PROTOCOL

Lectin Affinity Electrophoresis

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Abstract

Lectin affinity electrophoresis is a powerful technique to investigate the interaction between a lectin and its ligand. Affinity electrophoresis results from the reduced mobility of a charged species owing to its interaction with an immobile species. In this protocol, a two-dimensional lectin affinity electrophoresis experiment is described that affords separation of oligosaccharides. The first-dimension is composed of a weak, polyacrylamide, capillary tube gel containing a lectin. The example described involves a mixture of fluorescently labeled disaccharides. The mobility of only the lectin-binding disaccharide is reduced affording a separation in the first-dimension. The tube gel is then extruded and placed onto the second-dimension gradient polyacrylamide gel and subjected to electrophoresis. Mobility in the second-dimension is dependent on molecular size and visualization is by fluorescence under transillumination. This method is also applicable, with appropriate modifications, for the separation and analysis of glycopeptides and glycoproteins.

Index Entries: Lectin; affinity electrophoresis; electrophoresis; two-dimensional gel electrophoresis; carbohydrates; oligosaccharides; glycoproteins; glycopeptides; fluorescence; fluorescent conjugates.

1. Introduction

Lectin affinity electrophoresis has provided a convenient method to examine lectin-polysaccharide interactions since its introduction in 1977 by Horejsi et al. *(1)*. All affinity electrophoresis techniques rely on interaction between a mobile charged species and another molecular species. Numerous variations of this basic theme have been enumerated (for reviews, *see* refs. *2,3*) including:

1. Immunoelectrophoresis, the binding of antigen with antibody *(4,5)*;
2. Lipopolysaccharides with protein *(6,7)*; and
3. Protein with other protein *(8,9)*, and DNA with protein *(10)*.

In lectin affinity electrophoresis, a lectin (a carbohydrate binding protein) is employed to specifically bind a glycoprotein or glycopeptide *(11–14)* or an oligosaccharide *(15–18)*. Affinity electrophoresis can provide both qualitative and quantitative information concerning the interaction of lectin with its sugar-containing ligand *(19)*. First, a simple qualitative experiment can separate ligand–binding components in complex mixtures; this concept was exploited by Hirai and Taketa in 1992 *(12)* as a means to diagnose malignancies. Lectin affinity electrophoresis can also be used to separate liver and bone derived alkaline phosphatase isozymes *(20)*. Second, lectin affinity electrophoresis can be used as a separation method much like affinity chromatography. Lee et al. *(17)* used lectin affinity chromatography to separate two disaccharides of identical molecular weight that would be inseparable by conventional chromatographic techniques. Finally, lectin affinity electrophoresis can also provide a disassociation constant for ligand and lectin based on the concentration dependent reduced mobility of the ligand–lectin complex in both gel *(19)* and capillary electrophoresis *(21)*. Affinity electrophoresis has been used extensively to measure dissociation constants *(10,19)*.

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In the two-dimensional experiment described below *(18)*, a neutral disaccharide is converted to a charged molecule (and thus made mobile in an electric field) by covalently attaching a fluorescent label. This molecule is moved under electrophoresis through a polyacrylamide tube gel containing lectin; if the disaccharide and lectin interact, the disaccharide's mobility will be reduced. The position of the labeled disaccharide can be visualized directly in the tube gel or it can be run into a second dimension, polyacrylamide gel, affording size separation prior to visualization.

2. Materials

2.1. First Dimension: Lectin Affinity Gel Electrophoresis

1. Mini-PROTEAN II tube (tube dimensions 1.5 × 75 mm) cell is from Bio-Rad Laboratories (Richmond, CA). Alternatively, any capillary tube gel electrophoresis equipment can be used.
2. Running buffer: 0.3*M* Tris-HCl, pH 7.0.
3. 5.5% Resolving gel solution: 5.23% acrylamide (w/v) and 0.27% *N,N'*-methylene bisacrylamide (w/v) (BIS) in the running buffer, pH 7.0.
4. 50 m*M* Calcium chloride.
5. 50 m*M* Manganese chloride. (This solution must be prepared immediately prior to its use.)
6. 10% Ammonium persulfate (APS). (This solution must be prepared immediately prior to its use.)

