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LECTIN VARIABILITY IN *PHASEOLUS COCCINEUS*

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Key Word Index—*Phaseolus coccineus*; Leguminosae; affinity chromatography; immuno-affinity; lectin.

Abstract—Lectin variability within *Phaseolus coccineus* is revealed by non-denatured electrophoretic patterns and immunological labelling of total seed protein extracts, showing that the different cultivars and wild varieties studied can be classified into three main categories according to the number of isolectins (three, two or one) present in each extract. Attempts in the purification of these isolectins were performed on three different affinity systems in which ligands were thyroglobulin (known to purify the *P. vulgaris* isolectins), pig red cell membrane ghosts (stroma) or antibodies against the *P. vulgaris* cv. Contender E_2L_2 isolectin. The *P. coccineus* isolectins exhibit varied affinities towards thyroglobulin and stroma, the cathodic and anodic (pH 4.5) isolectins being respectively retained by the two systems, whereas the antibody affinity system is the only one able to purify the totality of the isolectins present in an extract.

INTRODUCTION

It has been shown in some varieties of *Phaseolus vulgaris* [1, 2] that the lectins are in fact a mixture of five tetramers (isolectins) derived from the combination of two subunits, erythroagglutinating (E) and leucoagglutinating (L), associated in a non-covalent way to form five different tetrameric molecules: E_4, E_3L, E_2L_2, EL_3 and L_4 [3, 4]. This situation exists only in 33/107 of the *P. vulgaris* cultivars and plant introductions studied by Brown *et al.* [5]. The other varieties are constituted by 3, 2 or 1 lectin protein.

Many authors have analysed different aspects of the purification, characterization and hemagglutination of the lectins inside the *Phaseolus* genus [6–8] or even inside a species [1, 5, 9]. Moreover, having tested hybridization between *P. vulgaris* and other members of the genus, Smartt [10, 11] could conclude, according to the behaviour of *P. vulgaris* and *P. coccineus* in hybridization, that they were genetically very close relatives. These two species have indeed quite a few features in common and it is suggested that lectins homologous with those of *P. vulgaris* may occur in *P. coccineus*. Consequently we decided for a first step to screen different *P. coccineus* cultivars and wild varieties for their lectin composition, and to purify them in order to compare them with the *P. vulgaris* cv. Contender isolectins.

Until now much less work has been done on the *P. coccineus* lectins. We can nevertheless mention the study of Nowakova and Kocourek [12] who isolated two lectins from scarlet runner seeds (*P. coccineus L.*) by ammonium sulphate fractionation and ion-exchange chromatography. Their M_rs were found to be 120 000 and the lectins, homogeneous on polyacrylamide gel electrophoresis (PAGE), were composed of identical subunits with an M_r of 34 000. The two lectins were able to agglutinate the human ABO system in a non-specific way, and one of them to stimulate mitosis of lymphocytes.

An interesting technique was used by Ochoa and Kristiansen [13] to isolate the *P. coccineus* var. Alubia lectin. As neither oligosaccharides nor glycoproteins were able to inhibit the agglutination of their extract, they prepared an affinity chromatography column with pig red cell membrane ghosts (stroma) as a ligand. The lectin they purified was homogeneous on electrophoresis, consisting of four similar subunits of M_r 28 000 each and containing 20% wt/wt carbohydrate. The native molecule M_r was 112 000.

RESULTS

Lectin variation in non-denaturing conditions

An example of a non-denaturing PAGE of some total seed protein extracts (2%) is shown on Fig. 1a. A second gel (Fig. 1b) with the same protein extracts (diluted 32 ×) was run in the same conditions, transfered on nitrocellulose and labelled with antibodies prepared against the E_2L_2 isolectin of *P. vulgaris* cv. Contender [14] (see Discussion). Controls were performed with pre-immunized rabbit IgG. This enabled us to identify the *P. coccineus* lectins and to compare different cultivars and wild varieties with one another and with the five *P. vulgaris* cv. Contender isolectins.

