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Improved Procedures for Purification of the *Bandeiraea simplicifolia* I Isolectins and *Bandeiraea simplicifolia* II Lectin by Affinity Chromatography

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Bandeiraea simplicifolia plant seeds contain a family of five α-D-galactopyranosyl-binding isolectins (BS I-A_4, A_3B, A_2B_2, AB_3, B_4) and *N*-acetyl-D-glucosamine-binding lectin (BS II). After P_i/NaCl extraction and ammonium sulfate fractionation BS II is specifically adsorbed onto *p*-aminobenzyl-1-thio-*N*-acetyl-β-D-glucosaminide-succinylaminohexylaminyl–Sepharose-4B. The BS I isolectins pass through this column and BS II is selectively eluted by P_i/NaCl containing 2 mM *N*-acetyl-D-glucosamine or by 0.1 M sodium acetate buffer pH 3.6. The material not bound to the column is loaded onto *p*-aminophenyl-β-D-galactopyranosyl-succinylaminohexylaminyl–Sepharose-4B. BS I-A_4 is specifically eluted in a sharp peak with P_i/NaCl containing 1 mM *N*-acetyl-D-galactosamine. Then, BS I-A_3B, A_2B_2, AB_3 and B_4 ar selectively eluted, in a single peak for each isolectin, with P_i/NaCl containing 3 mM, 8 mM, 15 mM and 50 mM methyl α-D-galactopyranoside, respectively.

The seeds of *Bandeiraea simplicifolia* contain a family of five α-D-galactosyl-binding isolectins (BS I) [1] as well as an *N*-acetyl-D-glucosamine-binding lectin (BS II) [2]. The five α-D-galactosyl-binding BS I isolectins (A_4, A_3B, A_2B_2, AB_3, B_4) are tetrameric structures composed of two different glycoprotein subunits designated A and B. The A subunit exhibits a primary specificity for *N*-acetyl-α-D-galactosaminide groups but also reacts with α-D-galactopyranosyl units, whereas the B subunit shows a sharp specificity toward α-D-galactopyranosyl groups [1]. *B. simplicifolia* I isolectins A_4 and B_4 formed precipitates with biopolymers containing multiple non-reducing α-D-galactopyranosyl end-groups; these include guaran (a galactomannan) [3] and type-B blood-group substances [4–6]. Biopolymers containing multiple, non-reducing β-D-galactopyranosyl end-groups, such as larch arabinogalactan, did not form precipitates with either isolectin. Of great interest, a *p*-azophenyl-α-D-galactopyranoside–serum-albumin conjugate did not precipitate with either A_4 or B_4, whereas *p*-azophenyl-β-D-galactopyranoside–serum-albumin reacted very weakly with A_4 but strongly with B_4 [7]. Furthermore, *p*-nitrophenyl β-D-galactopyranoside was six times more potent an inhibitor of B_4 guaran precipitation than A_4 guaran precipitation [7]. Proceeding from these observations, we believed it should be possible to purify the BS I isolectins by affinity chromatography on a column of *p*-aminophenyl-β-D-galactopyranosyl-succinylaminohexylamine–Sepharose-4 B. We also discovered that improved preparations of the BS I isolectins resulted from prior removal of the BS II lectin. To this end we developed an improved procedure for the isolation of BS II using *p*-aminobenzyl-1-thio-*N*-acetyl-β-D-glucosaminide-succinylaminohexylaminyl–Sepharose-4B, a versatile immunosorbent used for the isolation of *N*-acetyl-D-glucosamine-binding proteins [8–13].

Abbreviations. BS I-A_4, A_3B, A_2B_2, AB_3, B_4, *Bandeiraea simplicifolia* I isolectins A_4, A_3B, A_2B_2, AB_3, B_4; BS II, *Bandeiraea simplicifolia* II lectin; P_i/NaCl, 0.1 M phosphate, 0.15 M NaCl, 0.1 mM $CaCl_2$, pH 7.2.

