Ultrasensitive fluorometric detection of carbohydrates as derivatives in mixtures separated by capillary electrophoresis

[monosaccharides/oligosaccharides/3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde/capillary zone electrophoresis/laser detection]

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ABSTRACT Reducing monosaccharides and oligosaccharides, after reductive amination, were separated and detected as their 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) derivatives by capillary electrophoresis/laser-induced fluorescence. Under optimized conditions, the minimum detectable quantities for monosaccharide solutes were assessed at low attomole levels (0.5 μmol for the CBQCA derivative of galactose). The system has shown considerable promise for high-sensitivity analysis of both neutral and amino sugars in glycoproteins. Complex oligosaccharides, isolated from bovine fetuin by hydrazinolysis, were also successfully "mapped."

Numerous research problems of modern biochemistry are associated with complex glycoproteins. The importance of various sugar moieties in glycoproteins is wide-ranging, as they are known to be involved in processes as significant as protein targeting, cell-cell recognition, antigen-antibody interaction, and the function of receptor proteins (1). While there are urgent needs to analyze structurally extremely small quantities of various glycoproteins, bioanalytical methodologies in the carbohydrate field appear to lag behind similar capabilities that are being rapidly developed for other biological macromolecules.

In particular, mono- and oligosaccharides formed as a result of various cleavages of complex glycans (2–5) must be adequately resolved from each other before their determination at high sensitivity. A variety of modern chromatographic techniques have been applied in this task, including capillary gas chromatography (GC), supercritical fluid chromatography (SFC), and modern liquid chromatography (LC). Virtually all of these analytical techniques need conversion of carbohydrates to some derivatives, either sufficiently volatile for GC (6), soluble in supercritical fluids for SFC (7, 8), or with chromophoric moieties for detection in LC (9). To use electromigration separation techniques in carbohydrate analysis, it is further necessary to introduce charged moieties into most types of sugars.

Capillary zone electrophoresis (CZE) and other variants of capillary electrophoretic methods have developed into analytical tools of remarkable performance during the last several years (10–13). Unprecedented separation efficiencies of the order of a million theoretical plates have now been achieved through CZE with polynucleotides (14–16) and proteins (17–19), while the combination of capillary electrophoresis with laser-induced fluorescence measurements gives remarkable measurement sensitivities at the order of attomole (10^{-18} mole) or even lesser amounts (20–22) for the fragments of these biological macromolecules. Structural characterization of glycoproteins isolated in minute quanti-

ties obviously needs developing similar techniques for the high-sensitivity analysis of mono- and oligosaccharides.

To make carbohydrate mixtures amenable to CZE separation and high-sensitivity measurements, we have developed a procedure for the incorporation of the primary amine group into the carbohydrate molecule, while subsequent treatment with a unique reagent, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA), yields highly fluorescent isoindole derivatives detectable with either an argon-ion or helium/cadmium laser-based device. The reagent was developed originally for high-sensitivity determination of amino acids and peptides (22), with the excitation maxima close to 450 nm. Amino sugars react readily and quantitatively with CBQCA in the presence of cyanide, while 1-amino-1-deoxyalditols made by reductive amination (23) of reducing sugars do so likewise (Scheme I).

Scheme I

We report here the results obtained through electromigration experiments with monosaccharide and oligosaccharide complex mixtures based on the above analytical procedure. The minimal detectable quantities for monosaccharide solutes were at low attomole levels. Effectiveness of the overall analytical approach has been assessed for bovine fetuin, a previously studied glycoprotein of M_r 48,500. CZE with laser-induced fluorescence detection was utilized to "profile" significant degradation products of this glycoprotein.

MATERIALS AND METHODS

Apparatus. A home-built capillary electrophoresis/laser-induced fluorescence detection system as described (22, 24) was used for this study. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) 90 cm long (50- μm i.d.; 187- μm o.d.) were suspended between two electrodes immersed in

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Abbreviations: CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; CZE, capillary zone electrophoresis; CE, capillary electrophoresis.

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the reservoirs filled with an appropriate operating buffer solution. The high-voltage dc power supply (Spellman High Voltage Electronics, Plainview, NY) used is capable of delivering 0–30 kV. A Plexiglas box with an interlock system provided safety for the operator.

On-column fluorescence detection was carried out by utilizing an argon-ion laser (Omnichrom, Chino, CA) operated at the wavelength of 457 nm (blue line) as a light source. An on-column optical cell was made by removing the polyimide coating from a short section of the fused silica capillary. The incident laser beam is aligned to its optimum position by adjusting the position of collecting optics between the flow cell and the detector.

Fluorescence emission at 552 nm was collected through a 600- μ m fiber optic placed at a right angle to the incident laser beam. Signals isolated by a band-pass filter were monitored with a R928 photomultiplier tube and amplified with a model 128A lock-in amplifier (EG & G Princeton Applied Research, Princeton, NJ).

