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Sensitive Analysis of Oligosaccharides Derivatized with 4-Aminobenzoic Acid 2-(Diethylamino)ethyl Ester by Matrix-assisted Laser Desorption/ Ionization Mass Spectrometry

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A sensitive detection method for oligosaccharides using matrix-assisted laser desorption/ionization mass spectrometry has been developed. The method involves the derivatization of oligosaccharides with 4-aminobenzoic acid 2-(diethylamino)ethyl ester (ABDEAE) by reductive amination, a technique which was initially developed for high-sensitivity detection of oligosaccharides by electrospray ionization mass spectrometry (K. Yoshino, T. Takao, H. Murata and Y. Shimonishi, *Anal. Chem.* 67, 4028 (1995)). Experiments using a high-mannose-type *N*-linked oligosaccharide ($Man_8GlcNAc_2$) as a model oligosaccharide showed that derivatization with ABDEAE gave 400-, 80-, and 20-fold increases in molecular ion abundance over the underivatized oligosaccharide and the oligosaccharide derivatized with 2-aminopyridine and 4-aminobenzoic acid ethyl ester respectively. ABDEAE-derivatized maltoheptaose could be detected at a level of 10 fmol, which represents a 1000-fold increase in sensitivity over the underivatized oligosaccharide. Moreover, ABDEAE derivatization also allows in-source fragmentation to give the specific ions produced by glycosidic cleavage.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) represents a powerful tool for the measurement of high molecular weight biomolecules with high sensitivity. The technique should be especially useful for the analysis of carbohydrates, such as those derived from glycoconjugates, whose structural elucidation is essential for an understanding of their biological functions. Carbohydrates, especially in small amounts, do not ionize readily, and this presents significant problems for MALDI-MS, just as in the application of electrospray ionization (ESI) and fast atom bombardment (FAB)MS. Several studies have appeared recently, which involve the detection of free oligosaccharides with relatively high sensitivity and which are based on the selection of solvent type used for ESI-MS[1] or the use of added metal ions and unconventional matrices for MALDI-MS.[2] In other studies, we[3,4] and others[5–7] have reported the use of derivatives for the sensitive detection of oligosaccharides with ESI-MS. In our previous work we demonstrated that chemical derivatization with 4-aminobenzoic acid 2-(diethylamino)ethyl (ABDEAE) enhances the ionization efficency of oligosaccharides in the positive ESI mode by as much as 5000-fold compared with that for free oligosaccharides. The achievement of high sensitivity in the positive-ion mode can be attributed to the high proton affinity of the basic tail, a 2-(diethylamino)ethyl group. In this study, we demonstrate that derivatization with ABDEAE gives an oligosaccharide derivative that also ionizes efficiently in MALDI, and, moreover, gives fragment ions which are useful for sugar sequence analysis using linear-mode time-of-flight measurement.

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EXPERIMENTAL

Chemicals

ABDEAE hydrochloride (ABDEAE/HCl) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Sodium cyanoborohydride and 4-aminobenzoic acid ethyl ester (ABEE) were obtained from Sigma Chemical (St. Louis, MO). A high-mannose-type *N*-linked oligosaccharide ($Man_8GlcNAc_2$) derived from ribonuclease B (RNase B) is a product of Oxford GlycoSystems (Abingdon, UK). 2-Aminopyridine (PA)-$Man_8GlcNAc_2$ was obtained from Takara Shuzo Co. (Otsu, Japan). Dextran (m.w. 4000–6000) was from Funakoshi Ltd. (Osaka, Japan). Maltoheptaose and other reagents of analytical grade are products of Nacalai Tesque (Kyoto, Japan).

Derivatization and purification of oligosaccharides

ABDEAE-derivatized oligosaccharides were prepared as described previously.[3] ABDEAE/HCl (1 μmol) dissolved in methanol (3.5 μL) was added to sodium cyanoborohydride (350 μg) in a 1.5 mL polypropylene micro-centrifuge test tube (Treff AG, Degersheim, Switzerland). Glacial acetic acid (0.4 μL) was then added to the solution to form the reagent mixture. An aliquot of this solution (4 μL) was added to a solution of an oligosaccharide (1 nmol) in water (4 μL) and the total volume of the reaction mixture was made up to 20 μL with methanol. The reaction tube was vortexed and maintained at 80 °C. After 60 min, the reaction tube was cooled, and distilled water (100 μL) added. The resulting derivative was immediately separated from excess reagent by reversed-phase high performance liquid chromatography (RP-HPLC).[4] ABEE derivatives were prepared according to previously described methods[8,9] and purified using the same RP-HPLC conditions as those for ABDEAE derivatives.

