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3-Aminobenzamide and 3-aminobenzoic acid, tags for capillary electrophoresis of complex carbohydrates with laser-induced fluorescent detection

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Abstract

The efficiencies in derivatization of reducing carbohydrates were compared by capillary electrophoresis using maltose as a model with nine monoaminobenzene derivatives by reductive amination in the presence of sodium cyanoborohydride. We found that aminobenzene derivatives substituted at the 3-position showed good reactivity with reducing carbohydrates as expected from the reaction mechanism, although the fluorescence intensities and molar absorptivities of these derivatives were not as high as those of 2- and 4-aminobenzene derivatives. The reagents, 3-aminobenzamide and 3-aminobenzoic acid, which showed the highest reactivity, were applied to the labeling of carbohydrate chains obtained from some sialic acid-containing glycoprotein samples, and also high-mannose and hybrid-type oligosaccharides. Capillary electrophoresis of these labeled carbohydrate chains in an inner surface-modified capillary with (50% phenyl)methylpolysiloxane allowed excellent separation of sialic acid-containing carbohydrate chains derived from fetuin and thyroglobulin as well as high mannose-type and hybrid-type carbohydrates derived from bovine pancreas ribonuclease B, soybean agglutinin and hen ovalbumin. The lower limit of calibration was as low as the 10^{-16} mol (injected amount) with helium–cadmium laser induced detection.

Keywords: Derivatization; Electrophoresis; Aminobenzamides; Aminobenzoic acids; Carbohydrates

1. Introduction

The need for detailed structural analysis of the carbohydrate chains in glycoconjugates such as glycoproteins and glycolipids has become more obvious with the characterization of such molecules. The type and extent of glycosylation of glycoprotein drugs often contribute to their physicochemical and recognition properties, and other important biological functions [1]. In the industrial production of glycoprotein pharmaceuticals, the type of host cell mainly controls glycosylation to the core protein, but environmental factors also influence glycosylation [2]. Therefore, it is important to establish a method for the analysis of the heterogeneity in the carbohydrate chains of glycoprotein drugs.

Carbohydrates need to be labeled with fluorescent or chromophoric tags to achieve high sensitivity on detection, because most carbohydrate species have neither chromophores nor fluorophores. A number of

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photometric and fluorometric tags have been developed for the analysis of carbohydrates by capillary electrophoresis and high-performance liquid chromatography [3]. Labeling by reductive amination using a combination of aromatic amines and reducing reagents has been most frequently employed [4,5]. The method involving 2-aminopyridine (PA) as the fluorescent tag is the most popular one for the analysis of carbohydrate chains obtained from glycoprotein samples and is widely used for high-performance liquid chromatography and capillary electrophoresis [6,7]. Many reagents classified as monosubstituted aminobenzene derivatives, for example, 2-aminobenzamide [8], 4-aminobenzoic acid ethyl ester [9] and *N*-(4-aminobenzoyl)-L-glutamic acid [10], have also been reported as being useful tags. Sulfonic acid derivatives of aminonaphthalene and aminopyrene are also reagents in this category, and were developed for capillary electrophoresis and slab gel electrophoresis [11–15]. Carbohydrate chains labeled with these tags exhibited high sensitivity and high resolution on high-performance liquid chromatography, capillary electrophoresis and slab gel electrophoresis.

Reaction efficiency is another important factor when choosing a labeling reagent for the derivatization reaction. High reaction efficiency is especially required for the labeling of sialic acid-bearing carbohydrate chains that are labile under acidic conditions. It is important to ensure complete labeling without loss of sialic acid residues during the derivatization reaction. A high reaction efficiency should make it possible to use milder derivatization conditions without loss of sialic acids or other labile residues during derivatization.

In the present study, nine mono-substituted aminobenzene derivatives of aminobenzamide (ABA), aminobenzoic acid ethylester (ABEE) and aminobenzonitrile (ABN) were compared as to the reaction efficiency on the derivatization of reducing carbohydrates using maltose as a model by capillary electrophoresis.