2.2. Second Dimension: Discontinuous Gradient Gel Electrophoresis

7. Mini-PROTEAN II dual slab (7 × 8 cm) cell is from Bio-Rad. Alternatively, any vertical slab gel electrophoresis equipment can be used. A two chamber (5 mL each) gradient maker is from Hoefer Scientific Instrument (San Francisco, CA). Alternatively, a similar gradient maker from another manufacturer can be substituted. The transilluminator (model TL-365A) is from Spectronics Co. (Westbury, NY).
8. Upper buffer: 1.25*M* glycine and 0.2*M* Tris (hydroxymethyl)aminomethane (Tris), pH 8.5.
9. Lower buffer 0.1*M* boric acid, 0.1*M* Tris, and 0.01*M* disodium ethylenediaminetetraacetic acid (EDTA), pH 8.5.
10. Stacking gel buffer: stacking gel buffer is obtained by adjusting the pH of the lower buffer to 6.5 with 1*M* HCl.
11. 12% Resolving gel solution: 11.5% acrylamide (w/v), 0.5% BIS (w/v) in the lower buffer, pH 8.5.
12. 22% Resolving gel buffer: 20% acrylamide (w/v), 2% BIS (w/v) in the lower buffer, pH 8.5.
13. 5% Stacking gel: 4.75% acrylamide (w/v) and 0.25% BIS (w/v) in stacking gel buffer, pH 6.5.
14. Sample preparation: 5 μmol of disaccharide (α-D-Man-[1→3]-D-Man or α-D-Gal-[1→4]-D-Gal) and 100 μmol of 7-amino-1,3-naphthalenedisulfonic acid, disodium salt (Aldrich Chemical, Milwaukee, WI) are dissolved in water (0.5 mL). After being incubated at 60°C for 2 h, 100 μmol of sodium cyanoborohydride is added to the reaction mixture. The mixture is further incubated at the same temperature for 24 h. The disaccharide-AGA conjugate is desalted on a 1.5 × 30 cm Bio-Gel P 2 column eluted with distilled water. The sample is freeze-dried and used in the gel electrophoresis.

3. Method

3.1. First-Dimension: Lectin Affinity Gel Electrophoresis

1. Calcium chloride (20 μL of 50 m*M*), manganese chloride (20 μL of 50 m*M*), and concanavalin A (Con A, 5 mg) are added in 1 mL of 5.5% of degassed resolving gel solution. The solution is degassed for 30 min. Then 7 μL of 10% APS and 1 μL of *N,N,N',N'*-tetramethylethylenediamine (TEMED) are added to the solution.
2. Immediately dip one end of a new capillary tube into the above gel solution until the level of the gel reaches to within 5 mm of the other end of the capillary tube. Then take the capillary tube out of the solution and lay it horizontally on a flat surface. It takes about 20 min to polymerize the gel. About five tube gels can be prepared from 1 mL gel solution.
3. Use a syringe to carefully wash the unfilled portion of the tube gel twice with running buffer. Firmly insert a gel tube into the buffer reservoir into each of the tube holder positions in the tube gel electrophoresis apparatus.
4. Fill the upper buffer chamber with running buffer. About 60 mL buffer is needed for the

Bio-Rad apparatus. Expel any air bubbles from the top of each capillary tube, connectors, and sample reservoir by gently injecting running buffer to displace the air.

5. Place the upper buffer reservoir containing the tube gels on the lower buffer reservoir. Fill the lower buffer container with the running buffer (~800 mL buffer is needed in the Bio-Rad apparatus). Any trapped air bubbles at the end of the gel can be removed by a gentle stream of buffer using a syringe having a long curved needle.
6. Place a stirring bar in the lower buffer container. Put the electrophoresis unit on a magnetic stirrer and stir slowly. A mixture of 2 μg each of the two disaccharide-AGA conjugates in 2.5 μL of water is loaded with a Hamilton syringe, onto the top of one capillary tube gel. A mixture of phenol blue and phenol red markers in 2.5 μL of water is loaded onto another tube gel.
7. Connect the electrodes to the power supply and run the gel at 150V (constant voltage). When the blue marker migrates to 1 cm from the bottom of the tube, the first dimension is completed. Normally this takes about 40 min.