Materials. All monosaccharide and oligosaccharide standards, bovine fetuin glycoprotein, and CF₃COOH were purchased from Sigma. Fluorogenic reagent 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) was synthesized in our laboratory (22). Anhydrous hydrazine was received from Pierce. Sodium cyanoborohydride (NaBH₃CN) was a product of Aldrich. Potassium cyanide, sodium phosphate, sodium borate, and acetic anhydride were analytical grade reagents purchased from Mallinckrodt, while sodium bicarbonate was received from Fisher. Operating buffer solutions of the desired concentration were prepared by dissolving appropriate amounts of sodium phosphate and sodium borate in water and adjusting the pH with NaOH. All samples were prepared in aqueous solutions and kept frozen when not in use.

Reductive Amination of Neutral Carbohydrates. Standards of mono- and oligosaccharides (reducing carbohydrates) and the samples obtained from cleavages of glycoproteins were dissolved in water and placed in screw-cap vials or polypropylene plastic sample vials. Excess 2.0 M (NH₄)₂SO₄ or 4.0 M NH₄Cl and 0.4 M NaBH₃CN were added into the vials and mixed well. The tightly sealed vials were placed into a temperature-controlled heating block and kept at 100°C for 100–120 min. After completion of the reaction, the solutions were immediately cooled by putting the vials into an ice bath. Such mixtures can be used directly for derivatization by CBQCA or can be dried and redissolved. The identity of the reductive amination product was verified by ¹³C NMR with model Am-500 (Bruker, Karlsruhe, F.R.G.).

Preparation of Acetylated Oligosaccharides. The amounts up to 10 mg of bovine fetuin glycoprotein were introduced into a screw-cap vial, and a minimal amount of anhydrous hydrazine (Pierce) necessary to cover the sample was added. A closed vial was heated at 100°C for 12 hr. After hydrazine was removed under a stream of nitrogen, the vial was placed overnight in a desiccator containing sulfuric acid. The carbohydrates were subsequently isolated by an open glass column (1-cm i.d. $\times$ 40 cm) packed with Bio-Gel P-2 (Bio-Rad). With water as the mobile phase, the first fraction (about 2 ml) was collected and dried.

The residue was redissolved in water, and a portion of this solution was subjected to reacylation of the amino groups on the oligosaccharide chains by adding 200–500 μ l of saturated sodium bicarbonate solution and 50–200 μ l of acetic anhydride and allowing the solution to stand at room temperature overnight. The solution was then deionized by both an Amberlite IRA-900 anion exchanger and an Amberlite 200 cation exchanger (Sigma). The collected solution was freeze-dried and redissolved in water for further treatment.

Hydrolysis of Glycoproteins and Oligosaccharides. The procedures were carried out with either CF₃COOH or HCl as

described by Honda *et al.* (3, 9). A portion of the glycoprotein solution dissolved in water (20 μ g/ μ l) or oligosaccharides obtained by hydrazinolysis (24) was mixed with 4.0 M HCl (200 μ l) in a screw-cap vial. The vial was closed tightly and placed into a heating block and kept at 100°C for 6 hr. Another portion of sample solution was hydrolyzed by 2.0 M CF₃COOH at 100°C for 6 hr. After hydrolysis, the reaction mixture was cooled, and the acid was removed by evaporating the solution to dryness under either nitrogen stream or reduced pressure. The residue was redissolved in water and stored for further treatment.

A portion of this hydrolysate was deionized by passing it through a small column of Amberlite IRA-900 anion exchanger. Collected eluate was dried and redissolved in a small volume of water for the analysis of amino sugars.

Another portion of hydrolysate was treated by adding a saturated aqueous solution (100–500 μ l, depending on the sample concentration) of sodium bicarbonate and acetic anhydride (50–200 μ l) for reacylation of the amino groups of the amino sugars released from glycoproteins or oligosaccharides. The mixture was allowed to stand at room temperature overnight and deionized by passing it through Amberlite IRA-900 (anion exchanger) and Amberlite 200 (cation exchanger) columns. The combined solution was evaporated to dryness and redissolved in water for the following reductive amination.

Derivatization Procedures. The CBQCA-derivatizing solution was prepared by dissolving the reagent (22) in methanol (10 mM solution). Potassium cyanide was dissolved in water to provide a 20 mM solution. The derivatizations of amino sugars and reductively aminated carbohydrates were performed by mixing an aliquot of the sample solutions with 10–20 μ l of potassium cyanide solution and 5–10 μ l of CBQCA solution. The mixture was allowed to stand at room temperature for about 1 hr prior to sample introduction. An aliquot of the final hydrolysate of glycoprotein or oligosaccharides was derivatized by the same procedure. The derivatives were introduced into the CE system by either a hydrodynamic or electromigration technique.

