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Separation of fluorescent oligosaccharide derivatives by microcolumn techniques based on electrophoresis and liquid chromatography

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ABSTRACT

Various aldose oligosaccharides can be quantitatively derivatized into primary amines for subsequent reaction with fluorogenic reagents, such as 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde or 3-benzoyl-2-naphthaldehyde. Capillary electrophoresis (CE) and microcolumn liquid chromatography (LC), coupled with laser-induced fluorescence detection, were evaluated as a means of separating complex oligosaccharide mixtures. Whereas microcolumn LC and open-tubular CE appear confined in their utility to relatively small oligosaccharides, unprecedented results were obtained with polyacrylamide gel-filled capillaries on hydrolyzed malto-oligosaccharides and enzymatically degraded samples of chondroitin sulfate and hyaluronic acid.

INTRODUCTION

The characterization of complex oligosaccharide mixtures is among the most challenging problems in biochemical analysis. Structural studies of complex sugar molecules, originating from glycoproteins or other biological sources, have been complicated by the natural polydispersity and heterogeneity (sequence variability) within such biomacromolecules. Although various fragments can be generated from the polysaccharides by specific enzymatic and/or chemical cleavages, effective separation techniques are required to resolve the mixture constituents. Ideally, these should cover the molecular weight range from the monosaccharide units to the size of their original native structures.

Gel filtration chromatography [1,2], ion-exchange chromatography [3,4] and slab gel electrophoresis [5–7] are often employed in the separation and analysis of oligosaccharides such as those of glycosaminoglycan nature. The most obvious current needs in the analysis of complex oligosaccharide mixtures pertain to a lack of both adequate resolving power and a suitable detection principle. For smaller oligosaccharides, the general problems have led to the significant use of the gas-phase analytical techniques [8,9] and, more recently, capillary supercritical fluid chromatography [10,11].

Capillary electrophoresis (CE), in its different separation modes, has recently

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emerged as an advantageous method for certain naturally charged biopolymers. Whereas open-tubular systems appear promising in the separation of proteins [12–14], gel-filled capillaries have been particularly successful in dealing with mixtures of oligo- and polynucleotides [15–17]. The unfavorable mass-to-charge ratios of the latter polyions appear to be a problem for the open-tubular systems [18], but the sizing effect of gels together with extremely high separation efficiencies [15] have been demonstrated as beneficial for regularly shaped and negatively charged oligonucleotides. In order to extend the general benefits of CE to oligosaccharides, the solutes of interest must first be made to possess charged moieties in their structures. In addition, introducing a detectable moiety (a chromophore) into such molecules appears essential.

We have recently succeeded in attaching certain fluorogenic labels to amino sugars [19] and neutral saccharides following their conversion to primary amines [20]. Optimization studies on the reductive amination of selected aldoses are further reported here, while two fluorogenic reagents utilized previously in high-sensitivity amino acid and peptide analysis [21,22] are employed. CE runs in open tubes and gel-filled capillaries are compared in terms of the migration behavior of oligosaccharides. The capabilities of microcolumn liquid chromatography (LC) in packed fused-silica capillaries are also compared with CE. Finally, a preliminary application of gel-filled capillaries to complex mixtures of fragments from acidic polysaccharides is reported.

EXPERIMENTAL

Equipment

The capillary electrophoresis system used for work with both open-tubular and gel-filled capillaries was a laboratory-made instrument described previously [21]. Various lengths of fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. (187 μm O.D.) were used for all variants of electrophoresis. A high-voltage power supply (Spellman High Voltage Electronics, Plainview, NY, USA), capable of delivering 0–30 kV, was utilized. Different polarity modes had to be selected for open-tubular and gel electrophoresis. The reservoir connected with the positive high-voltage end served for injection, while the reservoir at the detector end was at the ground potential for open-tubular electrophoresis; reversal was needed for gel electrophoresis. On-column fluorescence detection was facilitated by a Model 543 argon ion laser (Omnichrom, Chino, CA, USA) operated at 457 nm. A detailed description of the instrumental set-up and optical alignment can be found in previous papers [19–21].

