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Isolation of a lectin from the pollen grains of *Petunia hybrida*

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SUMMARY

A lectin was isolated and partially characterized from the pollen grains of *Petunia hybrida*. The lectin, isolated by gel filtration on Sephacryl S-200 and affinity chromatography on Concanavalin A-agarose, was found to have a molecular weight (approximate) of 105 000 D. The lectin agglutinated rat, mouse, sheep and human erythrocytes and required Ca^{2+} and Mg^{2+} for haemagglutinating activity. Sugar specificity of the lectin could not be elucidated. Acid phosphatase activity was associated with haemagglutinating activity throughout the purification procedure.

Key-words: lectin, *Petunia*, pollen grains, self-incompatibility.

INTRODUCTION

Lectins have been shown to play an important role in a range of biological activities (see Sharon & Lis 1972, Lis & Sharon 1986; Sharon 1987). On the basis of many circumstantial evidences, lectins have been implicated in the reproductive biology of higher plants, particularly in self-incompatibility recognition (Dumas & Gaude 1981; Heslop-Harrison & Heslop-Harrison 1982; Sharma & Shivanna 1986; Speranza & Calzoni 1990). Although a few reports have appeared in recent years on the presence of lectins in pollen grains (Gaude *et al.* 1983; Tumosa 1984; Carratú & Giannattassio 1990), no attempts have so far been made to isolate and characterize pollen lectins. In this paper, we report the isolation of a lectin/lectin-like protein from the pollen grains of *Petunia hybrida*.

MATERIAL AND METHODS

Plants of *Petunia hybrida* Vilm. were grown under field conditions. Pollen collected from freshly dehisced anthers was pooled and stored over dry silica at −20°C until use (1–2 months).

Lectin isolation

The lectin, throughout the purification steps described below, was monitored for haemagglutinability using the haemagglutination assay.

Crude extract was prepared by homogenizing pollen (5·0 g) in 50·0 ml chilled Tris buffered saline (TBS—0·05M TRIS–HCl, 0·15 M NaCl, 0·001 M $CaCl_2.2H_2O$, 0·001 M $MgCl_2.6H_2O$, pH 7·4) using acid-washed sand and glass dust as abrasive in a chilled mortar. The homogenate was centrifuged (15 000 *g*, 30 min, 4°C) and the supernatant collected. The pellet was re-extracted with 25·0 ml TBS and the supernatants pooled.

The crude extract was brought to 0–60% saturation with crystalline ammonium sulphate. The 0–60% ammonium sulphate precipitate was collected by centrifugation (15 000 *g*, 20 min, 4°C), suspended in 5·0 ml TBS (0·01 M, pH 7·4), and dialysed (×2) against the same buffer for 24 h.

The dialysed protein sample was fractionated on a Sephacryl S-200 (Pharmacia) column (1·8 × 21·0 cm) equilibrated with TBS (0·02 M, pH 7·4). Proteins were eluted with TBS (0·01 M, pH 7·4) at a flow rate of 0·25 ml min^{-1} and fractions (2·0 ml each), collected immediately after the void volume ($V_0 = 26·0$ ml), were concentrated by lyophilization.

An aliquot (10·0 ml) of the Sephacryl S-200 fraction showing haemagglutinability was loaded on a Concanavalin A-agarose (Con A-agarose, Sigma) column (1·8 × 1·6 cm, bed volume 4·0 ml) equilibrated with TBS (0·02 M, pH 7·4) for affinity chromatography. The unbound protein was eluted by washing the column with four volumes (16·0 ml) of TBS. The bound protein fraction was eluted with the same buffer (two bed volumes) containing α-methyl-D-mannoside (0·5 M, Sigma) at a flow rate of 0·12 ml min^{-1}. Fractions (0·5 ml each) containing protein were pooled (3·0 ml), dialysed to remove sugar, and concentrated by lyophilization.

Proteins were estimated by Bradford's method (1976) using bovine serum albumin as the standard.

Acid phosphatase assay

Acid phosphatase (ACP) activity was assayed by the procedure of Johnson *et al.* (1973). A unit of ACP activity (EU) is defined as the amount of enzyme that liberates one nanomole of *p*-nitrophenol in 30 min at 37°C in an incubation mixture of 2·0 ml.

