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PURIFICATION OF A LECTIN FROM *PARKIA JAVANICA* BEANS

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Key Word Index—*Parkia javanica*; Leguminosae; beans; lectin; hemagglutination.

Abstract—A lectin was extracted from *Parkia javanica* beans and purified by chromatography. The purified lectin showed two forms of proteins, one major and one faint band, both in non-denaturing PAGE and SDS–PAGE. Both of them appeared to be single polypeptide chains with M_r, determined by SDS–PAGE, of 47 900 and 45 700. The purified lectin could agglutinate red blood cells of rabbit (68 267 unit mg^{-1} protein) and rat (267 unit mg^{-1} protein), but could not agglutinate red cells of human, sheep or goose. Its hemagglutinating activity was completely inhibited by methyl-α-D-mannosamine and mannose at 5 mM. Ca^{2+}, Mn^{2+} and Mg^{2+}, but neither EDTA nor EGTA, were effective activators of the purified lectin. The $K_{0.5}$ of Ca^{2+}, Mn^{2+} and Mg^{2+} was 5, 17 and 13 mM, respectively. The optimal pH for hemagglutination was 7. The purified lectin was stable in pH 7–10 but labile at temperatures over 50°.

INTRODUCTION

Lectins are carbohydrate-binding proteins bearing at least two binding sites which allow them to precipitate glycoconjugates and/or agglutinate cells [1–3]. Owing to these properties, they are widely used in biochemical and cellular studies [4–7]. Lectins, first identified in extracts of plant seeds, show selectivity in their agglutination of erythrocytes of different animal species, some showing human blood-type specificity [8]. Lectins are found in plants, mostly in leguminous seeds. They are localized in the cotyledons of the seeds, with a particularly high concentration during the resting state. Inside the cells, lectins are found in the protein bodies [9] sharing this location with the storage proteins [10, 11]. Most commercial lectins are isolated from plants found in the West, whereas plants in the tropics have not been adequately explored. Therefore, we have screened tropical plants in the south of Thailand for new lectins [12]. Beans of the legumes, *Parkia javanica*, have been found to possess high agglutinating activity for rabbit red blood cells and for both red cells and spermatozoa of rat [13]. Purification and some properties of the hemagglutinin from *Parkia javanica* beans are presented here.

RESULTS AND DISCUSSION

Extraction of lectin

Effective extraction of lectin from *Parkia* beans was achieved by homogenization in buffer and stirring at 4° for 12 hr. Stirring for 15 hr or longer caused a gradual decrease in the hemagglutination, suggesting that degradation of lectin occurred. Removal of lipid from the crude extract by petrol resulted in an increased hemagglutinating activity (376 unit mg^{-1} protein), about 1.4 times greater than that of the untreated fraction (271 unit mg^{-1} protein). Most of the lectin in the petrol extract was precipitated at 60% saturation of ammonium sulphate for 6 hr (Table 1). The amount of total protein in the extracted solution varied with the age of the beans used for extraction. Germination of *Parkia* beans for 2–7 days resulted in a decrease of total protein, indicating that proteins were utilized for germination. In comparison, specific hemagglutinating activity of the bean lectin increased as germination was prolonged for 7 days. The protein pattern in SDS–PAGE of the lectin extracted from the beans germinated for 1–7 days, revealed that the amount of lectin did not change in comparison with other proteins which showed degradation. Lectins, at the time of germination, have been reported to be much more resistant to degradation than storage proteins both *in vivo* and *in vitro* [14]. In wheat grains the lectin was restricted to the germ and its level remained high after germination for at least 34 days [15]. *Parkia* beans allowed to germinate for 7 days were suitable for hemagglutination studies.

Purification of lectin

Purification of lectin from the *Parkia* extract was carried out by column chromatography. After passing the precipitated fraction through a DEAE–cellulose column, the hemagglutinating activity was eluted by NaCl in one peak (Fig. 1). When this active peak was further purified by Sephadex G-100 column, the activity was retarded and eluted by 0.1 M maltose (Fig. 2). This adsorption was also reported in the isolation of *Parkia speciosa* lectin [16, 17]. The hemagglutinating activity of the active peak from

