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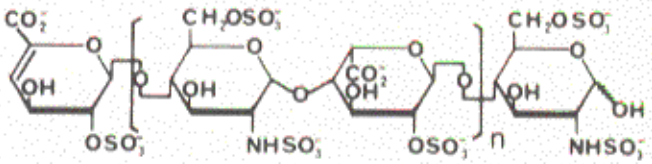
Isolation and recovery of acidic oligosaccharides from polyacrylamide gels by semi-dry electrotransfer

Acidic oligosaccharides derived from glycosaminoglycan heparin were separated by polyacrylamide gradient gel electrophoresis (PAGE). The gel could be visualized using Alcian Blue dye to give a pattern of highly resolved, well defined bands. The particular banding pattern obtained was the result of a heparinase catalyzed depolymerization which afforded oligosaccharide products that differed in size by one disaccharide unit. The separated oligosaccharides could be recovered prior to staining by electroelution onto a positively charged nylon membrane by a semi-dry transfer procedure. Subsequent elution and quantitative recovery of individual oligosaccharides from the membrane was achieved. By using multiple membrane layers a second separation dimension was obtained, resulting in increased oligosaccharide purity proportional to transfer depth. Preparative gradient polyacrylamide gel electrophoresis followed by semi-dry electro-transfer and recovery represents a novel method for the preparation of homogeneous acidic oligosaccharides.

1 Introduction

Proteoglycans (PGs) play an important role in a variety of diseases affecting the excretory system, respiratory system, circulatory system, and skeletal system, as well as the multisystem diseases of aging and cancer [1]. The chemistry and biology of a PG, however, is dominated by the polysaccharide or glycosaminoglycan (GAG) components for which it is named. These glycosaminoglycans are acidic, highly sulfated linear polysaccharides. There are many types of glycosaminoglycans; the major classes are: chondroitin sulfates, dermatan sulfate, heparan sulfate and heparin and keratan sulfate [1]. They are all structurally distinct and exhibit different biological activities. Heparin, the prototypical GAG, is widely used as an anticoagulant and antithrombotic agent and has many other recently discovered biological activities [1–3]. The basic structural feature of heparin is a predominant trisulfated disaccharide unit (Fig. 1). By using the appropriate enzyme, heparin lyase (heparinase EC 4.2.2.7), a uniform banding pattern of oligocaccharides can be obtained from GAGheparin [4]. Low molecular weight heparin-derived oligosaccharides display a variety of biological activities [5–8]. These activities are particularly pronounced in larger oligosaccharides having a degree of polymerization (dp) of between 8 and 20 saccharide units. Larger oligosaccharides are difficult to prepare at the level of purity required for structural analysis by spectroscopic techniques [9, 10], and for bioassay.

In the past, understanding and characterization of the GAG components of PGs have primarily been directed towards the identification of the class to which they belong, their molecular weight, their degree and position of sulfation and their charge density. We have been developing a method for sequencing these polysaccharides by enzymatically depolymerizing them into a mixture of oligosaccharides [7, 10, 11]. Gradient polyacrylamide gel electrophoresis (PAGE) is used to resolve these oligosaccharides into sharp, well defined bands [12]. Separation of these oligosaccharides is due principally to differences in molecular weight and charge density. High resolution was achieved by using a stacking gel for loading samples, a pore gradient resolving gel, and low voltage and temperature [12]. Once resolved, the oligosaccharide bands can be visualized by taking advantage of their ability to interact with organic cationic dyes such as Alcian Blue to form an insoluble colored complex or by silver staining. We have also recently developed a preparative gradient PAGE method in which 5–300 mg quantities of enzymatically depolymerized polysaccharides can be resolved into separate components (bands). These bands can be electro-transferred onto a positively charged, solid matrix consisting of RP Biotrace nylon membrane [13]. Recovery of resolved oligosaccharide components by elution from the membrane clearly represented the next important step in preparing oligosaccharides for further structural studies. Blotting and recovery of nucleic acids and proteins have been reported [14–17] but to our knowledge no such procedure has been reported for oligosaccharides or polysaccharides. Blotting was introduced first by Southern in 1975 by transferring DNA to nylon membrane. This was followed by RNA transfer (Northern blotting) and protein transfer (Western blotting). In 1979, electric current was introduced as a driving force for quick and efficient transfer [18]. Unlike nucleic acids and proteins, these polysaccharides are structurally complex, polydisperse, heterogeneous mixtures and therefore can not be easily prepared as homogeneous substances for sequencing. The ultimate aim of this work is to develop a Maxam-Gilbert type approach [19] to sequence these highly complex and important biopolymers.