MATERIALS AND METHODS

Bandeiraea simplicifolia seeds were obtained from Calbiochem (La Jolla, CA). Guaran was purified from crude guar gum (Meer Corp., New York, NY) by repeated alcohol precipitation from water [3]. The conjugates of bovine serum albumin and *p*-azophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, *N*,*N*′-diacetyl-β-chitobioside and *N*,*N*′,*N*″-triacetyl-β-chitotrioside were prepared as previously described [14,15]. *p*-Nitrophenyl α-D-galactopyranoside was obtained from Cyclo Chemical Co. (Los Angeles, CA). The synthesis of *p*-nitrophenyl *N*,*N*′-diacetyl-β-chitobioside and *N*,*N*′,*N*″-triacetyl-β-chitotrioside were previously described [16]. *p*-Aminobenzyl-1-thio-2-acetamido-2-

deoxy-β-D-glucopyranoside was made according to [8]. All other sugars were obtained from Pfanstiehl Laboratories (Waukegan, IL). Cyanogen bromide, acetonitrile and hexane-1,6-diamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sepharose 4B-200 and (1-ethyl-3,3-dimethylaminopropyl)-carbodiimide HCl were obtained from Sigma Chemical Co. (St Louis, MO); succinic anhydride was purchased from Matheson, Coleman and Bell (Cincinnati, OH).

Preparation of Succinylaminohexylaminyl-Sepharose-4B

Sepharose 4B (150 ml) activated with cyanogen bromide in acetonitrile (20 ml) [17] was reacted with hexane-1,6-diamine (35 g in 150 ml water adjusted to pH 10 with 12 M HCl), followed by treatment with succinic anhydride (15 g) at pH 6.0 [18].

Preparation of p-*Aminobenzyl-1-thio*-N-*acetyl-β-D-glucosaminide-succinylaminohexylaminyl–Sepharose-4B*

This affinity matrix (30 ml) was made according to [8,11] except that we used (1-ethyl-3,3-dimethylaminopropyl)-carbodiimide HCl as coupling agent. Unreacted carboxyl groups in the extension arm were blocked by reaction with ethanolamine (1.5 mmol) in the presence of (1-ethyl-3,3-dimethylaminopropyl)-carbodiimide HCl (3.0 mmol) at pH 4.6. These conditions generally lead to incorporation of 2 μmol ligand/ml gel [11].

Preparation of p-*aminophenyl-β-D-galactopyranoside-succinylaminohexylaminyl–Sepharose-4B*

p-Nitrophenyl β-D-galactopyranoside (250 mg) in methanol (25 ml), was reduced by hydrogen over a 10% palladium/charcoal catalyst at atmospheric pressure for 5 h. The suspension was filtered, the residue washed with methanol, and the filtrate evaporated to a syrup under reduced pressure. The succinylaminohexylaminyl–Sepharose-4B (40 ml) was resuspended in water (10 ml) and *p*-aminophenyl-β-D-galactopyranoside (0.6 mmol) in water (3 ml) was added. The slurry was adjusted to pH 4.6, (1-ethyl-3,3-dimethylaminopropyl)-carbodiimide HCl (1 mmol) was added, and the gel stirred at room temperature for 2 h and overnight at 4 °C. Free carboxyl groups were blocked by the addition of ethanolamine (1.5 mmol) and (1-ethyl-3,3-dimethylaminopropyl)-carbodiimide HCl (3.0 mmol).

Electrophoresis

Electrophoresis on polyacrylamide gels was performed at pH 8.9 in Tris/glycine buffer according to [19], and pH 4.3 in β-D-alanine/acetic acid using the chemical formulation described in [20]. Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to [21].

Qualitative Precipitation

For rapid screening of BS I and BS II activity we routinely employed a microcapillary test utilizing the ability of BS I isolectins to precipitate guaran (0.1 mg/ml in 0.01 M phosphate buffer, pH 7.2, containing 0.5 M NaCl), and BS II lectin to precipitate bovine serum albumin conjugates of *p*-azophenyl β-glycosides of 2-acetamido-2-deoxy-D-glucopyranose, *N,N'*-diacetylchitobiose or *N,N',N''*-triacetylchitotriose (1 mg/ml in P_i/NaCl).