3.2. Second-Dimension: Discontinuous Gradient Gel Electrophoresis

1. Wash and dry the glass plates, spacers, and comb. Assemble the slab gel sandwich. Make sure there is a good seal.
2. Degas 2.6 mL of 12% resolving gel and 2.5 mL of 22% resolving gel for about 30 min. Place the gradient maker on a stirrer and put a small stirring bar into the front chamber. Pour the 2.6 mL of 12% resolving gel into the back chamber and the 2.5 mL of 22% resolving gel into the front chamber of the gradient maker.
3. With stirring, add 15 μL of 10% APS and 2.5 μL of TEMED into the back chamber, 7.5 μL of 10% APS, and 2.5 μL of TEMED into the front chamber. Open the connection between the two chambers and the connection between the gradient maker and the gel apparatus. When the gel length is about 5.5 cm (1.5 cm from the top of the sandwich), stop pouring gel into the glass plates. Then carefully add about 1.5 cm water on the top of the gel. It takes about 20 min to polymerize the mini-slab gel.
4. After the gel polymerizes, remove the overlaying water and wash the surface of the gel twice with water. Then place a comb in the gel sandwich. This comb has a small well (4 mm long) for marker and a big well (7.3 cm long) for the tube gel from the first dimension.
5. APS (40 μL of 10% solution) and TEMED (2 μL) are added into 2 mL of 5% stacking gel solution. A syringe is then used to inject the gel onto the surface of the resolving gel and around the spacer. It takes about 20 min to polymerize the stacking gel.
6. Wash the wells twice with water by injecting water into the wells with a syringe. Fix the upper buffer chamber to the gel sandwich. Pour upper buffer (~120 mL is required in the Bio-Rad apparatus) into the upper buffer chamber. If the buffer does not leak through the seals, put the upper buffer chamber into the lower buffer container and pour lower buffer into the lower buffer container so that at least the bottom 1 cm of the gel is covered. Remove any air bubbles from the bottom of the gel by swirling the lower buffer with a pipet until the bubbles clear.
7. After the first dimension electrophoresis is complete, extrude the tube gel from the capillary tube using air pressure from a syringe filtered onto the tube gel with a silastic connector. Then load the extruded gel carefully on the mini-slab gel. Load marker dye solution in the small well.
8. Connect the electrodes to a power supply and run the gel at 150 V (constant voltage). When the blue marker migrates to 2 cm from the bottom of the slab-gel, the second dimension is complete. Normally this takes about 40 min.
9. Remove the slab-gel and put it on a transilluminator (at 365 nm) in a dark room. Turn on the UV light and take a picture.

Figure 1 demonstrates two-dimensional gel electrophoresis for the separation of disaccharide-AGA conjugates. A mixture of 2 μg each of α-D-Man-(1→3)-D-Man-AGA and α-D-Gal-(1→4)- D-Gal-AGA is analyzed. The first dimension (1.5 × 75 mm) is a 5.5% polyacrylamide gel containing 5 mg/mL of concanavalin A lectin. After separation in the first dimension, the gel is extruded and loaded onto a second gradient (12–22%) PAGE slab gel

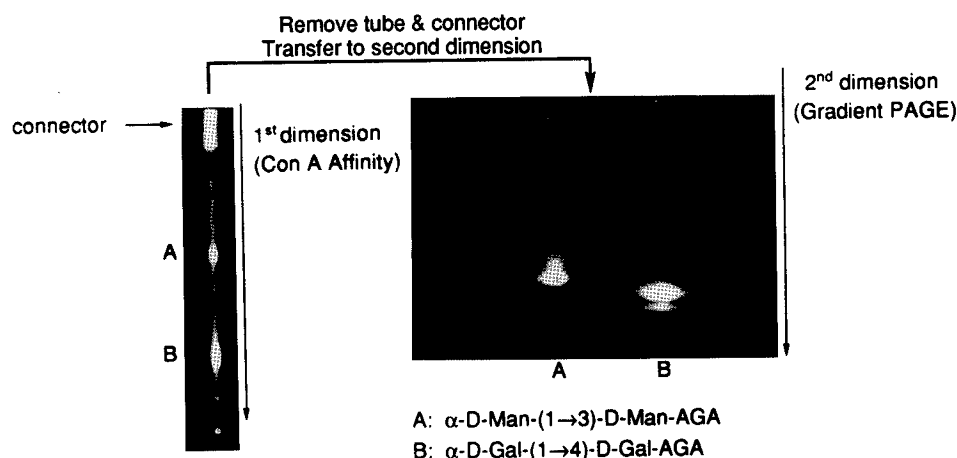

Fig. 1. Two-dimensional gel electrophoresis for the separation of disaccharide-AGA conjugates.

(7 × 8 cm). The gels were visualized by fluorescence using a transilluminator at 365 nm (λ_{ex}).