1 n = 2

2 n = 3

Figure 1. Major products of heparin lyase catalyzed depolymerization of bovine heparin ($n = 0, 1, 2, \ldots$) and the structures of oligosaccharide 1 and 2. The center disaccharide is the predominant repeating unit in heparin.

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Abbreviations: dp, degree of polymerization; **GAG**, glycosaminoglycan; **PAGE**, polyacrylamide gel electrophoresis; **PG**, proteoglycan; **SAX-HPLC**, strong anion exchange-high performance liquid chromatography; **TEMED**, *N,N,N',N'*-tetramethylethylenediamine

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2 Materials and methods

2.1 Materials

Bovine lung heparin (145.7 USP units/mg), sodium salt, was obtained from Sigma (St. Louis, MO, USA). Heparin lyase (heparinase, EC 4.2.2.7) was either purified from *Flavobacterium heparinum* [20] (5 units (μmol/min)/mg) or it was obtained from Sigma. Acrylamide (ultrapure), Tris, Alcian Blue dye, Bromophenol Blue dye and ammonium persulfate were obtained from Boehringer Mannheim, (Indianapolis, IN, USA). Glycine hydrochloride, disodium EDTA, Azure A dye, boric acid, sucrose, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Fisher Chemical Company (Fair Lawn, NJ, USA). All other chemicals were of reagent grade. Strong anion exchange-high pressure liquid chromatography (SAX-HPLC) columns, 4.6 mm × 25 cm (analytical) and 2.5 cm × 25 cm (preparative), both 5 μm particle size, were from Phase Separations (Norwalk, CT, USA). Spectropore dialysis tubing (M_r cut off 1000) was from Spectrum Medical (Los Angeles, CA, USA). Biotrace RP nylon and nitrocellulose membranes were obtained from Gelman Science (Ann Arbor, MI, USA) and 3MM paper from Whatman (Hillsboro, OR, USA). A 32 cm × 16 cm vertical slab gel unit (SE 620), 250 ml SG500 linear gradient maker apparatus and the TE70 semi-dry electrophoretic transfer unit were from Hoefer Scientific Instruments (San Francisco, CA, USA). The electrophoresis power unit model 1420B and Trans-Blot electrotransfer system were from Bio-Rad (Richmond, CA, USA). The UV-160 spectrometer was from Shimadzu (Tokyo, Japan).

2.2 Preparation of heparin-derived oligosaccharides

Bovine heparin (1 g) was depolymerized using heparin lyase [7] and the reaction was terminated at 80 % completion [10]. This resulted in a complex mixture of oligosaccharides, some of which contained sites where heparin lyase could still act [11]. Analytical SAX-HPLC demonstrated the presence of sizable amounts of a nonasulfated hexasaccharide (oligosaccharide 1) and a dodecasulfated octasaccharide (oligosaccharide 2). SAX-HPLC was performed as described previously [11]. Briefly, the column was equilibrated with 0.2 M sodium chloride at pH 3.5. Oligosaccharides or oligosaccharide mixtures were applied to the column. The sample on the column was eluted with a linear gradient of 0.2 to 2.0 M sodium chloride at pH 3.5. The column effluent was monitored by UV absorbance at 232 nm. Oligosaccharide 1 and 2 were prepared in milligram quantities using preparative SAX-HPLC [21]. Purified oligosaccharide 1 and 2 were 95 % and 85 % pure, respectively, by analytical SAX-HPLC analysis. Compositional analysis [7] was used to establish their structures as:

(1) ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S (1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S and

(2) ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S,

(ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; IdoA, iduronic acid; GlcN, glucosamine; and S, sulfate). The 500 MHz ^{1}H NMR spectra of these two oligosaccharides were consistent with their structure.