The microcolumn LC system consisted of a micropump (Brownlee Labs., Santa Clara, CA, USA), a 100-nl sample loop (Valco, Houston, TX, USA) a 90 cm × 250 μm I.D. fused-silica capillary (Polymicro Technologies) slurry-packed with Capcell C_{18} 5-μm silica particles (Shiseido, Tokyo, Japan) and a fluorescence detector. The detector used was a Schoeffel Model FS970 spectrofluorimeter. Gradient elution runs were done with a 10:1 flow splitter connected immediately after the mixing tee of the Brownlee pump. The flow-rate was adjusted to *ca.* 3 μl/min.

Fluorescence measurements under various reaction optimization conditions were carried out with a Perkin-Elmer (Norwalk, CT, USA) Model 650 spectrofluo-

rimeter equipped with a xenon arc lamp, powered by a Perkin-Elmer Model 150 power supply.

Chemicals

Chondroitin sulfate A (from bovine trachea), hyaluronidase (Type I-S, from bovine testes), all maltooligosaccharides and mannose and galactose were purchased from Sigma (St. Louis, MO, USA). Dextrin 15 (maltodextrin) and hyaluronic acid were obtained from Fluka (Ronkonkoma, NY, USA). The fluorogenic reagents, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) and 3-benzoyl-2-naphthaldehyde (BNA), were synthesized in our laboratory [21,22]. Additional chemicals used for capillary coating and the preparation of gel-filled capillaries were acrylamide, N,N′-methylenebisacrylamide, triethanolamine (TEA), triethanolamine chloride (TEA · HCl), ammonium persulfate and γ-methacryloxypropyltrimethoxysilane (all purchased from Sigma). Sodium cyanoborohydride ($NaBH_3CN$) was obtained from Aldrich (Milwaukee, WI, USA). Potassium cyanide and sodium borate were of analytical-reagent grade from Mallinckrodt (Paris, KY, USA).

Capillary coating and gel preparation

For capillary polyacrylamide gel electrophoresis, surface pretreatment is necessary prior to gel preparation [23]. A stock solution of acrylamide and N,N′-methylenebisacrylamide with concentration of 20% T and 30% C[a] was prepared for the subsequent variations of concentration. Preparation of the polyacrylamide gel was performed by a progressive polymerization (isotachophoretic method) developed in this laboratory [23].

Enzymatic digestion of glycosaminoglycans

Hyaluronidase (30 mg/ml in 0.15 *M* NaCl–0.1 *M* sodium acetate, pH 5.0, corresponding to 9000 units/ml) was prepared in cold solution prior to use. A 100-mg amount of chondroitin sulfate A was dissolved in 0.5 ml of 0.15 *M* NaCl–0.1 *M* sodium acetate (pH 5.0). Chondroitin samples were preincubated at 37°C for about 10 min before adding the hyaluronidase solution. To a preincubated sample vial containing 20 mg of chondroitin sulphate A, 400 μl of hyaluronidase solution was added. The mixture was kept at 37°C for 0.5 h. After digestion, 0.8 ml of solution was withdrawn and put immediately into a boiling water-bath for 10 min for enzyme deactivation. The solution was subsequently centrifuged and kept in a freezer for further treatment.

Approximately 8.0 mg of hyaluronic acid were dissolved in 1.5 ml of dionized water to obtain a clear solution, then 0.2 ml of this solution was mixed with 0.1 ml of hyaluronidase solution and incubated at 37°C for 30 min. The procedures for enzymatic treatment were the same as above.

Preparation of fluorescent carbohydrate derivatives

Before derivatization, all the mono- and oligosaccharides studied were dissolved in water and placed in screw-capped vials or polypropylene plastic sample

[a] C = g N,N′-methylenebisacrylamide (Bis)/% T; T = g acrylamide + g Bis per 100 ml of solution.

vials. Excess of 2.0 *M* ammonium sulfate or 4.0 *M* ammonium chloride and 0.4 *M* sodium cyanoborohydride were added to the vials and mixed. The tightly sealed vials were placed in a heating block and kept at 100°C for 100–120 min. After completion of this reaction, the solutions were immediately cooled by putting the vials in an ice-bath. Such mixtures can either be used directly for derivatization by a fluorogenic reagent, or dried and later redissolved.

