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THE ISOLATION OF LECTINS ON ACID-TREATED AGAROSE

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ABSTRACT

The ability of several D-galactose and *N*-acetyl-D-galactosamine-binding lectins to bind to Sepharose was investigated. Lectins from soybean, *Wistaria floribunda*, *Bauhinia purpurea alba*, and *Sophora japonica* could be isolated by affinity chromatography on acid-treated Sepharose 6B. These lectins would not bind to untreated Sepharose 2B, 4B, and 6B. The binding of *B. purpurea alba* and *S. japonica* was temperature-dependent. The *S. japonica* lectin would bind only at high pH. *Ricinus communis* toxin also showed a temperature-dependence of binding; acid-treated Sepharose 6B was a better affinity support for the toxin than was untreated Sepharose 4B. Lectins from lima bean, *Dolichos biflorus*, and kidney-bean phytohemagglutinin did not bind to Sepharose under any of the conditions studied.

INTRODUCTION

Several D-galactose and *N*-acetyl-D-galactosamine-binding lectins have been isolated and characterized in recent years[1–4]. The methods of isolation have incuded affinity chromatography on agarose gels[1], on insolubilized blood-group-active substances[2,3], and on monosaccharide derivatives coupled to agarose gels[5]. Some of the *N*-acetyl-D-galactosamine-binding lectins are weakly inhibited by D-galactose but do not bind to agarose gels[6]. Ersson *et al.*[7] found that the D-galactose-binding lectin of sunn-hemp seeds would not bind to agarose beads until the beads were subjected to mild acid hydrolysis. The use of affinity chromatography columns for the purification of plant lectins was recently reviewed by Lis *et al.*[8]. Differences in carbohydrate-binding specificity of several D-galactose-binding lectins were recently demonstrated by Irimura *et al.*[9].

During attempts to prepare D-galactose-binding lectins by affinity chromatography on Sepharose columns according to procedures published by others, we obtained disappointing results. We therefore investigated the behavior of several lectins on columns of Sepharose 2B, 4B, 6B, and acid-treated 6B.

EXPERIMENTAL

Materials and methods. — Seeds from *Bauhinia purpurea alba*, *Sophora japonica*, *Wistaria floribunda*, and *Dolichos biflorus* were obtained from F. W. Schumacher Co., Sandwich, Mass. 02563. Lima beans of the Thorogreen variety were obtained from W. Atlee Burpee Co., Philadelphia, Pa. 19132. Kidney-bean phytohemagglutinin (PHA-P) was obtained from Difco Laboratories, Detroit, Mich. 48232. Castor (*Ricinus communis*) beans were obtained from the Charles H. Lilly Co., Portland, Oreg. 97214. Soyafluff was obtained from Central Soya, Chicago, Ill. 60639. Ground seeds and Soyafluff were extracted, in the cold, overnight, with a buffer solution of 0.15M NaCl–15mM Na_3PO_4–0.05% NaN_3, pH 7.0 (PBS) at a ratio of 100 g per liter of PBS. The extracts were centrifuged and subjected to ammonium sulfate fractionation. The following precipitating fractions were used after dialysis for affinity chromatography: *B. purpurea alba*, 0.4–0.7 saturation[10]; *S. japonica*[6], 0.3–0.6; *W. floribunda*[11], 0.7–1.0; *D. biflorus*, 0–0.8; lima bean[12], 0.4–0.6; *R. communis*[1] 0.3–0.6; and Soyafluff[5], 0.4–0.8. In some cases, the dialyzed fraction was lyophilized before use.

Sepharose 2B, 4B, and 6B were products of Pharmacia Fine Chemicals, Inc. Piscataway, N.J. 08854. Acid-treated Sepharose 6B was prepared according to Ersson *et al.*[7] by washing the gel with cold 0.2M HCl and adding the acid-washed gel (50 ml) to 0.2M HCl (100 ml). This suspension was placed into a gently shaking water bath at 50° for 2 or 3.5 h. The gel was washed with PBS and was ready for use. Unless otherwise indicated, PBS was used for all column experiments.

Hemagglutination assays were performed with PBS-washed rabbit erythrocytes, and human type A and B erythrocytes. Blood was collected in tubes containing ethylenediaminetetraacetate rather than heparin because heparinized blood often gave variable and ambiguous results. Assays were set up in microtiter "U" plates at room temperature or 37° and incubated for 15 min to 24 h.

