Journal of Chromatography B, 879 (2011) 2928–2934

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid and sensitive analyses of glycoprotein-derived oligosaccharides by liquid chromatography and laser-induced fluorometric detection capillary electrophoresis

Takehiro Oyama, Masahiro Yodohsi, Ayako Yamane, Kazuaki Kakehi, Takao Hayakawa, Shigeo Suzuki*

Faculty of Pharmaceutical Sciences, Kinki University, Japan

ARTICLE INFO

Article history:
Received 30 April 2011
Accepted 21 August 2011
Available online 26 August 2011

Keywords:
Glycoprotein
N-linked oligosaccharides
High-performance liquid chromatography
Capillary electrophoresis with
laser-induced fluorometric detection

ABSTRACT

Asparagine-type oligosaccharides are released from core proteins as *N*-glycosylamines in the initial step of the action of the peptide N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase F). The released *N*-glycosylamine-type oligosaccharides (which are exclusively present at least during the course of the enzyme reaction) could therefore be derivatized with amine-labeling reagents. Here we report a method using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as a labeling reagent for glycosylamine-type oligosaccharides. We applied the method for the sensitive analysis of some oligosaccharide mixtures derived from well-characterized glycoproteins including human transferrin, α_1-acid glycoprotein, bovine fetuin, and ribonuclease B. NBD-labeled oligosaccharides were successfully separated on an amide-bonded column or a diol-silica column. This labeling method included the release of oligosaccharides from glycoproteins and derivatization of oligosaccharides in a one-pot reaction and was completed within 3 h. The method showed approximately fivefold higher sensitivity than that involving labeling with ethyl *p*-aminobenzoate (ABEE) in HPLC using fluorometric detection and a one order of magnitude higher response in ESI-LC/MS. We also applied this method for the sensitive analysis of glycoprotein-derived oligosaccharides by capillary electrophoresis with laser-induced fluorometric detection (LIF-CE). The limit of detection in HPLC and LIF-CE were 100 fmol and 4 fmol, respectively.

1. Introduction

The most abundant post-translational modification of proteins in nature is glycosylation. Understanding the function of glycans and their changes in relation to diseases has been complicated by challenges associated with their characterization. Particular carbohydrate sequences and linkages at each glycosylation site can vary considerably and contribute to heterogeneity. Their comprehensive characterization is only possible by high-resolution separation techniques following to sensitive fluorescent labeling. Several labeling methods with fluorogenic reagents have been developed for the analysis of asparagine-type oligosaccharides [1]. The methods using 2-aminopyridine (AP) [2] and ethyl *p*-aminobenzoate (ABEE) [3] are widely used for high-performance liquid chromatography (HPLC) with fluorometric detection. Takahashi and colleagues prepared ~600 AP-labeled asparagine-type oligosaccharides and completed three-dimensional mapping of their elution indices obtained on octadecyl-bonded silica (ODS)-, amide- and diethylaminoethyl (DEAE)-bonded columns [4–6]. The retention indices are available at the website [7]. 8-Aminopyrene-1,3,6-trisulfonate (APTS) is an important labeling reagent for high-resolution analyses of oligosaccharides by capillary electrophoresis (CE) because three sulfonate groups generate high mobility [8–11]. Moreover, the excitation maxima of APTS derivatives shift hyperchromically from 420 nm to 456 nm, which enables specific detection of the derivatives via laser-induced fluorometric-capillary electrophoresis (LIF-CE) using an argon laser (488 nm) as the light source [12]. We also reported 7-amino-4-methylcoumarin to be a sensitive labeling reagent for oligosaccharides, and applied fluorescence-detection HPLC of some oligosaccharides derived from glycoproteins [13]. These labeling methods are based on a common reaction scheme called "reductive amination". This requires two-step reactions (*i.e.*, Schiff base formation and reduction of the Schiff base to corresponding 1-aminoalditol derivatives), and usually involves tedious purification steps of the labeled oligosaccharides. These derivatization reactions must be carried out under acidic conditions, which are often conducted at relatively

Abbreviations: ABEE, ethyl *p*-aminobenzoate; AP, 2-aminopyridine; APTS, 8-aminopyrene-1,3,6-trisulfonate; Fmoc-Cl, 9-fluorenylmethyl chloroformate; PNGase F, peptide N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F; CE, capillary electrophoresis; LIF, laser-induced fluorometric detection.

* Corresponding author at: Department of Pharmacy, Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan. Tel.: +81 6 6721 2332; fax: +81 6 6721 2353.
E-mail address: suzuki@phar.kindai.ac.jp (S. Suzuki).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.08.026

low temperatures for a long time to avoid hydrolysis of the sialic acid residues.

We reported another type of fluorescent derivatization of *N*-linked oligosaccharides as released from glycoproteins using 9-fluorenylmethyl chloroformate (Fmoc-Cl), which is based on the labeling of glycosylamines generated by the action of PNGase F [14]. In general, *N*-glycosylamine-type oligosaccharides released by the action of PNGase F are chemically stable, and are exclusively present at least during the course of the enzyme reaction. However, they are subjected to hydrolysis to form free oligosaccharides by overnight incubation or treatment with acid. The glycosylamine-type oligosaccharides in the digestion mixture can be derivatized with Fmoc-Cl. Fmoc labeling has three main advantages. Firstly, Fmoc derivatives of glycosylamines have a single configuration (*i.e.* β-form) at the reducing end, and have no isomers that complicate the chromatograms. Second, Fmoc residues have strong fluorescence at 310 nm by irradiation at 266 nm. Thirdly, the Fmoc group is readily removed by incubation with morpholine in dimethylformamide at mild conditions. The main disadvantage is that Fmoc derivatives fluoresce with low wavelength light (266 nm), which often coincides with the faint fluorescence of contaminants in the reaction mixture.