The identity of the reductive amination product was verified by ^{13}C NMR spectrometry with a Model Am500 instrument (Bruker, Karlsruhe, Germany). As seen in Fig. 1, the chemical shift of the carbon at the reducing end becomes $\delta = 42$ ppm after introduction of the primary amine group, compared with the original value of $\delta = 98$ ppm. No signal was observed around $\delta = 98$–102 ppm, where the 1′-carbon of the pyranose structure would appear if the reaction were incomplete. The side-reaction of forming a dimer imine usually occurs only with relatively high-concentration sample solutions as observed in the present NMR experiments. With the use of very low sample concentrations for reductive amination, this phenomenon can be neglected. In addition, the dimer imine formed cannot be reacted with our fluorogenic reagent, CBQCA, which reacts only with primary amines.

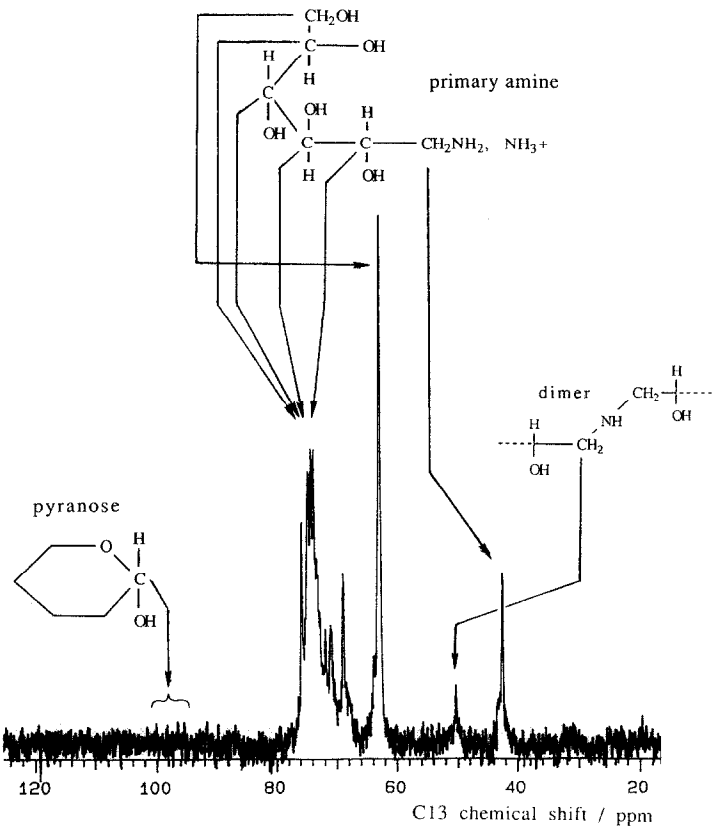


Fig. 1. ^{13}C NMR spectrum of glucose after reductive amination.

Optimization of this derivatization procedure was pursued with respect to temperature, reaction time and the molar ratio of reagents to the model sugars (glucose and galactose).

Oligosaccharides obtained from the enzymatic digestion experiments were treated similarly. A 200-μl volume of digested chondroitin sulfate solution or 300 μl of digested hyaluronic acid was mixed with 200 and 100 μl of a reducing agent [0.2 *M* $NaBH_3CN$ and 1 *M* $(NH_4)_2SO_4$], respectively. The solutions were kept at 100°C for 2–3 h, then cooled and stored in a freezer prior to the next derivatization step.

Derivatization by CBQCA for electrophoresis (or by BNA for microcolumn LC) were carried out as described previously [19–22]. Typically, 20-μl aliquots of the final sample solution were mixed with 20 μl of 20 m*M* KCN and 10 μl of 10 m*M* CBQCA (or BNA) in methanol. The mixtures were allowed to stand at room temperature for at least 1 h prior to injection. Sample introduction was carried out by the hydrodynamic (gravity) method for open-tubular CE and by the electromigration method for gel-filled capillaries.

RESULTS AND DISCUSSION

A lack of detectable features in carbohydrate molecules has been a major hindrance to high-sensitivity measurements within this important class of compounds. The use of reductive amination, followed by the attachment of a fluorophore to the reducing end of mono- and oligosaccharides [20], has enabled us to extend the detection possibilities down to the attomole level. It was further necessary to optimize the reduction amination conditions with respect to reaction temperature and time, and also the molar ratios of the reducing agent and ammonium ion to sugars. Mannose and galactose, two of the most common monosaccharides found in glycoproteins, were chosen as model compounds for these experiments. The fluorescence intensity was measured at least three times, while the entire procedures were repeated several times to ensure adequate precision.