The ABDEAE-derivatized oligosaccharides, which

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exhibit maximum absorption at 309.8 nm, were quantified by UV absorbance at 310 nm using a Waters Model 486 tunable absorbance detector (Milford, MA). In order to further confirm the amounts of ABDEAE-derivatized high-mannose-type oligosaccharides they were also submitted to acid hydrolysis with 40% trifluoroacetic acid at 100 °C for 3 h, and then the glucosamine in the hydrolysate was derivatized with phenylisothiocyanate (PITC) using methods described previously.[10] The resulting PITC derivative was applied to a reversed-phase column (Develosil $5C_{18}$-HG5, 2 mm i.d. × 250 mm, Nomura Chemicals, Aichi, Japan) and quantified at 254 nm using a Waters 616 HPLC system equipped with a 717-plus autosampler.

MALDI mass spectrometry

Positive-ion MALDI-MS was performed using a Voyager-RP or Voyager-Elite time-of-flight mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Framingham, MA). The spectra were obtained using a linear-mode measurement, where the ions, accelerated to 20 kV, with delayed-extraction,[11] or 30 kV, without delayed extraction, were passed respectively through a 2.0 m (Voyager-Elite) or 1.3 m (Voyager-RP) flight tube to the detector.

For sample preparation, ABDEAE (0.025–1 pmol/μL), ABEE (0.5–10 pmol/μL), PA (2–20 pmol/μL) derivatives and free oligosaccharides (10–250 pmol/μL) were dissolved in distilled water. 1 μL aliquots of these sample solutions were placed on concave flat surfaces of a stainless steel plate (2.7 mm diameter), mixed with the matrix solution the supernatant of 50% acetonitrile solution saturated with α-cyano-4-hydroxy cinnamic acid) and air dried. The ions were generated by irradiating the sample area with the output of a nitrogen laser (337 nm). Signal was recorded by modulating the position of the spot area of the incident laser light with a stepper motor.

RESULTS

The sensitivity of ABDEAE derivatives in MALDI-MS was tested by comparing ABEE-, PA- and underivatized $Man_8GlcNAc_2$ with the corresponding ABDEAE derivative (Fig. 1). α-Cyano-4-hydroxy-cinnamic acid had been selected as a matrix compound for this and other measurements of relatively low molecular weight oligosaccharides after testing various MALDI-MS matrix compounds. Using a 10 pmol sample of underivatized oligosaccharide (Fig. 1d), the analyte signal corresponding to the $[M+Na]^+$ ion was observed at *m/z* 1744.9. As the sample quantity was further reduced, the molecular ion signal became indistinguishable from the background (data not shown). The PA and ABEE derivatives showed similar signal-to-background ratios to that observed in Fig. 1d using 2 pmol and 500 fmol of sample, respectively (Fig. 1c and 1b). It should be noted that the PA derivative partially decomposed to give some unknown fragment ions at relatively high laser power. The ABDEAE derivative, however, gave a prominent molecular ion signal at *m/z* 1943.6 $[MH]^+$, even when the sample amount was decreased to 25 fmol (Fig. 1a). In this case, the signal-to-background ratio of the spectrum is similar to that of ABEE-, PA-, or underivatized $Man_8GlcNAc_2$, which required 20, 80, and 400 times, respectively, the sample size. Figure 2 shows a comparison of the spectra of ABDEAE-derivatized maltoheptaose and the underivatized material, obtained using sample sizes of 10 fmol and 10 pmol respectively (Figs 2a and 2b). The molecular ion

