

Lectin Affinity Electrophoresis for the Separation of Fluorescently Labeled Sugar Derivatives

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Lectin affinity electrophoresis was applied to the separation of charged, fluorescent conjugates of disaccharides. Four fluorescent conjugates were prepared by reductive amination of α -D-Man-(1 \rightarrow 3)-D-Man, α -D-Gal-(1 \rightarrow 4)-D-Gal, α -D-Gal-(1 \rightarrow 6)-D-Glc, and β -D-Gal-(1 \rightarrow 4)-D-Glc in the presence of 7-amino-1,3-naphthalenedisulfonic acid. These charged fluorescent-disaccharide conjugates all have identical molecular weight and in the absence of concanavalin A lectin failed to separate either by agarose or by polyacrylamide gel electrophoresis. In the presence of either free or immobilized concanavalin A, agarose gel electrophoresis and polyacrylamide gel electrophoresis could separate the fluorescent conjugate of α -D-Man-(1 \rightarrow 3)-D-Man from that of α -D-Gal-(1 \rightarrow 4)-D-Gal, α -D-Gal-(1 \rightarrow 6)-D-Glc, and β -D-Gal-(1 \rightarrow 4)-D-Glc. © 1992 Academic Press, Inc.

Recently, we demonstrated the utility of gradient polyacrylamide gel electrophoresis (PAGE) and capillary zone electrophoresis for analyzing and sequencing charged, fluorescent conjugates of neutral oligosaccharides (1,2). Gradient PAGE separates these conjugates the same way sodium dodecyl sulfate-PAGE separates proteins, on the basis of the molecular size of the oligosaccharide chain. In these earlier studies, we reported both the high resolving power of gradient PAGE and the high detection sensitivity using fluorescent-sugar conjugates. However, we also noted a limitation of this method, its failure to cleanly resolve oligosaccharide-fluorescent conjugates that have the same molecular size.

Lectin affinity electrophoresis combines the biospecific interaction between a lectin and a sugar with the

driving force of electrophoresis. This method has been used mainly for analyzing glycoproteins, related to the diagnosis of cancer (3,4) and inflammatory conditions (3). Rosaki and Foo (5) also demonstrated its utility for the diagnosis of diseases of the bone and of the hepatobiliary tract.

Lectin affinity electrophoresis has not been applied to the analysis of oligosaccharides due to the absence of fixed charges required to facilitate separation by electrophoresis and due to the lack of sensitive detection methods for most oligosaccharides (6). This paper describes the incorporation of the lectin concanavalin A into a gel matrix and the use of electrophoresis to separate sugar-fluorescent conjugates that have the same molecular weight. To sequence an oligosaccharide-fluorescent conjugate with gradient PAGE, using exoglycosidases, it must be free of other conjugates having different structures but similar molecular size. This new method might be of considerable use for purifying and analyzing structurally similar oligosaccharides released from glycoproteins and might represent a second dimension for the analysis of complex oligosaccharides by electrophoresis.

MATERIALS AND METHODS

Materials

The α -D-Man-(1 \rightarrow 3)-D-Man, α -D-Gal-(1 \rightarrow 4)-D-Gal, α -D(+)-melibiose [α -D-Gal-(1 \rightarrow 6)-D-Glc], *N*-acetylneuramin-lactose [mixture of Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4)-D-Glc and Neu5Ac-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)-D-Glc], sodium cyanoborohydride, neuraminidase from *Clostridium perfringens*, concanavalin A (Con A),² Con

² Abbreviations used: Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis; Gal, galactose; Glc, glucose; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; AGA, monopotassium 7-amino-1,3-naphthalenedisulfonic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, Tris(hydroxymethyl)aminomethane.

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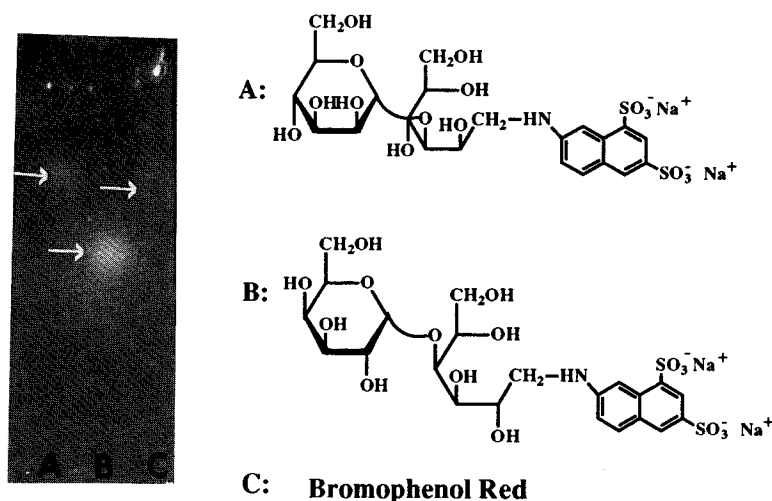


