ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 177, 330–333 (1976)

COMMUNICATIONS

An *N*-Acetyl-D-Glucosamine Binding Lectin from *Bandeiraea Simplicifolia* Seeds

A second lectin with a high activity for nonreducing 2-acetamido-2-deoxy-α-D-glucopyranosyl residues has been isolated from the seed extracts of *Bandeiraea simplicifolia* by affinity chromatography on chitin. The lectin is a glycoprotein of subunit molecular weight 30,000. Some properties of the new lectin are reported.

An α-D-galactopyranosyl binding lectin (1) was recently isolated and characterized from the seed extracts of *Bandeiraea simplicifolia*. While pursuing further studies on this lectin, a second protein capable of interacting with *p*-azophenyl 2-acetamido-2-deoxy-α- and -β-D-glucopyranoside BSA[1] conjugates was discovered. The new lectin, isolated from the same plant seed by affinity chromatography, shows a primary specificity for *N*-acetyl-D-glucosamine. Several other *N*-acetyl-D-glucosamine binding lectins have been cited earlier. Wheat germ agglutinin isolated by Burger *et al.* (2) and Allen *et al.* (3) and the *Ulex europeus* lectin studied by Matsumoto and Osawa (4) are two examples. But this is the first instance of a lectin which displays a high activity towards α-linked *N*-acetyl-D-glucosaminyl units. In this communication we report our preliminary findings on the second lectin present in the seed extracts of *B. simplicifolia*, its purification by affinity chromatography and some of its properties.

Materials and methods. *Bandeiraea simplicifolia* seeds were the generous gift of Calbiochem, La Jolla, California. Chitin used for affinity column was obtained from Pfanstiehl Laboratories, Waukegan, Illinois. BSA was obtained from Miles Laboratories, Kankakee, Illinois. Polyvinyl pyrrolidone was obtained from Calbiochem, La Jolla, California. All other chemicals used in this investigation were of reagent grade or the best quality available. Unless otherwise specified all experiments were conducted at 4°C. The sugars used in this study were available from previous studies or purchased from Pfanstiehl Laboratories, Waukegan, Illinois. The disaccharide 3-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-glucopyranose was kindly provided by Dr. R. U. Lemieux (5), University of Alberta, Canada. The *p*-azophenyl 2-acetamido-2-deoxy-(α and β)-D-glucopyranoside–BSA conjugates (α-GlcNAc-BSA or β-GlcNAc-BSA) were prepared by the method described by Iyer and Goldstein (6). *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and *p*-nitrophenyl-2-deoxy-α-D-glucopyranoside were reduced to the corresponding *p*-aminophenyl glucosides, diazotized, and coupled to bovine serum albumin.

Chitin prepared as described by Bloch and Burger (7) was packed into a column (28 × 2.2 cm) and washed exhaustively with phosphate-buffered saline (PBS; 0.1 M sodium phosphate, pH 7.0, containing 0.15 M NaCl). Finely ground *Bandeiraea simplicifolia* seeds (100 g) were extracted two times with methanol (2 × 300 ml; 1 h) and filtered on a Buchner funnel, and the filtrate was discarded. Polyvinylpyrrolidone (6 g) was added to the dry, methanol-extracted meal and the mixture was extracted twice with PBS–Ca buffer (phosphate buffer containing 0.1 mM calcium chloride; 2 × 300 ml; 2 h). The crude extract was subjected to ammonium sulfate fractionation as follows: fraction 1, 0–40% saturation; fraction 2, 41–55% saturation; fraction 3, 56–75% saturation. The appropriate quantity of ammonium sulfate was added to the crude extracts, and the solution was stirred gently for 2 h and centrifuged (15,000*g*; 15 min.). The precipitate obtained in each stage was dissolved in 0.15 M sodium chloride (30–50 ml) and dialyzed against PBS–Ca buffer, pH 7.0, until the dialysate was free of ammonium sulfate (Nessler's reagent). The three fractions were applied individually to the chitin column. After the absorbance at 280 nm reached the base line, the bound protein was eluted with PBS containing 1% *N*-acetyl-D-glucosamine. The protein fractions were pooled, dialyzed against PBS–Ca buffer to remove *N*-acetyl-D-glucosamine and concentrated by membrane ultrafiltration (Amicon Corp. PM-10 membrane), and the protein contents were determined by the microbiuret protein assay procedure of Janatova *et al.* (8). Elution with 0.5 N HCl [cf. Bloch and Burger (7)] served to remove additional protein not removed by *N*-acetyl-D-glucosamine. The results obtained are shown in Table I. The fraction eluted with 0.5 M HCl was reapplied to the affigel-10 column and the bound

[1] Abbreviations used: BSA, bovine serum albumin; WGA, wheat germ agglutinin; PBS, phosphate-buffered saline.