4. Notes

1. Because most of the lectins are stable around pH 7, the pH of the lectin affinity gel is adjusted to 7.0. Generally, this electrophoresis condition can be used for most lectins.
2. Sucrose, disodium EDTA, and boric acid should not be used in lectin affinity gel. Sucrose is a disaccharide, which can interact with some of the lectins. Some lectins need a metal ion (which can be chelated by EDTA) to retain their activity. Boric acid can form complex with two adjacent hydroxyl groups in a sugar and thus, may interfere with the interaction between the sugar and lectin.
3. Because the two disaccharides were labeled with a fluorescent tag, which has two negative charges, the disaccharide-tag conjugates could migrate in an electric field and can be visualized under UV light.
4. Time between the two dimensions should be managed well. Before the first dimension is complete, the second dimension should be ready to use. This will minimize the diffusion of the sample in the first dimension after it is done.
5. Make sure there are no air bubbles in the polymerized tube gel. Degassing well (30 min under water aspirator vacuum with stirring) and polymerizing slowly (use a minimum amount of APS and TEMED) can prevent this problem.
6. A gradient slab gel is used to optimize the size separation of oligosaccharides. An isocratic gel at an appropriate acrylamide density can also be used in the second dimension.
7. One-dimensional lectin affinity gel electrophoresis can be performed in either a tube or slab gel format. Visualization of the bands, within the first dimension, must be immediate since diffusion is rapid in the lectin containing gel since it has a low acrylamide concentration *(17)*.
8. Although it is possible to store the tube gel containing lectin for a short time at 4°C, it is best to use it immediately after it has been prepared.

5. Lectins, Lectin Stability, and Lectin Requirements in Affinity Electrophoresis

There are a large number of commercially available lectins (Table 1) in addition to the Con A lectin described in this protocol. These lectins have different specificities that can be exploited in analysis (Table 1). This protocol, with minor modifications, has been used effectively with *Triticum vulgaris* (wheat germ) and *Bandceiraea* lectins. The issues that must be raised in using this protocol with a new lectin include:

1. Lectin cost and availability;
2. Lectin specificity;
3. Lectin stability; and
4. Lectin requirements (i.e., metals) and compatibility with electrophoresis buffer.

Table 1
Specificities of Commercially Available Lectins

Lectins and source	Specificities
Anguilla anguilla[a]	α-L-Fuc
Lectin, AAA[b]	α-L-Fuc-(1→6)-D-GlcNAc
Lotus tetragonolobus lectin (LTL)[a]	α-L-Fuc
Tetragonolobus purpureas[a]	α-L-Fuc
Ulex europaeus (UEA1)[a]	α-L-Fuc
Agaricus bisporus[a]	β-D-Gal-(1→3)-D-GalNAc
Amaranthus caudatus lectin (ACL)[c]	β-D-Gal-(1→3)-D-GalNAc
Arachis hypogaea[a]	β-D-Gal-(1→3)-D-GalNAc
Banderiraea simplicifolia[a]	
BS-1	α-D-Gal, α-D-GalNAc
BS1-A_4	α-D-GalNAc
BS1-B_4	α-D-Gal
Bauhinia purpurea[a]	β-D-Gal-(1→3)-D-GalNAc
Caragana arborescens[a]	D-GalNAc
Codium fragile[a]	D-GalNAc
Dolichos biflorus agglutinin (DBA)[a]	α-D-GalNAc
Elderberry bark lectin (EBL)[c]	α-D-Gal-(2→6)-D-NeuNAc
Erythrina corallodendron[a]	β-D-Gal-(1→4)-D-GlcNAc
Erythrina cristagallii[a]	β-D-Gal-(1→4)-D-GlcNAc
Euonymus europaeus[a]	α-D-Gal-(1→3)-D-Gal
Glycine max[a]	D-GalNAc
Helix aspersa[a]	D-GalNAc
Helix ponnatia[a]	D-GalNAc
Jacalin[c]	β-D-Gal-(1→3)-D-GalNAc
Lectin, HPA[b]	α-D-GalNAc>α-D-GlcNAc >> α-D-Gal
Lectin, SBA[b]	α-D-GalNAc>β-D-GalNAc >> α-D-Gal
Maclura pomifera[a]	α-D-Gal, α-D-GalNAc
Momordica charantia[a]	D-Gal, D-GalNAc
Peanut agglutinin (PNA)[c]	β-D-Gal-(1→3)-D-GalNAc
Phaseolus limensis[a]	D-GalNAc
Pseudomonas aeruginosa (PA-1)[a]	D-Gal
Psophocarpus tetragonolobus[a]	D-GalNAc, D-Gal
Ptilota plumosa[a]	α-D-Gal
Ricinus communis agglutinin 1 (RCA1, RCA_{120})[c]	D-Gal, D-GalNAc
Sophora japonica[a]	β-D-GalNAc
Soybean agglutinin (SBA)[c]	α-D-GalNAc, β-D-GalNAc
Vicia villosa[a]	D-GalNAc
Vigna radiata[a]	α-D-Gal
Viscum album[a]	β-D-Gal
Wisteria floribunda[a]	D-GalNAc
Bandeiraea simplicifolia BS-2[a]	D-GlcNAc
Datura stramonium lectin (DSL)[c]	Chitobiose and chitotriose
Datura stramonium	$(D\text{-GlcNAc})_2$
Lectin, DSA[b]	*N*-acetyllactosamine
Lectin, PWM[b]	di-*N*-acetylchitobiose
Lycopersicon esculentum[a]	$(D\text{-GlcNAc})_3$
Phytolacca americana[a]	$(D\text{-GlcNAc})_3$
Solanum tuberosum[a]	$(D\text{-GlcNAc})_3$
Triticum vulgaris[a]	$(D\text{-GlcNAc})_2$ and NeuNAc
Ulex europaeus (UEA2)[a]	$(D\text{-GlcNAc})_2$
Vicia sativa[a]	D-Glc, D-Man
Wheat germ agglutinin (WGA)[c]	$(D\text{-GlcNAc})_2$ and $(D\text{-GlcNAc})_3$
Concanavalin A (Con A)[a]	α-D-Man, α-D-Glc
Lens culinaris[a]	α-D-Man
Lectin, GNA[b]	D-Man
Vicia faba[a]	D-Man, D-Glc
Pisum sativum[a]	α-D-Man
Lectin, MAA[b]	α-NeuNAc-(2→3)-D-Gal
Lectin, SNA[b]	α-NeuNAc-(2→6)-Gal or GalNAc
Cicer arietinum[a]	Fetuin
Mycoplasma gallisepticum[a]	Glycophorin