FIG. 1. Con A-agarose gel analysis of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and α -D-Gal-(1 \rightarrow 4)-D-Gal-AGA. Lane A, 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA; lane B, 1 μ g of α -D-Gal-(1 \rightarrow 4)-D-Gal-AGA; lane C, bromophenol red. Con A-Sepharose was added at a concentration of 1 mg/ml and 1% agarose was used.

A-agarose (10 mg Con A/ml gel), Con A-Sepharose (10–15 mg Con A/ml gel), and a horizontal submarine minigel electrophoresis unit (7.5 \times 10 cm) were from Sigma Chemical Co., St. Louis, MO. Monopotassium 7-amino-1,3-naphthalenedisulfonic acid salt (Amido-G-Acid, AGA) were from Aldrich, Milwaukee, WI. The monopotassium salt of AGA was used after recrystallization from double-distilled, deionized water. The 32 \times 16-cm vertical slab gel unit (SE 620), the TE70 semidry electrophoresis transfer unit, and Nylon 66 plus (positively charged nylon membrane) were obtained from Hoefer Scientific Instruments, San Francisco, CA. Agarose was from International Biotechnologies, New Haven, CT. Bio-Gel P-2, the electrophoresis power unit Model 1420B, and trans-blot electrotransfer system

were purchased from Bio-Rad, Richmond, CA. Glycine, tris(hydroxymethyl)aminomethane (TRIS), boric acid, glycerol, *N,N'*-methylene bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Fisher Chemical Co., Fair Lawn, NJ. The 3-mm paper was from Whatman, Hillsboro, OR. The transilluminator (TR-365A), uv-transmitting filter protector (UVT-150), and diffusing screen were purchased from Spectronics Co., Westbury, NY. All other chemicals were reagent grade.

Methods

Preparation of fluorescently labeled sugar. Oligosaccharide (6 μ mol) was dissolved in 600 μ l of AGA solution [50% (w/v) in water adjusted with sodium hydroxide to

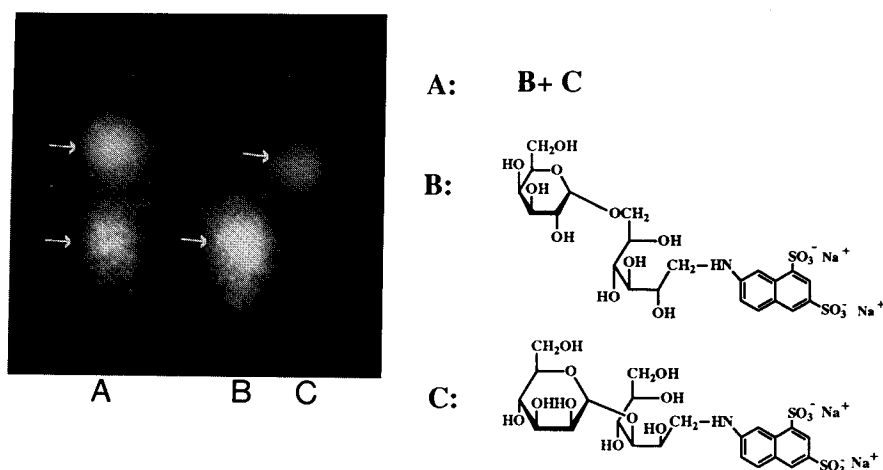


FIG. 2. Con A-agarose gel analysis of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA. Lane A, mixture of 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and 1 μ g of α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA; lane B, 1 μ g of α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA; lane C, 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA. Free Con A was added at a concentration of 10 mg/ml and 1% agarose was used.