Our studies show that the rate of reductive amination increases with temperature. The maximum fluorescence intensity was reached in about 100 min at 95°C; accordingly, we set 100°C as a standard for all subsequent experiments. Stable fluorescence was observed when the molar ratio of $NaBH_3CN$ to substrate was at least 2:1, while no adverse effects were seen at significantly larger ratios. However, a large excess of ammonium ion [from $(NH_4)_2SO_4$, NH_4Cl or another ammonium salt] was needed to ensure a good reaction yield. Owing to concern that the large excess of ammonium salt needed might yield a side-product with a fluorogenic reagent, it was deemed necessary to check for possible background fluorescence in the absence of sugar; a negligible blank response was observed. In practical measurements by CE–laser-induced fluorescence, a very small peak can be found that has no adverse effects on the analytical results. The results of optimization studies are summarized in Fig. 2.

In exploiting the separation potential of CE together with the extremely high sensitivity of laser-induced fluorescence detection for carbohydrates, we have incorporated both a potent fluorophore and a charged moiety into the molecules of interest by their reaction with CBQCA. Addition of borate to the buffer is also believed to contribute to the overall electrophoretic mobility of oligosaccharides. As seen in Fig. 3A, the maltodextrin oligosaccharides, ranging from one glucose unit to *ca.* the 15-

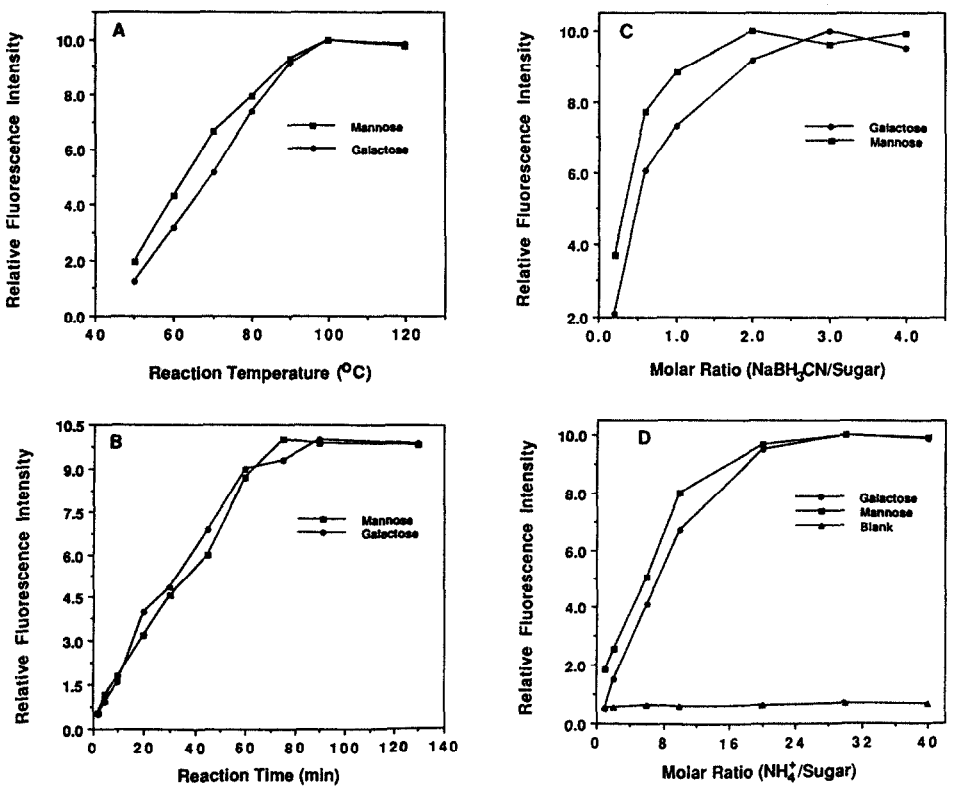


Fig. 2. Dependence of the relative fluorescence intensity of CBQCA-derivatized carbohydrates on the reaction conditions for reductive amination. Monosaccharides were reductively aminated (A) at various temperatures for 120 min and (B) at 95°C for different time periods. Experiments in (C) and (D) were performed by keeping the concentrations of ammonium ion (C) and reducing agent ($NaBH_3CN$) (D). constant, with a reaction temperature of 100°C and a reaction time of 120 min.

mer, can be distinguished by CE in the open-tubular format. Fig. 3B shows the relationship between CE migration times and the degree of polymerization (correlation coefficient 0.996), which corresponds to the peaks detected in Fig. 3A. Obviously, the resolution between the peaks decreases with increasing number of glucose units. The situation is reminiscent of the problems encountered in our previous studies with oligonucleotides [18] possessing an unfavorable mass-to-charge ratio. Our attempts to improve the resolution of large oligosaccharides through the use of mobile phase additives, such as cyclodextrins or detergents, have so far not been successful.