TABLE I

PURIFICATION OF LECTIN EMPLOYING AFFINITY CHROMATOGRAPHY ON CHITIN

Fraction	Protein applied (mg)	Protein isolated	
		1% *N*-acetyl D-glucosamine eluate (mg)	0.05 *N* HCl eluate (mg)
1 (0-40% saturated) $(NH_4)_2SO_4$	1596	12.7	102
2 (41-55% saturated) $(NH_4)_2SO_4$	1500	101.3	58.8
3 (56-75% saturated) $(NH_4)_2SO_4$)	1060	78.0	53.6

protein was eluted with 1% *N*-acetyl-D-glucosamine, establishing that the original acid elution served to displace tightly bound protein.

In order to ensure removal of any contamination by the α-D-galactopyranosyl binding lectin (1) of *B. simplicifolia*, fraction 3 was passed over a Biogel-melibionate affinity column (1).

Electrophoresis on polyacrylamide gels using the chemical formulations described by Reisfeld *et al.* (9) was performed at pH 4.3 in β-alanine-acetic acid. Gels were fixed in 12% trichloroacetic acid, stained with 0.05% coomassie blue, and destained and stored in 7% acetic acid. Treatment of the lectin with dimethyl suberimidate was by the method of Davies and Stark (10).

Antisera to the *N*-acetyl-D-glucosamine binding lectin were prepared by immunizing rabbits as previously reported for the α-D-galactopyranosyl binding lectin (1). The antibody thus formed, as well as antiserum against crude seed extract (1), were run against the purified lectin by the Ouchterlony double-immunodiffusion method.

Results and discussion. Polyacrylamide gel electrophoresis at pH 4.3 of ammonium sulfate fractions 1 and 2 (Table I) showed two bands: a major protein component (95%) and a minor protein band (Fig. 1). The second band does not appear in fraction 3 and for this reason fraction 3 was used for further study. Single precipitin bands were obtained by two-dimensional immunodiffusion when purified lectin was run against rabbit antiserum to purified lectin as well as against antiserum to the crude seed extract. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (11) gave a single protein band with an estimated subunit molecular weight of 30,000. Treatment of the lectin with dimethyl suberimidate followed by polyacrylamide gel electrophoresis showed four protein bands of approximate molecular weights 32,500, 70,000, 96,000, and 130,000. These data indicate a tetrameric structure for the lectin. The carbohydrate content as determined by the phenolsulfuric acid method of Dubois *et al.* (12) (methyl α-D-galactopyranoside as standard) showed 4% carbohydrate associated with the protein.

The lectin (2 mg/ml) was tested for its agglutinating properties against human types A, B, and O erythrocytes. The lectin did not agglutinate any of the erythrocytes.

The precipitin reaction between the *N*-acetyl-D-glucosamine binding lectin and α- and β-GlcNAc-BSA was studied by the procedure described by So and Goldstein (13) using the ninhydrin method of Schiffman *et al.* (14). The results shown in Fig. 2 indicate that both conjugates give a precipitin-like curve, with the α-GlcNAc-BSA conjugate bringing down more of the lectin in the precipitate than the β-GlcNAc-BSA conjugate.

The specificity of the second lectin isolated from the seed extracts of *B. simplicifolia* was examined by

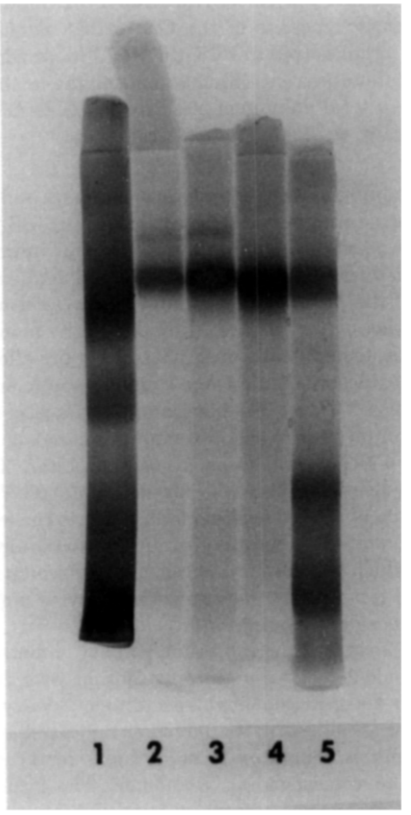

FIG. 1. Polyacrylamide disc gel electrophoretic patterns. Protein samples polymerized in stacking gel were subjected to electrophoresis for 2 h; 5 mA/tube in β-alanine-acetic acid buffer pH 4.3. 1, ammonium sulfate fraction 56-75%; 2, 0-40% fraction 1, 1% sugar eluate; 3, 41-55% fraction 2, 1% sugar eluate; 4, 56-75% fraction 3, 1% sugar eluate; 5, fraction eluted with 0.05 N HCl.