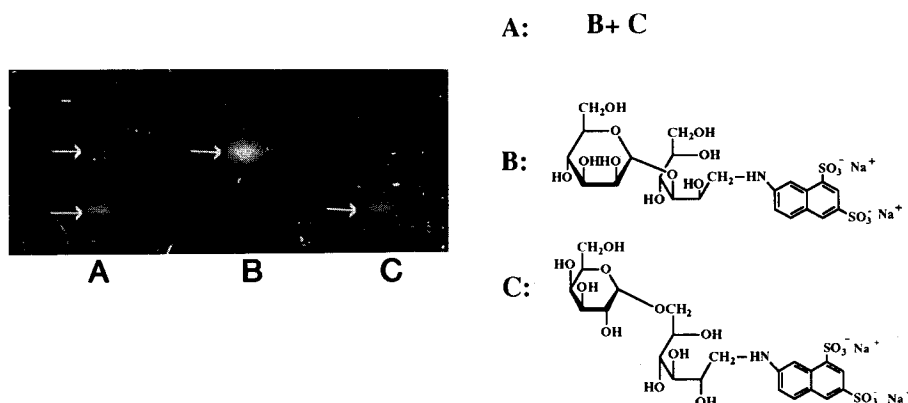


FIG. 3. PAGE analysis of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and α -D-Gal-(1 \rightarrow 6)-D-Gal-AGA. Lane A, mixture of 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and 1 μ g of α -D-Gal-(1 \rightarrow 6)-D-Gal-AGA; lane B, 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA; lane C, 1 μ g of α -D-Gal-(1 \rightarrow 6)-D-Gal-AGA. Free Con A was added at a concentration of 10 mg/ml and 12% (w/v) isocratic polyacrylamide gel was used.

pH 6.2]. After being heated at 80°C for 60 min, sodium cyanoborohydride (30 μ mol) was added (the pH changed <0.1 unit). The mixture was heated for overnight at 70°C using a heating block. After the reaction was complete, the products were desalted on a 2.5 \times 50-cm Bio-Gel P-2 column eluted with distilled water. The samples were freeze-dried and reconstituted in 100 μ l of distilled water before loading on the preparative gel.

Purification of sugar-AGA conjugates. Oligosaccharide-AGA conjugates were purified by preparative PAGE using a linear gradient of 12 to 22% (w/v) acrylamide that contained 0.5–2% (w/v) *N,N'*-bisacrylamide. The preparation of gels essentially followed the procedure of Al-Hakim and Linhardt (7). Electrophoresis was performed for 7 h at 400 V (constant voltage). Oligosaccharide-fluorescent conjugates were transferred from gels to positively charged nylon membranes by

semidry electrotransfer and the sugar-fluorescent conjugates were recovered from the membranes by washing with a 2 M solution of sodium chloride. Sodium chloride was removed using a Bio-Gel P-2 column eluted with distilled water.

Lectin affinity electrophoresis in an agarose gel. Electrophoresis was carried out in a 1% agarose gel. Agarose (150 mg) was added to 150 ml of buffer composed of 50 mM Tris, 1 mM calcium chloride, 1 mM manganese chloride, 1 mM magnesium chloride, at pH 7.0 and melted in a microwave oven at 600 W for 5 s. The melted agarose was transferred to a 40°C water bath and Con A was added to a concentration of 10 mg/ml. Immobilized Con A in the form of Con A-Sepharose or Con A-agarose was also incorporated into some of the agarose gels in an effort to improve resolution. These immobilized lectin preparations were added in place of soluble Con A (1 packed gel vol of immobilized Con A/3 vol of 1% aga-

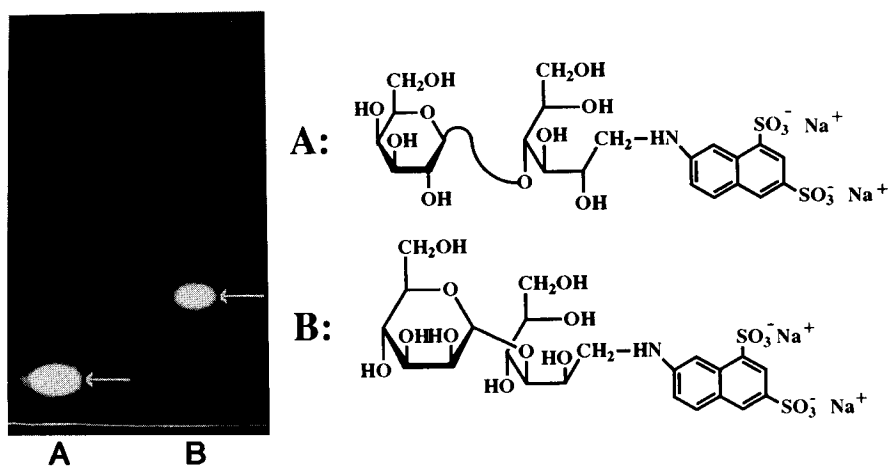


FIG. 4. PAGE analysis of β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA and α -D-Man-(1 \rightarrow 3)-D-Man-AGA. Lane A, β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA was produced by neuraminidase digestion of 1 μ g of *N*-acetylneuramin-lactose-AGA conjugates; lane B, 1 μ g of α -D-Man-(1 \rightarrow 3)-D-Man-AGA. Free Con A was added at a concentration of 10 mg/ml and 12% (w/v) isocratic polyacrylamide gel was used.