Here we report a method using NBD-F as a fluorometric reagent for labeling glycosylamine-type oligosaccharides. We applied the method for sensitive detection of glycoprotein-derived oligosaccharides in HPLC and CE with LIF detection. NBD-labeled oligosaccharides were successfully analyzed on normal-phase, chemically bonded silica columns. The described method showed approximately fivefold higher sensitivities than oligosaccharides labeled with ABEE or Fmoc-Cl. We also applied LIF-CE for the analysis of NBD-labeled oligosaccharides. Sensitivity was high and the detection limits were at the fmol level.

2. Experimental

2.1. Materials

Peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase (PNGase F; EC 3.5.1.52, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and ethyl *p*-aminobenzoate (ABEE) were obtained from Tokyo Kasei (Chuo-ku, Tokyo, Japan). α_1-Acid glycoprotein (human), transferrin (human), ribonuclease B (bovine pancreas), and sodium cyanoborohydride were obtained from Sigma–Aldrich Japan (Shinagawa-ku, Tokyo, Japan). Fetuin (bovine) was obtained from Gibco (Invitrogen, Chuo-ku, Tokyo, Japan). Ovalbumin was purified from chicken eggs according to the method reported by Kekwick and Cannan [15]. Other reagents and solvents used in the present study were reagent grade or HPLC grade, and purchased from Wako (Dosho-machi, Chuo-ku, Osaka, Japan).

2.2. Release of N-Linked oligosaccharides and NBD derivatization

Release of *N*-linked oligosaccharides from a glycoprotein sample followed by labeling with NBD-F was undertaken in a one-pot reaction. Briefly, a sample of glycoprotein (100 μg) dissolved in 10 μL 20 mM phosphate buffer (pH 8.0), and solution heated at 100 °C for 5 min. After cooling, 1 μL of PNGase F (0.2 unit) was added to the mixture, and incubated at 37 °C for 2 h. After the mixture was heated on a boiling water-bath for 2 min, a freshly prepared solution of 0.3 M NBD-F in acetonitrile (5 μL) was added, and the mixture heated at 70 °C for 5 min. Water (100 μL) and dichloromethane (200 μL) were added to the mixture. The solution was shaken vigorously and the dichloromethane layer carefully removed. The same procedure for extraction with dichloromethane was repeated twice. Finally, the aqueous layer containing NBD-labeled glycosylamine-type oligosaccharides was evaporated to dryness by a centrifugal evaporator (Tomy, CC-101). The residue was dissolved in 100 μL of water and a portion (typically 5 μL) was used for the analysis by HPLC and CE. Dried samples were stable for at least several months at −20 °C.

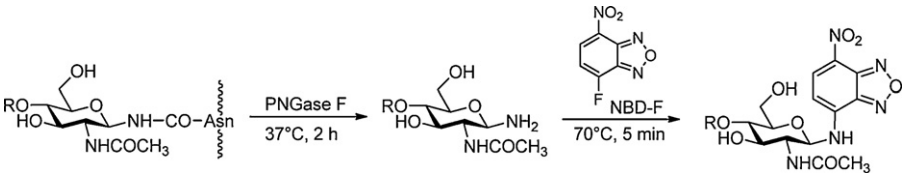
2.3. Derivatization of glycoprotein-derived oligosaccharides with Fmoc-Cl and ABEE

Derivatization of the released glycosylamine-type oligosaccharides with Fmoc-Cl has been described [14]. ABEE derivatization and purification of the labeled oligosaccharides were carried out according to the method of Wang et al. [3]. Briefly, PNGase F digests of a glycoprotein (100 μg) were mixed with 1 μL of acetic acid. The reaction mixture was dried and dissolved in each 20 μL aliquot of the freshly prepared solution of ABEE (165 mg or 1 mmol) dissolved in 1 mL of a mixture of acetic acid–methanol (41:250, v/v) and sodium cyanoborohydride (35 mg) in methanol (1 mL). The reaction mixture was kept at 80 °C for 60 min. After cooling, the solution was dissolved in 100 μL of water. Excess ABEE was removed by extraction twice with each 200 μL aliquot of ethyl acetate. The aqueous phase obtained was evaporated to dryness using a centrifugal evaporator. The residue was dissolved in 100 μL of water and a portion (5 μL) used for the analysis by HPLC.

2.4. HPLC analyses

For HPLC analyses we used a Prominence system (Shimadzu, Nakagyo-ku, Kyoto, Japan). This consisted of a UFLC LC-20AD pump, a SIL-20AC auto sampler, a DGU-20A3 degasser, a CTO-20A column oven, and a RF10AXL fluorescence detector controlled with a LCMSstation® system. NBD-, Fmoc- and ABEE-labeled oligosaccharides derived from transferrin were separated on an Amide80 column (Tosoh, Tokyo, Japan; 4.6 mm i.d., 250 mm) thermostated at 40 °C using a linear gradient formed by 0.1% (v/v) acetic acid in acetonitrile (solvent A) and an aqueous solution of 0.2% acetic acid/3% triethylamine (solvent B) at a flow rate of 1.0 mL/min. The column was initially equilibrated and eluted with 23% solvent B for 5 min. Then a mixing ratio of solvent B was increased linearly to 44% over 75 min and then further increased to 95% over 10 min. The column was equilibrated under initial conditions for 15 min before the next injection. NBD-labeled acidic oligosaccharides from glycoproteins were separated under identical conditions except for the gradient program: 20–25% B for 5 min, 25–35% B from 5 to 80 min, and 35–95% B from 80 to 90 min. NBD derivatives of neutral oligosaccharides from glycoproteins were separated on an Inertsil diol column (GL Science, Tokyo, Japan; 4.6 mm i.d., 250 mm) at a flow rate of 1.0 mL/min with a linear gradient using 0.1% trifluoroacetic acid (TFA; solvent A) and 95% acetonitrile containing 0.1% TFA (solvent B); 99% solvent B for 5 min, 99–75% solvent B from 5 to 15 min, 75–50% from 15 to 75 min, and 50% to 1% solvent B from 75 to 80 min. Fluorometric detection was undertaken at 470 nm (excitation)/540 nm (emission) for NBD derivatives, 266 nm (excitation)/310 nm (emission) for Fmoc derivatives, 310 nm (excitation)/370 nm (emission) for ABEE derivatives.