Among the electrophoretic and immunologic patterns obtained, three main categories of lectin patterns can be distinguished according to the number of lectin bands detectable in an extract. Most of the cultivars and wild varieties we studied are classified in those 3 categories (see Table 1) represented by 5, 3 or 1 lectin band. Cultivars X 410 and 'alubia' are apart as they are the only ones which present two lectin bands. As *P. vulgaris* cv. Contender contains five isolectins of very close charge, some of the *P. coccineus* cultivars also contain five isolectins but slightly displaced in comparison to the latter (see Fig. 1). A few other cultivars and wild varieties exhibit only three bands corresponding to the lectins, the ones of cultivar X 421 and of the wild varieties NI 813 and 818, being slightly more condensed together. Finally, one lectin band characterizes the remaining cultivars and the wild variety NI 726. No cultivar has yet been characterized by a lack of lectins of this type as it was the case for the *P. vulgaris* Pinto III cultivar [7].

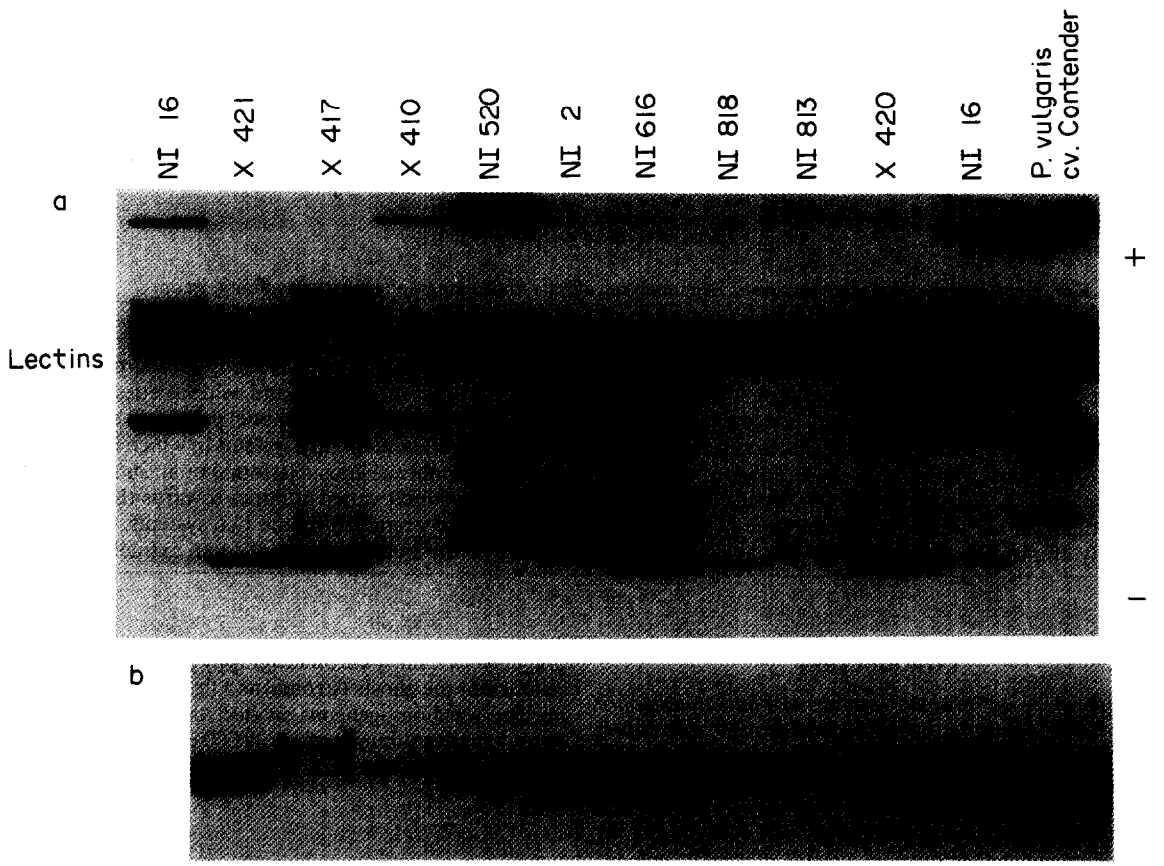

Fig. 1. (a) Non-denatured pH 4.5 PAGE of some *Phaseolus coccineus* total seed protein extracts. (b) Total seed proteins of the same *P. coccineus* extracts labelled with anti-*P. vulgaris* cv. Contender E_2L_2 antibodies after transfer on nitrocellulose paper.

Table 1. *Phaseolus coccineus* cultivars and wild varieties studied

Phaseolus coccineus	Number of lectin bands 5	3	1
subsp. *coccineus*	NI 16	NI 2	NI 579
	132	15	726w
	191	90	
	608	229	
	X 417	616	
		813w	
		818w	
		X 420	
		421	
subsp. *polyanthus*			NI 373
			519
			520
			758
subsp. *formosus*			NI 552