Analytical disc electrophoresis was performed with 7.5% poly(acrylamide) gels. The lectin (100 μg) in 10% sucrose–5mM Tris–38mM glycine (50 μl pH 8.3), was deposited on the gels and an electrical current at 3 mA/gel was applied for 1 h at 10° with the just described Tris–glycine buffer in the reservoir. Gels were stained with Coomassie Blue for protein and with the periodic acid–Schiff reagent for carbohydrate.

Electrophoresis in 0.1% of sodium dodecylsulfate was performed in 7.5% poly(acrylamide) gels. The lectin (4.0 mg) was dissolved in 2% sodium dodecyl sulfate–100mM Na_3PO_4 (pH 7.0, 200 μl). The sample was heated for 2 h at 60°, and then diluted with buffer to give a concentration of 0.1% of sodium dodecyl sulfate; 25–100 μl of sample were applied to the gels. The electrophoresis was performed at room temperature at 2 mA/gel for 20 min, followed by 8 mA/gel for 3 h with 100mM Na_3PO_4 (pH 7.0) buffer in the reservoir.

Analytical gel electrophoresis at pH 4.5 was performed in 7.5% poly(acrylamide) gels with the buffer system of Reisfeld *et al.*[13] for 1 h at room temperature and 6 mA/gel.

Chromatography columns having water jackets, obtained from Pharmacia Fine Chemicals, Inc., were used to study the effects of temperature upon the affinity chromatography of some of the lectins.

RESULTS

The results of our affinity chromatography experiments are summarized in Table I. After electrophoresis of the various lectins in the presence of sodium dodecyl sulfate, all bands that stained with Coomassie Blue also stained with the periodic acid–Schiff reagent, indicating the presence of carbohydrate in all polypeptide components detected.

TABLE I

RESULTS OF AFFINITY CHROMATOGRAPHY OF LECTINS ON VARIOUS SEPHAROSE ADSORBENTS

Lectin	*Temp.* (°)	*Sepharose type*				*Yield* (%)[b]
		2B	*4B*	*6B*	*AT-6B*[a]	
B. purpurea	24	–[c]	–	–	–	
	11				–	
	4	–	–	–	+[d]	8
R. communis$_{I}$[e]	24		+		+	
	9		+			
R. communis$_{II}$[e]	24		–		+	
	9		–			
S. japonica	24	–	–	–	–	
	11				+	7
	4	–	–	–	+	7
Soybean	24	–	–	–	+	10
	11				+	10
	4	–	–	–	+	10
W. floribunda	24	–	–	–	+	15
	11				+	15
	4	–	–	–	+	15
PHA, lima bean, and *D. biflorus*	24	–	–	–	–	
	4	–	–	–	–	

[a]Acid-treated Sepharose 6B. [b]Relative to the load on the column (w/w). [c]No binding or weak binding of hemagglutinating activity. [d]Tight binding of hemagglutinating activity. [e]The combined yield for $RCA_I + RCA_{II}$ at 9° on Sepharose 4B was 36%; at 24° on Sepharose AT-6B, it was 36% of column load.

Affinity chromatography of the lectin of B. purpurea alba. — Attempts to isolate the lectin from *Bauhinia* on columns of Sepharose 6B, 4B, and 2B were unsuccessful. Columns of Sepharose 6B, which had been acid-treated for 2 h, were prepared for affinity chromatography. These columns provided weak adsorbents for the lectin. Hemagglutinating activity was detected in the protein peak obtained by washing the

column with PBS; the remaining hemagglutinating activity was slowly eluted from the column during washing with PBS. Sepharose 6B, which had been acid-treated for 3.5 h, provided a better affinity adsorbent. A significant hemagglutinating activity was still detected in the nonadsorbed protein peak, however. The residual, bound lectin could be eluted with 0.2M D-galactose. Lowering the column temperature from 24° to 11° resulted in a tighter binding of the lectin; the lectin was eluted from the column more slowly with PBS, and a greater yield of lectin was obtained in the peak eluted with D-galactose. Lowering the column temperature to 4° resulted in a doubling of the yield of lectin. Some hemagglutinating activity was still present in the non-adsorbed fractions, accounting for 5–10% of the hemagglutinating activity present in the original material. The results of one experiment is shown in Fig. 1. The purity of the lectin was analyzed by gel electrophoresis, and the results are shown in Figs. 5 and 8.

