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Improved capillary electrophoretic separation and mass spectrometric detection of oligosaccharides

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Abstract

We have developed a CE method for the separation of structural isomers of oligosaccharides labeled with N-quaternized benzylamine. Oligosaccharides with reducing ends were derivatized with benzylamine by reductive amination followed by quaternization to yield a fixed cation label. The benzylamine-derivatized oligosaccharides were analyzed by CE–UV in ammonium acetate buffer and off-line matrix-assisted laser desorption ionization (MALDI) MS. The method was applied to a 1 nmol sample of a model oligosaccharide (LNDFH 1). From this sample a 38 fmol diluted standard was detected. The quaternization of benzylamine-labeled products significantly improved CE separation of neutral oligosaccharides along with several structural isomers. Two hexasaccharide isomers (LNDFH I and LNDFH II) were baseline resolved using an ammonium acetate buffer. This method was also applied successfully to the profiling of oligosaccharides released from the glycoprotein RNase B. The release of 6 pmol of glycans followed by workup showed the detection of all components, with one component corresponding to 100 fmol. Micropreparative collection of CE enabled successful off-line CE–MALDI-MS without additional sample clean up. This report provides a simple and rapid method to separate and analyze oligosaccharides.

Keywords: Derivatization, electrophoresis; Oligosaccharides; Glycoproteins; Proteins

1. Introduction

The complete characterization of the glycan is essential for understanding the biological functions of glycoproteins [1–3]. However, the separation of oligosaccharide mixtures released from glycoproteins is often challenging due to their heterogeneity. Structural isomers are of particular concern as they frequently suffer from poor chromatographic separation.

Capillary electrophoresis (CE) can play an important role in the analysis of oligosaccharide mixtures because of its inherent high resolving power and sensitivity [4–7]. The small volume and sample amounts necessary for analyses are ideally suited to the sample limitations common to oligosaccharides analyses. However, the CE analysis of oligosaccharides remains far from routine. One major limitation is the oligosaccharides' lack of chromophoric activity in the operational range of existing detectors. To overcome this problem, oligosaccharides are often labeled with UV active and/or fluorescent labels for increased sensitivity [8,9]. The most common method for labeling oligosaccharides is via reductive amination [10]. 2-Aminopyridine was the first introduced as an oligosaccharide label, however, since then many more have been used successfully. In addition to their chromophoric activities, other

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features have been common to these compounds. They typically couple to the oligosaccharides through an aromatic amine group. They also contain acidic groups and/or slightly basic amines. Widely used labels such 1-aminopyrene-3,6,8-trisulfonate (APTS) [11,12], 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) [13,14], and 2-aminopyridine [8,15] exemplify these features. However, aromatic amines are not the best for coupling the labels to the oligosaccharide as the benzene group reduces the nucleophilicity of the nitrogen. Furthermore, the labels do not increase the sensitivity during mass spectrometry (MS) detection. With the multiple anionic groups, the MS analyses are even complicated by the presence of other charge states in the negative mode and by the coordination of variable numbers of alkali metals and/or protons in the positive mode.

More nucleophilic amines such as benzylamine (BA) and 9-aminofluorene [16] are benzylic amines with high UV absorbance. They are more effective for coupling to oligosaccharides because the electron pair on the nitrogen is not delocalized into the aromatic system, thereby rendering the nitrogen more nucleophilic. Recent studies in this laboratory have shown them to be effective labels for optical spectroscopic detection [16]. As little as a few picomol of oligosaccharides can be labeled and detected by UV. Recent studies have also shown that N-quaternization of BA derivatives enhances their MS detectability. Broberg et al. reported that the N-quaternized BA label resulted in a 10-fold increase in matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectral sensitivity compared to the underivatized oligosaccharide [17]. As low as 50 fmol of the derivative could be detected by MS.

In this work, we used N-quaternized BA as an oligosaccharide-labeling reagent for oligosaccharide analysis with CE and MS. Monosaccharides and small oligomers like tri- and tetrasaccharides have been labeled with fluorescent labels and were separated by CE [18–20]. To the best of our knowledge, labels with sequestered positive charge have not been used for oligosaccharides in CE separation. These labels have two important advantages. The sequestered charge is known to enhance MS-detectability of oligosaccharides; but, it also may improve the electrophoretic behavior of the compound during CE separation.