RESULTS AND DISCUSSION

After successful use of CBQCA for the high-sensitivity detection of amino acids and peptides (22) by capillary electrophoresis/laser-induced fluorescence, it appeared worthwhile to try to extend this approach to amino-modified carbohydrates. Although reductive amination is a well-known organic reaction, its analytical applications have been rare (25–27). Reaction of an aldehyde with ammonia or primary or secondary amines in the presence of a reducing agent, sodium cyanoborohydride (NaBH₃CN), leads to the alkylated amines as in Scheme II.

Scheme II

To achieve optimum detection sensitivity and reproducibility, it was necessary to optimize reductive amination conditions with respect to reaction temperature, reaction time, and the molar ratios of the reducing agent and ammonium ion to sugars (J.L., O.S., and M.N., unpublished work). The maximum fluorescence intensity was reached in about 100 min at 95°C; accordingly, we set 100°C as a standard for all of the following experiments.

Under the optimized conditions, detection limits were examined for three most common monosaccharides (man-

FIG. 1. Electropherogram of three common monosaccharides derivatized by CBQCA after amination. Peak assignments were from left to right: first, mannose; second, glucose; third, galactose. The concentration was 3.0 nM for each. Electrophoretic conditions were: buffer, 10 mM Na_2HPO_4 /10 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$, pH 9.40; capillary, 50- μm i.d. $\times$ 90 cm (60-cm effective length); injection by electromigration, 5 kV for 5 s; applied voltage, 22 kV (15 μA).

nose, glucose, and galactose). Fig. 1 shows an electropherogram of this simple mixture with each monosaccharide at 3 nM. When a 2.1-nl sample was injected, the detection limits corresponded to 2.3 amol for mannose, 1.3 amol for glucose, and 0.5 amol for galactose (signal-to-noise ratio = 3). The relationship between the peak height and concentration of standard monosaccharides was found to be linear over 4 orders of magnitude (10^{-9} – 10^{-5} M) with satisfactory correlation coefficients ($R^2 = 0.998$ for all three sugars).

The detection methodology reported here is several orders of magnitude more sensitive than the best previously reported results with liquid chromatography (2, 3, 9) and CE (28). UV, conventional fluorescence, or amperometric detection used with such separation methods usually feature detection limits at picomole levels. The high sensitivity of measurements reported here is mainly due to the use of a suitable fluorogenic reagent that is compatible with the spectral characteristics of the argon-ion laser.

The use of highly sensitive laser detection complements the resolving power and rapidity of CZE. Without buffer optimization efforts, large numbers of theoretical plates were readily achieved for mono- and oligosaccharides. The values ranged from 100,000 to 400,000 theoretical plates per m. Fig. 2 shows the separation of a 12-component monosaccharide mixture in about 20 min. In agreement with the experience of another laboratory (28), we find using borate as a buffer additive to be beneficial. Complexation of hydroxy groups with borate ion, by magnifying small steric differences be-

FIG. 2. Electrophoretic separation of a derivatized monosaccharide mixture. Sample concentrations were 6.2 μM for glucosamine and galactosamine, 5.5 μM for galacturonic acid, and 4.4 μM for other sugars. Peak assignments were: 1, D(+)-glucosamine; 2, D(+)-galactosamine; 3, D-erythrose; 4, D-ribose; 5, D-talose; 6, D-mannose; 7, D-glucose; 8, D-galactose; 9, impurity; 10, D-galacturonic acid; 11, D-glucuronic acid; 12, D-glucosaminic acid; and 13, D-glucose 6-phosphate. Electrophoretic conditions were: buffer, same as in Fig. 1; capillary, 50- μm i.d. $\times$ 88 cm (58-cm effective length); hydrodynamic sample injection, 5 s; applied voltage, 20 kV (12 μA).

tween closely related isomers, enhances resolution in addition to contributing to the charged character of these solutes.

To evaluate the capability of the method with respect to determining oligosaccharides with a single reducing center, studies were carried out on standard oligosaccharides of various chain lengths. Fig. 3 shows an electropherogram of standard solutes at 1.5 μM concentration. In a common phosphate/borate buffer (10 mM/10 mM, pH 9.40), the oligosaccharides appear at predictable time intervals. Not surprisingly, resolution decreases with the increasing number of glucose units. This situation is reminiscent of the problems encountered in open tubular CZE of oligonucleotides (16), suggesting that gel-filled capillaries may be essential to analyzing larger molecules, as they have been shown to be in the field of polynucleotide separations (14).