Similar results were achieved with microcolumn LC in the reversed-phase mode. In order to enhance the hydrophobicity of the oligosaccharide solutes, BNA [22] rather than CBQCA [21] was used for derivatization following the reductive amination step. While the lower oligosaccharides are separated efficiently on a C_{18} stationary phase, resolution difficulties arise at approximately ten monosaccharide units, as seen in Fig. 4. Attempts to optimize gradient elution at this point have not been successful.

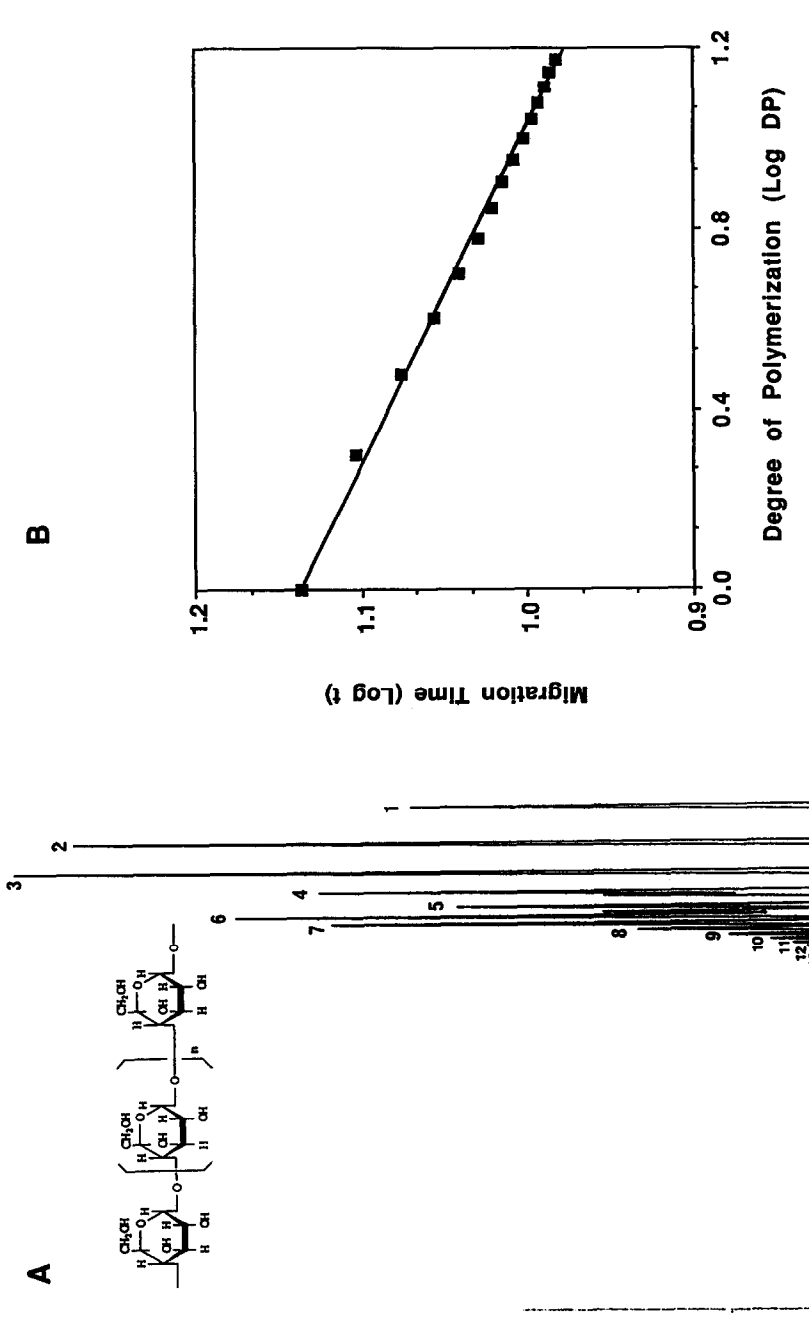


Fig. 3. (A) Electrophoretic separation of a partially hydrolyzed polysaccharide (Dextrin 15) in the open-tubular system. (B) Correlation (log–log) between molecular weight and migration time. Capillary 88 cm [58 cm effective length × 50 μm I.D.; buffer, 10 m*M* Na_2HPO_4–10 m*M* $Na_2B_4O_7 \cdot 10H_2O$ (pH 9.40)]; applied voltage, 20 kV (12 μA).