This research provides the first example of a fixed positive charge label for CE separation with photometric and mass spectrometric detection. As a major part of this study we also reexamined the buffer systems for improved separation and MS analyses. Borate buffers of various concentrations are now considered to be the most effective for CE separation of oligosaccharides because borate forms a relatively stable complex with oligosaccharides and provides a charge to the oligosaccharides. Borate and phosphate buffers were used to separate APTS- and ANTS-labeled oligosaccharides with excellent results [5,21,22]. However, borates and phosphates will affect the MS analyses and may also counteract the positive charge on the label.

2. Experimental

2.1. Materials

Unless stated otherwise, the chemicals used in the derivatization reaction were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Solvents were of HPLC grade. Oligosaccharides were purchased from Oxford Glycosystems (Rosedale, NY, USA). Evaporation of small amounts of solvent was done on a Centrivap Concentrator (Labconco, Kansas City, MO, USA) at 45 °C. Porous graphitized carbon (PGC) cartridges for desalting were purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Derivatization of oligosaccharides with benzylamine

The oligosaccharide (1 nmol) was dissolved in nanopure (18 Ω, resistance) water (50 μl). In a separate microcentrifuge tube, benzylamine (10 μl) was dissolved in methanol (100 μl). To this solution was added $NaCNBH_3$ (3 mg, 0.05 mmol) followed by glacial acetic acid (5 μl). The resulting reagent solution was added immediately to the above solution of the oligosaccharide in water. The reaction mixture was heated to 80 °C for 2 h, cooled to room temperature, and was evaporated in vacuo. The

labeled oligosaccharide was dissolved in nanopure water (100 μl) and was desalted by a method adapted from the literature [23]. A PGC cartridge was washed with an aqueous solution of 80% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid (15 ml total) at a flow-rate of about 3 ml/min. The solution of the labeled oligosaccharide was applied to the PGC cartridge. Subsequently the cartridge was washed by gravity with nanopure water (15 ml total) at a flow-rate of about 1 ml/min followed immediately by an aqueous solution of 60% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid (15 ml total). The UV-active fractions were collected and concentrated in vacuo.

2.2.1. General procedure for quaternization

The benzylamine-labeled oligosaccharide (1 nmol) was dissolved in methanol (100 μl) in a micro centrifuge tube. To the solution was added $NaHCO_3$ (3 mg) followed by CH_3I (20 μl). The reaction mixture was warmed to 30–40 °C for 3 h. The solvent was evaporated in vacuo and the residue was desalted with a PGC cartridge (see above).

2.3. Capillary electrophoresis

Capillary electrophoresis was performed on a Hewlett-Packard ^{3D}CE (Agilent Technologies, Pleasanton, CA, USA). Separations were performed in 56 cm effective length×50 μm I.D. bare fused-silica capillary with extended light path geometry. The sample was loaded by pressure injection (50 mbar) for 4 s. Analysis was carried out at a temperature of 25 °C with an applied voltage of +20 kV. Running buffers were 50, 75, and 100 m*M* borate buffer (pH 9.2) and ammonium acetate buffer (pH 4.5). Detection was carried out by on-column measurement of UV absorption at 230 and 248 nm. Between runs, the capillary was washed with 1.0 *M* sodium hydroxide for 10 min using 900 mbar of air (0 V), followed by reconditioning with the running buffer for 10 min at the same pressure and voltage. The eluent (<1 μl) was collected directly into 10 μl of methanol into which the electrode was inserted. The fraction collector was programmed to collect at the onset of the peak.

2.4. Release of N-linked glycans from ribonuclease B

PNGase F (1U) and 0.5 ml 200 m*M* NH_4HCO_3 buffer was added to the RNase B aqueous solution (20 μg in 0.5 ml water solution) and incubated overnight at 37 °C. A 4.0 ml 95% aqueous ethanol solution was added and the mixture chilled to −20 °C for 30 min to precipitate the proteins. The supernatant was removed from the mixture and evaporated to dryness. Nanopure water was added and the oligosaccharide mixture was desalted by the PGC cartridge using the procedure described above.