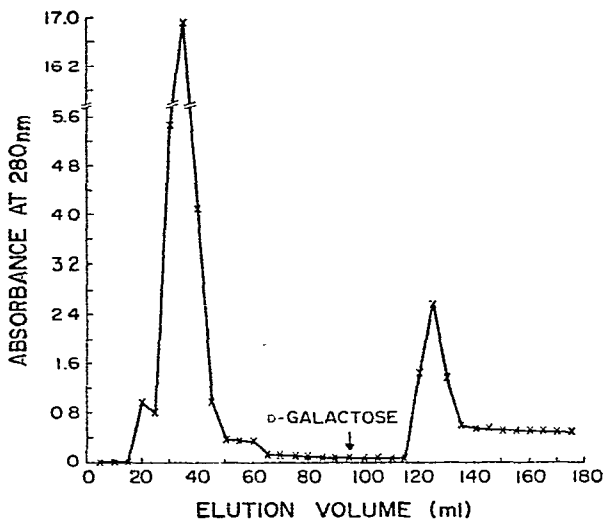

Fig. 1. Affinity chromatography of *B. purpurea* lectin on Sepharose 6B treated with acid for 3.5 h. Crude lectin (200 mg) in PBS (4.7 ml) was applied to a column (1.6 × 16.5 cm) at 4°. The column was washed with PBS and at the point indicated elution was started with PBS–0.4M D-galactose. Flow rate was 3.6 ml/h.

Affinity chromatography of the lectin of R. communis. — Isolation by affinity chromatography of *Ricinus* lectin (RCA_I) and *Ricinus* toxin (RCA_{II}) was performed initially on Sepharose 4B at room temperature. Whereas RCA_I bound to the column and could be eluted with 0.1M lactose, the RCA_{II} toxic lectin bound weakly and was detected in all the fractions obtained by washing the column with phosphate buffer. Lowering the column temperature to 9° resulted in separating the toxin from the major non-binding protein-fraction. Continuous washing with PBS slowly eluted the toxin. The RCA_I lectin bound tightly to the column at 9°, as expected (see Fig. 2).

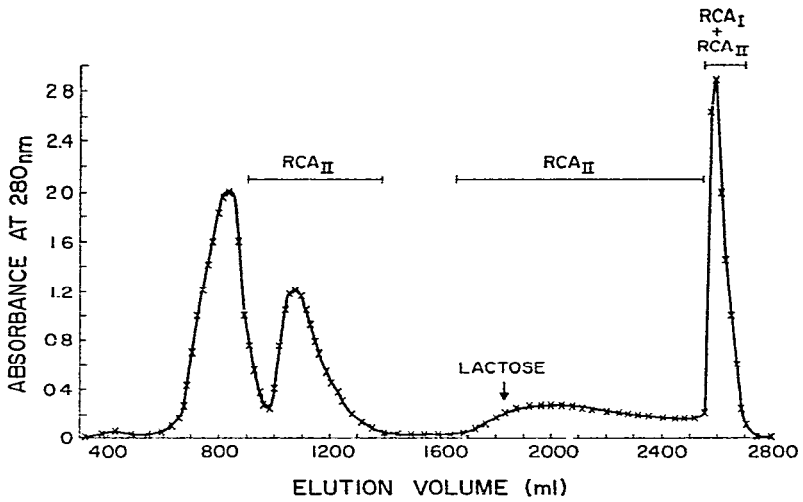

Fig. 2. Affinity chromatography of *R. communis* lectins on Sepharose 4B. Crude lectin (1300 mg) in PBS (98 ml) was applied to a column (5.0 × 41.0 cm) at 9°. The column was washed with PBS and at the point indicated elution was started with PBS–0.2M lactose. Flow rate was 25.1 ml/h.

