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Analysis of fluorescently labeled oligosaccharides by capillary electrophoresis and electrospray ionization mass spectrometry

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Abstract

Neutral oligosaccharides were fluorescently conjugated with 7-amino-1, 3-naphthalenedisulfonic acid. A mixture of fluorescently labeled chitobiose, chitotriose, and chitotetrose were successfully separated by preparative capillary electrophoresis (CE) and the individual components characterized by electrospray ionization-mass spectrometry (ESI-MS). By combining fluorescent labeling with CE, the use of highly specific exoglycosidases and ESI-MS, a more structurally complex *N*-linked glycan was analyzed.

Introduction

Glycosylation plays an important role in biological processes such as antigenicity, secretion, and clearance of glycoproteins (Drickamer & Taylor 1998, Reuter & Gabius 1999). Some of the functional diversity of the glycoproteins can be attributed to their oligosaccharide components. The glycosylation profile of a recombinant protein can significantly affect its biological activity and is particularly important in human therapeutic applications (Kobata 1998, Kornfield 1998, Dennis *et al.* 1999). Thus, the identification of individual and closely related glycoprotein-derived oligosaccharides remains a necessary but demanding challenge. In recent years, different strategies for the preparation and sequencing of oligosaccharides from glycoproteins have been developed, i.e., size exclusion chromatography (Bigge *et al.* 1995), high pH ion-exchange chromatography (Lee 1990) and gel and capillary electrophoresis (Lee *et al.* 1991, 1992, Jackson 1997, Guttman & Ulfelder 1997). Recently, we reported a method to release and purify oligosaccharides that were *N*-linked to glycoproteins obtained from Sf-9 insect cells (Wolff *et al.* 1999). These released oligosaccharides were fluorescently labeled by reductive amination with monopotassium 7-amino-1,3-naphtalenedisulfonic acid (AGA) and analyzed by capillary electrophoresis (CE) in combination with sequential exoglycosidase digestion. Definitive assignment of structure was not possible based on CE alone. The current study investigates the potential of ESI-MS in combination with preparative CE and exoglycosidase digestion to characterize a mixture of fluorescently conjugated oligosaccharides.

Materials and methods

Fluorescent labeling of oligosaccharides

Monopotassium 7-amino-1,3-naphthalenedisulfonic acid (AGA) (Aldrich) was recrystallized from deionized water for the fluorescent labeling of the oligosaccharide. Chitobiose (1 mg) (Figure 1A), chitotriose (1 mg) (Figure 1B), and chitotetrose (1 mg) (Figure 1C), undecasaccharide (20 μg) (Figure 1D), and nonasaccharide (50 μg) (Figure 1E) were dissolved with approximately 20 times molar excess of AGA in 100 μl of 15% acetic acid and the mixture was incubated for 1 h at room temperature. AGA reacts with