2.5. Mass spectrometric analyses

The derivatization procedure was monitored by MALDI-MS. Mass spectra were recorded on an external source HiResMALDI (IonSpec, Irvine, CA, USA) equipped with a 4.7-Tesla magnet [24,25]. The instrument was equipped with a pulsed nitrogen laser (337 nm). MALDI-TOF mass spectra were also recorded in the linear mode by using a Proflex III (Bruker Daltonics). 2,5-Dihydroxybenzoic acid was used as a matrix (5 mg/100 μl in ethanol). A saturated solution of NaCl in methanol was used as dopant. A NH_4 resin was used to remove alkali metals and produce the protonated species. The solution of the labeled oligosaccharide (1 μl, 1 pmol) was applied first to the MALDI probe followed by sodium dopant (1 μl, 1 nmol) to obtain the sodium-coordinated quasimolecular ion. For the protonated parent, the sample was treated with NH_4 resin prior to the addition of the matrix solution (1 μl). The sample was dried under a stream of air and subjected to mass spectrometric analysis.

2.5.1. Preparation of the ammonium resin

The cation-exchange resin (ammonium form) was prepared by a previously published procedure [26]. An H^+-cation-exchange resin (100–200 mesh, Bio-Rad, NY, USA) was stirred in a 1 *M* ammonium acetate solution for 12 h. The product was filtered and washed with 1 *M* ammonium acetate solution, deionized water, acetone, and hexane. The resin was dried and stored for future use.

3. Results and discussion

3.1. Derivatization of oligosaccharides with benzylamine

In order to evaluate and optimize the derivatization procedure, maltoheptaose was used as the model oligosaccharide. The labeled compound was subsequently quaternized as described in the experimental section. The derivatization of a sugar aldehyde by reductive amination with benzylamine is shown in Fig. 1. The formation of the intermediate imine is fast so that the reductive step was performed in the same pot. We obtained the best results with a solution of the sugar in MeOH–water (9:1) that was treated with excess benzylamine in the presence of a catalytic amount of acetic acid and an excess of $NaCNBH_3$ at 80 °C for 2 h. The elevated temperature facilitated the elimination of water during imine formation. The volatile reagents were removed by evaporation in vacuo. The evaporation was repeated once to ensure complete removal of unreacted benzylamine prior to quarternization.

To remove the salts, we used a PGC cartridge as described in the experimental section. The PGC cartridge allowed for quick and efficient desalting. The desalted fractions of the BA-labeled sugar were combined and dissolved in MeOH. N-Quaternization was performed in the presence of methyl iodide and $NaHCO_3$ at room temperature overnight. At 30–40 °C the reaction could be reduced to 3 h. Care should be taken that the methyl iodide does not evaporate from the vial as this would lead to incomplete quaternization.

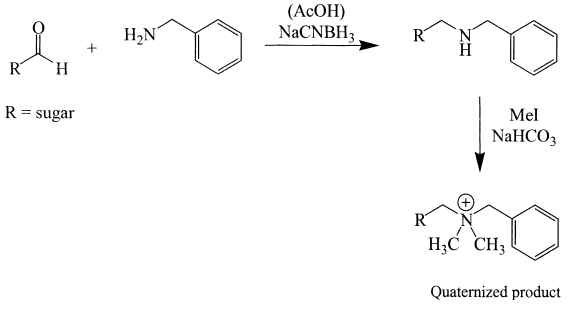

Fig. 1. Derivatization of an oligosaccharide aldehyde with benzylamine and subsequent quaternization.

The MALDI-Fourier transform (FT) MS spectrum of the BA-labeled maltoheptaose is shown in Fig. 2a. The progression of the quaternization reaction was conveniently monitored by MS. The spectrum of an incomplete reaction is shown in Fig. 2b. The sodiated starting material, the monomethylated intermediate, and the quaternized product are clearly visible. In our hands the quaternization reaction was quantitative (Fig. 2c).

3.2. CE Separation of structural isomers of oligosaccharides

3.2.1. Effect of sequestered charge on resolution

The effect of BA labeling and quaternization on the CE of compounds of different sizes is illustrated by maltopentose and maltoheptaose (Fig. 3). The two compounds belong to the same oligomeric series with 5 and 7 residues, respectively. The effect of the BA label was readily apparent. Without the BA no signals were observed in the CE separation with UV detection at 230 and 248 nm (Fig. 3a). With BA labels (unquaternized), detection and separation of two sugars were achieved in ammonium acetate buffer (Fig. 3b). The signals correspond to 200 nmol of material and the difference in elution time was 1.129 min. N-Quaternization of the benzylamine decreased slightly the migration time of both compounds (Fig. 3c) and increased the separation to 1.141 min. Similar observations were made with other pairings including maltohexaose and maltoheptaose. The difference in elution time of BA labeled oligosacchrides (Δt) was 1.144 min, which increased to 1.152 min with quaternization.