Table 1. Purification of lectin from *Parkia javanica* beans

Steps	Hemagglutination				
	Total protein (mg)	Specific activity (unit mg^{-1} protein)	Total activity ($\times 10^3$ unit)	Yield (%)	Purification (-fold)
Homogenate	6330	271	1720	100	1
Petrol extraction	2430	376	913	53	1
60% $(NH_4)_2SO_4$ pptn	1410	545	769	45	2
DEAE-cellulose	183	2630	480	28	10
Sephadex G-100	3	10 500	32	2	39
D-Mannose agarose	1	21 200	24	2	78

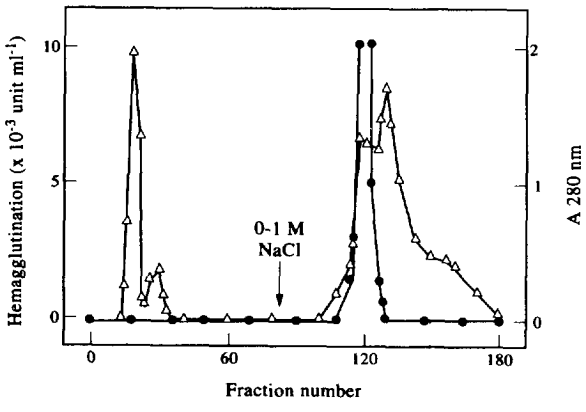

Fig. 1. DEAE-cellulose profile of lectin extract from *Parkia javanica* beans. ●—●, Hemagglutination; △—△, $A_{280\,nm}$.

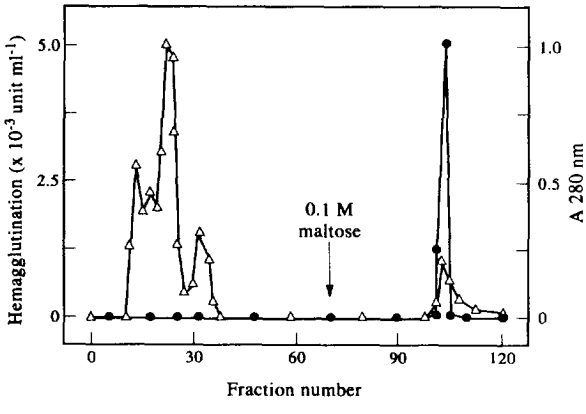

Fig. 2. Purification of active fractions from the DEAE-cellulose column on Sephadex G-100. ●—●, Hemagglutination; △—△, $A_{280\,nm}$.

Sephadex G-100 column was effectively inhibited by mannose, suggesting that the agglutinating factor was probably a lectin specific for mannose. For this reason, affinity on a column of D-mannose agarose was used in the final purification step of lectin preparation. By a combination of D-mannose agarose and elution with

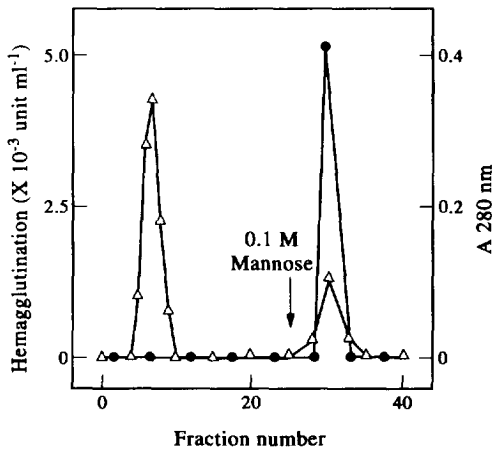

Fig. 3. Chromatography on D-mannose agarose of the active fractions from the gel filtration chromatography. ●—●, Hemagglutination; △—△, $A_{280\,nm}$.

0.1 M D-mannose, a single and symmetrical protein peak was observed (Fig. 3). During the purification, the active fractions were subjected to SDS-PAGE to monitor the improvement of purity. Figure 4 illustrates that chromatography on DEAE-cellulose and Sephadex G-100 columns effectively removed other proteins from the lectin fraction. The active peak from the final D-mannose agarose column showed one major protein band and one faint band in SDS-PAGE (Fig. 4). A similar protein pattern of the same active peak was also shown in non-denaturing PAGE. This observation indicated that the purified lectin was probably homogeneous. Table 1 demonstrates the results of lectin isolated utilizing 370 g of the beans. The lectin was purified 78-fold with a yield of 2% of the crude extract. Specific hemagglutinating activity of the purified lectin for rabbit red blood cells was 21 200 unit mg^{-1} protein.