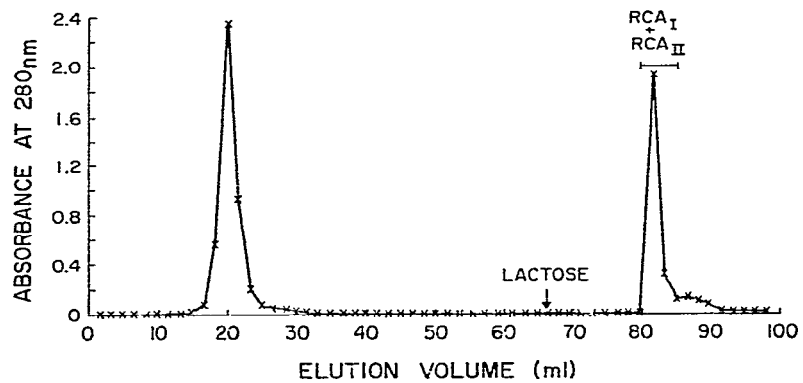

Fig. 3. Affinity chromatography of *R. communis* lectins on Sepharose 6B treated with acid for 2 h. Crude lectin (12.7 mg) in PBS (0.85 ml) was applied to a column (0.9 × 28.0 cm) at 24°. The column was eluted with 0.1M lactose. Flow rate was 2.9 ml/h. Fractions containing RCA_I and RCA_{II} are indicated.

Chromatography of the *Ricinus* lectins at 24° on Sepharose 6B that had been treated with acid for 2 h resulted in a good binding of both RCA_I and RCA_{II}. No hemagglutinating activity was detected in the preliminary fractions. Both lectins were co-eluted with 0.1M lactose (see Fig. 3) and were subsequently separated by gel filtration[1] on Sephadex G-150. The position of elution of RCA_I and RCA_{II} was determined by pooling the fractions showing hemagglutinating activity and by subjecting these

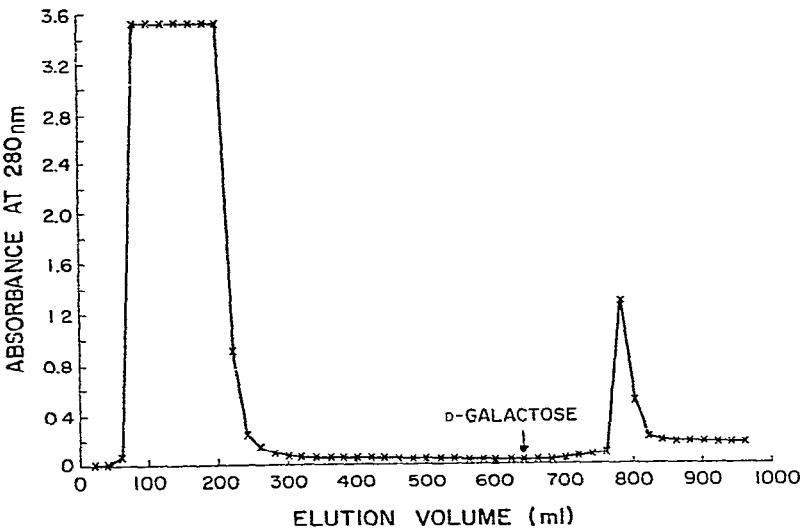

Fig. 4. Affinity chromatography of *S. japonica* lectin on Sepharose 6B treated with acid for 2 h. Crude lectin (500 mg) in 0.05M NaCl–0.05M Tris, (21 ml, pH 8.7) was applied to a column (2.6 × 34.0 cm) at 4°. The column was washed with the NaCl–Tris buffer, and at the point indicated elution was started with NaCl–Tris–0.2M D-galactose. Flow rate was 13.3 ml/h.

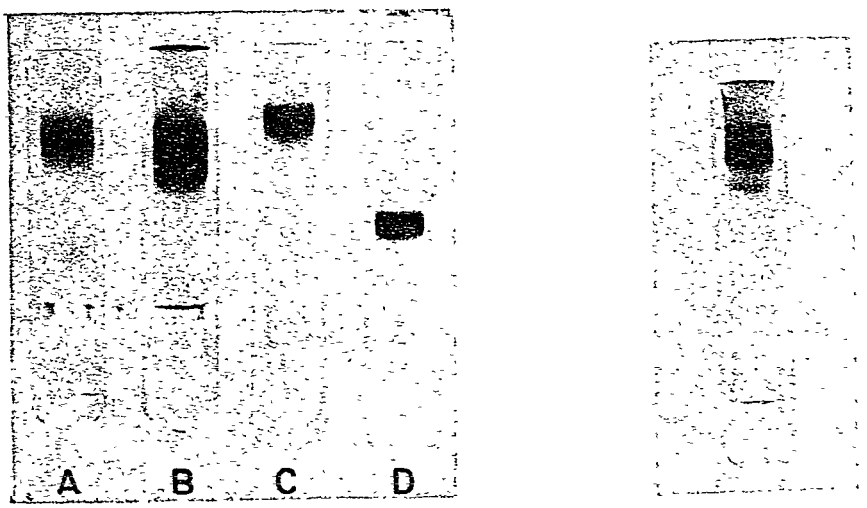

Fig. 5. Poly(acrylamide)-gel electrophoresis of lectins at pH 8.3 performed as described in Methods. The gels were stained with Coomassie Blue for protein: A, *B. purpurea*; B, *S. japonica*; C. soybean; and D, *W. floribunda*.