The most pronounced effects of quaternization were observed with isomers. LNDFH I and LNDFH II are isomeric hexasaccharides that vary by the position of the fucose. The CE analysis of the two benzyl amine labeled compounds in ammonium acetate buffer is shown in Fig. 4a. Only a single peak was observed whose identity was confirmed by MS to be a mixture of the two isomers. With N-quaternization, complete baseline separation of the two compounds was observed (Fig. 4b). A difference in elution time of 0.414 min was obtained.

Similarly favorable results were obtained with the isomeric pentasaccharides LNFP I and LNFP II. Separation of the pentasaccharides was observed

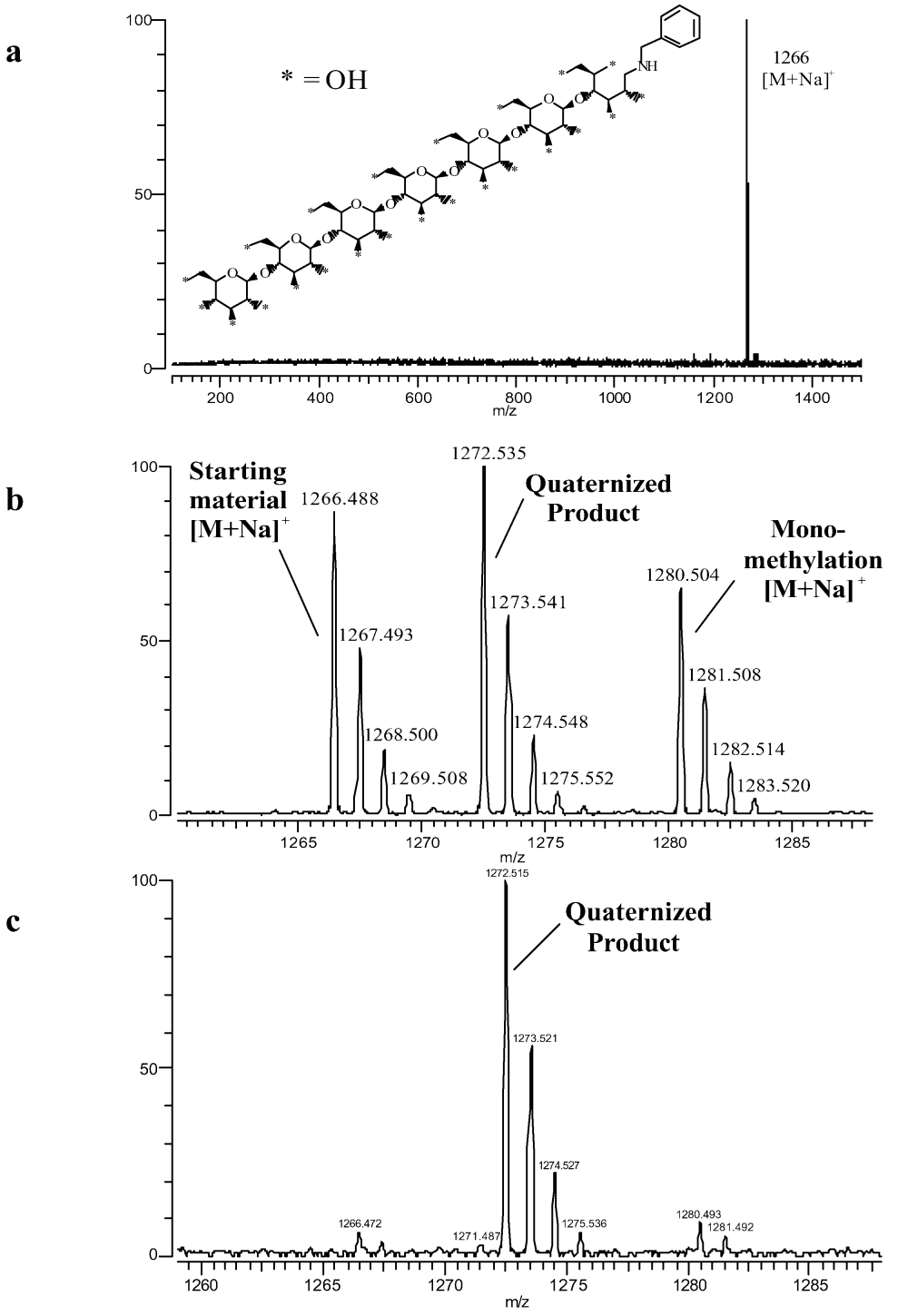

Fig. 2. (a) MALDI-FT mass spectrum of benzylamine labeled maltoheptaose (m/z 1266, $[M+Na]^+$). Mass spectrum of a reaction mixture during N-quaternization of BA labeled maltoheptaose (b) after 1 h and (c) after 2 h.

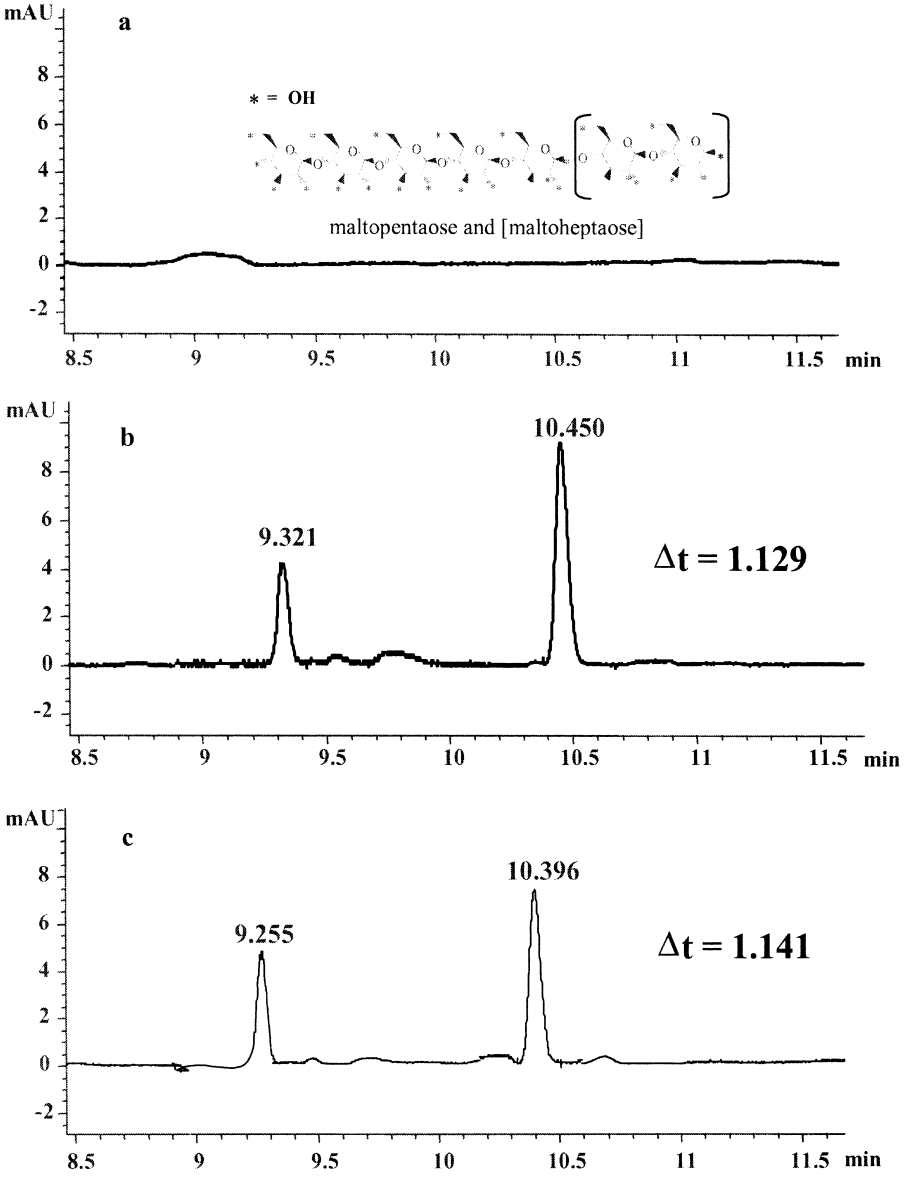

Fig. 3. CE separation of maltopentaose and maltoheptaose. Conditions: ammonium acetate buffer (pH 4.8), +20 kV potential, pressure injection (50 mbar for 4 s). E1ectropherogram of (a) native sugars (b) benzylamine labeled sugars, (c) N-quaternized BA labeled sugars.

only upon quaternization (Fig. 4c), although baseline resolution could not be obtained.