Properties of the purified lectin

The lectin purified from *Parkia* beans showed one major protein band and a faint one in SDS-PAGE with M_r of 47 900 and 45 700, respectively (Fig. 4, lane 6). The same electrophoretic pattern was also observed in the

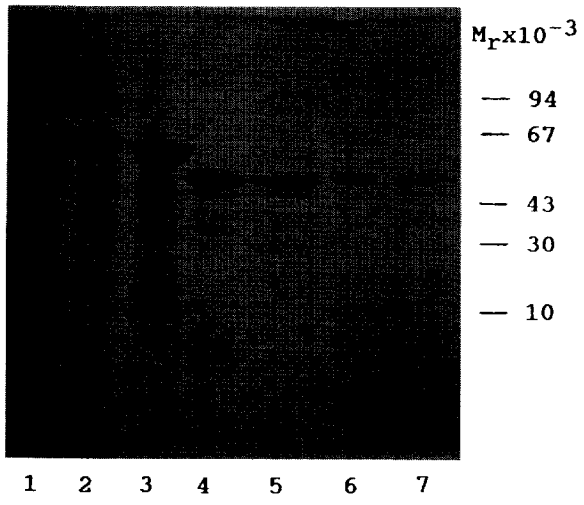

Fig. 4. SDS–PAGE of lectin from *Parkia javanica* beans at various stages of purification. 1, Crude extract; 2, petrol extraction; 3, 60% $(NH_4)_2SO_4$ precipitation; 4, DEAE–cellulose; 5, Sephadex G-100; 6, D-mannose agarose; 7, D-mannose agarose without reduction by 2-mercaptoethanol.

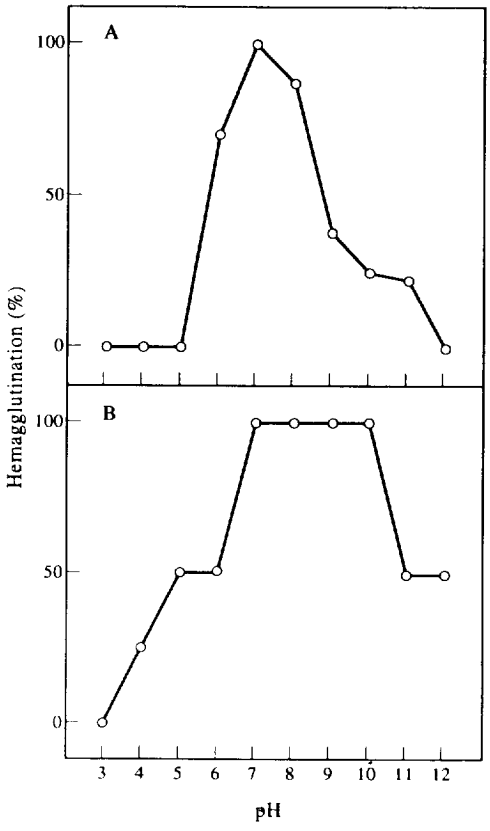

Fig. 5. pH profile of the purified *Parkia javanica* lectin. (A) pH dependences; (B) pH stability.

absence of the sulphydryl reagent, 2-mercaptoethanol (Fig. 4, lane 7). When the purified lectin was subjected to non-denaturing PAGE, a similar protein pattern composed of two protein bands was observed. The M_r of the two bands estimated from non-denaturing PAGE was 43 000 (the major band) and 39 000 (the faint band). These M_r values differed slightly from those estimated by SDS–PAGE because their separation in non-denaturing PAGE depended on net charge, shape and size of the proteins. Each of the two protein bands were glycoproteins as indicated by periodic acid–Schiff's staining and being adsorbed by Sephadex G-100 and D-mannose agarose during purification steps. Thus, the lectin purified from *Parkia javanica* beans probably consisted of two forms of proteins in unequal amounts. Both of them were monomeric structures consisting of single polypeptide chains. A limited number of lectins, including *Parkia speciosa* lectin, with a single polypeptide have been reported [16, 18–20] whereas most of the lectins studied to date have at least two subunits or more [21–23]. Lectins consisting of two or more forms have been found in taro [24] and garden pea [25].