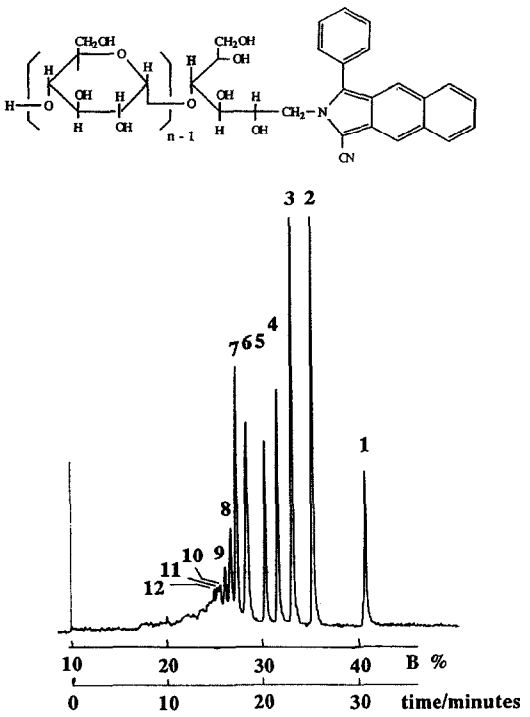


Fig. 4. Chromatogram of the individual BNA-derivatized components of Dextrin 15. Microcolumn: 90 cm × 250 μm I.D. packed with Capcell-C_{18}. Gradient elution: solvent A, water; solvent B, acetonitrile; linear gradient at a flow-rate of 3 μl/min.

Similarly to the success in separating polynucleotides [15], polyacrylamide gel-filled capillaries could be an alternative approach to separating larger oligosaccharides. Initial uses of a relatively low gel concentration (6% T and 3% C) were not satisfactory. However, the utilization of more concentrated gel matrices improved the resolution of oligosaccharides dramatically. Employing 10% T/3% C polyacrylamide gels and Tris–borate–urea buffers led to good separations of higher oligosaccharides (Fig. 5A). Their separation is now based strictly on the molecular size of the separated solutes, as seen in Fig. 5B. The relationship between migration times and the degree of polymerization is linear (correlation coefficient 0.997). Although there is some sacrifice in the speed of analysis compared with the open-tubular approach, the benefits for larger oligosaccharides are obvious when using the gel-filled capillaries. It is commonly thought that the most useful range of oligosaccharide fragments, originating from glycoprotein cleavages, should cover up to about 25 monosaccharide units. However, requirements in other biochemical and pharmaceutical problems may go well beyond that range.

The separation of acidic oligosaccharides such as the glycosaminoglycan-derived entities is yet another important task in purification and analysis for biosynthetic and structural studies. Enzymatic digestion of glycosaminoglycans, such as hyaluronic acid, chondroitin sulfate and heparin, is frequently used to produce smaller