Haemagglutination assay

Initially, red blood cells (RBC) from mouse, rat, rabbit, sheep and humans (A, B, O types) were used for testing the haemagglutinating activity of the crude extract. The crude extract was also tested with trypsin-treated rat RBC. In subsequent purification steps, only rat RBC were used for monitoring the activity of lectin.

Haemagglutinability was measured by the serial dilution technique in Laxbro microtitre plates with RBC suspension (2%, v/v in TBS (0·01 M, pH 7·4) and the endpoint determined visually. Inhibition studies were carried out by pre-incubating the sample with each of the following mono- and di-saccharides (0·1 M and 0·2 M): D-arabinose, L-arabinose, D-fucose, D-galactose, *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyl-D-glucosamine, D-lyxose, D-mannose, α-methyl-D-mannoside, D-melibiose, *N*-acetyl neuraminic acid, raffinose, rhamnose, L-sorbose, D-xylose, cellobiose, D-fructose and lactose.

TBS was used as control in each case. Two replicates were maintained for each assay, one at room temperature (28 ± 2°C) and another at 4°C. The plates were incubated for 1 h and observed for haemagglutinating activity. Haemagglutinating activity was expressed in haemagglutination units (HU) defined as the inverse of the highest dilution at which the extract shows detectable agglutination. Haemagglutinability of the sample, after affinity chromatography on Con A-agarose, was also tested in the absence of Ca^{2+} and Mg^{2+} in order to study the metal ion requirement of the lectin.

Polyacrylamide gel electrophoresis

The protein sample was electrophoresed on polyacrylamide slab gel (7·5%, 13·5 × 13·5 cm) by the method of Laemmli (1970). The gels were stained with Coomassie

Table 1. Haemagglutinating activity of the lectin at each step of purification

Fraction	Source of RBC	Haemagglutination units (HU)
Crude extract	Sheep	1
	Rat	2
	Rat (trypsinized)	2
	Mouse	2
	Rabbit	—
	Human	4*
Ammonium sulphate precipitate (0–60%)	Rat	2
Sephacryl S-200		
F I	Rat	4
F II	Rat	2
Con A-agarose		
F I	Rat	16

*The same titre was obtained with A, B, O and AB blood groups.

blue, and silver stain (Davis *et al.* 1986) to detect total protein, with periodic acid-Schiff's reagent (PAS) (Glossman & Neville 1971) for glycoprotein, and with Fast Garnet GBC using α-napthyl acid phosphate as substrate to detect activity bands of ACP (Scandalios 1969).

RESULTS

Haemagglutination assay

The haemagglutinating activity of the lectin at various stages of purification is summarized in Table 1. Rabbit RBC were not agglutinated by the crude extract; sheep RBC were agglutinated only by undiluted extract (1 HU); both mouse and rat RBC (Fig. 1a and b) were agglutinated by undiluted and $\frac{1}{2}$ dilution (2 HU) of the extract. Agglutination of human RBC (4 HU) was not blood group-specific. The extract also induced lysis of human and rat RBC, although the latter lysed only when incubated for about 3 h at 28 ± 2°C or overnight at 4°C. Trypsinization of rat RBC had no effect on the haemagglutinating activity of the crude extract.

The ammonium sulphate precipitate (0–60%) did not show enhancement of haemagglutinability. Following molecular sieving on Sephacryl S-200, maximum haemagglutinating activity was found to be associated with two fractions ($V_e = 34{\cdot}0$ ml and 40·0 ml, respectively), designated F I and F II. The fraction F I gave a titre of 4 HU while F II showed a titre of 2 HU.

None of the sugars tested were effective in inhibiting haemagglutination caused by either fraction. The molecular weights (approximate), as determined from molecular sieving, for F I and F II were 105 000 and 33 200 D, respectively.