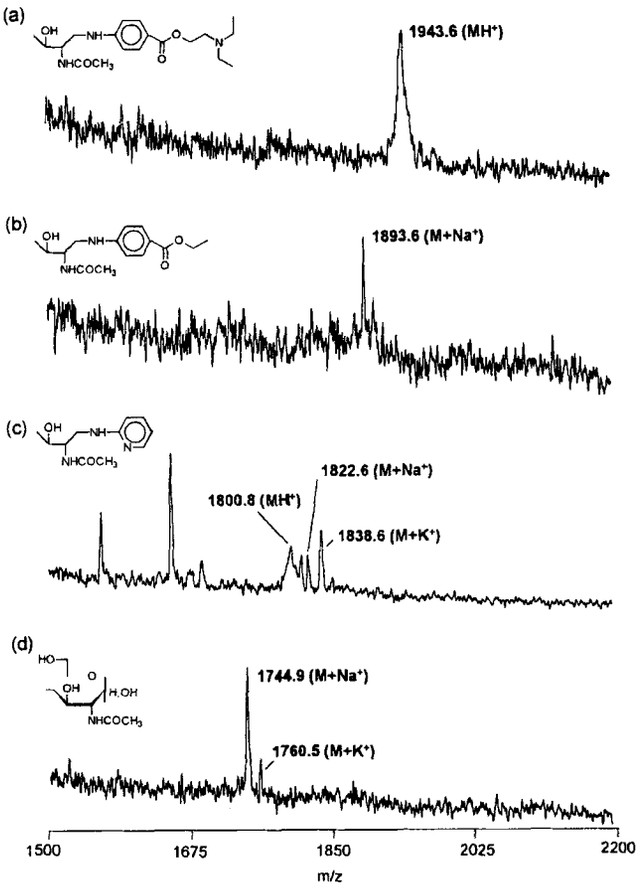

Figure 1. MALDI mass spectra of ABDEAE-$Man_8GlcNAc_2$ (25 fmol) (a), ABEE- (500 fmol) (b), PA- (2 pmol) (c), and free oligosaccharide (10 pmol) (d). The spectra were obtained with delayed extraction in the linear mode using a Voyager-Elite instrument.

signals (MH^+ for the ABDEAE derivative and $[M+Na]^+$ for the free sugar) were observed with similar signal-to-background ratios and indicate that the detection limit for the ABDEAE-derivatized oligosaccharides is in the order of 10 fmol. These data show that the use of an ABDEAE-derivatized oligosaccharide represents a 1000-fold improvement in sensitivity over an underivatized oligosaccharide.

MALDI mass spectra, obtained using a 200 fmol sample of the ABDEAE maltoheptaose, showed prominent fragment ions, which are produced either during the laser irradiation of the sample or during the acceleration process in the ion source. They correspond to ions which have retained the ABDEAE moiety, a positively-charged site, and have all resulted from glycosidic cleavage (Fig. 3a). On the other hand, these fragment ions were not observed in the spectrum of the ABEE derivative, even when 10 pmol of sample was employed (Fig. 3b). This result indicates that a positive charge, most probably localized on the basic diethylaminotail-group, promotes charge-remote fragmentation.

Finally, this method was applied to the sensitive detection of a considerably higher molecular weight oligosaccharide. ABDEAE-dextran (m.w. 4000–6000) showed a broad peak on a reversed-phase column which most probably represents the molecular-weight distribution of the mixture (data not shown). The MALDI-MS spectrum of this derivatized material gave an array of molecular ion signals ranging between 4000 and 5700, at intervals of 162 mass units, corresponding to glucosyl units (Figure 4).

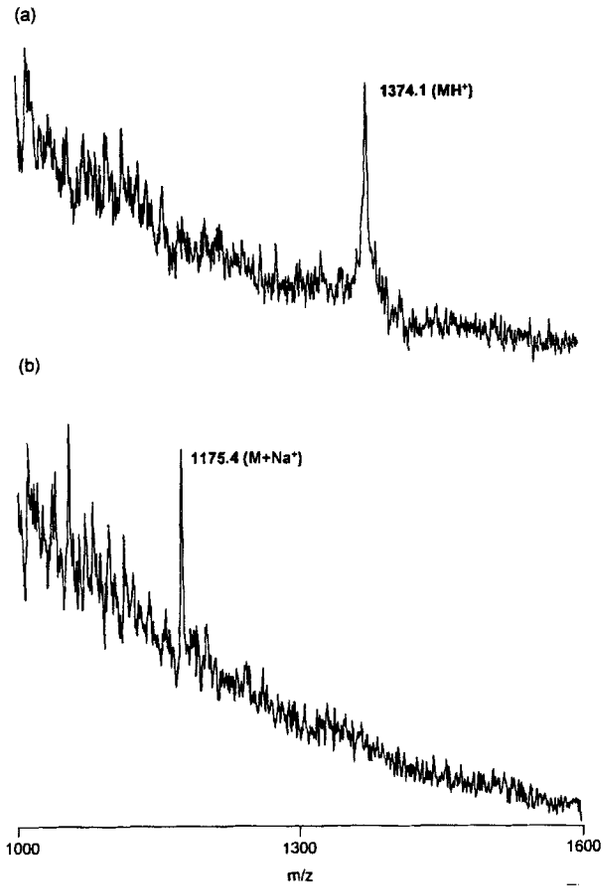

Figure 2. MALDI mass spectra of ABDEAE- (10 fmol) (a) and free maltoheptaose (10 pmol) (b). The spectra were obtained with delayed extraction using the linear mode of a Voyager-Elite instrument.