Classified according to the number of lectin bands revealed by non-denaturing PAGE and immunological labelling of total seed protein extracts as seen in Fig. 1. Samples X 410 and 'alubia' are separated as they contain two lectin bands (w = wild).

By means of a very simple immunochemical technique we were thus able to reveal the presence of lectins and their variability within the *P. coccineus* species.

Lectin purification

Three different affinity chromatography systems (thyroglobulin-Sepharose 4B, stroma-Sephadex G 25, anti-*P. vulgaris* lectin IgG-Sepharose 4B) have been used in order to isolate the *P. coccineus* lectins.

Non-denatured electrophoretic patterns of lectins purified on those three systems are shown on Fig. 2a for a five lectin type sample, NI 16. Indeed, taking into account the fact that thyroglobulin exhibits great affinity towards the five *P. vulgaris* cv. Contender isolectins [15] we started by using this method to purify the lectins of a five lectin bands sample. But after elution of the lectins we realized that only the three cathodic isolectins could be retained by this type of adsorbent (Fig. 2a, T) even after passing the non-adsorbed material on the column again. Another adsorbent, stroma, was then used. In this case the anodic isolectins were retained. Besides, the two eluting solutions used here did not release the same isolectins, water eluting the most anodic ones and pH 2.5 buffer the others (Fig. 2a, S). Finally, an immunoaffinity column containing IgG

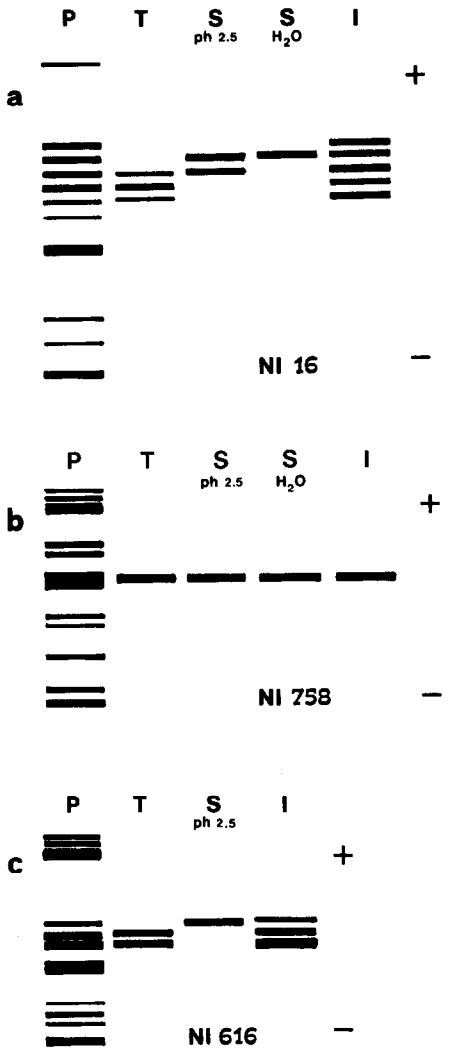

Fig. 2. Non-denatured pH 4.5 PAGE of *P. coccineus* isolectins purified on different affinity systems: T = thyroglobulin, S = stroma, I = IgG, P = total proteins. (a) five lectin bands sample; (b) one lectin band sample; (c) three lectin bands sample.