The fractions F I and F II were passed through a Con A-agarose column and eluted with α-methyl-D-mannoside. The eluted F I fraction showed eight-fold enhancement of

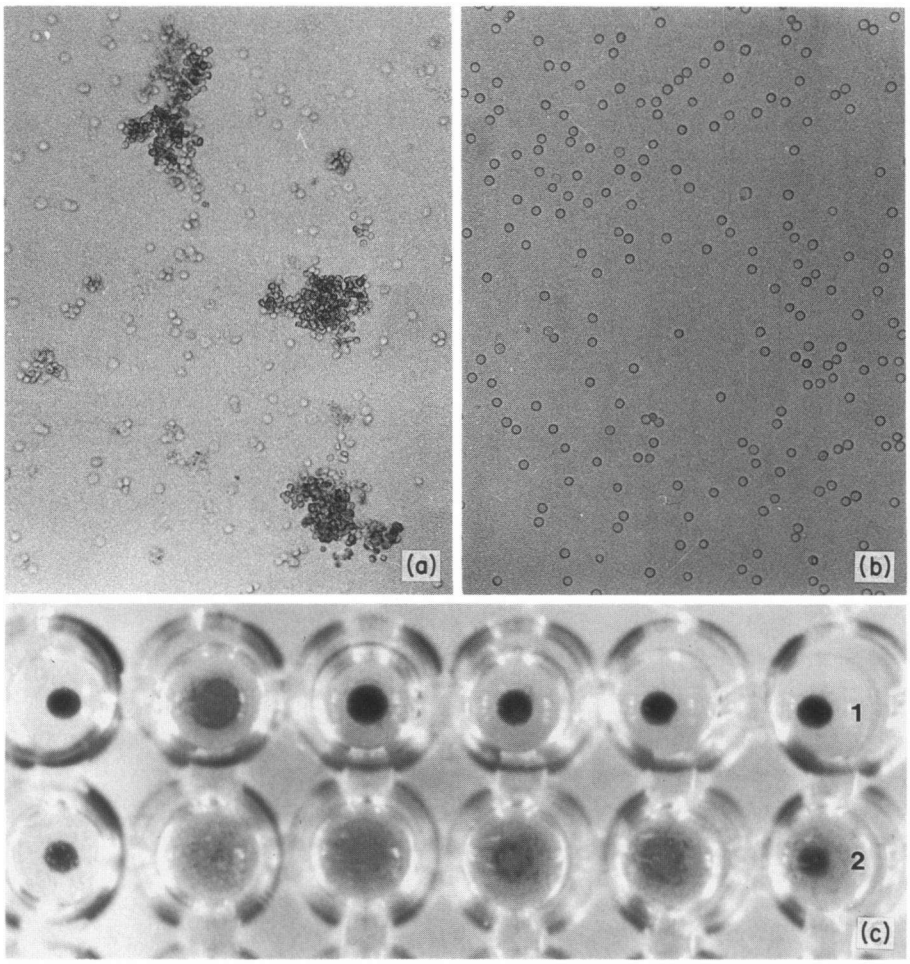

Fig. 1. Haemagglutinating activity of pollen extract with rat RBC (a), (b). Light micrographs showing agglutination of rat RBC by crude extract. (a) RBC incubated with undiluted extract, (b) RBC incubated with buffer (control); (c) serial dilutions of fraction F I protein eluted with α-methyl-D-mannoside in the absence (row 1) and presence (row 2) of Ca^{2+} and Mg^{2+}. The first well from the left in each row is control (only buffer). Only undiluted fraction (second well) shows some agglutination in row 1 whereas all wells (except control) show agglutination in row 2.

haemagglutinating activity (16 HU) over that of crude extract. However, no haemagglutinating activity could be detected in the F II fraction eluted with α-methyl-D-mannoside.

In the absence of Ca^{2+} and Mg^{2+}, the F I fraction protein showed poor haemagglutinability: 1 HU as compared to 16 HU in the presence of metal ions (Fig. 1c).

Acid phosphatase activity

ACP activity was associated with haemagglutinating activity throughout all the purification steps. Table 2 outlines the steps in the purification, the amount of total protein, and the ACP and haemagglutinating activity present in the sample at each step. Approximately, 1770-fold purification of ACP was achieved parallel to about 9000-fold purification of the lectin.

Table 2. Steps in the purification of lectin for pollen of *Petunia hybrida*

Fraction	Total protein (mg)	Acid phosphatase		Lectin	
		Specific activity EU mg protein^{-1}	Fold purification	Specific activity HU mg proten^{-1}	Fold purification
Crude extract (dialysed)	2775	$4{\cdot}4 \times 10^3$	1·0	4·3	1·0
Ammonium sulphate precipitate (0–60% saturation)	951	$11{\cdot}0 \times 10^3$	2·5	6·9	1·6
Sephacryl S-200	42	$14{\cdot}3 \times 10^4$	32	$1{\cdot}54 \times 10^2$	36
Con A-agarose	0·72	785×10^4	1770	$40{\cdot}0 \times 10^3$	9260

Polyacrylamide gel electrophoresis

The fraction F I protein, after chromatography on Con A-agarose, revealed two bands of ACP activity which represented two distinct isozymes of ACP. These bands corresponded with the two bands observed on staining with Coomassie blue and PAS. Silver stain revealed a third band just below the bands of the isozymes.