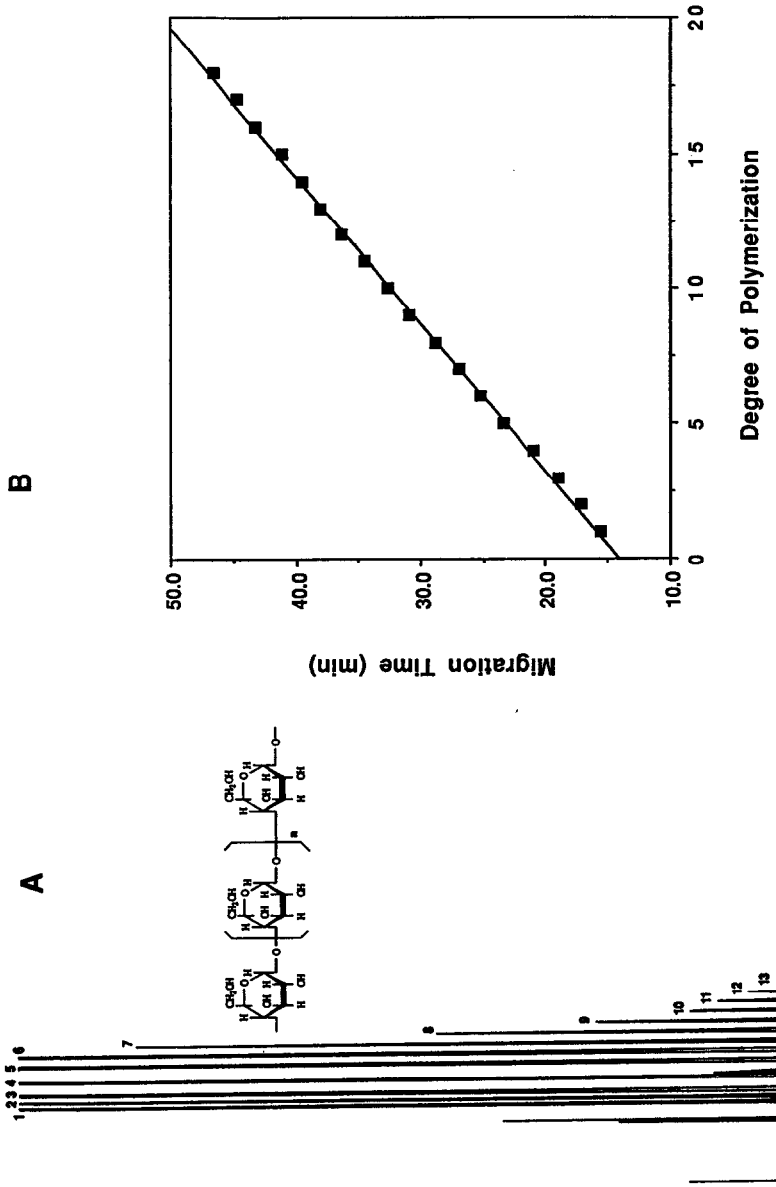
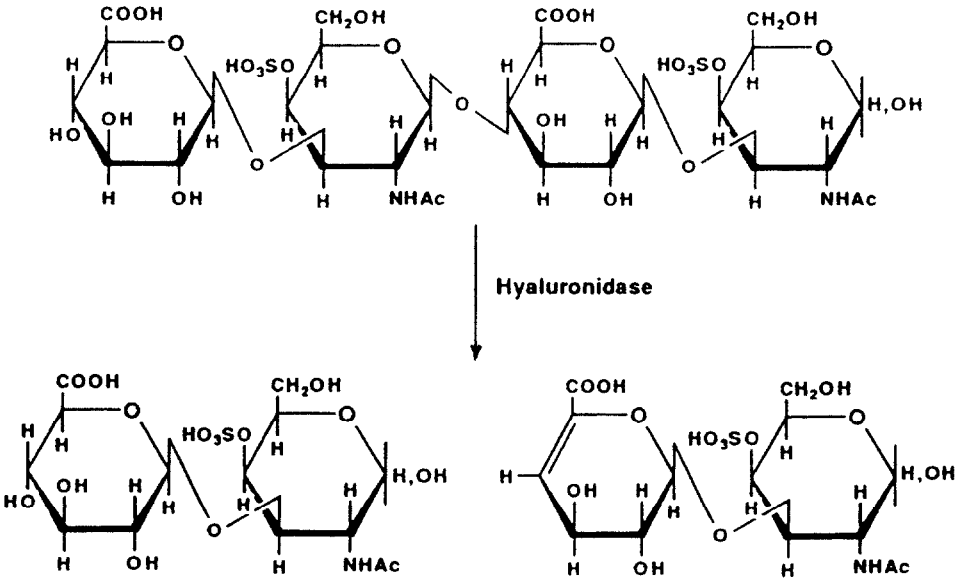


Fig. 5. (A) Electrophoretic separation of a partially hydrolyzed Dextrin 15 in a polyacrylamide gel-filled column. (B) Correlation between molecular masses of the oligomers from maltodextrin and their corresponding migration times. Gel concentration, 10% T, 3% C; capillary, 26 cm (19 cm effective length) × 50 μm I.D.; buffer, 0.1 *M* Tris–0.25 *M* borate–7 *M* urea (pH 8.33); applied field, 269 V/cm (20 μA).