rose). The Con A and agarose gel mixture was poured immediately into a 7.5×10 -cm minigel horizontal electrophoresis unit and a well-forming comb was inserted. After the gel had cooled, the comb was removed and samples of disaccharide-fluorescent conjugates ($1 \mu\text{l}$ at 1 mg/ml) were mixed with $1 \mu\text{l}$ of 50% (v/v) glycerol solution and loaded carefully into the bottom of each well. Electrophoresis was performed at 100 V (constant voltage) for 1 h at 5°C .

Lectin affinity electrophoresis in a polyacrylamide gel. Electrophoresis was performed in 12% (w/v) isocratic polyacrylamide resolving gel that contained 11.5% (w/v) *N,N'*-acrylamide, 0.5% (w/v) *N,N'*-bisacrylamide, 1 mM calcium chloride, 1 mM manganese chloride, 1 mM magnesium chloride, and 1% (w/v) glycerol, in resolving buffer (lower buffer chamber) made from 0.1 M boric acid, 0.1 M Tris, pH 8.3. Soluble Con A (10 mg/ml), Con A-Sepharose, or Con A-agarose (1 packed gel vol of immobilized Con A/3 vol polyacrylamide gel solution) was combined with 35 ml of 12% polyacrylamide gel solution and $200 \mu\text{l}$ of freshly prepared 10% (w/v) ammonium persulfate solution. Polymerization was initiated by the addition of $20 \mu\text{l}$ of TEMED.

The 10 ml of stacking gel prepared from 4.75% (w/v) *N,N'*-acrylamide and 0.25% (w/v) *N,N'*-bisacrylamide in resolving buffer (adjusted to pH 6.3 with hydrochloric acid), $35 \mu\text{l}$ of 10% (w/v) ammonium persulfate solution, and $10 \mu\text{l}$ of TEMED. The upper buffer chamber was filled with a buffer made from 1.25 M glycine and 0.2 M Tris, pH 8.3. Disaccharide-fluorescent conjugates ($1 \mu\text{l}$ at 1 mg/ml) were combined with $1 \mu\text{l}$ of 50% (w/v) glycerol solution and loaded into each well. Electrophoresis was performed for 4 h at 400 V (constant voltage) with cooling using circulating tap water.

Photography. The pictures of the gels were taken immediately following electrophoresis in a dark room on the transilluminator (at 365 nm) using a diffusing screen between the lamp and the gel. ATMX-100 black and white 35-mm film (Kodak), a uv filter, and an aperture setting of f2.8, with automatic shutter speed, were used.

RESULTS AND DISCUSSION

Preparation of Disaccharide-AGA Conjugates

Studies in our laboratory have focused on the separation and detection a number of linear oligosaccharide model compounds in an effort to devise a method for oligosaccharide sequencing using gel electrophoresis. This approach relies on the syntheses of charged, fluorescent-oligosaccharide conjugates that are first purified and then treated with specific exoglycosidases and the shifts in band mobility on gradient PAGE are used to determine their sequence (1). The gradient PAGE method used to separate these conjugates, however, has

certain limitations, particularly in the separation of small linear oligosaccharides having the same molecular size.

Several particularly difficult separation problems were selected to test an approach that uses lectin affinity electrophoresis. The oligosaccharides examined were disaccharides: α -D-Man-(1 \rightarrow 3)-D-Man, α -D-Gal-(1 \rightarrow 4)-D-Gal, α -D-Gal-(1 \rightarrow 6)-D-Glc, and β -D-Gal-(1 \rightarrow 4)-D-Glc [derived from a commercially available mixture of Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4)-D-Glc and Neu5Ac-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)-D-Glc]. Con A is known to strongly interact with α -D-Man at the nonreducing terminus of one of these disaccharides while not interacting with α - or β -D-Gal residues in the nonreducing termini of the other three disaccharides (8-10).

Each oligosaccharide was conjugated to AGA by reductive amination, purified by preparative gradient PAGE, and recovered in $>70\%$ yield by semidry electrotransfer as previously described (1). The AGA conjugate of β -D-Gal-(1 \rightarrow 4)-D-Glc was prepared by treating a mixture of the trisaccharide-AGA conjugates, Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA, and Neu5Ac-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA (comigrating on preparative PAGE) with neuraminidase from *Clostridium perfringens*. Analyses of all three AGA-disaccharide conjugates, by analytical gradient PAGE, gave single fluorescent bands, each migrating to the same position on the gradient polyacrylamide gel (data not shown).