[a]Sigma Chemical Company.
[b]Boehringer Mannheim.
[c]Vector Laboratory.

Cost and availability issues are best addressed by using a capillary tube gel affinity electrophoresis first dimension. This minimizes the amount of lectin required (slab gel affinity electrophoresis requires 25-fold more lectin than tube gel electrophoresis). Lectin specificity (Table 1) establishes the oligosaccharide mixtures that can be separated and analyzed. If lectin stability is a concern, lectins stabilized through immobilization onto gel matrices can be incorporated directly into the first dimension (affinity) gel *(17)*. Alternatively, agarose (having a low gelling temperature) can be used to immobilize lectin in the first (affinity) dimension *(17)*. The metal requirement of lectins

for interaction with sugars poses a serious complication in lectin affinity electrophoresis. A required metal ion must be compatible with buffer, gel matrix, and must not interfere with the current flow in electrophoresis. The design of appropriate buffer systems should begin by using established electrophoresis buffers *(22)*.

6. Glycoproteins and Glycopeptide Analysis by Lectin Affinity Electrophoresis

The protocol described, analyzes fluorescently labeled oligosaccharides by lectin affinity electrophoresis. Glycopeptides, similarly labeled with a charged fluorescent molecule can be separated using the identical protocol. A label containing an isothiocyanate group can be reacted with an amino terminus of a glycopeptide, or oligosaccharide can first be chemically or enzymatically *(23)* released from peptide and then labeled (*see* Section 2.2., step 14). The acrylamide concentration of the second dimension gel used in size separation might need to be reduced (i.e., 10%) corresponding to increased oligosaccharide size. Alternatively, acidic oligosaccharides, such as ones containing sialic acid, can be directly analyzed. Detection in the second dimension can be accomplished using periodic acid schiff base staining (PAS) *(24)*. Glycoproteins often have sufficient charge to carry their separation under electrophoresis. Before directly analyzing such a glycoprotein, the buffer pH must be carefully considered as the pH chosen should result in the mobility of glycoprotein without causing the mobility of lectin (this can be prevented by covalent immobilization of lectin [*see* Section 5.]). The second-dimension (size separation) can be performed in the presence of sodium dodecyl sulfate (SDS) if desired and visualization can rely on PAS *(24)* or standard protein staining based on Coomassie dye or silver.

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