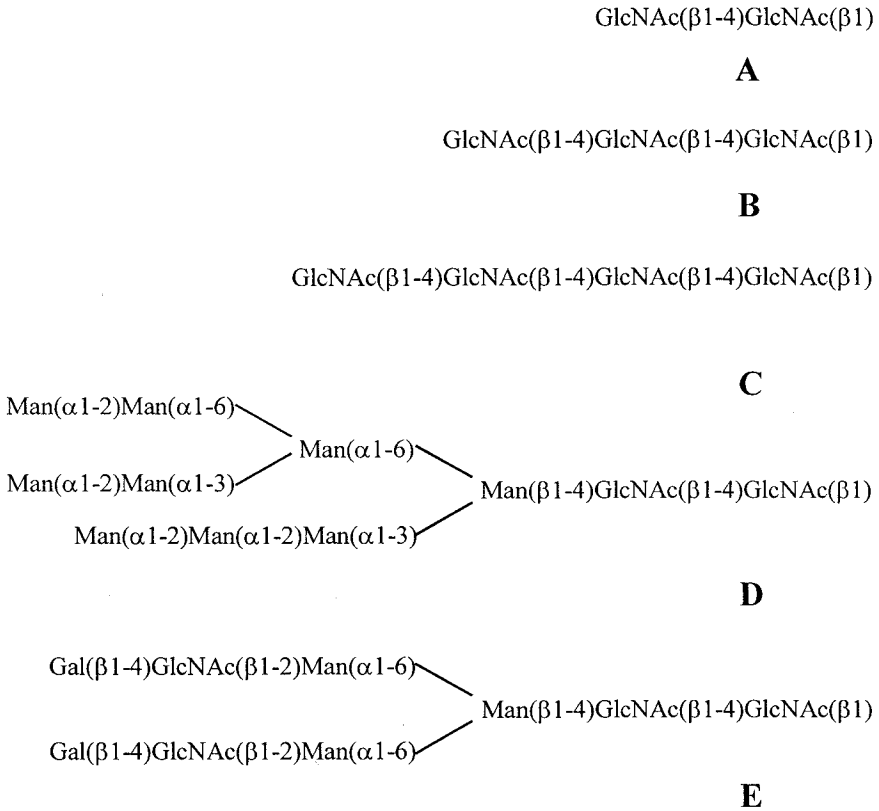

Fig. 1. *N*-Glycan derived oligosaccharide standards: chitobiose (A); chitotriose (B); chitotetrose (C); asialo-, galactosylated biantennary nonasaccharide (D); oligomannose undecasaccharide (E).

the reducing termini of the oligosaccharide to form a Schiffs base, which can be stabilized by reduction with sodium cyanoborohydride. Therefore, 100 μl of 1.0 M sodium cyanoborohydride in water was added to the mixture and incubated for 12 h at 45 °C. Excess AGA was removed by gel permeation chromatography on a Sephadex G-25 (Sigma) column (45 cm × 1.5 cm i.d.) with detection at 247 nm.

Analytical capillary electrophoresis

The experiments were performed with a capillary electrophoresis system (Dionex, Sunnyvale, CA) at 23 kV by fluorescent detection (λ_{ex} of 250 nm and λ_{em} of 450 nm). Separation and analysis were carried out in a reversed polarity mode using a fused silica (externally coated except where the tube passed through the detector) capillary tube (37 cm length × 50 μm i.d.). The separation buffer contained 20 mM sodium phosphate pH 3.5. The sample was injected by pressure injection (5 s, 0.3 atm.) resulting in an injection volume of 0.5 μl.

Preparative capillary electrophoresis

Individual glycan peaks were separated by a P/ACE 5500 System (Beckman Instruments, Fullerton, CA) and a capillary cartridge containing an uncoated fused silica capillary (57 cm length × 75 μm i.d.). The electrophoretograms were acquired using the system Gold software package (Beckman Instruments, Fullerton, CA). Prior to every run the capillary was conditioned with 0.5 M NaOH (0.5 min 1.3 atm.) and rinsed (2 min, 1.3 atm.) with the separation buffer (5 mM sodium phosphate pH 3.5). All separations were performed at 18 °C, with a potential of −20 kV (reversed polarity mode). Samples were injected by pressure (10 s at 0.03 atm.) and detected by UV absorbance at 247 nm. Before the individual glycans were separated, initial runs were carried out to establish the migration times of the chitobiose, chitotriose and chitotetrose. After verifying adequate precision, fraction collection runs were performed. The electrophoresis was run shortly before chitobiose was detected, then the power was stopped, the autosampler moved to an elution

vial containing 10 μl separation buffer and the voltage reestablished until shortly before chitotriose was detected. Chitotriose was then collected in a separate vial, followed by chitotetrose. The fraction collection runs were repeated 19 times allowing the collection of a sufficient amount of sample for the ESI-MS after desalting using MicroSpin columns (Sephadex G-10, Amersham Pharmacia Biotech, Piscataway, NJ) and freeze-dried.

Enzyme digestion of the oligosaccharides

Approximately 40 μg of the fluorescently conjugated nonasaccharide ($Gal_2GlcNAc_2Man_3GlcNAc_2$) was treated with 80 mU/ml of β-galactosidase from *Streptococcus pneumoniae* (Oxford GlycoSystems, Bedford, MA) for 18 h at 37 °C in a total volume of 200 μl 10 mM sodium acetate buffer pH 6.0. After 18 h, the reaction mixture was boiled for 5 min, dialyzed using a 1000 MWCO against 100 volumes of water, freeze-dried, and analyzed by ESI-MS.