Fig. 6. Poly(acrylamide)-gel electrophoresis of *S. japonica* lectin at pH 4.5 performed as described in Methods. The gel was stained with Coomassie Blue for protein.

fractions to gel filtration on Sephadex G-150. The effluent fractions were analyzed for protein and for hemagglutinating activity. The hemagglutinin having the lower molecular-weight was assumed to be (without proof) the toxic lectin[1], RCA_{II}.

Affinity chromatography of the lectin of S. japonica. — The hemagglutinating activity from *Sophora* failed to bind to columns of Sepharose 6B, 4B, 2B, and acid-treated 6B at room temperature with PBS as the column buffer. When the column buffer was changed to 0.15M NaCl–0.05M Tris (pH 8.7), the hemagglutinating activity was weakly adsorbed at room temperature to Sepharose 6B that had been treated with acid for 2 h. Operation of the column under these conditions at 11° gave very good results. No detectable hemagglutinating activity was eluted with the buffer; the lectin, however, could be eluted with 0.2M D-galactose. The results of one experiment are presented in Fig. 4. The peak eluted with D-galactose showed the presence of two protein bands when examined by gel electrophoresis (Fig. 5). When the electrophoresis was performed at pH 4.5, three protein bands were observed (Fig. 6). Electrophoresis in the presence of sodium dodecyl sulfate showed a major protein band and some minor components (Fig. 8).

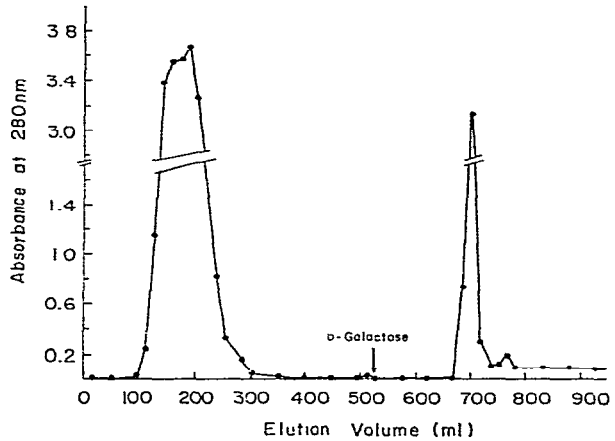

Fig. 7. Affinity chromatography of soybean lectin on Sepharose 6B treated with acid for 2 h. Crude lectin (538 mg) in PBS (43 ml) was applied to a column (2.6 × 36.5 cm) at 24°. The column was washed with PBS and at the point indicated elution was started with PBS–0.2M D-galactose. Flow rate was 17 ml/h.

Affinity chromatography of soybean lectins. — The hemagglutinating activity present in Soyafluff did not bind to columns of Sepharose 6B, 4B, or 2B at room temperature nor at 4°. Chromatography on Sepharose 6B that had been treated with acid for 2 h resulted in complete binding of the hemagglutinating activity at room temperature, 11°, and 4°. The bound lectin was eluted with 0.2M D-galactose (see Fig. 7). Electrophoresis of the D-galactose-eluted peak showed the presence of one major and one minor protein band (Fig. 5). Electrophoresis in the presence of sodium dodecyl sulfate showed a single protein band (Fig. 8). When increasing amounts were

applied to the affinity column, 2 minor protein peaks eluted with D-galactose appeared after the major lectin fraction. These minor components may represent isolectins.