prepared against the *P. vulgaris* cv. Contender E_2L_2 isolectin as a ligand enabled us to purify all of the five *P. coccineus* lectins (Fig. 2a, I).

In the same way we used those three methods in order to purify lectins from total seed proteins of cultivars and wild varieties exhibiting three lectin bands (Fig. 2c). Again, thyroglobulin seemed to retain preferentially the cathodic isolectins (although for samples NI 15 and X 421 all three isolectins could be eluted together from this column), whereas the anodic ones were generally eluted from the stroma column. Using the IgG column, the three isolectins could be eluted together. Figure 2b shows the electrophoretic patterns of a lectin purified on those three affinity systems from a sample characterized by one lectin band.

These results clearly reveal the heterogeneous behaviour of the *P. coccineus* lectins towards the thyroglobulin and stroma affinity systems, the immunoaffinity

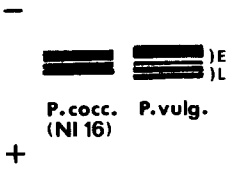

Fig. 3. SDS electrophoresis of *P. coccineus* isolectins purified from a five lectin bands sample, and *P. vulgaris* cv. Contender isolectins given as a comparison.

chromatography being until now the only system able to purify all of the isolectins of an extract.

SDS electrophoresis of purified lectins

In many cases, when purified lectins migrate in the presence of SDS three bands or more can be visualized (Fig. 3), their M_rs being estimated between 29 000 and 29 500. It is more difficult to distinguish them individually in comparison with the *P. vulgaris* cv. Contender subunits visualized in the same conditions (M_r 28 000–30 000). But in agreement with the observed results in *P. vulgaris*, in many cases more than two bands are distinguished, revealing a more complex situation for the polypeptidic constitution of lectins [5, 16], and especially for subunit L of the five tetramers lectin system which shows a greater heterogeneity than the E subunit.

Hemagglutination

Hemagglutination tests have been done on total seed protein extracts, but not on purified lectins. All *P. coccineus* cultivars as well as the wild varieties tested agglutinate the human ABO erythrocytes (data not shown) in a non specific way. Quantitative data of the total seed protein agglutination can not lead to a classification of our cultivars as we can not relate these results with the electrophoretic patterns.

DISCUSSION

An immunochemical technique was used in order to reveal the *P. coccineus* lectins, but making use of anti-lectin antibodies prepared against the *P. vulgaris* E_2L_2 isolectin. This choice was made assuming that, the two species being genetically very close, most of their lectin antigenic determinants are certainly identical due to the related lectin genes of Legumes [17–19]. The possibility that some isolectins escape identification is then extremely low, although we cannot rule out this possibility categorically because lectins of different specificities which do not cross-react may occur in the same plant [20].

As expected the biochemical and biological properties of the *P. coccineus* lectins are quite similar to those of *P. vulgaris*. There are however a few different features to be pointed out. As for *P. vulgaris*, most of the *P. coccineus* lectins are constituted of isolectins with different chemical and biological properties. Besides, as is the case for *P. vulgaris*, lectins are subject to many genetic variations [2]. In opposition to *P. vulgaris*, we notice the weak capacity of a thyroglobulin-Sepharose 4B column to purify all of the *P. coccineus* lectins. In most cases only part of the isolectins of a cultivar can be purified by such a column.