2.3 Preparation of polyacrylamide gels

The resolving gel buffer and lower chamber buffer contained 0.1 M boric acid, 0.1 M Tris and 0.01 M disodium EDTA at pH 8.3 and were prepared as previously described [12]. Gradient polyacrylamide resolving gel was prepared from two resolving gel buffer solutions, one containing 11.52 % w/v acrylamide, the other containing 20.02 % w/v acrylamide with 0.48 and 2 % w/v *N,N'*-methylenebisacrylamide and 1 and 15 % w/v sucrose, respectively. Gels were poured vertically between glass plates (16 cm × 32 cm) separated by 0.75, 1.5 and 3 mm spacers [12]. For the 3 mm preparative gel (140 mL total resolving gel volume), gradients were poured by adding 70 mL of 12 % solution to the reservoir and 70 mL of 22 % solution to the mixing chamber of the linear gradient maker (0.75 and 1.5 mm gels were similarly prepared using 17 mL and 35 mL total volume, respectively). Ammonium persulfate, 300 μL of 10 % in water, was added to the reservoir and 150 μL to the mixing chamber, followed by addition of 30 μL TEMED to both reservoir and mixing chamber. The solution in the mixing chamber was continuously mixed using a magnetic stirrer. The valve between the reservoir and the mixing chamber was open. Polyacrylamide solution from the mixing chamber, passing by gravity into two channels leading to the top of the glass plates, formed a linear gradient from the bottom up to the top. The unpolymerized gel was overlaid with water and polymerization occurred from top to bottom. After polymerization had completed, the water layer was removed. A 10 mL solution of stacking gel made of 4.75 % w/v acrylamide and 0.25 % w/v *N,N*-methylenebisacrylamide in stacking gel buffer (which was identical to the resolving gel buffer but was adjusted to pH 6.3 with hydrochloric acid), containing 10 μL of TEMED and 150 μL of 10 % w/v of ammonium persulfate, was added to the top of the resolving gel. A comb (well formers) was inserted. After polymerization, the comb was removed and each well was rinsed with stacking buffer and then with upper chamber buffer composed of 0.2 M Tris and 1.25 M glycine at pH 8.3.

2.4 Electrophoresis of oligosaccharide samples

Samples (in distilled water at concentrations described in the text) were combined with an equal volume of 50 % w/v sucrose in distilled water containing trace quantities of Phenol Red (10 μg/mL) and Bromophenol Blue (10 μg/mL) and loaded into the bottom of each well using a microsyringe. Electrophoresis was performed for 16 h at 400 V while cooling with circulating tap water (T = 10–15 °C). The gel was removed from the glass plates and stained with Alcian Blue, 0.5 % w/v, in 2 % v/v aqueous acetic acid for 30 min. Destaining was carried out with several 200 mL volumes of 5 % v/v aqueous acetic acid.

2.5 Electro-transfer to nylon membrane

2.5.1 Transfer using wet Trans-Blot system

Electrophoresis was carried out as described above using 3 mm and 1.5 mm gels. The unstained gel was placed into a Trans-Blot apparatus containing four layers of RP nylon membrane placed directly against the gel [13]. Voltages of 5 to 25 V over 1 to 8 h were used in transfer experiments. The ionic strength of the transfer buffer (0.025 to 0.25 M Tris-glycine, pH 8.3) was also varied in the electroelution experiments.

2.5.2 Transfer using semi-dry unit

The resolving gel was cut to the same size as the transfer apparatus (14 × 16 cm) and soaked in transfer buffer consisting of Tris base (5.82 g), glycine hydrochloride (4.35 g) and methanol (200 mL) made up to 1 liter with double distilled water and having a pH of 9.2. Several layers of blotting paper and transfer membranes were cut to the same size as the gel and soaked in the transfer buffer. Two pieces of saturated blotting paper (3 mm) were placed on the top of the Mylar mask, centering them over the opening. Four layers of nylon membrane (Biotrace RP) were placed on the top of the blotting papers. The soaked gel was placed directly on the nylon membranes followed by a piece of blotting paper, thus constructing a transfer sandwich. Caution was taken to prevent air bubble formation between layers by adding a few drops of buffer and sweeping each layer with a gloved finger to ensure good contact between various layers. The cover was placed over the transfer sandwich and electro-transfer was performed at 7–10 V for 2 h. After completion of the transfer process, the transfer sandwich was removed carefully without disturbing the layers. Thin slices of membranes and gel (3 mm) were cut from the edges and the center of the gel sandwich. These strips were stained as described above (the nylon membrane was stained for 2 min and destained for 2 min) to give the exact location of bands on the nylon membrane and the transfer efficiency by measuring the residual oligosaccharide sample in the gel.