The LC–MS system comprised a quadrupole-ion-trap and time-of-flight mass spectrometer (LC-QIT-TOF-MS; Shimadzu). The detection conditions were: probe voltage, 4.5 kV; detector voltage, −1.7 kV; capillary temperature, 200 °C; flow rate of nebulizer gas, 1.5 L/min; mass range, *m*/*z* 1200–2400 for neutral saccharides and *m*/*z* 800–2000 for acidic saccharides; ion trapping time, 30 ms; and scan rate, 5 s/scan. A mixture of ABEE-labeled glucose oligomers was used for the calibration of molecular masses.



Scheme 1. Enzymatic release of glycosylamine-type oligosaccharides by the action of PNGase F and their derivatization with 4-fluoro-7-nitrobenzofurazan (NBD-F).

2.5. Capillary electrophoresis of NBD-labeled oligosaccharides

Beckman Coulter P/ACE MDQ equipped with an argon ion laser GLA3050A (Showa Optronics, Tokyo, Japan) was used with 32Karat (ver. 8.0) software for data processing and system controlling (Beckman Coulter, Brea, CA, USA). A polydimethylsiloxane-coated capillary, Inert Cap I® (50 μm i.d.; 50 cm in total and 40 cm for effective length; GL Sciences) was used. A detection system setup for fluorescein (488 nm (excitation)/522 nm (emission)). The capillary was washed with water (20 psi, 2 min), and then filled with 100 mM borate buffer (pH 8.3) containing 5% polyethylene glycol 2000 (20 psi, 0.5 min). A NBD-labeled sample was injected at 1 psi for 10 s, and separated by applying the voltage at −15 kV.

3. Results and discussion

3.1. Reaction scheme

PNGase F has been used for releasing asparagine-linked oligosaccharides from glycoproteins. If a glycoprotein containing asparagine-linked oligosaccharides is incubated with PNGase F, the amide linkage between an oligosaccharide and asparagine in a peptide sequence is hydrolyzed to form β-glycosylamine oligosaccharide and aspartic acid [16]. β-Glycosylamine-type oligosaccharides are gradually converted to free oligosaccharides. The stability of the glycosylamine is sensitive to the pH of the reaction solution. Glycosylamine oligosaccharides are stable at weak alkaline media (pH ≈ 8.0), and the rate of hydrolysis from the glycosylamine to free oligosaccharide is very slow [17]. We previously reported the conversion of glycosylamines to the corresponding Fmoc derivatives by an *in situ* derivatization reaction. Here we propose NBD-F as an alternative reagent for labeling glycosylamines because the reaction of amines with this reagent proceeds under the slightly basic conditions. Moreover NBD-labeled glycosylamine fluoresces at 480 nm or irradiating with an argon laser beam which is frequently used for as a light source for fluorimetric detection. We re-examined the reaction conditions and applied them to oligosaccharides of various glycoprotein specimens. The overall reaction scheme is depicted in Scheme 1.

3.2. Optimization studies for the labeling of glycosylamine oligosaccharides released from glycoprotein samples

The NBD derivatization method was optimized by using human serum transferrin as a model glycoprotein. This glycoprotein contains biantennary oligosaccharides with two sialic acids as a main oligosaccharide (approximately >85%) [18]. As described above, glycosylamines derived from glycoproteins are stable in weak alkaline media. Labeling of amines with NBD-F also preferentially proceeds in alkaline media. Therefore, we optimized reaction conditions by changing the concentration and pH of alkaline phosphate buffer for the glycosylamine-releasing reaction with PNGase F followed by NBD labeling.

Recovery of NBD-oligosaccharides was evaluated by the peak area of the main oligosaccharide of transferrin obtained by HPLC analyses (Fig. 1). The buffer pH was optimal at 8.0. The concentration of buffer was not so critical but seemed to be optimal at 20–100 mM. Under optimized conditions using 20 mM phosphate (pH 8.0), the reaction rate of NBD labeling was enhanced with reagent concentration, and almost reached a plateau at 300 mM NBD-F solution. Slight decrease of the products at higher buffer concentration at high temperature indicates the decomposition of