2. Experimental

2.1. Reagents and materials

2-, 3- and 4-ABEE, and 2-, 3- and 4-ABA were obtained from Nacalai Tesque (Nakagyo-ku, Kyoto). 2-ABN was obtained from Sigma–Aldrich Japan (Tokyo, Japan), and its two isomers were obtained from Wako (Osaka, Japan). 3-AB was obtained from Tokyo Kasei (Tokyo, Japan). All the tagging reagents were the analytical grade and used after recrystalization. Dextran oligomers were obtained from Seikagaku Kogyo (Tokyo, Japan). Hen ovalbumin was prepared from fresh hen eggs, and purified by repeated crystallization by the method of Keckwick and Cannan [16]. Thyroglobulin was prepared from fresh porcine thyroids by the method of Ui and Tarutani [17]. Soybean agglutinin was prepared from soybean flour (not roasted, Sigma) according to the method reported by Dorland et al. [18]. Fetuin (bovine) was from GIBCO Labs. (Grand Island, NY, USA). Ribonuclease B (bovine pancreas) and sodium cyanoborohydride were from Sigma–Aldrich Japan.

Peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGase F, EC 3.2.2.18) was from Boehringer Mannheim (Tokyo, Japan). *Endo-N*-acetyl-β-glucosaminidase was a gift from Dr. Takegawa of Kagawa University. Neuraminidase (*Arthrobacter ureafaciens*) was a gift from Drs. Tsukada and Ohta (Marukin Shoyu, Uji, Kyoto, Japan). Sodium dodecyl sulfate (SDS) and polyethylene glycol (PEG 70 000) were of biochemical grade and obtained from Wako. Concanavalin A (Con A)-Sepharose was obtained from Pharmacia (Uppsala, Sweden). All other carbohydrate samples and reagents were of the highest grade commercially available or of HPLC grade, and used without further purification. A fused-silica capillary (75 μm) was purchased from Waters Japan (Tokyo, Japan). A DB-17 capillary column (100 μm I.D.) for gas chromatography was obtained from J&W Scientific (Folsom, CA, USA). The inner surface of the latter capillary was chemically modified with (50%-phenyl)methylpolysiloxane (0.1 mm film thickness). The detection window (5 mm wide) for UV absorption or fluorescent detection was made by carefully removing the polyimide coating with a razor. Water purified with a Milli-Q purification system (Millipore) after double distillation of deionized water was used throughout the work.

2.2. Apparatus

Capillary electrophoresis was performed with a

P/ACE 5010 (Beckman Instruments, Tokyo, Japan). The separation was monitored in the on-column detection mode with a Beckman UV absorption monitor at 200 nm or a laser-induced fluorescence (LIF) detection system with a 25 mW helium–cadmium ion laser with an excitation filter of 325 nm and an emission band-path filter of 450 nm. A fused-silica capillary column [57 cm (effective length 50 cm)×75 μm I.D.] and a DB-17 capillary column (100 μm I.D.) of the same length were employed. The operation temperature was maintained at 25°C. UV absorption spectra were recorded with a Shimadzu UV-160 spectrophotometer with a 1 cm quartz cell. Fluorescence spectra were recorded with a Hitachi F-4010 fluorescence spectrometer with a 1 cm quartz cell using the 100-times diluted solutions employed for the observation of UV absorption spectra. Separation by capillary electrophoresis was performed in 50 m*M* borate buffer (pH 9.3) containing 150 m*M* SDS, 200 m*M* borate buffer (pH 10.5) or 50 m*M* phosphate buffer (pH 2.5). Tris–borate buffer (pH 8.5, 50 m*M*) was also employed for the analysis of the carbohydrate chains labeled with 3-AB involving a DB-17 capillary.

2.3. Selection of the labeling reagent

The derivatization reaction of maltose, as a model carbohydrate, with an equimolar mixture of 2-, 3- and 4-ABA was performed according to the similar procedures reported by Okafo et al. [19], and described bellow (Section 2.6). The reaction was also performed using an equimolar mixture of ABEE isomers and ABN isomers, respectively. Finally, the derivatization reaction of maltose with an equimolar mixture of 3-ABA, 3-ABEE and 3-ABN was performed. The yield of the derivatization with 3-ABA was tentatively assigned as 100%, and the relative yields of the derivatization with other reagents were calculated based on the peak areas, with correction as to the relative molar responses to the 3-ABA derivative at 200 nm. To confirm that mutual reactions among the reagents did not occur, reactions without maltose were also performed.