Agglutination Assay

Hemagglutination assays were performed in a microtitrator plate (Cooke) using twofold serial dilutions of the protein in 50 μl 0.15 M NaCl. A 50-μl sample of a 3% suspension of group A or B human red blood cells (washed three times with saline) was added and, after 1 h at room temperature, the degree of agglutination was assessed on a serological scale (0–4) [22]. 1 unit of agglutinating activity was defined as the amount of protein required to cause half-maximal agglutination.

Glycosylhydrolase Activities

Glycosidase activity was monitored using *p*-nitrophenyl glycoside substrates according to [23].

Assay Procedures

Protein concentration was determined by absorbance at 280 nm, using $A_{1\,cm}^{1\,mg/ml} = 1.41$ for BS I isolectins [3] and $A_{1\,cm}^{1\,mg/ml} = 1.03$ for BS II lectin [24].

RESULTS AND DISCUSSION

Extraction and Ammonium Sulfate Fractionation

100 g finely ground, decoated *Bandeiraea simplicifolia* seeds were extracted by magnetic stirring five times (5 × 10 min) with 200 ml methanol, and once (10 min) with 200 ml methylene chloride and filtered. The filtrates were discarded. Methanol extraction removes large quantities of chromophoric substances, which bind irreversibly and non-specificially, to the affinity chromatography gel thereby reducing its efficiency. Methylene chloride extraction removes lipids and facilitates extraction of the lectins with saline buffer. This procedure gave a dry, light brown

meal (50 g) with a 50% loss in weight. All subsequent procedures were carried out at 4°C. The dry meal (50 g) was extracted with P_i/NaCl (400 ml) (0.1 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.1 mM $CaCl_2$ and 0.01% $Na_2S_2O_4$) by stirring for 2 h. The presence of sodium dithionite in the buffer prevents the oxidation of phenolic compounds to quinones, which tend to polymerize and condense with reactive groups of protein, e.g. $-SH$ and $-NH_2$ groups. The P_i/NaCl extract was centrifuged for 30 min at $10000 \times g$ and the sediment was re-extracted as before with P_i/NaCl buffer (300 ml). Solid ammonium sulfate was added to 30% saturation. After gentle stirring for 2 h precipitated proteins were centrifuged at $10000 \times g$ for 30 min and discarded. The BS I and BS II lectins were precipitated by adding solid ammonium sulfate to 75% saturation. (By using a 30–75% ammonium sulfate fraction instead of the 40–75% fraction used previously [2,24], the yield of BS II lectin was increased 2–3-fold.) After gentle stirring overnight, the precipitate, collected by centrifugation at $10000 \times g$ for 30 min, was resuspended in P_i/NaCl (60 ml) and dialyzed against five changes of P_i/NaCl to remove salts and some remaining chromophoric substances. Centrifugation at $10000 \times g$ for 30 min gave a clear brown solution (≈ 200 ml). This material precipitated guaran and *N*-acetyl-D-glucosaminide conjugates.

Specific Adsorption and Elution of the BS II Lectin

The 30–75% ammonium sulfate fraction (≈ 200 ml) in P_i/NaCl, was loaded on the *p*-aminobenzyl-1-thio-*N*-acetyl-β-D-glucosaminide-succinylaminohexylaminyl – Sepharose - 4B column (1.4 × 13 cm) with a flow rate of approximately 50 ml/h. The column was washed with 750 ml P_i/NaCl and fractions of 7 ml were collected. The column eluate gave a copious precipitate with guaran but did not precipitate the *p*-azophenyl-β-*N*-acetyl-D-glucosaminide–serum-albumin conjugate, indicating that under these conditions the column was not overloaded with the BS II lectin. The BS II lectin was specifically and completely eluted in a sharp peak with P_i/NaCl containing 2 mM *N*-acetyl-D-glucosamine (100 ml) (Fig. 1). No further protein was eluted by increasing the concentration of *N*-acetyl-D-glucosamine to 50 mM. At this point, addition of 0.05 M HCl (100 ml) removed a small amount of brown chromophoric material. The column was then re-equilibrated with P_i/NaCl. Fractions having an absorbance at 280 nm of > 0.25 were pooled, dialyzed against several changes of water to remove *N*-acetyl-D-glucosamine and salts, and freeze-dried to afford a white powder (350 mg).