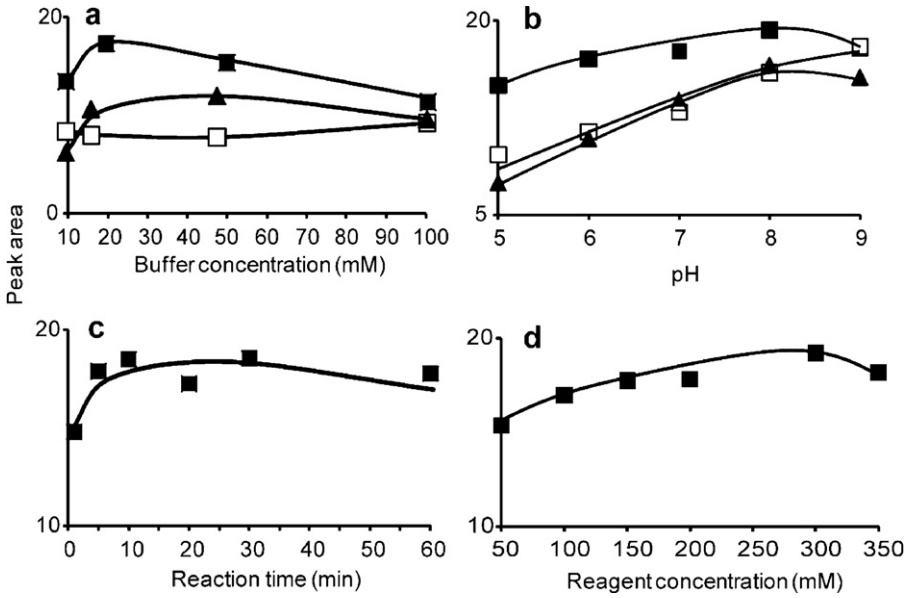


Fig. 1. Effects of buffer concentration (a), pH (b), reaction time (c), and reagent concentration (d) on NBD derivatization of a biantennary oligosaccharide at 70 °C. Unless otherwise depicted in plots, transferrin-derived oligosaccharides (5 μg as a glycoprotein) in each buffer (pH 8.0, 20 μL) were mixed with an acetonitrile solution of NBD-F (300 mM, 5 μL), and the mixture incubated at 70 °C for 10 min. (■) Sodium phosphate, (□) sodium borate, and (▲) ammonium phosphate.

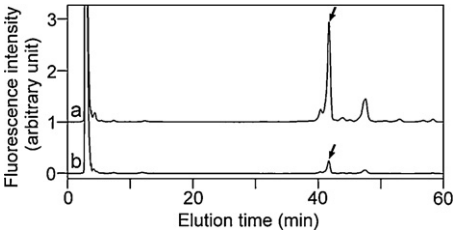


Fig. 2. HPLC analyses of free oligosaccharides as ABEE derivatives as remaining in a reaction mixture after NBD labeling (b) with reference (a). Analytical condition: column; Amide80 (4.6 mm i.d. × 250 mm), eluent; (A) acetonitrile containing 0.1% acetic acid, and (B) 0.2% acetic acid containing 0.2% triethylamine, gradient program; 20% (B) for 5 min, 20–40% (B) for 75 min, 40–95% (B) for 10 min, flow rate; 1.0 mL/min, fluorometric detection; 310 nm (excitation)/370 nm (emission), amount of injection; 5 μg as a glycoprotein.

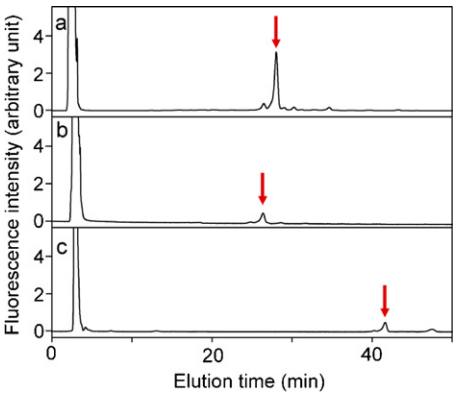


Fig. 3. Comparison of the sensitivity of NBD-labeled (a), Fmoc-labeled (b) and ABEE-labeled (c) oligosaccharides derived from human transferrin. Analytical conditions were identical as those for Fig. 2. Fluorometric detection; (a) 470 nm (excitation)/540 nm (emission), (b) 266 nm (excitation)/310 nm (emission), (c) 310 nm (excitation)/370 nm (emission), amount of injection; 5 μg as a glycoprotein.

the NBD derivatives [19]. The NBD labeling seemed to be completed within 5 min at 70 °C using 300 mM NBD-F. A 2 h reaction of PNGase F in 20 mM phosphate buffer (pH 8.0) for the generation of oligosaccharide glycosylamines and labeling by adding 300 mM NBD-F and heating at 70 °C for 5 min was chosen as the optimal conditions for derivatization. The repeatability of this method is also assayed based on the peak area of samples independently prepared from 50 μg of transferrin. We found 4.1% ($n = 5$) as RSD.

For the next experiment, we estimated the amount of unlabeled oligosaccharides remaining in the reaction mixture. The reaction mixture of PNGase F digestion was divided into two equal portions. To one portion was added acetic acid to form free oligosaccharides; it was then labeled with ABEE under standard conditions. The other portion was converted to NBD-labeled glycosylamine-type oligosaccharides as described above. The reaction mixture was further derivatized with ABEE. We estimated the proportion of NBD-unlabeled oligosaccharides from comparison of these two peak intensities of ABEE-labeled oligosaccharides obtained by HPLC (Fig. 2). ABEE derivatives of major biantennary oligosaccharides of transferrin appeared at 42 min. Compared with the upper trace of the direct derivatization mixture, ABEE derivatives after PNGase F digestion/NBD-F labeling were 9.2% of that for the upper trace. That is, >90% of oligosaccharides were released as glycosylamine and labeled with NBD-F. We believe that 9.2% of transferrin glycans are converted to free oligosaccharides in the process of PNGase F digestion because derivatization with NBD-F is sufficiently fast. Bynum et al. recently reported that an online reaction system using solid-phase reactors that immobilize PNGase F produces quantitative amounts of glycosylamine-type oligosaccharides [20]. That is, optimizing PNGase F digestion may enhance the recovery of NBD-labeled oligosaccharides in our method.

3.3. Comparison of the detection sensitivities of NBD-labeled oligosaccharides with those of Fmoc-Cl and ABEE

The sensitivity of detection of NBD-labeled glycosylamine-type oligosaccharides was compared with the sensitivity of detection obtained by previously reported PNGase F/Fmoc-Cl derivatives and the reductively aminated derivatives with ABEE. Glycosylamine oligosaccharides from transferrin by PNGase F digestion were divided into three equal portions and derivatized with NBD-F, Fmoc-Cl and ABEE. The resultant chromatograms obtained on an Amide80 column are shown in Fig. 3. The main peak corresponding to a disialylated biantennary oligosaccharide was detected at 28 min, 27 min, and 42 min for NBD-, Fmoc- and ABEE derivatives, respectively. NBD derivatives had the most intense signal. The peak area of NBD derivatives was 4.4 times more intense than that for Fmoc derivatives, and 5.7 times more intense than that for ABEE derivatives.

