Isolation and properties of a lectin from the seeds of hairy vetch (*Vicia villosa* Roth)*

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(Received 17 December 1980/Accepted 30 January 1981)

The lectin of the seeds of hairy vetch (*Vicia villosa* Roth), which selectively binds murine cytotoxic T-lymphocytes, was purified by simple affinity-chromatographic procedures on two different *N*-acetyl- α -D-galactosaminyl-carriers. The lectin thus obtained is homogeneous on polyacrylamide-gel electrophoresis both in acid and alkaline media and has a mol. wt. of approx. 120 000. The lectin molecule appears to comprise four subunits of equal electrophoretic mobility, contains 4.3% of covalently bound neutral sugar and 0.72 Mn and 0.94 Zn atoms respectively. The anti-(blood-group A₁) specific erythroagglutinating activity of the lectin can be detected at a limit concentration of 15 μ g/ml and is inhibitable most effectively by *N*-acetyl-D-galactosamine.

The unique property of the seed lectin of hairy vetch (*Vicia villosa* Roth), which binds selectively to T 145, a distinguishing surface glycoprotein of murine cytotoxic lymphocytes (Kimura & Wigzell, 1978), was shown by Kimura *et al.* (1979*a,b*). Those authors described a successful application of the lectin to separation, by affinity chromatography, of murine cytotoxic lymphocytes from natural killer cells and 'K'-cells. They isolated the lectin by affinity chromatography of a seed-protein fraction on blood-group A substance coupled to Sepharose 2B.

The importance of a practical application of the lectin in experimental immunology prompted us to devise a simple purification procedure for the lectin and to characterize its molecular properties, which, to our knowledge, have never been reported.

Materials and methods

The seeds of *V. villosa* Roth cv. Negra were obtained from the Uniform Agricultural Co-operative Bilov, near Plzeň, Czechoslovakia.

Separon H-1000 (a macroporous hydroxyalkyl methacrylate-ethylene dimethacrylate co-polymer) was a product of Laboratorní přístroje, n.p. (národní podnik), Prague, Czechoslovakia; it was glycosylated with free *N*-acetyl-D-galactosamine in dioxan, with dry HCl as catalyst, as described by Filka *et al.*

(1978). *N*-(ϵ -Amino hexanoyl)-D-galactosamine coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) was prepared as described by Allen & Neuberger (1975). The water-soluble *O*- α -D-galactosyl-polyacrylamide co-polymer used for affinity electrophoresis was prepared as described by Hořejší *et al.* (1978).

Isolation of lectin

Preparation of crude protein fraction. Seeds of *V. villosa* were soaked overnight in 0.15 M-NaCl, homogenized, suspended in 5 litres of the same salt solution and left at 4°C for 10 h. The suspension was then acidified to pH 4.0 with 2 M-HCl and the seed slurry, together with the precipitated proteins, were removed by centrifugation (1500 g for 30 min). The supernatant was saturated with solid (NH₄)₂SO₄ to 80% saturation, the precipitate separated by centrifugation, suspended in 4 vol. of water and extensively dialysed. The precipitate formed during dialysis was removed and the supernatant freeze-dried.

Affinity chromatography. The crude protein fraction corresponding to 250 g of seeds was dissolved in 20 ml of 0.15 M-NaCl, the insoluble material removed by centrifugation (1500 g for 30 min) and the supernatant applied to a column (1.8 cm $\times$ 20 cm) of *O*-(*N*-acetyl- α -D-galactosaminyl)-Separon H-1000 equilibrated with 0.15 M-NaCl. The column was washed with the same solution at a flow rate of 16 ml/h until non-adsorbed proteins had been com-

* This is paper 50 of the series 'Studies on Lectins'.

pletely removed. The lectin was then released either by a 0.1 M-solution of *N*-acetyl-D-galactosamine or, alternatively, by 0.4 M-borate buffer, pH 8.0. Fractions containing the lectin were collected, dialysed and freeze-dried.