2.6 Elution and recovery of membrane-bound oligosaccharides

Stacked layers of nylon membrane were aligned with stained nylon strips. Bands were located using these stained strips as a guide. Strips along the entire width of the unstained nylon membrane stack were cut using a sharp razor. Each stack of nylon strips, containing a single band, was immersed in a test tube containing 2 mL of 2 M sodium chloride and placed on a shaker for three hours at room temperature. The salt solution containing recovered oligosaccharide was dialyzed five times in M_r 1000 cutoff bags, each time against 100 volumes of water, and the sodium chloride-free sample was freeze dried and, after weighing, reconstituted in distilled water. The UV absorbance of the oligosaccharide solutions was determined at 232 nm.

2.7 Binding capacity of nylon membrane

A 96 well microtiter plate was divided into six ten-well rows with each well in every other row containing a 0.28 cm^2 RP nylon membrane disc. Heparin (250 µg, 200, 150, 100, 75, 50, 25, 10, 5 and 0 µg in 10 µL of distilled water) was added to each ten-well row. Distilled water (90 µL) was added to rows one and two, transfer buffer (90 µL) was added to rows three and four and 2M sodium chloride (90 µL) was added to rows five and six. After 4 h at 20 °C a 5 µL aliquot was removed from each well and added to 995 µL of Azure A dye reagent (0.02 % w/v in distilled water). The concentration of unbound heparin in each well was determined by measuring absorbance at 620 nm.

2.8 Binding strength of nylon membrane

An RP-nylon membrane (5 × 10 cm) was dotted (the area of the dot was approximately 0.3 cm^2) with 1000 µg, 750, 500, 250, 200, 150, 100, 75, 50, 25, 10 and 5 µg of heparin. The membrane was placed into a sandwich comprised of a gel containing no sample, dotted RP-nylon, nitrocellulose, RP-nylon, nitrocellulose, RP-nylon, nitrocellulose and RP-nylon. The sandwich was placed in the semi-dry transfer apparatus. Transfer was performed for 2 h at 7–10 V. The same experiment was then repeated using a transfer time of 4.5 h.

3 Results and discussion

3.1 Discontinuous gradient PAGE for the separation of acidic carbohydrates

Studies in our laboratory on the concentration gradient of polyacrylamide monomer and cross-linker, the use of a discontinuous buffer system and low voltage with cooling have demonstrated this to be a useful system for resolving complex mixtures of heparin oligosaccharides [12]. The work by our laboratory and others has also demonstrated that PAGE and discontinuous gradient PAGE is generally applicable in the separation of a wide variety of acidic carbohydrates. In addition to heparin [12], other GAGs and GAG-derived oligosaccharides have been fractionated using PAGE, including heparan sulfate [12, 13] chondroitin and dermatan sulfate [22–24] and keratan sulfate [25]. Because the electrophoretic mobility of a sample is directly proportional to charge and field strength and inversely proportional to viscosity of medium and size of molecules, various gradient concentrations and pore sizes were investigated for better resolution [12]. It was concluded that due to the nature of these polydisperse samples, a gradient of pore sizes which extended over the entire length of the gel was required for a good separation. Once a high resolution preparative separation was achieved it was necessary to establish methods to recover the resolved oligosaccharides from the gradient gel. Initial attempts focused on cutting the bands out, grinding the gel strips and soaking them in buffer. Although some oligosaccharide could be recovered using this method, the yield was low. Electroelution by transfer onto a polymer membrane was first attempted using nitrocellulose but the binding of the polyanionic heparin oligosaccharides to nitrocellulose was too weak to make this a useful approach.

3.2 Electrophoretic transfer systems

Electro-transfer of heparan-sulfate derived oligosaccharides from a polyacrylamide gel to a positively charged nylon membrane was recently reported [13]. This approach was developed for analytical applications. Gradient gels are particularly difficult to dry (they tend to crack) making autoradiography problematic. Electro-transfer of radiolabelled oligosaccharide out of the gel onto a membrane permits their easy detection by autoradiography. In the present work, two electro-transfer systems were investigated. The Trans-Blot wet transfer cell and a semi-dry transfer apparatus were used to recover oligosaccharide from the gel. The Trans-Blot wet transfer resulted in sample loss, band broadening and long transfer times. These problems were especially pronounced when a large amount of sample is used (such as 5–300 mg of

oligosaccharide mixture typically loaded onto a preparative gel). The large volume of buffer present in the transfer tank may have resulted in sample loss through ion-exchange mediated dissociation from the positively charged nylon membrane. The compression required to hold the sandwich assembly together distorted the gel. When combined with the high voltage used during the wet electrotransfer the resolution between the transferred bands was reduced. Decreasing the voltage and the ionic strength of the buffer did not markedly improve the resolution nor did it increase transfer efficiency.