3.4. HPLC analyses of oligosaccharides derived from glycoproteins

This PNGase F/NBD labeling method was applied to the HPLC analysis of oligosaccharide mixtures derived from various glycoproteins. We carefully checked the separation conditions, and found an Amide80 column produced good resolution of acidic oligosaccharides.

Fig. 4(a) shows the separation of NBD derivatives of *N*-linked oligosaccharides of human transferrin. To confirm the structures of some oligosaccharides, the derivatives were analyzed by liquid chromatography–electron spray ionization–mass spectrometry (LC–ESI-MS). Peak 1 clearly showed a pseudomolecular ion at m/z 1192 of $[M-2H]^{2-}$ corresponding to a disialylated biantennary oligosaccharide at 28 min as the major peak. Peak 2 showed a molecular ion at m/z 1265 corresponding to a fucosylated disialobiantennary oligosaccharide. Peak 3 at m/z 1046 corresponded to a monosialylated biantennary oligosaccharide.

The method was also applied to the analysis of bovine serum fetuin. Oligosaccharides of this glycoprotein mainly comprise a series of triantennary oligosaccharides containing linkage isomers in one of the lactosamine branches with 2–4 residues of α-2,3- or α-2.6-linked *N*-acetylneuraminic acid [21–23]. Fig. 4(b) shows the result of separation. Large peaks (7 and 8) observed at ∼30 min indicated m/z 1520 corresponding to trisialylated oligosaccharides. Fetuin contains more than four species of trisialylated triantennary oligosaccharides. The results indicated that the resolution of Amide80 was not sufficient for the complete resolution of the linkage isomers of trisialylated oligosaccharides. Peak 4 and peak 6 (data not shown) showed the same molecular ions, m/z 1192, corresponding to disialylated oligosaccharides. Peak 5 indicated tetrasialylated oligosaccharides (m/z 1666).

α_1-Acid glycoprotein (AGP; human) has bi-, tri-, and tetraantennary oligosaccharides. Some of the oligosaccharides are fucosylated at the terminal lactosamine residues to form a sialyl Lewis^X structure [24,25]. AGP oligosaccharides were separated based on their molecular size (Fig. 4(c)). Disialobiantennary oligosaccharides (peak 9, m/z 1192) were observed at 28 min as a large peak. Trisialotriantennary oligosaccharides (peaks 10 and 11, m/z 1520) appeared at 29 min and 31 min, respectively, and monofucosylated trisialotriantennary oligosaccharides (peak 12, m/z 1593) appeared at 32 min. Small peaks observed at 33 min to 35 min indicated a series of tetrasialotetraantennary oligosaccharides (peak 13, m/z 1848) and monofucosylated tetrasialotetraantennary oligosaccharides (peak 14, m/z 1921). The complex distribution of the same molecular ion signals may be due to the linkage isomers of *N*-acetyl