Analytical methods

Discontinuous polyacrylamide-gel electrophoresis was performed in an apparatus designed by Davis (1964) in alkaline (Steward *et al.*, 1965) or acidic (Reisfeld *et al.*, 1962) buffers. Electrophoresis in alkaline medium was for 1.5 h at a current density of 4 mA/tube (75 mm × 5 mm) or, in acidic medium, for 2 h and 7 mA per tube. Gels were stained with Amido Black 10B. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969) in a 7.5% (w/v) gel. The polyacrylamide molecular weight of subunits was calculated from their electrophoretic mobilities in comparison with those of lysozyme and its covalent oligomers (Payne, 1973). Reduction of the presumed S-S bonds was performed with 2-mercaptoethanol. Affinity electrophoresis on polyacrylamide gels was performed by the method of Hořejší *et al.* (1977), with α -D-galactosyl ligands immobilized in the form of a soluble *O*- α -D-galactosyl-polyacrylamide copolymer (Hořejší *et al.*, 1978).

Thin-layer gel chromatography (t.l.g.c.) on Sephadex G-200 (Superfine grade) in 0.15 M-NaCl in the presence or absence of 0.2 M-D-mannose was performed in the Pharmacia t.l.g.c. apparatus.

For amino acid analysis, lectin samples were hydrolysed at 110°C in sealed tubes under N₂ with 6 M-HCl for 20 and 70 h respectively, and analysed on an amino acid analyser (Model AAA 881; Mikrotechna, Prague, Czechoslovakia). Cysteine and methionine were determined as cysteic acid or methionine sulphone respectively, after oxidation of a sample with performic acid (Moore, 1963); tryptophan was determined independently (Spies & Chambers, 1949).

Total neutral-sugar content was determined by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) with D-glucose as reference sugar. The metal content of the lectin was determined on a Varian Techtron model AA-175 atomic-absorption spectrophotometer. Measurements of the sedimentation velocity of the lectin were performed on a MOM 3170 ultracentrifuge at a speed of 59 780 rev./min.

The erythroagglutinating activity of the lectin and inhibitory activity of sugars was assayed by a test-tube serial-dilution method as described previously (Hořejší & Kocourek, 1978).

Results

For the isolation of anti-(blood-group A₁)-specific lectin from the seeds of *V. villosa*, affinity chromato-

graphy on an *N*-acetyl-D-galactosaminyl derivative of Separon H-1000 or *N*-(ϵ -aminohexanoyl)-D-galactosamine coupled to Sepharose 4B were used. The adsorbed lectin could be eluted either by 0.1 M-*N*-acetyl-D-galactosamine solution or by 0.4 M-borate buffer, pH 8.0. Elution of bound lectin with glycine/HCl buffer, pH 3.0, was unsuccessful. The yield of the purified lectin was 220 mg/kg of seeds.

The affinity-purified lectin yielded a single sharp band on discontinuous polyacrylamide-gel electrophoresis in both alkaline and acidic media. Electrophoresis in the presence of sodium dodecyl sulphate of both reduced and non-reduced samples showed one intense zone corresponding to a mol.wt. of 30 000 and a very weak diffuse one corresponding to an approx. mol.wt. of 14 000. The intensity of the weak zone was not influenced by reduction with 2-mercaptoethanol.

T.l.c. on Sephadex G-200 (Superfine grade) revealed the presence of a single component with an elution volume corresponding to a mol.wt. of 95 000. When chromatography was performed in the presence of D-mannose, the mobility of the lectin corresponded to mol.wt. of 120 000. On ultracentrifugation the lectin yielded a single symmetrical peak; its sedimentation coefficient (*s*_{20,w}) was 6.2 S (in 0.05 M-Tris/HCl buffer, pH 7.6).

Preparations of the lectin obtained by affinity chromatography on both types of affinity carriers by elution with either *N*-acetyl-D-galactosamine or the borate buffer did not differ in electrophoretic

Table 1. Amino acid composition of the *V. villosa* lectin

Amino acid	Composition	
	Residues/molecule of mol.wt. 120 000	Mol/100 mol
Lysine	35.8	4.31
Histidine	19.8	2.38
Arginine	24.7	2.97
Aspartic acid	93.8	11.29
Threonine	69.7	8.39
Serine	61.4	7.39
Glutamic acid	84.7	10.19
Proline	57.1	6.87
Glycine	47.9	5.76
Alanine	56.9	6.85
Half-cystine	None	
Valine	68.0	8.18
Methionine	2.2	0.26
Isoleucine	45.8	5.51
Leucine	72.6	8.74
Tyrosine	23.4	2.82
Phenylalanine	54.0	6.50
Tryptophan	13.1	1.58

Table 2. *Inhibition of erythroagglutinating activity of the V. villosa lectin by sugars*

The inhibitory activity of sugars is expressed in minimum concentrations of sugars (mM) inhibiting erythroagglutination by 0.03% lectin solution.