The second electro-transfer system examined was a semi-dry transfer unit which was recently introduced onto the market. This unit does not use a buffer tank, and required low voltage and a considerably shorter transfer time. Electro-transfer using this apparatus resulted in the rapid and complete transfer of heparin-derived oligosaccharides from both analytical (1.5 mm) and preparative (3 mm) gradient polyacrylamide gels to positively charged nylon membranes. Visualization of bands by staining the membrane with Alcian Blue could be achieved in a few minutes. For transferring larger quantitites of oligosaccharide samples (particularly from 3 mm preparative gels), multiple layers of positively charged nylon membranes were required to avoid membrane saturation. These membranes, described by the manufacturer as having a capacity of 500 μg of protein per cm^2, bound only 100–200 μg of heparin/cm^2 in transfer buffer. Examination of each membrane layer within the sandwich suggested that once the first layer was saturated with the sample, oligosaccharide being electro-transferred from the gel penetrates through the saturated first layer into the next layer. However, when a membrane contained less than a saturating concentration of heparin (<100 μg/cm^2), then, under the conditions used in the semi-dry electro-transfer (7–10 V for up to 4.5 h), nearly all the sample remains tightly bound to that membrane.

3.3 Preparative gradient PAGE

A mixture of heparin-derived oligosaccharides of different sizes ranging from disaccharide (dp 2) to oligosaccharide of dp≈28 can be resolved into well defined bands using preparative gradient PAGE (Fig. 2). Although gradient PAGE of heparin oligosaccharides, with high resolution and well defined bands, has been described previously in some detail [12], we modified the reported procedure slightly to obtain enhanced resolution of the especially large sample sizes used in preparative PAGE. By making a 12 to 22 % total acrylamide gradient, a high resolution separation was achieved through the entire gel (Fig. 2). The smiling effect, prevalent in the 3 mm preparative gels, was reduced by using circulating tap water as coolant during electrophoresis. Decreasing the voltage also helped to optimize the separation.

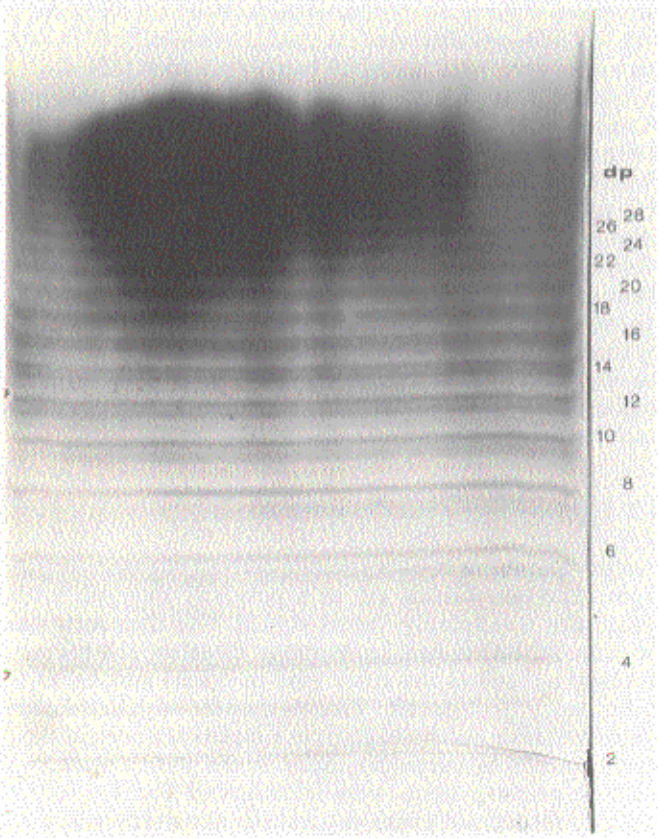

Figure 2. Stained 3 mm preparative gradient polyacrylamide gel containing 3 mg of oligosaccharide sample. Major bands are labelled as to their dp.