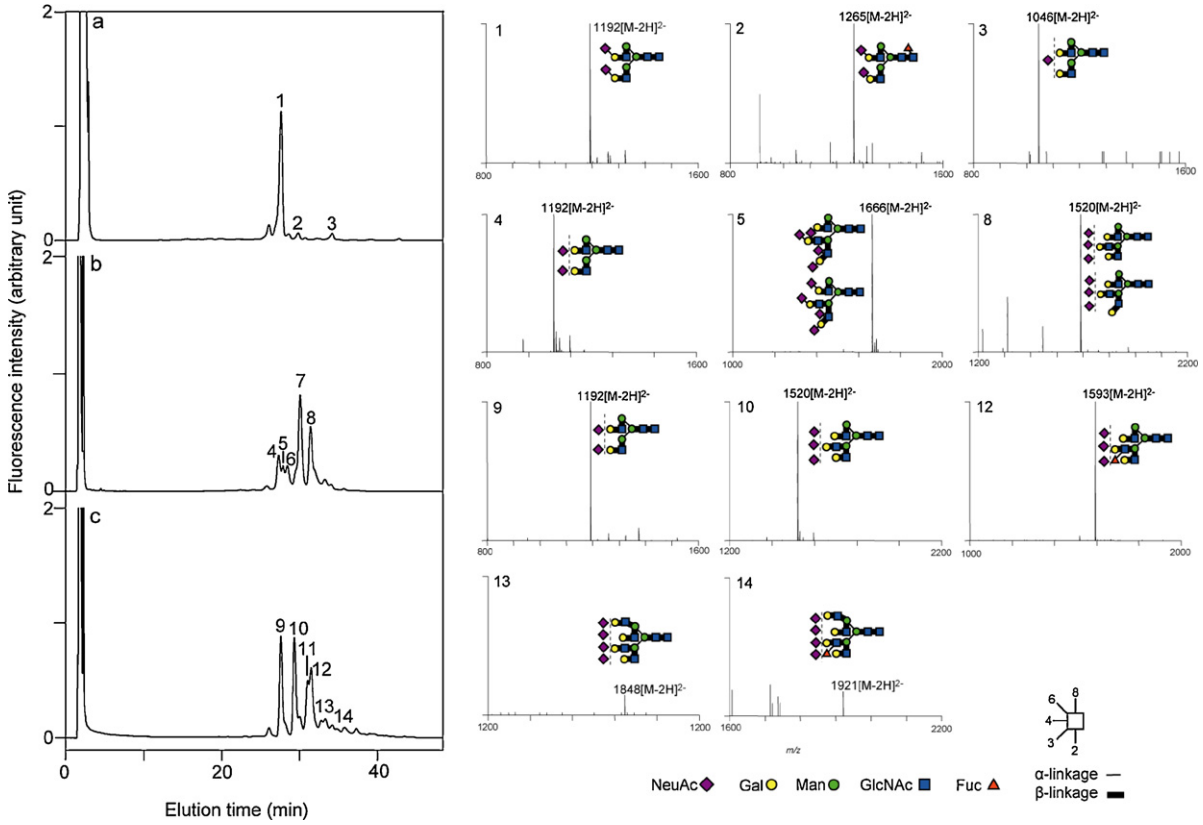


Fig. 4. Application of the PNGase F/NBD method for the HPLC analysis using fluorometric detection of oligosaccharides from human serum transferrin (a), fetal calf serum fetuin (b) and human α_1-acid glycoprotein (c) with ESI-MS spectra of each peak. All MS signals of oligosaccharides with *m/z* values were obtained as the adduct ion of trifluoroacetic acid ($[M+CF_3CO_2]^-$). Analytical conditions: column; Amide80 (4.6 mm i.d. × 250 mm), eluent; (A) acetonitrile containing 0.1% acetic acid (B) 0.2% acetic acid containing 0.2% triethylamine, gradient program; 20–25% (B) for 5 min, 25–35% (B) for 75 min, 35–95% (B) for 10 min, flow rate; 0.8 mL/min, fluorometric detection; 470 nm (excitation)/540 nm (emission), amount of injection; 5 μg as a glycoprotein.

neuraminic acid (NeuAc) residues. NBD derivatization produced pseudomolecular ions of oligosaccharides containing sialic acid at high sensitivity. This may be an advantage for sensitive analyses of minute amounts of carbohydrates in glycoprotein samples.

The described method was also applied to the analysis of carbohydrate chains derived from glycoprotein samples containing neutral oligosaccharides (Fig. 5). We employed a diol column instead of an amide column for the separation of neutral oligosaccharides. This separation mode seemed to be useful for the analysis of complex-type asialooligosacchardies and high mannose-type oligosaccharides.

Ribonuclease B (bovine pancreas) has been reported to contain a series of high mannose-type oligosaccharides, including $Man_{5-9}GlcNAc_2$ [26]. Use of the diol column led to five peaks for these oligosaccharides. In contrast to previously described acidic oligosaccharides, NBD derivatives of a series of high mannose-type oligosaccharides were detected as adduct ions with TFA, $[M+CF_3CO_2]^-$. From the peak identification based on their molecular mass, ribonuclease B oligosaccharides were separated according to molecular size. Moreover, small shoulder peaks observed at the front of peak 3 indicated the presence of linkage isomers of $Man_7GlcNAc_2$ in the terminal α-1,2-linked mannose.

Ovalbumin contains a series of high mannose-type oligosaccharides and bisected hybrid-type oligosaccharides [27–29]. Separation of these oligosaccharides is shown in Fig. 5(b). Peak identification based on the molecular mass of peaks indicated the elution order of the oligosaccharides to be $Man_5GlcNAc_2 < Man_3GlcNAc_5$, $Man_4GlcNAc_4 < Man_6GlcNAc_2$, $Man_4GlcNAc_4 < Man_5GlcNAc_4 < Man_5GlcNAc_6$, $Man_4Gal_1GlcNAc_5$ and/or $Man_5GlcNAc_5$ and $Man_7GlcNAc_2$.

All the data shown above were obtained by injection of 5 μg glycoproteins. Therefore, for example, monofucosylated biantennary oligosaccharides of transferrin occupying ~2% in total of oligosaccharides indicate ~2.5 pmol as the injection amount. Therefore, the lower limit of detection (signal-to-noise (S/N) ratio = 5) in HPLC using fluorometric detection for these oligosaccharides was estimated to be 100 fmol. The sensitivities of NBD-labeled oligosaccharides were compared with those of Fmoc- and ABEE-labeled oligosaccharides in negative ESI-MS analyses: they were 12 times and 10 times higher than those for Fmoc and ABEE derivatives, respectively. The enhanced sensitivity of NBD derivatives indicated the usefulness of this method. However, the resolution of NBD derivatives of oligosaccharides in HPLC was not enough. We therefore applied LIF-CE to the analysis of NBD-labeled glycosylamine-type oligosaccharides.

3.5. Application to CE with LIF detection

LIF-CE was applied to the analysis of NBD-labeled glycosylamine-type oligosaccharides. NBD derivatives fluoresce by irradiating light at 470 nm. The maximum oscillation frequency of an argon ion laser is 488 nm. Therefore, a high sensitivity in LIF-CE using an argon laser was expected for NBD-labeled oligosaccharides.

Various modes have been proposed for the separation of labeled oligosaccharides by CE. These include plain capillary zone electrophoresis for size/charge resolution; micellar electrokinetic chromatography based on pseudo-partition chromatography; and borate complex mode for *in situ* formation of negatively charged borate complexes. We chose the borate complex mode in the