Affinity chromatography of the lectin of W. floribunda. — In addition to a weakly agglutinating mitogen (mol. wt. 70,000), *Wistaria* contains[11] a hemagglutinin (mol. wt. 136,000) inhibited by D-galactose. Attempts to isolate this lectin by affinity chromatography on Sepharose 6B, 4B, and 2B were not successful. All hemag-

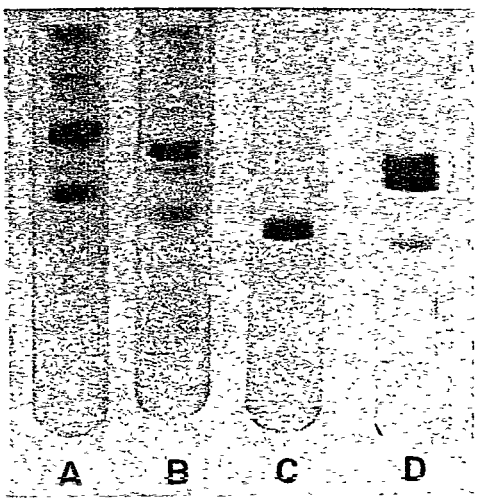

Fig. 8. Poly(acrylamide)-gel electrophoresis of lectins in sodium dodecyl sulfate performed as described in Methods. The gels were stained with Coomassie Blue for protein: A, *B. purpurea*; B, *S. japonica*; C, soybean; and D, *W. floribunda*.

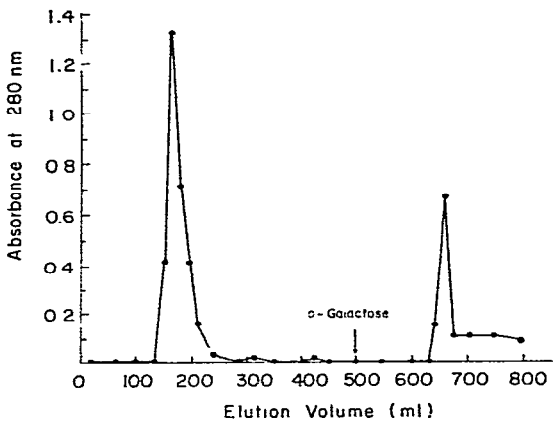

Fig. 9. Affinity chromatography of *W. floribunda* lectin on Sepharose 6B treated with acid for 2 h. Crude lectin (72 mg) in PBS (8.3 ml) was applied to a column (2.6 × 35 cm) at 24°. The column was washed with PBS and at the point indicated elution was started with PBS–0.2M D-galactose. Flow rate was 15 ml/h.

glutinating activity was washed off the columns with PBS. When chromatography was performed on a column of Sepharose 6B that had been treated with acid for 2 h, all hemagglutinating activity remained bound to the column at room temperature, 11°, and 4°. The lectin was eluted with 0.2M D-galactose (see Fig. 9). Gel electrophoresis in the presence or absence of sodium dodecyl sulfate showed the presence of a single major protein component in the lectin fraction (Figs. 5 and 8). Gel filtration on Sephadex G-150 of the D-galactose-eluted protein showed the presence of a single protein peak having a mol. wt. ~140,000.

Affinity chromatography of the lectins of PHA, lima bean, and D. biflorus. — All efforts to isolate these lectins on Sepharose columns were unsuccessful.

DISCUSSION

Lectins have come into wide use in recent years to study the carbohydrate structures of cell surfaces and isolated glycoproteins[14,15]. The value of lectins in such studies warrants the development of methods for their isolation. The studies reported here have resulted in a very simple procedure for the isolation of soybean agglutinin. Previous procedures required the preparation of affinity columns by synthetic means[5,16]. Contaminating isolectins may be separated from the major lectin by ion-exchange chromatography, as reported by others[16,17]. Alternatively, our data indicate that under appropriate conditions it may be possible to separate the isolectins on acid-treated Sepharose 6B by taking advantage of differential affinities for the acid-treated gel.

Poretz *et al.*[2] recently described a procedure for the isolation of the *S. japonica* lectin by adsorption to crosslinked, blood group-active substance. In agreement with these workers, we also found that the hemagglutinating activity of this lectin was maximum at pH 8.5 and above. After failing to observe a binding of the lectin to acid-treated Sepharose 6B at pH 7.0, we equilibrated the column with a pH 8.7 buffer. The importance of pH is reflected in the binding of the lectin to the affinity column at pH 8.7 but not at pH 7.0. As did Poretz *et al.*[2], we also found evidence of lectin aggregation when the lectin was submitted to electrophoresis. However, the presence of isolectins could not be ruled out. Since obtaining the results reported here, we have examined other fractions of the seed extract resulting from ammonium sulfate fractionation. About 30% of the total hemagglutinating activity of the extracts was present in the fraction precipitating at an $(NH_4)_2SO_4$ saturation of 0.6–0.8. This activity had a pH optimum of 8.5–9.0 for hemagglutination and it could be isolated by the same method as that reported here for the hemagglutinating activity precipitating at an $(NH_4)_2SO_4$ saturation of 0.3–0.6. Hence, by selecting a seed-extract fraction that precipitates at an $(NH_4)_2SO_4$ saturation of 0.3–0.8, it is possible to obtain an increase in the yield of lectin of 30% (when compared to the yield given in Table I) upon affinity chromatography. Terao and Osawa[19] have previously reported the isolation of lectins from *S. japonica* by affinity chromatography on Sepharose 6B. The relationship of their three active fractions to the lectin reported here is not clear.