Compared to the purification of BS II on a chitin column [24], the advantages of the present method are: good yield, rapid elution by an inexpensive eluent, high reproducibility and a stable, high-capacity affinity matrix, which may be used repeatedly (Table 1). It is noteworthy that BS II can be eluted at pH 3.6 in 0.1 M acetate buffer.

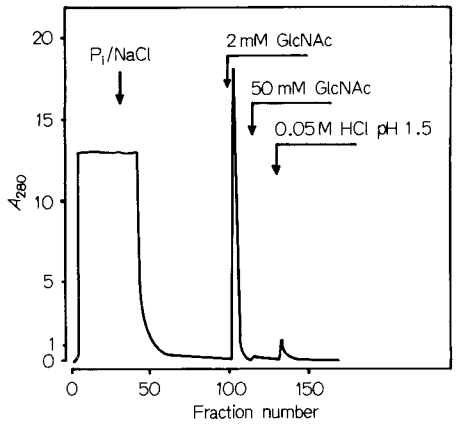

Fig. 1. *Affinity chromatography on* p-*aminobenzyl-1-thio*-N-*acetyl-β-D-glucosaminide-succinylaminohexylaminyl–Sepharose-4B column (1.4 × 13 cm; flow rate of 50 ml/h) of the 30–75% ammonium sulfate fraction (200 ml) in P_i/NaCl.* The column was washed with P_i/NaCl (750 ml) and was eluted with P_i/NaCl containing 2 mM D-GlcNAc (100 ml), 50 mM D-GlcNAc (100 ml) and with 0.05 M HCl, pH 1.5. Fractions of 7 ml were collected

Purity and Properties of BS II Lectin

Polyacrylamide gel electrophoresis at pH 4.3 gave two protein bands. It is believed that the minor, high-molecular-weight component represents an aggregated form of the BS II lectin, a speculation strengthened by the visualization of a single protein-staining component upon polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate and 2-mercaptoethanol. These results are in accord with our previous studies [2,24].

The BS II lectin, eluted with either 2 mM *N*-acetyl-D-glucosamine or 0.1 M acetate pH 3.6, precipitated the *N*-acetyl-D-glucosamine; *N*,*N*′-diacetylchitobiose and *N*,*N*′,*N*″-triacetylchitotriose–serum-albumin conjugates [26] and was devoid of α and β-D-glucosidase, α and β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, α-D-mannosidase *N*-acetyl-α-D-galactosaminidase and β-D-galactosaminidase and β-L-fucosidase activities.

Specific Adsorption and Elution of the BS I Isolectins

The P_i/NaCl eluate (fractions 1–45) from the *p*-aminobenzyl-1-thio-*N*-acetyl-β-D-glucosaminide-substituted column, which gave a precipitate with guaran, was loaded onto the *p*-aminophenyl-β-D-galactopyranosyl-succinylaminohexylaminyl-Sepharose-4B column (1.4–19 cm) with a flow rate of 50 ml/h. The