ESI mass spectrometry analysis

The negative-ion mass spectra were obtained by using a Micromass, Inc. (England) Autospec equipped with an electrospray interface. Samples were initially dissolved in water/acetonitrile (1:1, v/v) containing 14 mM NH_4OH which was also used for the mobile phase. A Harvard syringe pump was used to deliver the mobile phase at a flow rate of 20 μl min^{-1}. Samples were introduced into the system through 20 μl injection loop on a Rheodyne (Cotati, CA) injection port. Nitrogen was used both as bath and nebulizer gas at flow rates of 250 l h^{-1} and 14 l h^{-1}, respectively. The electrospray ion source was held at 80 °C and the spray needle was held at 7.7 kV. Tetramethylammonium iodide in acetonitrile was used as the calibrant (Hop 1996). The spectra shown are 30–40 scans of one representative injection. The manufacturer's software (OPUS) was used to acquire and process data.

Results and discussion

A hypermannosylated undecasaccharide (Figure 1D) was first examined because this glycan is both large, containing 11 saccharide units and represents the major glycan structure found in glycoproteins prepared in insect cell culture. Undecasaccharide was conjugated with AGA and approximately 80 ng of the conjugate was analyzed by ESI-MS. The resulting spectrum (data not shown) displayed only the signal corresponding to the doubly charged ion $[M\text{-}2H^+]^{2-}$ at 1084 consistent with the expected molecular-ion. No fragmentation was observed confirming the utility of ESI-MS for glycan analysis.

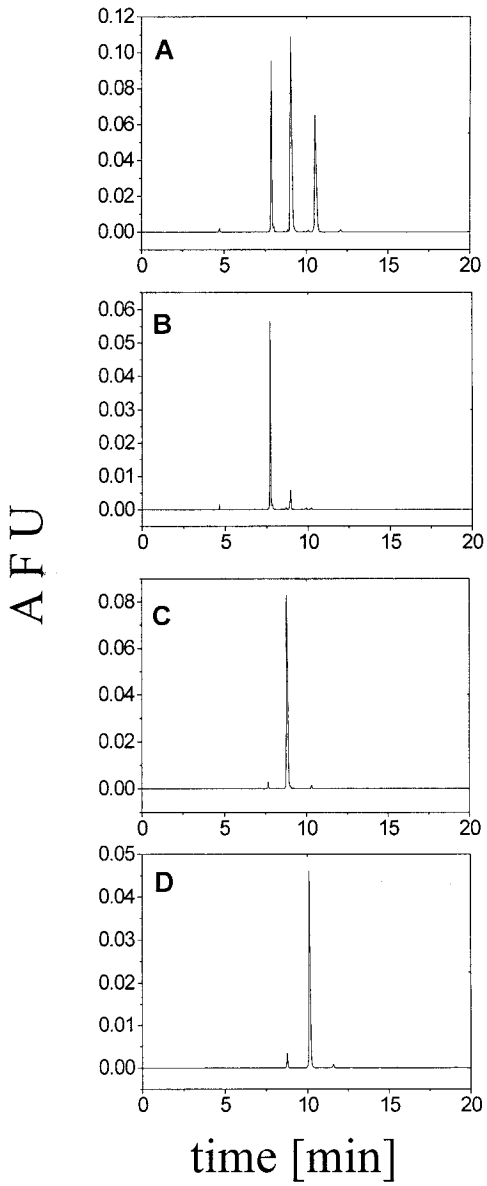

Fig. 2. Capillary electrophoretogram of AGA conjugated glycans: Mixture of chitobiose, chitotriose, chitotetrose (A); chitobiose separated from the mixture shown in (A) by preparative capillary electrophoresis (B); chitotriose separated from the mixture shown in (A) by preparative capillary electrophoresis (C); chitotetrose separated from the mixture shown in (A) by preparative capillary electrophoresis (D). AFU corresponds to absolute fluorescence units.

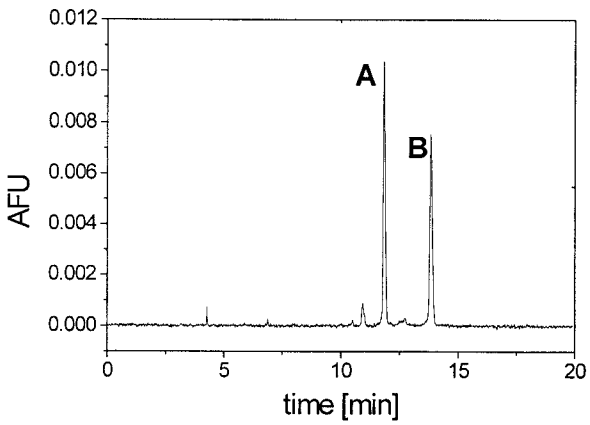

Fig. 3. Capillary electrophoretogram of AGA conjugated nonasaccharide digested with galactosidase (A) co-injected with undigested conjugated nonasaccharide (B).