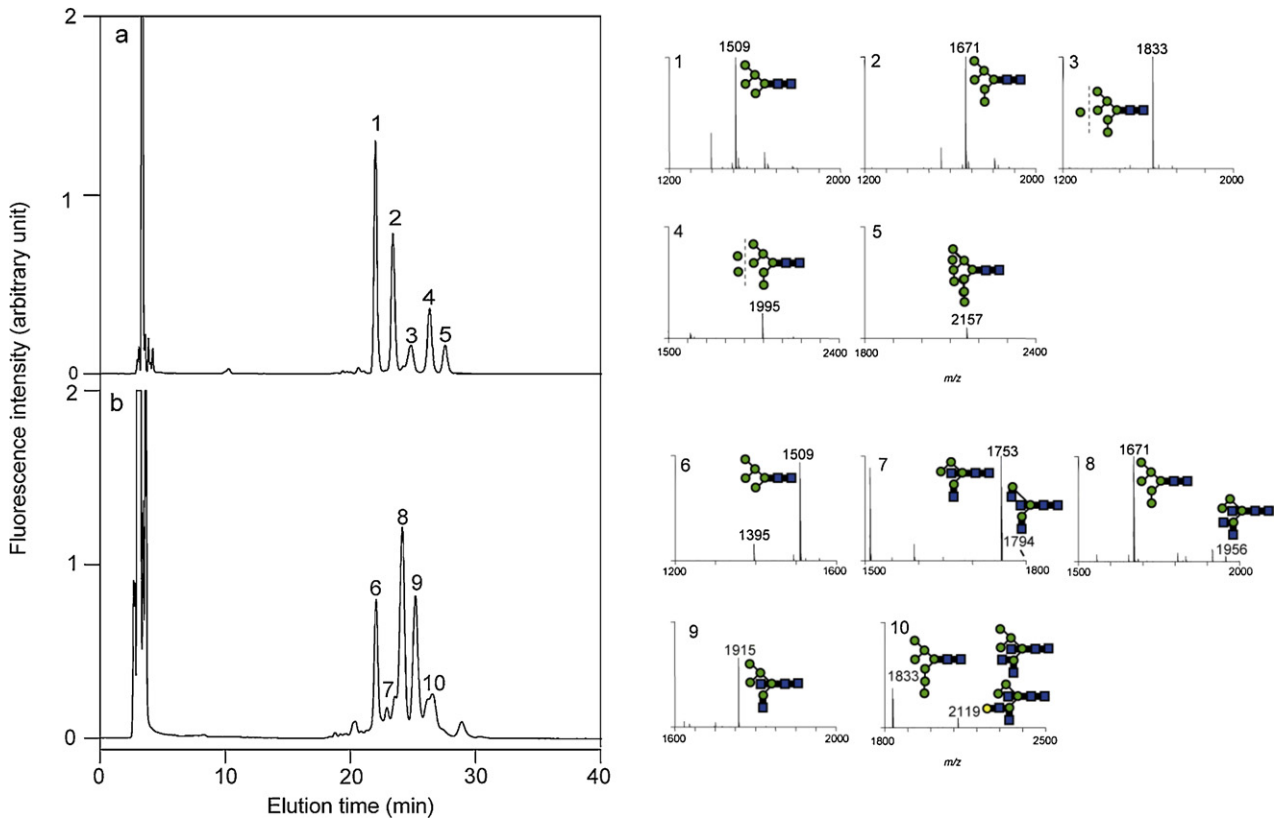


Fig. 5. Application of the PNGase F/NBD method for the HPLC analysis of oligosaccharides from bovine ribonuclease B (a) and ovalbumin (b). Analytical condition: column; Inertsil diol (4.6 mm × 250 mm), eluent; (A) 0.1% TFA, and (B) 95% acetonitrile containing 0.1% TFA, flow rate; 1.0 mL/min, gradient program; 99% (B) for 5 min, 99–75% (B) for 10 min, 75–50% (B) for 60 min, 50–1% (B) for 5 min, fluorometric detection; 470 nm (excitation)/540 nm (emission), amount of injection; 5 μg as a glycoprotein.

present study. NBD derivatives of oligosaccharides were separated as anionic borate complexes on neutrally coated capillary using 100 mM borate (pH 8.3) as the electrophoresis buffer. The neutrally coated capillary suppresses the generation of electroendoosmotic flow. Therefore, NBD-oligosaccharides move to the anode based on the number of acidic groups (*i.e.*, number of NeuAc residues) and the association with borate ions.

Fig. 6 shows the separation of NBD-labeled glycosylamine-type oligosaccharides derived from human transferrin, bovine fetuin, and human α_1-acid glycoprotein. Derivatives were separated based on the number of sialic acid residues. Tetrasialo-, trisialo- and disialooligosaccharides appeared at ~10–11 min, 12 min and 14 min, respectively. Further resolution seemed to be based on the difference in oligosaccharide structures. Transferrin-derived oligosaccharides indicated a large major peak corresponding to disialobiantennary oligosaccharides at 14 min, and small peaks observed at 14.2 min were assignable to fucosylated disialobiantennary oligosaccharides. Fetuin indicated two peaks each at tetrasialo-, trisialo- and disialooligosaccharide fractions. Splitting of the peaks at each fraction was probably due to the difference in linkage (*i.e.*, NeuAcα2 → 3 and NeuAcα2 → 6). These results showed very similar electropherograms to those of APTS-labeled oligosaccharides derived from fetuin [30]. Peak assignments shown in the figures were estimated from comparison of those electropherograms. As described above, α_1-acid glycoprotein contains biantennary, triantennary and tetraantennary oligosaccharides with 2–4 residues of NeuAc with or without fucose in one of the branches. Therefore, resolution of the oligosaccharides derived from this glycoprotein indicated a complex electropherogram. The limit of detection of NBD-labeled oligosaccharides was also estimated from a main peak of transferrin-derived oligosaccharides: it was 4 fmol (S/N ratio = 20).