The standard sample of 3-ABA Mal was obtained using Mal (3.4 mg, 10 μmol) according to the procedure described below. The isolated 3-ABA Mal was confirmed by fast atom bombardment mass spectrometry using glycerol as matrix, and showed an abundant peak at m/z 484 $(M-1+Na)^+$.

2.4. Optimization of the derivatization reaction

A dried sample of maltohexaose (100 nmol), as a model oligosaccharide, was dissolved in 5 μl of a solution of 3-ABA (0.35 *M*) in dimethylsulfoxide–acetic acid (7:3, v/v) containing 1 *M* $NaBH_3CN$. The mixture was kept at different temperatures with varying reaction periods. After cooling the mixture, an aqueous solution (10 μl) of the internal standard (3-ABA Mal, 10.0 μmol/ml) was added to it. The excess reagent and the reaction solvent were removed according to the procedures described above, the combined fluorescent fractions being concentrated, and a portion of the solution was analyzed by capillary electrophoresis, with a UV detector monitoring the absorption at 200 nm.

2.5. Labeling of an oligosaccharide specimen or a mixture of carbohydrate chains released from a glycoprotein sample

A solution (0.7 *M*, 5 μl) of an amine reagent in a mixture of dimethylsulfoxide (seven volumes) and acetic acid (three volumes) was added to a dried carbohydrate sample (less than 100 nmol) or a dried mixture obtained through the enzyme reaction described above in a screw-capped polypropylene tube (1.5 ml volume). A solution (5 μl) of freshly prepared 2 *M* sodium cyanoborohydride in the same solution was added to the mixture, and then the mixture was kept for 1 h at 50°C. After cooling to room temperature, the reaction mixture was diluted with water (200 μl). Ethyl acetate (200 μl) was added to the mixture and then the mixture was shaken vigorously. The organic phase was removed carefully and the aqueous phase was evaporated to dryness. The residue was dissolved in a small volume of water (200 μl) containing 20% methanol and 0.2% acetic acid and applied to a small column (30 cm×1.0 cm I.D.) of Sephadex G-10 previously equilibrated with the same solvent. The fractions eluted early, which contained the labeled oligosaccharide, were collected with monitoring the fluorescence and evaporated to dryness. The residue was dissolved in a small volume of water (500 μl) and then a portion was used for capillary electrophoresis.

2.6. Release of asparagine-linked oligosaccharides from glycoprotein or glycopeptide samples with PNGase F

An aqueous solution (50 μg/50 μl) of a glycopeptide or glycoprotein sample was mixed with 50 μl of 50 m*M* phosphate buffer (pH 7.5) in a screw-capped polypropylene tube (1.5 ml volume). One unit (5 μl) of PNGase F was added to the solution, and then the mixture was kept at 37°C for 48 h. After the reaction had been terminated by heating the mixture for 3 min at 100°C, the reaction mixture was evaporated to dryness in a centrifugal evaporator (CC-101, Tomy, Tokyo, Japan) at room temperature, and the residue was used for the derivatization reaction.

2.7. Release of sialic acids from sialic acid-containing carbohydrate chains with neuraminidase

The method was essentially the same as described previously and was briefly as follows [20]. An aqueous solution (20 μl) of a mixture of labeled carbohydrate chains was diluted with 10 m*M* acetate buffer (pH 5.0, 20 μl). An aqueous solution (10 μl) of neuraminidase (10 munits) was added and the mixture was incubated overnight at 37°C. After keeping the mixture for 3 min in a boiling water bath, it was centrifuged and a portion of the supernatant solution was used for analysis by capillary electrophoresis.