3.2.2. Effect of the running buffer pH on the electrophoretic migration

MS analysis of CE-separated oligosaccharides typically employ ammonium acetate buffer because borate buffers, the commonly preferred buffer for oligosaccharides, interfere with MS analysis. We examined both buffer systems to determine the separation efficiency of the quarternized compounds.

With N-quaternization, the BA labeled structural isomers LNDFH I and LNDFH II were completely separated in ammonium acetate buffer at pH 4.8.

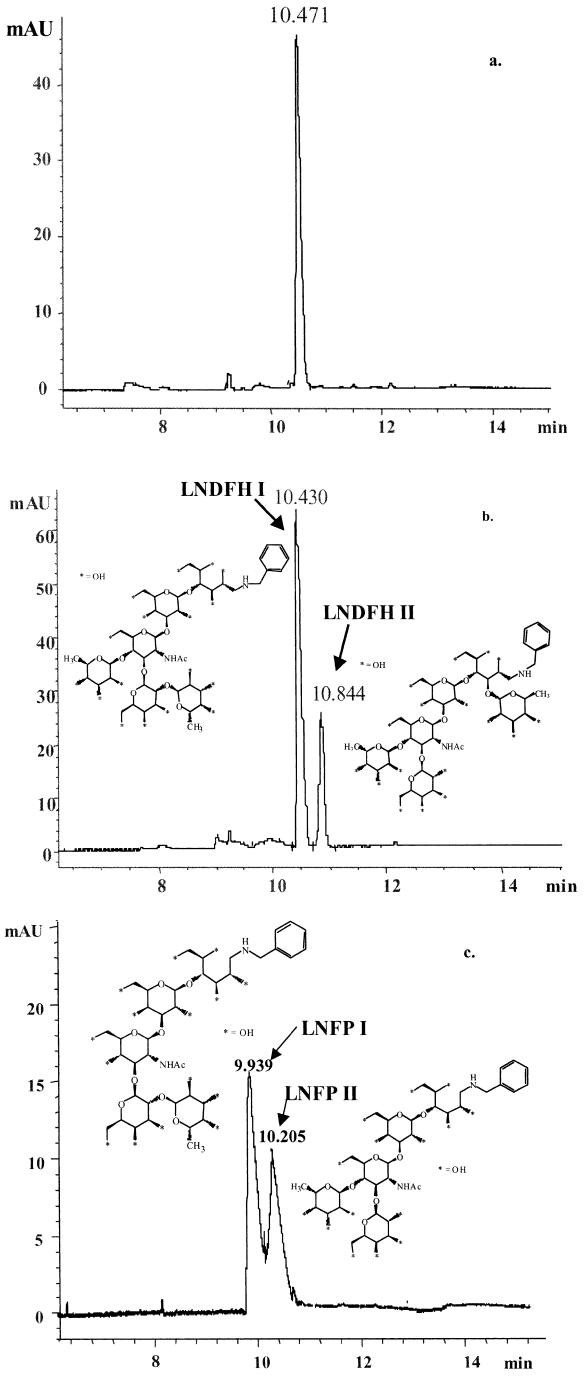

Fig. 4. (a) CE separation of benzylamine-labeled LNDFH I and LNDFH II in ammonium acetate buffer (pH 4.8). (b) CE separation of N-quaternized, BA-labeled LNDFH I and LNDFH II. (c) CE separation of N-quaternized, BA-labeled LNFP I and LNFP II.