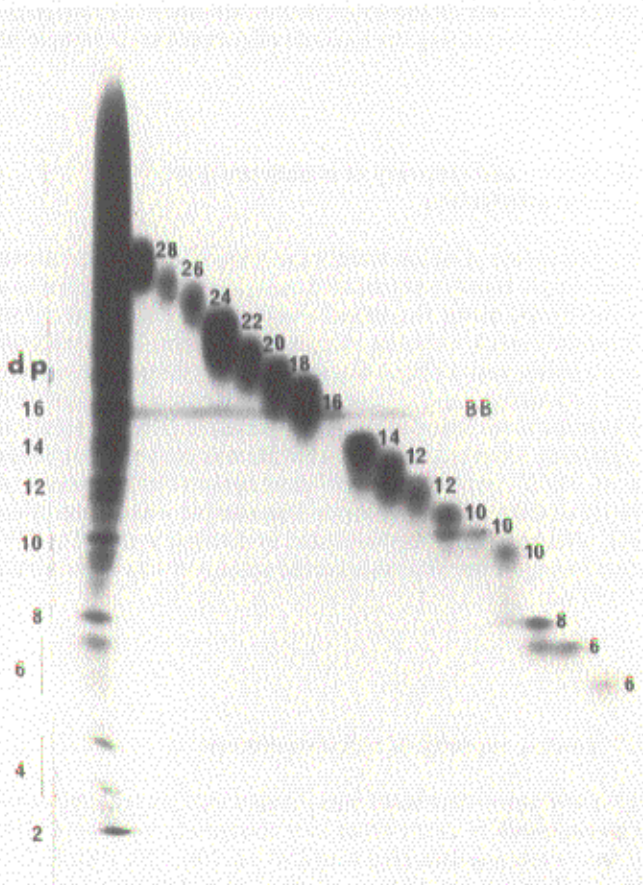

Figure 3. Stained analytical gradient polyacrylamide gel (1.5 mm) of oligosaccharides (50 to 75 μg) recovered from a preparative gel using electro-transfer. On the left is the depolymerized oligosaccharide mixture. Recovered oligosaccharide samples are loaded (left to right) from highest (dp of 28) to lowest molecular weight (dp of 6). The dp is labelled to the right of each band. The dp 8 band and the dp 6 band, immediately to its right, are oligosaccharides 2 and 1, respectively. Bromophenol Blue is labelled as BB.

Figure 2 shows a stained preparative 3 mm gel which contained 3 mg of oligosaccharide mixture, representing only 1 % of the sample loaded on the gel used in the transfer experiment. The simple banding pattern observed is primarily the result of the presence of a predominant trisulfated disaccharide repeating unit present in bovine lung heparin (porcine mucosal heparin has a lower concentration of this trisulfated disaccharide [7]). Each major band differs by a single disaccharide unit and thus represents an oligosaccharide ladder of dp 2, 4, 6 . . . 28 [12]. Typically, between 5 and 300 mg of sample was loaded onto a preparative gel; thus each band might represent up to 13 mg of a single predominant oligosaccharide of defined size. Figure 3 shows bands which were recovered from the preparative gel and reapplied to an analytical gel (each containing 50–75 µg) together with 150 µg of the mixture from which they were obtained. These bands, representing predominately single oligosaccharide components, were recovered first by semi-dry electro-transfer of the samples from a polyacrylamide gel to a positively charged nylon membrane followed by the location of bands through the staining of strips cut from the membrane. Studies on the binding capacity of the positively charged nylon membrane demonstrated that while up to 200 µg of heparin/cm² could bind in transfer buffer, virtually no heparin bound to this membrane in the presence of 2 M sodium chloride. The bands were eluted from the membrane with salt, exhaustively dialyzed and freeze-dried. Application of several of these recovered oligosaccharide to analytical SAX-HPLC resulted in a single major peak at the expected elution time.