In contrast to *S. japonica*, all the other lectins examined had maximum hemagglutinating activity at pH 7.0–7.5, including the PHA, lima bean, and *D. biflorus* lectins. In some respects, our results are in contrast to those reported by others. Irimura and Osawa[10] reported the isolation of *Bauhinia* lectin by chromatography on Sepharose 6B; Toyoshima and Osawa[18] reported the isolation of *Wistaria* lectin (mol. wt. 136,000) by chromatography on Sepharose 6B; and Nicolson *et al.*[1] reported the isolation of RCA_I and RCA_{II} by chromatography on Sepharose 4B. In our laboratory, *Bauhinia* and *Wistaria* lectins could not be isolated by affinity chromatography on any of the Sepharoses that are commercially available. Also, the RCA_{II} lectin bound only weakly to Sepharose 4B and was slowly eluted from the column with PBS. These lectins were adsorbed well only by acid-treated Sepharose 6B, the binding of *Bauhinia* lectin to this adsorbent being temperature dependent.

The discrepancy between our results and those of others[1,10,18] may be due to differences that exist between various commercial lots of Sepharose. From our results, it would appear that the binding of some lectins to Sepharose is dependent upon the accessibility of certain types of D-galactose oligosaccharide chains in the gel. Their presence and accessibility may vary from lot to lot of Sepharose. By acid-treating the Sepharose, the necessary residues are made available for binding by the lectins. We chose Sepharose 6B for acid-treatment, as did Ersson *et al.*[7], because of its greater bead-stability relative to Sepharose 2B and 4B.

Affinity chromatography on Sepharose 2B and 4B was also studied in the hope that a more loose bead-structure might provide an unhindered access to the carbohydrate residues required for lectin binding, thus giving results similar to those obtained with acid-treated Sepharose 6B. However, similar results were not obtained.

The purity of the isolated lectins was analyzed by electrophoresis in the presence or the absence of sodium dodecyl sulfate. The significance of the minor components observed remain to be determined. They may represent minor contaminants, isolectins, or proteolytic fragments of the parent lectin. We occasionally saw a minor protein peak that was eluted by D-galactose after the major peak from *Bauhinia*. This minor component may correspond to the faint, fast-moving band seen upon electrophoresis in the absence of sodium dodecyl sulfate (Fig. 5). *Wistaria* lectin gave a single protein band when subjected to electrophoresis in the absence of sodium dodecyl sulfate, and gave a single protein peak when subjected to gel filtration on Sephadex G-150. However, electrophoresis in the presence of sodium dodecyl sulfate showed the presence of a major and a minor component. This minor component may represent a proteolytic fragment of the parent lectin that has retained the ability to form part of the active lectin molecule. The ammonium sulfate fraction from *Wistaria* used in this work was reported[11] to contain a weakly hemagglutinating mitogen having a mol. wt. of 70,000. Because this mitogen has subunits having a mol. wt. nearly identical to that of the strongly hemagglutinating lectin[18] (mol. wt. 140,000), we would not have detected, by disc electrophoresis in the presence of sodium dodecyl sulfate, its presence in our purified lectin. However, the absence of this mitogen is indicated by our results with gel-filtration on Sephadex G-150 and by

our results of disc electrophoresis performed in the absence of sodium dodecyl sulfate. Apparently, the mitogen has a binding specificity such that it did not bind to the affinity column. Because of its weak hemagglutinating-activity, we did not detect it in the preliminary fractions from the column either.

In addition to demonstrating apparent differences in specificities and in binding affinities among the various lectins studied, the data reported here also emphasize the importance of studying and specifying such parameters as pH and temperature when developing and reporting systems for the affinity chromatography of lectins.

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