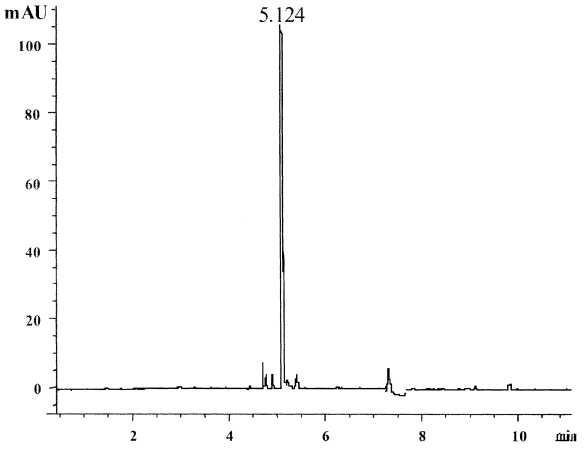

Fig. 5. CE separation of N-quaternization of BA-labeled LNDFH I and LNDFH II in 50 m*M* borate buffer (pH 9.4).

However, with the borate buffer we found that separation could not be obtained. Fig. 5 shows the CE analysis of the N-quaternized LNDFH I and LNDFH II mixture while employing a borate buffer at pH 9.4. A single peak is observed with no apparent separation. Varying a number of experimental parameters including the buffer concentration, sample injection pressure, running voltage, and capillary temperature did not improve the separation (data not shown).

3.3. Separation of complex mixtures

A mixture of oligosaccharides was prepared by mixing together several components from disaccharides to heptasaccharides. The MALDI-FT MS spectrum of the mixtures is shown in Fig. 6a. All peaks correspond to molecular ions, namely the quaternized benzylamine-labeled oligosaccharides. The disaccharide, the pentasaccharides, and the hexasaccharides represent isomers.

The CE separation of the mixture in acetate buffer at pH 4.8 is shown in Fig. 6b. The derivatized oligosaccharides were collected after CE separation and were identified by MALDI-MS. Peak 1 is due to a disaccharide mixture, cellobiose and gentiobiose that differ only by the β-1,4- and β-1,6-linkages, respectively. The CE separation of the two compounds could not be obtained even after quaternization. In general, this method appears not to work

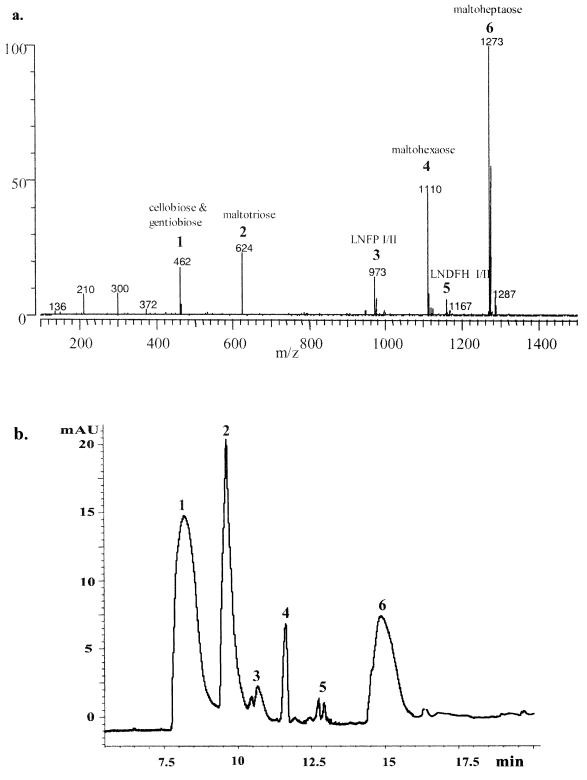

Fig. 6. Off-line CE MALDI analysis of the mixture of nine benzylamine labeled oligosaccharides. (a) MALDi-FT mass spectrum of N-quaternized BA attached to nine oligosaccharides. (b) CE electropherogram of N-quaternized BA-labeled oligosaccharides.

well with isomers of mono- and disaccharides but with larger oligomers such as penta and hexasaccharides. Peaks 2, 4, and 6 show maltotriose, hexaose, and heptaose, respectively, illustrating that oligomers are readily separated. Peak 3 is a doublet corresponding to LNFP I and LNFP II. Although complete separation was not accomplished, the two compounds were readily elucidated. Peak 5 is due to LNDFH I and LNDH II illustrating complete baseline separation.