Hemagglutinating activity

The purified lectin could mediate agglutination of red blood cells of rabbit (68 267 unit mg^{-1} protein) more effectively than those of rat (267 unit mg^{-1} protein), while no hemagglutination of human, sheep or goose red cells was obtained. Trypsin treatment of rabbit red cells prior to the hemagglutination test increased the specific hemagglutinating activity of *Parkia* lectin up to 64-fold. Preincubation of human red cells with trypsin did not facilitate hemagglutination. Concanavalin A can agglutinate human red cells and its activity increased when preincubation of the cells with trypsin was performed [24]. Moreover, the hemagglutinating activity of the purified lectin was activated by divalent cations. The $K_{0.5}$ of Ca^{2+}, Mn^{2+} and Mg^{2+} was 5, 17 and 13 mM, respectively. Chelating agents such as EDTA and EGTA at 40 mM had no influence on the activity. In addition, the hemagglutinating activity of *Parkia* lectin rapidly declined when the lectin was pre-heated to over 50°. Activity was reduced to one half at 55° and was completely lost at 70°, indicating that its activity depends on the native protein conformation of the lectin. This finding suggests that the activity is related to cations in the lectin, like metal ions in concavanalin A which protect it from proteolytic and temperature degradation [26–28]. Since metal ions were found to bind strongly to lectins, treatment with EDTA failed to remove cations [29, 30].

Similarly to the hemagglutinating activity of lectin from *Artocarpus integer* seeds [31], that of the lectin purified from *Parkia javanica* beans depended on pH. The optimal value was pH 7 (Fig. 5A). The lectin was stable in the pH range from 7 to 10 (Fig. 5B). More acidic or basic pHs decrease both stability and activity.

Several sugars were found to bind specifically with the purified lectin and inhibited its hemagglutinating activity. Both methyl-α-D-mannosamine and mannose were effective inhibitors, with the same concentration (5 mM) causing 100% inhibition of hemagglutination (Table 2). Malt-

Table 2. Minimal concentrations of sugars inhibiting hemagglutination 100% at the score of 2

Sugars	Concentration (mM)
Methyl-α-D-mannosamine	5
Mannose	5
Maltose	10
Glucose	50
N-Acetyl glucosamine	100
Fructose	NI
Saccharose	NI
Galactose	NI*
N-Acetyl-D-mannosamine	NI*
N-Acetyl galactosamine	NI*

NI = Inhibition less than 100% at 200 mM.
NI* = No inhibition at 200 mM.

ose (10 mM), glucose (50 mM) and *N*-acetyl glucosamine (100 mM) were less potent inhibitors. It is interesting that sugars which bind to lectin specifically contain mannose in their molecules similar to those that bind with *Lens culinaris*, concanavalin A [32] and *Parkia speciosa* lectin [16]. Fructose and saccharose showed less than 100% inhibition of hemagglutination at 200 mM, whereas galactose, *N*-acetyl-D-mannosamine and *N*-acetyl galactosamine showed no inhibitory effect at 200 mM (Table 2).

EXPERIMENTAL

Chemicals. DEAE–cellulose, Sephadex G-100, D-mannose agarose, D-mannose, methyl-α-D-mannosamine, *N*-acetyl glucosamine, maltose, *N*-acetyl galactosamine, *N*-acetyl-D-mannosamine, trypsin and soybean trypsin inhibitor were purchased from Sigma.

Preparation of lectin extract from Parkia beans. Extraction of lectin from *Parkia* beans was performed at 4° according to the method of ref. [13]. Ungerminated *Parkia* beans were allowed to germinate for 7 days. The beans were washed, chopped and homogenized in 3 times their vol. of buffer A (5 mM K-Pi buffer, pH 7.4, 0.9% NaCl), using a Waring blender. The suspension was stirred for 12 hr and then filtered through cheesecloth. After centrifugation of the filtrate at 2000 *g* for 15 min, the supernatant was extracted with an equal vol. of cold petrol twice at room temp. and subsequently allowed to stand for separation at −20°. The aq. phase was spun at 2000 *g* for 15 min and then pptd with 60% satd $(NH_4)_2SO_4$ for 6 hr. After centrifugation at 22 500 *g* for 30 min, the pellet was resuspended in buffer A and dialysed overnight against the same buffer before further purification.