2.8. Group separation of 3-AB labeled carbohydrate chains derived from thyroglobulin on a small column packed with Con A-Sepharose

An aqueous solution of a mixture of 3-AB labeled carbohydrate chains obtained from thyroglobulin (40 μl, 250 μg as protein), according to the procedures described above, was applied to a small column (5 cm×0.5 mm I.D.) of Con A-Sepharose 4B, which had been previously equilibrated with 10 m*M* Tris–HCl buffer (pH 8.0) containing 0.15 *M* NaCl, and 1 m*M* calcium and manganese ions [21]. After equilibration for 30 min at room temperature, the column was eluted with the equilibration buffer (7 ml), the same buffer containing 10 m*M* methyl α-D-glucopyranoside (7 ml), and finally the same buffer containing 100 m*M* methyl α-D-mannopyranoside (7 ml). After each fraction had been concentrated to about 1 ml under reduced pressure, the concentrate was applied to a Sephadex G-25 column (30 cm×1 cm I.D.) equilibrated with 10 m*M* NH_4HCO_3. The eluate showing fluorescence was collected and evaporated to dryness. The residue was dissolved in water (50 μl) and used for analysis by capillary electrophoresis.

3. Results and discussion

3.1. Selection of a labeling reagent

The reagents used in the present study and their abbreviations are presented in Fig. 1. Some of these reagents are widely used for the efficient labeling of reducing carbohydrates. Their photometric and fluorometric characteristics are summarized in Table 1.

2-ABA showed very intense fluorescence at 419 nm and was recently reported to be a fluorescent tag for the HPLC of carbohydrates [8]. 4-ABEE was originally reported to be a tag for the mass spec-

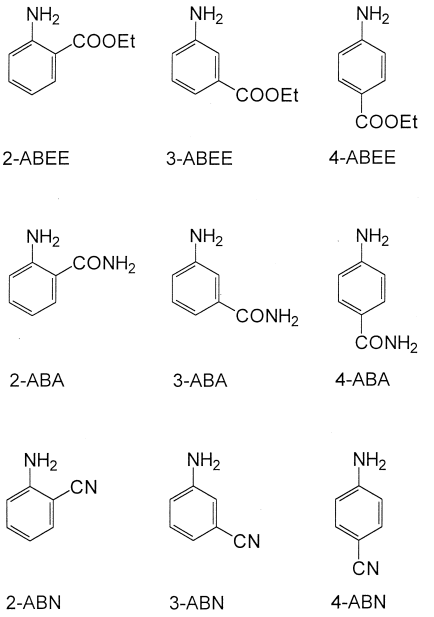

Fig. 1. Monosubstituted aminobenzene derivatives as candidates for the derivatization of reducing carbohydrates.

Table 1
Photometric and fluorometric characteristics of the mono-substituted aminobenzene derivatives[a]

Amino-benzene	Photometric characteristics		Fluorometric characteristics		
	λ_{max} (nm)	Molar absorptivity	Excitation (nm)	Emission (nm)	Relative intensity
2-ABA	311	3620	319	419	2270
3-ABA	306	3000	306	452	100
4-ABA	276	15150	281	358	190
2-ABEE	325	3570	330	419	970
3-ABEE	309	2360	302	410	27
4-ABEE	277	17100	290	354	84
2-ABN	315	5500	316	383	190
3-ABN	306	2770	309	392	25
4-ABN	270	17400	387	343	130

[a] ABA, aminobenzamide; ABEE, aminobenzoic acid ethyl ester; ABN, aminobenzonitrile. The data were obtained in a methanol solution.

trometry of carbohydrates and exhibits strong absorption at 277 nm, which is advantageous for photometric detection [9]. The spectrophotometric and fluorometric data for the labeled carbohydrates with these reagents are not consistent with the data in Table 1, as it has been reported that 4-ABEE derivatives showed an absorption maximum around 320 nm [22]. However, we can see general photometric and fluorometric characteristics of the labeling reagents in this table.

Reducing carbohydrates can be labeled by reductive amination using these aminobenzene derivatives through Schiff base formation, as shown in Fig. 2, which is the rate-determining step in derivatization.