DISCUSSION

The haemagglutinability of the lectin was not inhibited by any of the saccharides tested at any stage of purification. This may be due to several reasons: (a) the range of sugars tested did not include the one specific for the lectin; (b) the lectin binds more readily with complex oligosaccharides—for some lectins no monosaccharide inhibitors are known and they are effectively inhibited by oligosaccharides only (see Sharon 1987); (c) the lectin preferably recognizes glycopeptides rather than just carbohydrate sequences of proteins—the lectins from *Vicia graminea, V. villosa* and *Agaricus bisporus* (see Sharon 1987) are inhibited by glycopeptides; or (d) interaction of the lectin with the RBC may not be sugar specific—a lectin has been isolated from *Escherichia coli* which is L-histidine-specific (see Balding 1981). Gaude *et al.* (1983) also reported that neither the haemagglutinating activity of *Populus* pollen diffusate nor the adhesion of RBC to *Brassica* pollen grains could be inhibited by the sugars tested. Recently, Carratú & Giannattassio (1990) have also reported that simple sugars do not inhibit pollen haemagglutinating activity.

The extract of *Petunia* pollen clearly contained more than one lectin as exhibited by the haemagglutinability of different Sephacryl S-200 fractions. Moreover, haemagglutinating activity of only one of the fractions (F I) was eluted with α-methyl-D-mannoside from the Con A-agarose column. The haemagglutinating activity of the F II fraction was not recovered from the Con A-agarose column indicating that most of it remained bound to Con A-agarose and the binding was not sugar-specific; the interaction between F II fraction protein and Con A may be hydrophobic. Con A has been reported to show considerable hydrophobicity (Davey *et al.* 1976; Ochoa *et al.* 1979).

The different haemagglutinating proteins in the extract may have been contributed by different plants since pollen pooled from plants of a heterogeneous population was used. Elution of haemagglutinating activity of F I protein with α-methyl-D-mannoside suggests the glycoprotein nature of the lectin. The *Vicia graminea* lectin also binds completely to Con A-sepharose and can be eluted with α-methyl-D-mannoside (Duk & Lisowska 1981). A lectin from *Ricinus communis* beans has been shown to form an insoluble complex with Con A which could be dissociated only upon addition of Con A-specific sugars (Podder *et al.* 1974).

The F I fraction protein, eluted with α-methyl-D-mannoside, showed a 16-fold reduction in haemagglutinating activity in the absence of Ca^{2+} and Mg^{2+}, suggesting that the lectin requires Ca^{2+} and Mg^{2+} for activity. Other lectins, including Con A, are known to have a requirement for Ca^{2+}, Mg^{2+}, Mn^{2+} or other metal ions (see Sharon & Lis 1972).

The third band revealed, following electrophoresis of F I fraction protein, on gel stained with silver nitrate was not detectable on gels stained with PAS. This may be attributed to its very low concentration (as indicated by its absence in gels stained with Coomassie blue) or the absence of covalently bound sugars in this protein; the former possibility is more applicable since the protein was eluted with α-methyl-D-mannoside.

Acid phosphatase activity was checked at each stage of purification since these enzymes are known to be glycoproteins that bind to Con A (Lorenc-Kubis & Bøg-Hansen 1981). It

is difficult to ascertain, from the data available at present, whether the haemagglutinating and acid phosphatase activities are associated with the same or different protein molecule(s). The two activities may reside on the same molecule as many lectins that possess enzyme activity have been reported. Haemagglutinins from seeds of mung bean and other legumes have been shown to possess α-galactosidase activity (Hankins *et al.* 1980). Nevertheless, the possibility of the haemagglutinating and enzyme activities being associated with different protein species is difficult to rule out since a third distinct band (which did not show acid phosphatase activity) was observed on silver-stained gel. Experiments dealing with the effects of inhibition of enzyme activity on haemagglutinating activity and vice versa would help in elucidating the nature of the protein involved.

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