I and *Phaseolus vulgaris* lectins (Murphy & Goldstein, 1979; Leavitt *et al.*, 1977), cannot be excluded.

Two lectins that have different biological and physicochemical properties but are immunologically related have been purified from *Robinia pseudoacacia* seeds. They are neither isolectins nor a dimer–tetramer pair. Since the carbohydrate content appears to be one of the main differences in their composition, it might play a role in their respective properties, and it would be of interest to determine if their polypeptide chains are identical or not.

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