The same is seen for a stroma column, although the isolectins purified on this type of column are different from those purified on thyroglobulin. In the five isolectin cultivars, only the cathodic ones are retained by thyroglobulin. This is to be considered in relation with the fact that the most cathodic lectins of *P. vulgaris* (E_4, E_3L) exhibit the greatest affinity towards thyroglobulin, in comparison with the anodic ones [21]. On the other hand only the anodic isolectins show affinity for the stroma column. The heterogeneous behaviour of the different isolectins towards thyroglobulin as well as stroma could be due, according to Ochoa and Kristiansen [13], to varied adsorption mechanisms. By reversing the elution order (water, pH 2.5–pH 2.5, water) the band pattern particular to each eluant is found suggesting that the isolectins can be fixed to the stroma by different mechanisms. According to these authors "the capacity of distilled water to break the complex lectin-stroma is attributed to a weakening in hydrophobic bridges which take place in the formation of this complex" and they suggest that "heterogeneous binding sites on stroma" account for the behaviour of lectins on the adsorbent.

Another explanation could come from the presence in the crude extract of oligosaccharides, polysaccharides and glycoproteins which could interfere with the binding of lectins to immobilized glyco-conjugates but not to an immunoadsorbent. Indeed, a method (perhaps not the only one) able to purify all of the *P. coccineus* lectins is to employ an immunoaffinity technique making use of anti-lectin antibodies.

Quite a few *P. vulgaris* cultivars contain five tetrameric isolectins, E_4, E_3L, E_2L_2, EL_3 and L_4, issued from the combination of two different subunits, E and L, and it seems that it is also the case for some *P. coccineus* cultivars. However, for the latter the five isolectins are electrophoretically more separated than in the case of *P. vulgaris*. This can only arise from a more important difference between the two subunits (M_r or charge). SDS electrophoresis shows that the M_r difference between the two or three *P. coccineus* subunits is smaller than for the *P. vulgaris* subunits. The difference must then result from a greater charge difference. Besides, we have observed a certain number of cultivars supposed to be *P. coccineus* which lectin region at pH 4.5 is clearly constituted by the superposition of the five *coccineus* bands and the five *vulgaris* bands (data not shown). Hybridization between *P. vulgaris* and *P. coccineus* are in progress in order to check that the electrophoretic mobility difference of the five bands in the two species is really genetic.

One of the *P. coccineus* cultivar (X 417) is characterized by the predominance of the most anodic tetramer (pH 4.5) corresponding to the tetramer L_4 of *P. vulgaris*. This cultivar could be interesting for the study of lectin biosynthesis regulation. Supposing there exists two genes coding for the two subunits, we would have a cultivar where one out of the two genes is very weakly expressed. On the other hand, supposing the two subunits are formed by the same gene but that post-transcriptional modifications give rise to the two different subunits, this cultivar could be interesting for the investigation of post-transcriptional modifications. Such a cultivar has also been observed in *P. vulgaris* [6].

There exist a few *P. vulgaris* cultivars, Pinto III for example, that contain no lectins of this type at all [2, 21]. The lectins they may contain have physico-chemical and biological properties different from the traditional ones. No such cultivar has yet been detected in *P. coccineus*.

The immunodetection technique on acrylamide gels after transfer on nitrocellulose paper (Western blots) seems very useful in determining the presence of lectins and analysing them in different cultivars. The technique is rapid and needs no previous purification. It could very likely be extended to different species, perhaps even to all legumes because of the lectin genetic, and consequently immunologic, relationship in the whole family [17–19].

The last question is about the significance of the extreme lectin heterogeneity inside a species. Does this heterogeneity have a biological or physiological importance for the lectin function in the plant? In this view it should be interesting to perform genetic studies and to compare lectinic genomic DNA of the different cultivars and species.

EXPERIMENTAL

Seed samples. Phaseolus coccineus seeds were kindly provided by Dr. R. Marechal (Faculté des sciences agronomiques de l'Etat, Phytotechnie des régions chaudes, B-5800 Gembloux), except for 'alubia' provided by Dr. J. L. Ochoa (Centro de Investigaciones biologicas de baja California, La Paz, Mexico).