fragments for subsequent separation. Testicular hyaluronidase has been the most common enzyme used extensively for the preparation of oligosaccharidic fragments. In general, chondroitin sulfate and hyaluronic acid can be converted by hyaluronidase into oligosaccharides with N-acetyl-D-hexosamine residues at their reducing terminals, which could further be utilized for chemical modification, and D-glucuronic acid residues with non-reducing terminals. A scheme for depolymerization involving a tetrasaccharide from chondroitin sulphate may proceed as follows:



Enzymatic cleavages of such glycans should yield a final mixture of repeatable oligosaccharides with sizes ranging from a disaccharide to oligosaccharides with various chain lengths, depending on the exposure of the starting material to the enzyme. In our initial experiments, CE of chondroitin sulfate A-derived oligosaccharides with low-concentration gels (6% T and 3%) failed to provide adequate component resolution. However, significant improvements were achieved with more concentrated gel matrices (18% T and 3% C) and buffers containing Tris–borate–EDTA or Tris–borate–urea. Fig. 6 demonstrates a representative separation of chondroitin sulfate-derived oligosaccharides with the use of Tris–borate–EDTA buffer; however, further optimization of the buffer composition and gel concentration appears desirable. Technical problems associated with high-concentration gels and their reproducible preparation inside the fused-silica capillaries must still be overcome.

Problems of separating glycosaminoglycan-derived oligosaccharides may also be caused by their general structural features. Normally, glycosaminoglycans are negatively charged, linear polysaccharides built up from disaccharide repeating units. However, structural diversity may be conferred on the macromolecule by variations in both the degree and position of sulfated groups. For example, chontroitin-4- and -6-sulfate are often associated with each other biochemically and during isolation steps. Consequently, oligosaccharides derived from chondroitin sulfate could be a

Fig. 6. Electrophoretic separation of the enzymatically digested chondroitin sulfate A in a polyacrylamide gel-filled capillary. Gel concentration, 18% T, 3% C; capillary, 32 cm (23 cm effective length) × 50 μm I.D.; buffer, 0.1 *M* Tris–0.25 *M* borate–2 m*M* EDTA (pH 8.48); applied field, 178 V/cm (7 μA).

mixture of hybrid structures containing both 4- and 6-sulfated disaccharide units. As indicated in Fig. 6, the splitting of each peak may be associated with the difference in positions of the sulfated groups, which could not be distinguished by open-tubular electrophoresis and a relatively low-concentration gel column. Small differences in the migration rates of hybrid oligosaccharides are expected particularly for solutes with a greater number of repeating units. It is also possible that the oligosaccharide peaks reflect conformational differences. It has been speculated that different mobilities of chondroitin-4- and -6-sulfate fragments with identical molecular weights may be related to a subtle difference in chain conformation [5], making the analysis of glycosaminoglycan-derived oligosaccharides a far more complex problem when compared with the separation of polynucleotide fragments.

Fig. 7 shows an additional example of the separation of acidic oligosaccharides in a polyacrylamide gel-filled column (15% T and 3% C). Hyaluronic acid has a similar structure to chondroitin sulfate, except for a lack of sulfation in the former.

Fig. 7. Electrophoretic separation of enzymatically digested hyaluronic acid in a polyacrylamide gel-filled capillary. Gel concentration, 15% T, 3% C. Capillary, 39 cm (25 cm effective length) × 50 μm I.D.; buffer as in Fig. 6; applied field, 282 V/cm (10 μA).

Disaccharides from hyaluronic acid cleaved by hyaluronidase contain just a singly charged group (a carboxyl group), while two charged moieties are associated with chondroitin sulfate. Better resolution of the oligosaccharide bands (compared with the results for chondroitin sulfate) was achieved with hyaluronic acid. It has been indicated [24] that hyaluronidase catalyzes transglycosylation in addition to hydrolysis and may thus generate oligosaccharides having sequences that were not present in the original substrate. This is likely to make the separation patterns even more complex. Direct measurement of the rate of enzymatically catalyzed transglycosylation relative to that of the hydrolysis in depolymerization of glycans has so far not been feasible.

Although the present studies on the separation of oligosaccharides in gel-filled capillaries must be viewed as preliminary, they clearly show potential for applications to biochemical problems hitherto unsolvable by other analytical techniques. We feel that capillary electrophoresis with laser-induced fluorescence detection is eminently suited to deal with the unique and difficult problems in carbohydrate research.

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