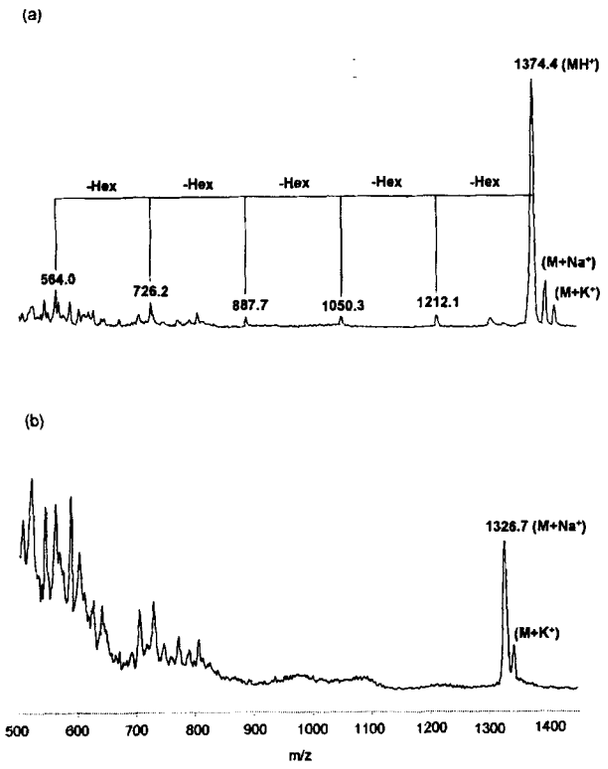

Figure 3. MALDI mass spectra of ABDEAE- (200 fmol) and ABEE-maltoheptaose (10 pmol). Hex denotes hexose. The spectra were obtained using the linear mode of a Voyager-RP instrument.

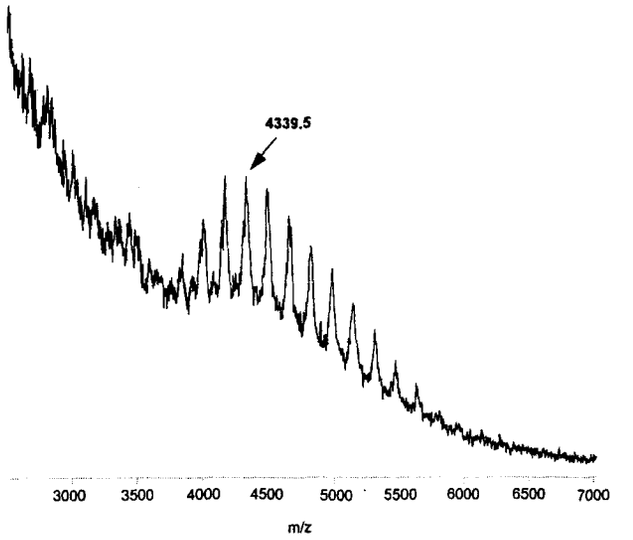

Figure 4. MALDI mass spectrum of ABDEAE-dextran (m.w. 4000–6000). The spectrum was obtained using the linear mode of a Voyager-RP instrument.

CONCLUSION

The data presented in this paper illustrates the high sensitivity of ABDEAE-derivatized oligosaccharides in MALDI-MS. Recently, chemical derivatization with reagents which contain a quaternary ammonium group has been demonstrated to be effective for increasing the sensitivity for oligosaccharides in MALDI-[6] and/or ESI-MS.[5–7] A sensitivity for the ABDEAE-derivatized oligosaccharides of the order of 10 fmol was obtained with a signal-to-background ratio of 5:1 (Figure 2a), which is nearly comparable to the detection limit in ESI-MS for oligosaccharides derivatized by ABDEAE[3,4] or by several cationic compounds such as (carboxymethyl)trimethylammonium chloride hydrazide (Girard's Reagent T),[6] 4-aminophenethyltrimethylammonium acetate[6] and trimethyl-(*p*-aminophenyl)ammonium chloride.[5–7] This represents at least a 1000-fold improvement in sensitivity over the underivatized oligosaccharide in MALDI-MS (Figure 2). The sensitivity attained by derivatization with ABDEAE or the above compounds can be attributed to the high proton affinity of the 2-(diethylamino)ethyl group or to the positive charge on the quaternary ammonium group. Moreover, ABDEAE derivatization allows in-source fragmentation to provide additional ions, which can be used for structural confirmation of oligosaccharides, at the sub-picomole level in a normal linear-mode measurement (Figure 3a). This high sensitivity for derivatized oligosaccharides is clearly an advantage for structural characterization of oligosaccharides such as those available only in limited quantities as well as for detection of large polysaccharides.

Acknowledgments

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