Separation of Disaccharide-AGA Conjugates by Con A-Agarose Gel Electrophoresis

Samples were applied to two different agarose gels. One contained immobilized Con A in the form of Con A-agarose (Fig. 1) and the other contained free Con A (Fig. 2). Both free and immobilized Con A are known to bind terminal sugars, α -D-Man, α -D-Glc, and α -D-GlcNAc, in oligosaccharide chains. To demonstrate the utility of agarose gels for lectin affinity electrophoresis, the fluorescent conjugates of α -D-Man-(1 \rightarrow 3)-D-Man, α -D-Gal-(1 \rightarrow 4)-D-Gal, and α -D-Gal-(1 \rightarrow 6)-D-Glc were analyzed. After performing electrophoresis for 1 h, both the free Con A and the immobilized Con A gels (at Con A concentrations of 1 mg/ml) separated the two disaccharide-AGA conjugates that had the same molecular weight but different lectin binding affinities (Figs. 1 and 2). The electromobility of α -D-Man-(1 \rightarrow 3)-D-Man-AGA conjugate was considerably lower than the electromobilities of α -D-Gal-(1 \rightarrow 4)-D-Gal-AGA and α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA conjugates (Figs. 1 and 2). These results were consistent with our expectations based on the known binding affinities of Con A for similar disaccharides. It should be pointed out that in the absence of Con A, no separation could be achieved between these disaccharides using agarose gel electrophoresis (data not shown). One major problem with the use of immobi-

lized Con A-agarose was the severe tailing and diffusion of bands associated with both interacting, and noninteracting disaccharide-AGA conjugates. Such diffuse banding is not unusual in agarose gel electrophoresis.

Separation of Disaccharide-AGA Conjugates by Con A PAGE

In an effort to solve the problems of band diffusion encountered during the lectin affinity electrophoresis on agarose, we also examined a Con A PAGE system. The polyacrylamide gel (Fig. 3) gave far better resolution of a mixture of α -D-Man-(1 \rightarrow 3)-D-Man-AGA and α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA conjugates than did the agarose gel (Fig. 2). This suggests that much of the band broadening occurring in the agarose system was the result of diffusion in the low density agarose gel. The α -D-Man-(1 \rightarrow 3)-D-Man-AGA conjugate showed considerably less mobility on the Con A containing polyacrylamide gel than the α -D-Gal-(1 \rightarrow 6)-D-Glc-AGA conjugate, again as expected. Treatment of a mixture of the trisaccharide conjugates, Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA and Neu5Ac-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA, with neuraminidase afforded a single disaccharide conjugate, β -D-Gal-(1 \rightarrow 4)-D-Glc-AGA, which also migrated faster than α -D-Man-(1 \rightarrow 3)-D-Man-AGA (Fig. 4).

The inclusion of immobilized Con A, either in the form of Con A-agarose or Con A-Sepharose, did not significantly affect the resolution of the polyacrylamide gel while in the absence of added Con A all three disaccharide-AGA conjugates comigrated (data not shown).

Con A affinity electrophoresis appears to be useful for the separation and analyses of simple disaccharide-AGA conjugates of the same molecular size. The incorporation of either free or immobilized Con A into the gel affords resolution of disaccharide-fluorescent conjugate having α -D-Man at its nonreducing terminus from

ones having either α -D-Gal or β -D-Gal at their nonreducing termini. Con A affinity polyacrylamide gel electrophoresis results in sharper banding and higher resolution than does Con A affinity agarose gel electrophoresis, probably due to band diffusion in the weaker agarose gel. Even in polyacrylamide gels, the band intensity decreases with time following electrophoresis. Delay in photographing the gel shown in Fig. 3 decreased the band intensities compared to the gel shown in Fig. 4 (containing an identical amount of sample in each lane). Additional studies are required to examine whether this method is widely applicable to a wide variety of lectins that bind sugars with different specificities. Preliminary studies have demonstrated that it is possible to combine lectin affinity in a tube polyacrylamide gel containing free Con A with gradient PAGE on a slab gel to afford two-dimensional separation of oligosaccharide-AGA conjugates. Such an approach might be useful for the exoglycosidase-based sequencing of more complicated glycoprotein- and glycolipid-derived oligosaccharides.

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