Protein extract. Total proteins were extracted from cotyledon flour in 10 vol. 1% NaCl, stirred for 10 min at 4° and centrifuged at 15 000 rpm for 30 min; the supernatant was then dialysed and lyophilized.

Hemagglutination. Erythroagglutination was performed following the Salk method [22] using the Cooke Engineering Company microdilution material. Total seed protein extracts were diluted in PBS pH 7.4 at a concn of 10 mg/ml.

Affinity chromatography. A thyroglobulin-Sepharose 4B column (2.5 × 40 cm) was prepared as in ref. [15], the lectin elution buffer being 0.05 M gly–HCl pH 3 containing 0.5 M NaCl. A second column (3 × 40 cm) which adsorbent consisted of pig red cell membrane ghosts (stroma) was prepared according to the method described in ref. [13]. Elution of the lectins was performed with H_2O and 0.2 M gly–HCl pH 2.5. A third affinity column (1.5 × 25 cm) was prepared as in ref. [23] with antibodies against the E_2L_2 isolectin of *P. vulgaris* cv. Contender as a ligand. The lectins were eluted with 0.2 M gly–HCl pH 2.5. Purification of the lectins was performed at 4°. After elution the lectins were dialysed against distilled H_2O and lyophilized.

Electrophoresis. Non-denaturing polyacrylamide gel electrophoresis (PAGE) was adapted from the method described in ref. [24] in 7.5% polyacrylamide gel, and the samples were dissolved in 20% sucrose, 5% NaCl at a concn of 0.2% for purified lectins and 2% for total proteins. Denaturing PAGE in the presence of SDS was done following the method of ref. [25] in 17% polyacrylamide gels with 0.1% SDS. Samples were dissolved in 2.5% SDS and 1% mercaptoethanol at a concn of 0.1% for purified lectins and 0.5% for total proteins, and denatured at 100° during 20 min.

Electrophoretic transfer on nitrocellulose paper (Western blot). After the first migration (PAGE pH 4.5) where the seed extracts are diluted 32 times from a 2% sample, the polyacrylamide gel and the nitrocellulose paper were put in contact and squashed between blotting paper, aluminium sheets as the electrodes, and two glass plates. Migration for the transfer proceeds from the anode to the cathode, the nitrocellulose paper being placed between the gel and the cathode, and lasts 2 hr at 150 mA, the buffer impregnating the blotting paper consisting of 0.7 M β-ala-AcOH (pH 4.5), containing 25% MeOH. Following the migration, the nitrocellulose paper can be kept in the same buffer before being used for the immunological reaction.

Immunological labelling on nitrocellulose paper. The nitrocellulose paper was first washed for 5 min in 10 mM NaOAc, 0.1 M NaCl, pH 4.5, and then in the same buffer containing 1% BSA during 30 min. It was washed again for 5 min in 1% PBS-BSA containing 0.5% Tween 20 (PBT). Rabbit antibodies against the *P. vulgaris* cv. Contender E_2L_2 isolectin [14] were dissolved in this last soln at a dilution of 1/500 considering that the stock IgG concn corresponded to $E_{280} = 30$. The incubation took place during 30 min (0.05 ml IgG/cm^2 paper) and was followed by two washings with PBT (2 × 10 min). A second incubation with goat antibodies against rabbit IgG coupled with alkaline phosphatase (Sigma 825) (2 μl/ml PBT) was performed during 30 min, and again followed by two washings with PBT (2 × 15 min). Finally the nitrocellulose paper was incubated for 5 min in a 33 mM Tris–HCl pH 9.5 buffer and the lectins were revealed after incubation during a few min in the same buffer (50 ml) containing 300 mg Na-α-naphthylphosphate and 50 mg fast red TR-salt (Serva 21314). The nitrocellulose paper was then rinsed in H_2O. Controls were performed using IgG from pre-immunized animals.

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