3.3.1. Analysis of N-linked oligosaccharides derived from ribonuclease B

N-Linked oligosaccharides of ribonuclease B released by PNGase F were derivatized with benzylamine, followed by quaternization. N-Linked

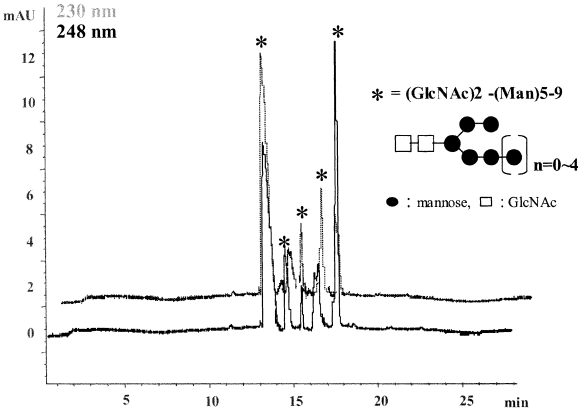

Fig. 7. CE separation of BA-labeled N-linked glycans released from RNase B.

oligosaccharide profiles obtained by CE are shown in Fig. 7. Subsequently CE fractions were analyzed by MALDI to identify each peak (label). It is apparent that this method can be applied to the profiling of oligosaccharides released from glycoproteins.

3.4. Detection limit and quantitation

The detection limit of BA-labeled compound was evaluated using LNDFH I as the standard analyte. A 1-nmol sample was treated and diluted to 160 fmol, 104 fmol, and 38 fmol. The response was linear over an order of magnitude with the detection limit potentially well below 30 fmol. The electropherogram of benzylamine labeled LNDFH I in the femtomole level produced a signal-to-noise ratio of over 100. Linear correlation between peak area or peak height and molar amount of benzylamine labeled sugar was obtained (Fig. 8). With sample injection of about 4 nl (injection pressure is 200 mbar), this amount of sugar detected is equal to 38.10 fmol. Commonly used derivatization reagents ANTS and AA (anthranilic acid) have been shown to be effective in the femtomolar level detection limit but it was obtained by injection of diluted solutions of sugars derivatized at much higher concentrations (~μmol) [11,13]. With the BA label we have previously shown that as little as a few picomoles of oligosaccharides can be labeled [16].

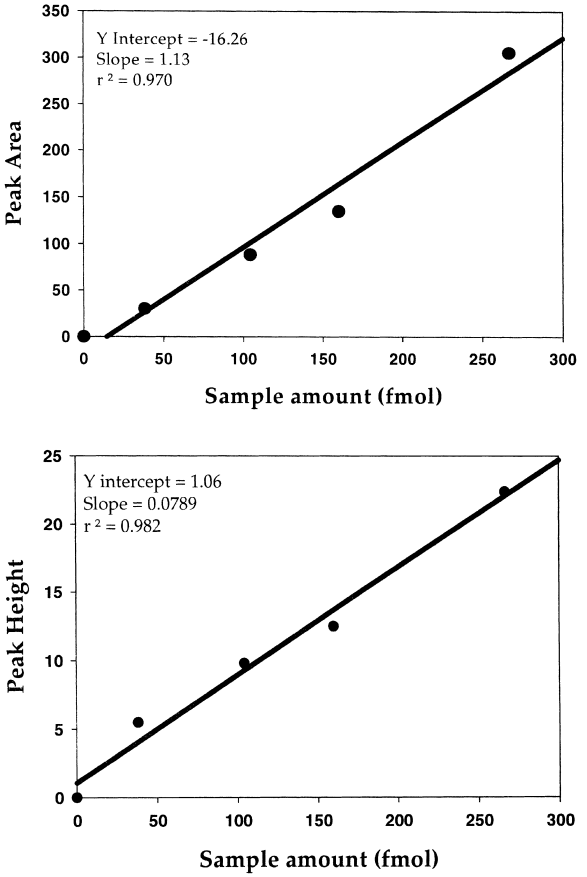

Fig. 8. Linear correlation between (a) peak area and (b) peak height and total amount (fmol) of BA-labeled LNDFH I.

4. Conclusion

This work demonstrates the CE separation of BA-labeled sugars with an ammonium acetate buffer. The use of BA (N-quaternization) as a UV tag for CE separation and detection was investigated. The label could be efficiently introduced into oligosaccharides. The quaternization of BA labeled products enabled not only CE migration of neutral oligosaccharides but also separation of structural isomers of oligosaccharide. CE separation and MS analysis were enhanced by ammonium acetate. The major advantages of this method are its simplicity and sensitivity. This technique could potentially provide new opportunities for the analysis of complex oligosaccharides of closely related structures.

Acknowledgements

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