Because the labeling reaction starts with the attack of the lone pair of amino groups, yielding a Schiff

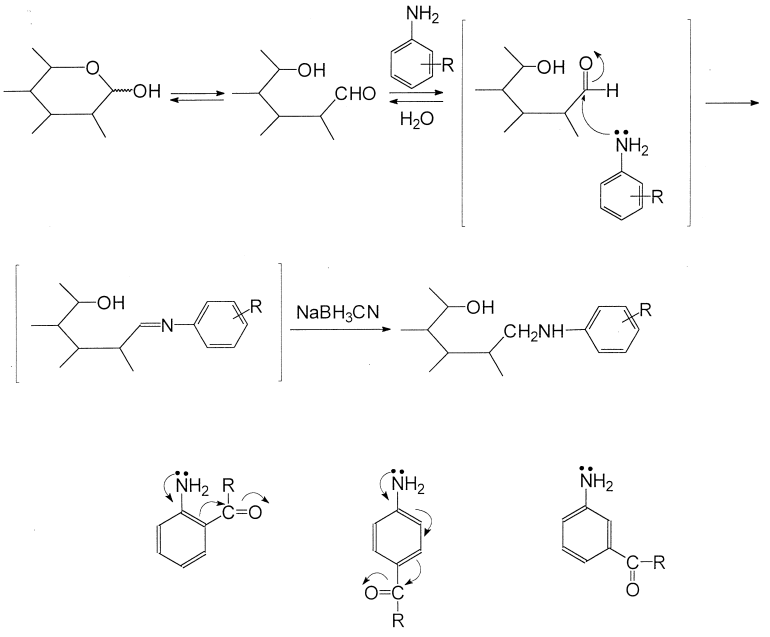

Fig. 2. Mechanism of the labeling of reducing carbohydrates with monosubstituted aminobenzene in the presence of sodium cyanoborohydride.

base (Fig. 2, upper panel), a higher electron density at the amino groups is favorable. The presence of an electron attractive group at the 2- or 4-position of the benzene ring decreases the electron density of the amino groups (Fig. 2, lower panel). In contrast, monoaminobenzene derivatives substituted at 3-position are expected to show a smaller effect on the electron density at the amino group. Therefore, monoaminobenzene derivatives substituted at the 3-position seem to be advantageous for the Schiff base formation, although no papers were found describing comparative studies on the reactivities of these reagents. We also examined labeling using 3-methoxyaminobenzene as the labeling reagent, which has an electron-donating group. However, dextran hydrolyzate labeled with this reagent showed poor resolution (data not shown), and no further studies were performed.

Bigge et al. reported excellent efficiency on fluorescent labeling of glycans with 2-ABA and 2-anthranilic acid employing twenty-seven reducing oligosaccharides with a tritium-labeled galactose residue at the C-6 position [8]. They also reported that the labeling reaction with 2-ABA proceeded quantitatively and that the yields corresponded to those with the classical method of alkaline reduction with sodium borohydride for the glycans examined. However, since 3-ABA should be the most reactive of the aminobenzene derivatives, based on mechanistic considerations, it might permit milder derivatization conditions decreasing the degradation of carbohydrate chains during derivatization reactions.

Using an equimolar mixture of mono-aminobenzene derivatives substituted at the 2-, 3- or 4-positions, and maltose as a model oligosaccharide, the relative reactivity of each reagent was directly compared by the ratio of the derivatives produced on reductive amination. An example of derivatization of maltose with an equimolar mixture of aminobenzamide derivatives (Fig. 3) indicates that each reagent shows a different derivatization efficiency.

We have also examined the relative reactivity of each reagent using chitobiose and lactose as model oligosaccharides, and observed similar results (data not shown). Although detection was performed photometrically, the 3-ABA derivative (peak 1) gave the most intense peak and thus showed the highest reaction efficiency. 2-ABA and 4-ABA (peaks 3 and 2, respectively) gave almost the same peak areas. The relative molar responses of the derivatives labeled with 2-ABA and 4-ABA, compared to 3-ABA were determined to be 1.08 and 1.05 at 200 nm, respectively. The relative efficiencies, calculated using the relative responses and areas, showed that 3-ABA was about four times more reactive than 2-ABA, and that 4-ABA showed 31% of the relative reactivity to that of 3-ABA under the present conditions. The same strategy was also applied for the other reagents and the results are summarized in Table 2.