Table 1. *Affinity chromatography of* Bandeiraea simplicifolia *lectins*

Ligand (columns dimensions)	Elution conditions in P_i/NaCl	Yield from 50 g dry meal	
		product	yield
p-Aminobenzyl 1-thio-*N*-acetyl-β-D-glucosaminide (13 × 1.4 cm)	2 mM D-GlcNAc (100 ml)	BS II	mg 350
Chitin (28 × 2.2 cm) [2, 24]	1% D-GlcNAc	BS II	75 – 150
Sephadex G-75 [24]	1% Me-α-D-Glc	BS II	25 – 75
p-Aminophenyl β-D-galactopyranoside (19 × 1.4 cm)	1 mM D-GalNAc (100 ml)	BS I-A_4	155
	3 mM Me-α-D-Gal (100 ml)	BS I-A_3B	200
	8 mM Me-α-D-Gal (100 ml)	BS I-A_2B_2	200
	15 mM Me-α-D-Gal (100 ml)	BS I-AB_3	90
	50 mM Me-α-D-Gal (100 ml)	BS I-B_4	85
Melibionate Bio-gel (20 × 2.6 cm) [1, 25]	1.4 mM D-GalNAc (300 ml)	BS I-A_4	
	6.8 mM D-GalNAc (350 ml)	BS I-A_3B	
	5 mM Me-α-D-Gal (250 ml)	BS IA_2B_2, AB_3, B_4	50 – 150
Insolubilized type A + H hog mucin (10 × 2.1 cm) [1, 25]	P_i/NaCl (300 ml)	BS I-B_4 passed through AB_3 slightly retarded	
	50 mM Me-α-D-Gal (150 ml)	BS I-A_2B_2	

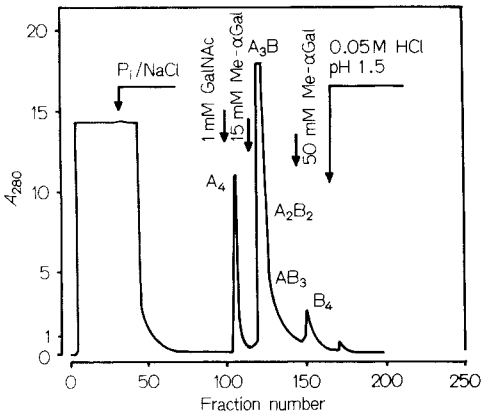

Fig. 2. *Affinity chromatography on* p-*aminophenyl-β-D-galactopyranosyl-succinylaminohexylaminyl–Sepharose-4B column (1.4 × 19 cm; flow rate of 50 ml/h) of the unbound eluate (≈ 220 ml) from the* p-*aminobenzyl-1-thio-*N-*acetyl-β-D-glucosaminide-substituted column.* The column was washed with P_i/NaCl (750 ml) and was eluted successively with P_i/NaCl containing 1 mM D-GalNAc (100 ml). 15 mM methyl α-D-galactopyranoside (150 ml) and 50 mM methyl α-D-galactopyranoside (100 ml), and finally with 0.05 M HCl (100 ml). Fractions of 7 ml were collected

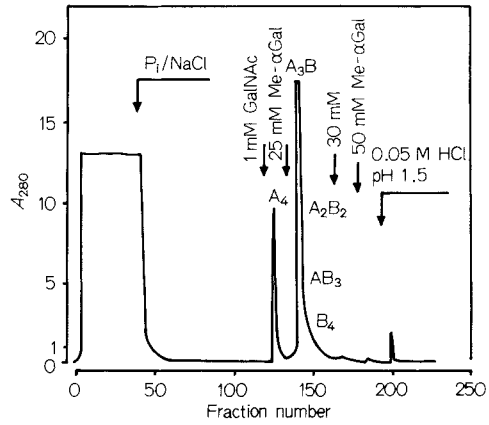

Fig. 3. *Affinity chromatography on* p-*aminophenyl-β-D-galactopyranosyl-succinylaminohexylaminyl–Sepharose-4B column (1.4 × 19 cm; flow rate of 50 ml/h) of the unbound eluate (≈ 220 ml) from the* p-*aminobenzyl-1-thio-*N-*acetyl-β-D-glucosaminide-substituted column.* The column was washed with P_i/NaCl (750 ml) and was eluted successively with P_i/NaCl containing 1 mM D-GalNAc (100 ml), and with methyl α-D-galactopyranoside (200 ml, 25 mM, 100 ml each of 30 mM and 50 mM) and finally with 0.05 M HCl pH 1.5 (100 ml). Fractions of 7 ml were collected