The most common glycoproteins appear to contain two neutral sugars, mannose and galactose, and two amino sugar derivatives, *N*-acetylglucosamine and *N*-acetylgalactosamine. The neutral sugars can be easily released from a glycoprotein by hydrolysis with 2.0 M CF_3COOH acid in 6 hr at 100°C. For a complete release of hexosamines, more strongly acidic conditions are needed. Usually, a glycoprotein is hydrolyzed by 4.0 M HCl for 6 hr at 100°C, generating the amino sugars completely from their *N*-acetylated derivatives. If the resultant amino acids and peptides are removed

FIG. 3. Electropherogram of standard oligosaccharides derivatized by CBQCA after amination. The sample concentration was 1.5 μ M each. Peak identifications were: 1, maltoheptaose; 2, maltohexaose; 3, maltopentaose; 4, maltotetraose; 5, maltotriose; 6, maltose; and 7, glucose. Electrophoretic conditions are the same as described in Fig. 2.

from the mixture, hexosamines can be directly determined by our fluorescent derivatization approach, while neutral sugars do not interfere. Fig. 4A shows the electropherogram obtained from a hydrolysate of bovine fetuin in 4.0 M HCl. Under these conditions, the peaks for glucosamine and galactosamine are clearly indicated, while the third major peak likely corresponds to N-deacetylated sialic acid, which we did not investigate in the present study. A simultaneous analysis of neutral and amino sugars would necessitate reacylation of hexosamines prior to the reductive amination and subsequent use of our fluorogenic reagent, CBQCA. This analysis could be performed by further hydrolyzing oligosaccharides isolated from the polypeptide chain. It also provides an alternative way for verifying the presence of oligosaccharides. The results obtained from this approach are presented in Fig. 4B–D. The electropherogram of CF_3COOH hydrolysate reveals the presence of mannose, galactose, and hexosamines (Fig. 4C), but two amino sugars do not seem to be well detected because of their incomplete release by CF_3COOH (2, 9). Fig. 4D shows the results from fetuin oligosaccharides hydrolyzed by HCl, giving clearly the four major monosaccharide components (identified through standards).

An exciting possibility of a further use of the CE/laser fluorescence system is in high-sensitivity oligosaccharide “mapping” (an analogy to peptide mapping in protein studies). Complex oligosaccharide mixtures could be separated and detected following isolation of glycan entities and their further fragmentation. Although the use of enzymes, such as glycopeptidase F (5) and Pronase (29), for releasing carbohydrates from polypeptide matrix is known to be beneficial, hydrazinolysis represents a simple route for N-glycosides, giving as the major product free reducing oligosaccharides (30). For the sake of simplicity, we used hydrazinolysis in this work.

An electrophoretic oligosaccharide “map” obtained through hydrazinolysis of bovine fetuin is shown in Fig. 5. The four major peaks observed are likely to correspond to the oligosaccharides proposed for fetuin by others (5), although we have presently no means to verify these structures. The

FIG. 4. Analysis of monosaccharides released from 5 mg of bovine fetuin by acidic hydrolysis. (A) Peak assignments were: 1, glucosamine; 2, galactosamine; 3, unknown. Amino sugars were released with 4 M HCl at 100°C for 6 hr and subsequently derivatized by CBQCA. Electrophoretic conditions were: buffer, 20 mM Na_2HPO_4 /20 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$, pH 9.50; capillary, 50- μ m i.d. $\times$ 90 cm (60-cm effective length); hydrodynamic injection, 10 s; applied voltage, 20 kV (24 μ A). Peak assignments for B–D were: 1, N-acetylglucosamine; 2, N-acetylgalactosamine; 3, mannose; 4, fucose; 5, galactose; 6, galacturonic acid (internal standard). (B) Standard mixture of monosaccharides with 10-s hydrodynamic injection. (C) Hydrolysate from 2.0 M CF_3COOH treatment with 6-s hydrodynamic injection. (D) Hydrolysate from 4.0 M HCl treatment with 15-s hydrodynamic injection.

FIG. 5. Analysis of N-linked oligosaccharides released from 5 mg of bovine fetuin by hydrazinolysis. Electrophoretic conditions were: buffer, 20 mM Na_2HPO_4 /20 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$, pH 9.50; capillary, 50- μm i.d. $\times$ 90 cm (60-cm effective length); hydrodynamic injection, 15 s; applied voltage, 20 kV (25 μA).

extra minor peaks may also be accounted for according to the recent information (31) on this glycoprotein. Acquisition of standard compounds and mass-spectroscopic studies will be needed to identify positively the various structural arrangements in these sugar molecules.

The injection volumes and the carbohydrate amounts introduced into the CE/laser fluorescence system described above correspond to subpicogram amounts of glycoproteins. While the potential for experiments with extremely small samples and carbohydrate analysis [possibly, at the single-cell level (32)] is clearly indicated, future developments must concentrate on improving the methods for isolation and specific fragmentation of glycan entities at such low levels.

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