A parallel investigation into the separation and recovery of structurally defined oligosaccharides from a 1.5 mm gradient polyacrylamide gel was performed. A mixture containing 1 mg of hexasaccharide 1 and 1 mg of octasaccharide 2 (purified using SAX-HPLC) was prepared. This mixture was applied to the polyacrylamide gel, separated by electrophoresis and electrotransferred (semi-dry) to a 5-layer sandwich of positively charged nylon membrane; oligosaccharides were recovered using a salt solution as described previously and desalted by dialysis. Figure 4 shows that this artificial mixture of hexasaccharide 1 and octasaccharide 2 can be efficiently separated and recovered. Analysis by UV absorbance at 232 nm demonstrated that 95 % of the sample had been recovered. Analytical SAX-HPLC, using hexasaccharide 1 and octasaccharide 2 standards, confirmed the identity of the recovered oligosaccharides.

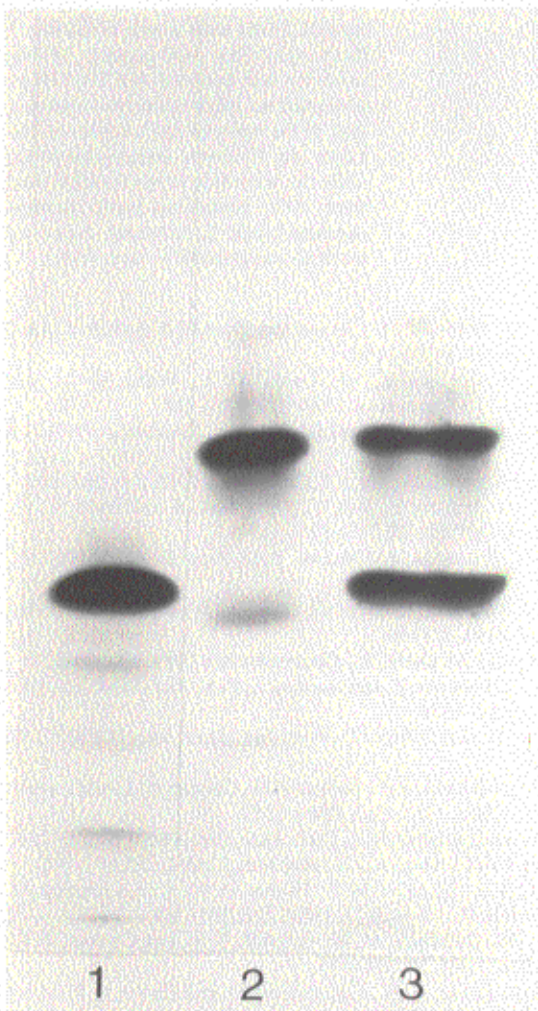

Figure 4. Stained gradient polyacrylamide gel (1.5 mm) of oligosaccharides 1 and 2 and the mixture from which they were recovered. Oligosaccharides 1 (100 µg, lane 1) and 2 (100 µg, lane 2) were fractionated and recovered by electro-transfer from an equimolar mixture (lane 3).

3.4 Kinetic studies with successive layers of membranes

An added advantage to this approach for preparing heparin-derived oligosaccharides became clear when analyzing the results of the kinetic study (Fig. 5). As octasaccharide 2 (250 µg) was electro-transferred from a 1.5 mm gel, not only was it completely recovered but it was further purified as it transferred through successive layers of membrane. Once one layer was saturated, octasaccharide 2 moved through it and into the next layer. Since contaminating oligosaccharides (< 15 %) are at lower concentrations than octasaccharide 2, they did not saturate the first nylon layer, remained tightly bound, and thus failed to transfer to lower membrane layers. As the experiments on heparin demonstrated, at the low ionic strength under which the transfer took place and in the semi-dry environment of the sandwich, once the oligosaccharide is bound to the membrane (at concentrations below saturation) it does not release. This represents a second dimension in the separation and results in further purification of the sample. This second separation process does not depend on the size of the molecule (as demonstrated in Fig. 4) but it is controlled by a number of milliequivalents of oligosaccharide contained within each band. Thus the major component of any mixture will always transfer away from contaminants, into the membrane layers furthest from the gel.

4 Concluding remarks

A preparative gradient PAGE method for the separation of heparin-derived oligosaccharides is described. These oligosaccharides can be electro-transferred to a positively charged nylon membrane and then recovered. The strength of this method lies in its simplicity and general applicability for the recovery of acidic oligosaccharides. In addition, this paper reports the preliminary observation that a second dimension operates in this separation and recovery system. Thus the purity of the oligosaccharide increased with transfer depth. Work is currently in progress to extend this method to other GAGs.