column was washed with 750 ml P_i/NaCl and fractions of 7 ml were collected. The eluate from this column did not precipitate with guaran, indicating that the column was not overloaded with the BS I isolectins. The BS I-A_4 isolectin was specifically and completely eluted in a sharp peak (fraction I) with P_i/NaCl containing 1 mM *N*-acetyl-D-galactosamine (100 ml) (Fig. 2). At this point, addition of 15 mM methyl α-D-galactopyranoside in P_i/NaCl (150 ml) eluted A_3B, A_2B_2 and AB_3 in a single peak (fraction III). By increasing the concentration of methyl α-D-galactopyranoside to 50 mM the B_4 isolectin was specifically eluted (fraction III). The column was then washed with 0.05 M HCl to remove some chromophoric material and re-equilibrated with P_i/NaCl.

Fractions I, II and III were dialyzed against water and freeze dried or dialyzed against P_i/NaCl until free of sugar and stored at −20 °C until used. Both the concentration and volume of the methyl α-D-galactopyranoside are very important considerations in the elution of only the A_3B, A_2B_2 and AB_3 isolectins. For example, by using 25 mM methyl α-galactoside in P_i/NaCl (200 ml), the A_3B, A_2B_2, AB_3 and also the B_4 isolectins were eluted in a single peak (Fig. 3).

Stepwise elution of the column with methyl α-D-galactopyranoside (100 ml each of 3 mM, 8 mM

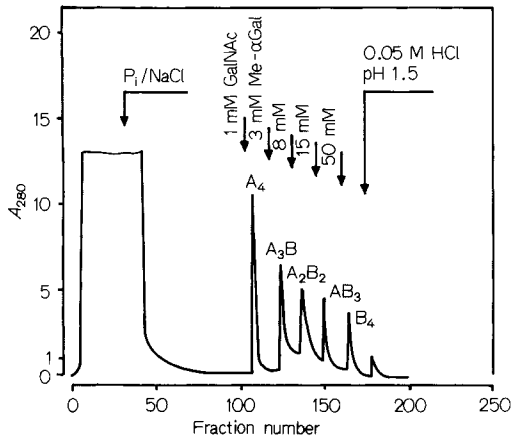

Fig. 4. *Affinity chromatography on* p-*aminophenyl-β-*D-*galactopyranosyl-succinylaminohexylaminyl–Sepharose-4B column (1.4 × 19 cm; flow rate of 50 ml/h) of the unbound eluate (≈ 220 ml) from the* p-*aminobenzyl-1-thio-*N-*acetyl-β-*D-*glucosaminide-substituted column.* The column was washed with P_i/NaCl (750 ml) and was eluted successively with P_i/NaCl containing 1 mM D-GalNAc (100 ml) and with methyl α-D-galactopyranoside (100 ml each of 3 mM, 8 mM, 15 mM and 50 mM), and finally with 0.05 M HCl (100 ml). Fractions of 7 ml were collected

15 mM anf 50 mM) successively eluted A_3B, A_2B_2, AB_3 and B_4, respectively, as separate peaks (Fig. 4). The characteristics of each new column must be determined. Table 1 lists the yields of each BS I isolectin.

Compared to the method described previously [1, 3, 25], the advantages of the present procedure are: (a) a single matrix suffices for the isolation and purification of all the isolectins, (b) the specific sugar ligand (*p*-nitrophenyl β-D-galactopyranoside) used in the synthesis of the affinity column is commercially available. (c) rapid elution by a relatively inexpensive commercially available sugar (methyl α-D-galactopyranoside) and, (d) a stable long-lived affinity matrix (over a year).

Our studies also indicate that the length of the extension arm between the insoluble matrix and sugar ligand is important. In a previous study we found that an affinity column consisting of *p*-aminophenyl α-D-galactopyranoside coupled to cyanogen-bromide-activated Sepharose failed to bind the BS I isolectins [3].

Purity and Properties of BS I Isolectins

The five *B. simplicifolia* isolectins were separated by polyacrylamide gel electrophoresis at pH 8.9. In addition to the five major bands a number of satellite bands were also discernible, a result in accord with previous studies [1, 7] The isolectins were devoid of any glycosylhydrolase activity.

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