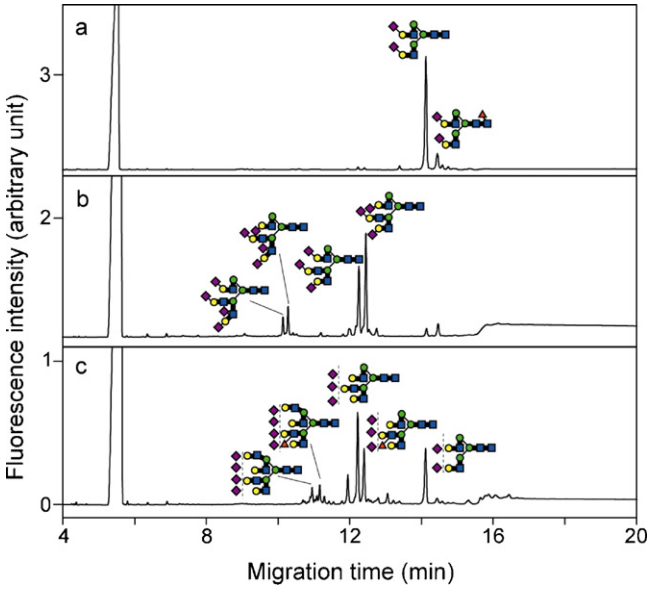


Fig. 6. Capillary electrophoresis with laser-induced fluorometric detection of oligosaccharides derived from human serum transferrin (a), fetal calf serum fetuin (b), and human α_1-acid glycoprotein (c). Analytical condition: capillary, InertCap® 1 (50 cm, 100 μm i.d.; effective length 40 cm); buffer, 100 mM borate (pH 8.3); capillary temperature, 25 °C; sample injection, 1 psi for 10 s; applied voltage, −15 kV.

4. Conclusion

The described method comprises digestion of PNGase F for the generation of glycosylamine-type oligosaccharides followed by fluorescent derivatization with NBD-F. This method has two

characteristic features: (i) sensitive analyses of *N*-linked oligosaccharide with easy operations attainable in a one-pot reaction within 3 h; and (ii) excitation maxima of NBD derivatives match LIF-CE using an argon laser. Sensitivity using fluorescence-detection HPLC was 4-times and 6-times higher than that previously reported for Fmoc- and ABEE-labeled oligosaccharides, respectively. The sensitivity of NBD derivatives in LC–ESI-MS was one order of magnitude higher than those labeled with ABEE and Fmoc. The sensitivity of NBD-labeled oligosaccharides was in the fmol range using LIF-CE. Therefore, the described method shows promise in the profiling of *N*-linked oligosaccharides in various biological samples as well as quality control in the manufacturing processes of glycoprotein pharmaceuticals.

Acknowledgments

This work was supported by the "High-Tech Research Center" Project for Private Universities: a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, 2007–2011.

References

[1] D.J. Harvey, J. Chromatogr. B (2010), doi:10.1016/j.jchromb.2010.11.010.
[2] S. Hase, T. Ikenaka, Y. Matsushima, Biochem. Biophys. Res. Commun. 85 (1978) 257.
[3] W.T. Wang, N.C. LeDonne Jr., B. Ackerman, C.C. Sweeley, Anal. Biochem. 141 (1984) 366.
[4] N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, N. Takahashi, Anal. Biochem. 171 (1988) 73.
[5] N. Takahashi, H. Nakagawa, K. Fujikawa, Y. Kawamura, N. Tomiya, Anal. Biochem. 226 (1995) 139.
[6] N. Takahashi, J. Chromatogr. A 720 (1996) 217.
[7] N. Takahashi, K. Kato, http://www.glycoanalysis.info/galaxy2/.
[8] A. Guttman, Nature 380 (1996) 461.
[9] F.T. Chen, R.A. Evangelista, Electrophoresis 19 (1998) 2639.
[10] T.S. Raju, J.B. Briggs, S.M. Borge, A.J. Jones, Glycobiology 10 (2000) 477.
[11] N. Callewaert, S. Geysens, F. Molemans, R. Conteras, Glycobiology 11 (2001) 275.
[12] F.-T.A. Chen, in: P. Thibault, S. Honda (Eds.), Methods Mol. Biol., Humana Press, 2002, p. 105.
[13] M. Yodoshi, A. Tani, Y. Ohta, S. Suzuki, J. Chromatogr. A 1203 (2008) 137.
[14] S. Kamoda, M. Nakano, R. Ishikawa, S. Suzuki, K. Kakehi, J. Proteome Res. 4 (2005) 146.
[15] R.A. Kekwick, R.K. Cannan, Biochem. J. 30 (1936) 227.
[16] T.H. Plummer Jr., J.H. Elder, S. Alexander, A.W. Phelan, A.L. Tarentino, J. Biol. Chem. 259 (1984) 10700.
[17] H.S. Isbell, H.L. Frush, J. Am. Chem. Soc. 72 (1950) 1043.
[18] Y. Satomi, Y. Shimonishi, T. Hase, T. Takao, Rapid. Commun. Mass Spectrom. 18 (2004) 2983.
[19] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[20] M.A. Bynum, H. Yin, K. Felts, Y.M. Lee, C.R. Monell, K. Killeen, Anal. Chem. 81 (2009) 8818.
[21] R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver, B. Bendiak, Anal. Biochem. 182 (1989) 1.
[22] S. Suzuki, S. Honda, Electrophoresis 19 (1998) 2539.
[23] E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson, H. Van Halbeek, J. Biol. Chem. 263 (1988) 18253.
[24] M. Nakano, K. Kakehi, M.H. Tsai, Y.C. Lee, Glycobiology 14 (2004) 431.
[25] B. Fournet, J. Montreuil, G. Strecker, L. Dorland, J. Haverkamp, J.F.G. Vliegenthart, P. Binette, K. Schmid, Biochemistry 17 (1978) 5206.
[26] A. Koller, J. Khandurina, J. Li, J. Kreps, D. Schieltz, A. Guttman, Electrophoresis 25 (2004) 2003.
[27] T. Tai, K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, A. Kobata, J. Biol. Chem. 280 (1975) 8569.
[28] T. Tai, K. Yamashita, S. Ito, A. Kobata, J. Biol. Chem. 252 (1977) 6687.
[29] K. Yamashita, Y. Tachibana, A. Kobata, J. Biol. Chem. 253 (1978) 3862.
[30] A. Guttman, F.-T.A. Chen, R.A. Evangelista, Electrophoresis 17 (1996) 412.