Sugar	Inhibition
N-Acetyl-D-galactosamine	0.1
D-Galactosamine hydrochloride	2.6
p-Nitrophenyl β -D-galactopyranoside	2.6
2-Deoxy-D-galactose	3.7
Methyl α -D-galactopyranoside	6.5
Melibiose	7
D-Arabinose	8.2
Raffinose	8.4
Lactose	14
Methyl β -D-galactopyranoside	26
D-Galactose	28
D-Mannose	28

mobility, subunit composition or their behaviour on Sephadex G-200 t.l.c.

The amino acid composition of the lectin is shown in Table 1. The lectin is rich in aspartic acid and glutamic acid, threonine and serine, and it also contains 4.3% of neutral sugar. Atomic-absorption spectroscopy revealed the presence of 0.72 atom of Mn and 0.94 atom of Zn per molecule of the lectin (of presumed mol.wt. 120 000).

Affinity electrophoresis on polyacrylamide gel containing immobilized D-galactosyl residues showed that the interaction of the lectin with sugars was stronger in the acidic than in the alkaline pH regions.

The affinity-purified *V. villosa* lectin agglutinates only human blood-group-A₁ erythrocytes at the minimum concentration of 15 μ g/ml; other types of human blood-groups-ABO-system erythrocytes were not agglutinated. The erythroagglutinating activity was most inhibited by N-acetyl-D-galactosamine; values characterizing inhibitory potency of several other sugars are given in Table 2.

Discussion

Seed lectins of various *Vicia* species display a diversity in binding specificities unusual within a single genus. Thus lectins of *V. ervilia* (bitter vetch) (Fornstedt & Porath, 1975), *V. faba* (broad bean) (Allen & Neuberger, 1976) and *V. sativa* (common vetch) (Falasca *et al.*, 1979) bind D-mannose and D-glucose and are non-specific towards erythrocytes of the ABO-blood-groups system, similar to a lectin contained in seeds of *V. cracca* (tufted vetch) (Aspberg *et al.*, 1968). This lectin, however, occurs in the seeds together with an anti-(blood-group A₁)-specific lectin inhibitable by N-acetyl-D-galacto-

samine (Rüdiger, 1977). Interactions of another group of *Vicia* lectins cannot be inhibited by simple sugars [e.g. the anti-(blood-group N)-specific lectin of *V. graminea* (no common name) (Prigent & Bourrillon, 1976)].

The anti-(blood-group A₁) lectin of *V. villosa* described in the present paper appears to be most similar to the specific lectin of *V. cracca*: (1) its molecule comprises the same number of subunits of similar size, and its amino acid composition and neutral sugar contents are also closely similar; (2) it is highly anti-(blood-group A₁)-specific and its activity can be inhibited most actively by N-acetyl-D-galactosamine; its interactions with cross-linked dextrans are also weak, as is the case for *V. cracca* lectin (Karhi & Gahmberg, 1980). There is, however, a very striking difference in the binding affinities of the two anti-(blood-group A₁)-specific lectins under different conditions of pH. As revealed by the affinity-electrophoresis results, the binding affinity of *V. villosa* lectin to carbohydrates is considerably weaker in alkaline than in acidic media, whereas the opposite is true for the *V. cracca* lectin (Hauzer *et al.*, 1979). This behaviour explains why the *V. villosa* lectin adsorbed to the N-(ϵ -amino-hexanoyl)-D-galactosamine derivative of Sepharose could not be eluted by acid buffers as described for the *V. cracca* lectin (Rüdiger, 1977). In contrast with the situation in *V. cracca*, we did not observe the presence of a non-specific lectin in *V. villosa* seeds.

Our preliminary studies (L. Grubhoffer, M. Tichá & J. Kocourek, unpublished work) indicate that some variance in binding activities may be found even in different cultivars within the species *V. villosa* itself. Out of eight different cultivars tested, one was completely inactive, whereas the others differed in the degree of their anti-(blood-group A₁) erythroagglutinating activities. This fact points anew to the possibility of using lectins as genetic or taxonomic markers, but at the same time to the necessity of a proper identification of biological sources of lectins, especially as far as the highly diversified cultivars of agricultural plants are concerned.

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