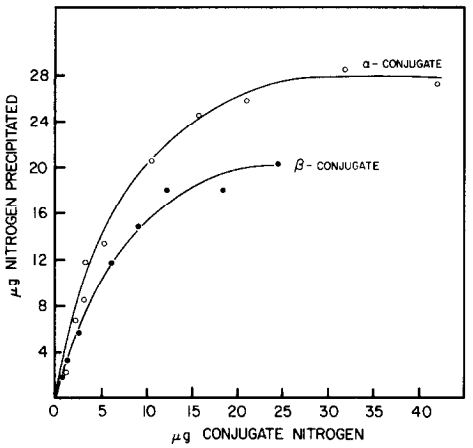

FIG. 2. Precipitin reaction between 2-acetamido-2-deoxy-D-glucopyranosyl binding lectin from *B. simplicifolia* seeds and *p*-azophenyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside-BSA conjugates. Increasing amounts of the GNAc-BSA conjugates (2–40 μg) dissolved in PBS–Ca pH 7.0 were added to duplicate tubes containing buffer and the lectin (180 μg) in a total volume of 500 μl. ○, α-GlcNAc-BSA conjugate; ●, β-GlcNAc-BSA conjugate.

determining to what extent various sugars and their derivatives inhibited the lectin–β-GlcNAc-BSA precipitin reaction. Table II presents the inhibition data in terms of micromoles of inhibitor required for 50% of the precipitin reaction. *N*-Acetyl-D-glucosamine was by far the best inhibitor of the monosaccharides tested, being over 400 times more effective than D-glucose. Of the three common amino sugars examined (*N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-mannosamine), only *N*-acetyl-D-glucosamine was a good inhibitor. These data indicate the necessity for an equatorial *N*-acetyl group at the C-2 position for binding to the lectin. An equatorial C-4 hydroxyl group is also required, a fact which is further confirmed by comparing methyl α-D-glucopyranoside with methyl α-D-galactopyranoside, a noninhibitor.

Comparison of the inhibition potency of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside with its β-anomer and *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside with its β-anomer indicate that the α-anomer is bound six to eight times more avidly than the corresponding β-anomer. The situation with disaccharides is complex because both maltose and cellobiose are equivalent as inhibitors and *N,N'*-diacetyl chitobiose [β-D-GlcNAc-(1→4)-GlcNAc] is about twice as potent as the disaccharide with a nonreducing α-linked *N*-acetyl-D-glucosaminyl residue [α-D-GlcNAc-(1→3)-D-Glc]. Of fundamental importance is the fact that *N,N',N''*-triacetyl chitotriose is approximately equivalent to *N,N'*-diacetyl chitobiose as an inhibitor. These data set this lectin apart from wheat germ agglutinin (3, 4) and the potato lectin (15), both of which possess combining sites that are complementary to sequences of three or four β-(1→4)-linked *N*-acetyl-D-glucosaminyl units.

TABLE II

INHIBITION OF THE *B. simplicifolia* LECTIN–β-GlcNAc-BSA CONJUGATE PRECIPITIN REACTION BY VARIOUS SUGARS AND THEIR DERIVATIVES

Sugar inhibitors	Micromoles of inhibitor required for 50% inhibition
2-Acetamido-2-deoxy-D-glucopyranose	0.017
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	0.010
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	0.080
p-Nitrophenyl 2-acetamido-2-deoxy-α-D-glucopyranoside	0.005
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside	0.03
N-Acetyl-D-mannosamine	17.0
N-Acetyl-D-galactosamine	0% at 50 μmol
D-Glucose	7.4
Methyl α-D-glucopyranoside	1.3
Methyl β-D-glucopyranoside	16.0
Methyl α-D-galactopyranoside	0% at 100 μmol
3-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-glucopyranose	0.01
Maltose	1.8
Cellobiose	1.6
N,N'-diacetyl chitobiose	0.0045
N,N',N''-triacetyl chitotriose	0.0062

Further details on the physical, chemical, and biological properties of this new lectin will be forthcoming.

Acknowledgments. This study was supported by National Institute of Health Grant AM 10171 and United States Public Health Service Grant GM-00187.

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Received March 31, 1976