Purification of lectin. All chromatographic steps were conducted at 4°. The lectin extract was dialysed against buffer B (5 mM K-Pi buffer, pH 7.4) and then loaded onto a column (11 × 2.6 cm) of DEAE–cellulose equilibrated with the same buffer. After washing with buffer B, the bound proteins were eluted from the column by a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 40 ml hr^{-1} (Fig. 1). Fractions (3 ml each) containing the highest hemagglutinating activity were pooled, concd and dialysed against buffer A. The dialysate was then chromatographed on a Sephadex G-100 column (80 × 1.4 cm) equilibrated with buffer A. The column was washed with 200 ml of this buffer before elution with 0.1 M maltose at a flow rate of 30 ml hr^{-1} (Fig. 2). Active fractions (3 ml each) were pooled, concd, dialysed against buffer A, and subsequently loaded onto a 2 ml D-mannose agarose column, equilibrated with buffer A. After washing with buffer A (50 ml), the bound lectin was eluted with 0.1 M D-mannose at a flow rate of 10 ml hr^{-1} and sample fractions of 1 ml each were collected (Fig. 3).

Gel electrophoresis. Non-denaturing PAGE was carried out on 6–9% slab gel using the method of ref. [33]. SDS–PAGE was monitored according to the method of ref. [34] using a slab gel with a linear gradient of 8–12% polyacrylamide. Protein bands were stained with Coomassie Brilliant Blue R-250 dye. The M_r calibration in non-denaturing PAGE was performed according to the procedure described in Sigma Tech. Bulletin No. MRK-137 (10–86).

Preparation of red blood cells. Whole blood was freshly collected from rabbit and immediately mixed with 5 mM EDTA (blood:EDTA = 5:1). After centrifugation at 300 *g* for 10 min at 4°, the packed red cells were washed with buffer A twice. The pellet was resuspended in the same buffer and made up to 2% suspension of red blood cells.

Hemagglutination test. Lectin soln (50 μl) was incubated with an equal vol. of 2% red blood cells in a well of a microtiter plate at room temp. for 1–2 hr. Positive hemagglutination was obtained when the red cells did not sediment to the bottom of the well forming a red button. Agglutinating activity (unit) was determined using 2-fold serial dilution of lectin [35]. The titer was the highest dilution that still caused agglutination. The activity was the reciprocal of the titer. Specific agglutinating activity was the activity per mg of protein.

Sugar inhibition test. The purified lectin was diluted with buffer A to the concn that caused hemagglutination of rabbit red cells at the score of 2. The test was repeated using the diluted lectin in the presence of various concns of a test sugar (25 μl in buffer A) or in the presence of 25 μl of buffer A (control). The minimal concn of sugar that fully inhibited hemagglutination was recorded.

Heat stability test. Portions of the purified lectin were heated for 20 min at 30–80°. The heated soln was rapidly cooled in ice and assayed for agglutinating activity in comparison with the control which was kept at −20°. Results were expressed as percentage of the control without heating.

pH *stability test.* The purified lectin was adjusted to different pH values from 3 to 12, by adding 40 μl of Universal buffer (Britton and Robinson type) which was prepared according to ref. [36]. An aliquot of the mixture was taken for measurement of the final pH using pH paper. After being left for 1 hr at room temp., the samples were adjusted back to pH 7.4 with 30 μl of 0.5 M

Tris–HCl, pH 7.7, and assayed for agglutinating activity. Results were expressed as percentage of the control, which was the lectin soln diluted with buffer A instead of the Universal buffer and left standing for 1 hr at room temp.

Effect of pH. An aliquot (5 μl) of the purified lectin was adjusted to different pH values, from 4 to 12, by adding 40 μl Universal buffer, and 5 μl of 20% red cell suspension was added. Hemagglutination was then recorded. The final pH of the mixture was estimated using pH paper. The results were expressed as percentage of the control, which was the agglutinating activity of lectin in buffer A at pH 7.4.

Effect of divalent cations and chelating agents. The effect of divalent cations and chelating agents on hemagglutinating activity was monitored. The activity of the purified lectin was determined in the presence of various concns of Ca^{2+}, Mn^{2+}, Mg^{2+}, EDTA or EGTA.

Trypsin digestion. One millilitre of a 10% suspension of rabbit red cells was treated with an equal vol. of 1 mg ml^{-1} trypsin at room temp. for 1 hr. The reaction was terminated by addition of 1 ml soybean trypsin inhibitor (1 mg ml^{-1}). The red cells were packed, washed in buffer A many times and then made up to 2% suspension. Hemagglutinating activity of the treated cells was determined by comparison with that of untreated cells.

Germination. Dry *Parkia* beans were immersed in H_2O overnight. The beans were buried in wet sand and allowed to germinate for 1–7 days. Germinated beans were taken and prepared for the lectin extract.

Protein concn was determined according to the method of ref. [37] using bovine serum albumin as standard.

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