Table 1. ESI-MS analysis of fluorescently labeled oligosaccharides.

Sample	$[M\text{-}2H^+ + Na^+]^-$	$[M\text{-}2H^+]^{2-}$
Conjugated chitoboise	732	355
Conjugated chitotriose	935	456
Conjugated chitotertrose	1138	558
Conjugated undecasaccharide		1084
Conjugated nonasaccharide	1949	963
Conjugated heptasaccharide		801

M, molecular weight of fully protonated oligosaccharides; conjugated chitobiose M = 710, conjugated chitotriose M = 912, conjugated chitotetrose M = 1116, conjugated nonasaccharide M = 1928, conjugated undecasaccharide M = 2170, conjugated heptasaccharide (obtained from glycosidase digestion of conjugated nonasaccharide) M = 1604.

Next, the application of preparative CE to the isolation of individual glycan conjugates from a mixture was examined. An artificial mixture of the three conjugated neutral oligosaccharides, chitobiose, chitotriose and chitotetrose, conjugated with AGA, was prepared and separated by preparative CE. Each conjugate was recovered with a high level of purity, as confirmed by analytical CE using fluorescence detection (Figure 2). The individual glycans, subjected to ESI-MS, afforded clean spectra (not shown) with <100 ng of sample. The signal at 732 (Table 1) corresponds to the singly charged ion $[M\text{-}2H^+ + Na^+]^-$, and the one at 355 to the doubly charged ion $[M\text{-}2H^+]^{2-}$. The ESI-MS spectra of conjugated chitotriose and chitotretrose showed singly charged ions $[M\text{-}2H^+ + Na^+]^-$ at 935 and 1138, respectively, and doubly charged ions $[M\text{-}2H^+]^{2-}$ at 456 and 558, respectively (Table 1). No fragmentation was observed in these spectra.

A complex biantennary nonasaccharide (Figure 1E) was next fluorescently conjugated with AGA and subjected to ESI-MS. A clean spectrum was obtained with approximately 200 ng of the conjugated nonasaccharide (not shown). A doubly charged ion $[M\text{-}2H^+]^{2-}$ was observed at 963 and a smaller signal corresponding to a single charged ion $[M\text{-}2H^+ + Na^+]^-$ was observed at 1949 (Table 1). The conjugated nonasaccharide was subjected to exogalactosidase digestion using the β-galactosidase from *Streptococcus pneumoniae* (EC 3.2.1.23). After 18 h at 37 °C, CE of the reaction mixture showed the disappearance of the starting material, and the formation of a single major compound having a shorter migration time, indicating a reduced mass/charge ratio, as shown by co-injection of the starting conjugated nonasaccharide and its galactosidase digestion product (Figure 3). This galactosidase digestion product is the corresponding conjugated heptasaccharide resulting from the removal of the two galactose units at the non-reducing termini of the conjugated nonasaccharide. The ESI-MS spectrum of the galactosidase digestion mixture showed the presence of a signal at 801 (Table 1), corresponding to the doubly charged ion $[M\text{-}2H^+]^{2-}$ of the heptasaccharide afforded through the removal of two galactose units from the conjugated nonasaccharide. Preparative CE was not required for this sample as the galactosidase digestion was complete forming only a single compound, the biantennary heptasaccharide.

In conclusion, a variety of glycans could be conjugated to AGA and efficiently fractionated using preparative CE. ESI-MS could then be applied for the characterization of fluorescently labeled oligosaccharides ranging from di- to undecasaccharide, requiring sample amounts of as little as 80 ng. Off-line CE/MS analysis offers an advantage to direct (on-line) CE/MS, as it permits the use of exoglycosidase sequencing in the analysis of glycan mixtures. Moreover, digestion of fluorescently labeled oligosaccharides with exoglycosidases permits the further structural characterization particularly when used in conjunction with ES/MS analysis. The additional structural data, obtained in this method by ESI-MS, decreases reliance on precise retention/migration time measurements required by other methods that involve separations based solely on chromatography or electrophoresis. Studies are underway to examine the application of a direct (on-line) interface between ESI-

MS to CE for the sequential enzymatic analysis of fluorescently labeled oligosaccharides derived from recombinant glycoproteins obtained from insect cells.

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