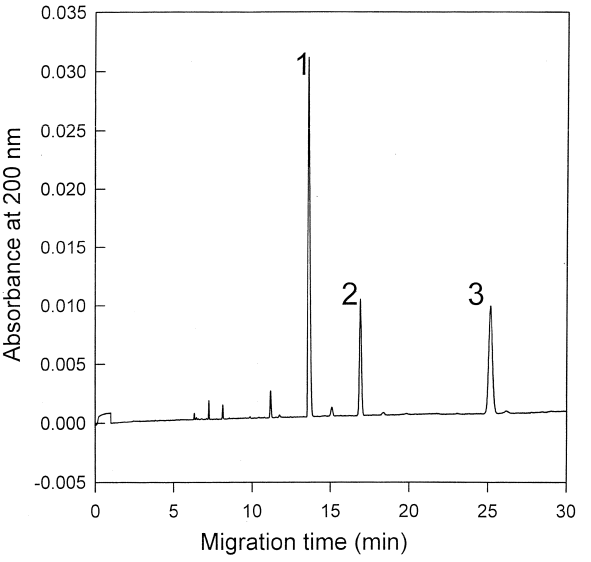

Fig. 3. Analysis of maltose after labeling with an equimolar mixture of 2-ABA, 3-ABA and 4-ABA. (1) 3-ABA derivative; (2) 4-ABA derivative and (3) 2-ABA derivative. Analytical conditions: 50 m*M* phosphate buffer (pH 2.5) in a fused-silica capillary [57 cm (50 cm effective length)×50 μm I.D.]. Applied potential: 25 kV. Detection: UV absorption at 200 nm.

While 3-ABA gave the highest reaction efficiency in all cases, the other reagents studied were also useful tags for most reducing carbohydrates. Since there are a huge number of carbohydrate chains in nature, it is not practical to determine the yield for all oligosaccharides using each reagent. Furthermore, carbohydrate chains released from various glycoproteins are generally composed of extensively complex mixtures. Therefore, we propose the use of competitive methods for evaluation of the relative reactivity.

For analysis by capillary electrophoresis, carbohy-

Table 2
Relative yields on reductive amination of maltose with mono-substituted aminobenzene derivatives

Aminobenzene derivative	Relative yield (%)[a]		
	Aminobenzoic acid ethyl ester (ABEE)	Aminobenzamide (ABA)	Aminobenzonitrile (ABN)
2-Substituted	0.43	27.1	4.62
3-Substituted	56.9	100[b]	13.8
4-Substituted	10.3	31.2	0.45

[a] The yields are the averages of three experiments.
[b] The yield of 3-ABA derivative of maltose which showed the highest yield was 98±3% (n=3) using the standard 3-ABA Mal, and tentatively assigned as 100%.

drates should have electric charges for their effective resolution [23]. Although the 3-ABA derivative has one imino group per molecule, the donation of a negative charge to the derivative will make it possible to finely adjust the mobility in the electrophoretic process by controlling the pH of the running buffer. Thus, an analogous compound, 3-aminobenzoic acid (3-AB), was also examined as a candidate labeling reagent, and it showed a similar reaction efficiency to that of 3-ABA (data not shown).

3.2. Optimization of the labeling reaction

The procedures for the labeling reaction involving 3-ABA and 3-AB were similar to those described by Okafo et al. [19]. The effects of the reaction temperature and reaction time on the labeling of maltohexaose with 3-ABA are shown in Fig. 4.

The yield was almost constant after 20 min and above 50°C. Hence, the reaction time and reaction temperature were set at 30 min and 50°C, respectively. Removal of the excess reagent and reaction solvent (dimethylsulfoxide) by chromatography on a small column of Sephadex G-10 was necessary to achieve better resolution on capillary electrophoresis. The recoveries in the purification step were almost quantitative, with 3-ABA or 3-AB labeled maltohexaose as the model (data not shown).

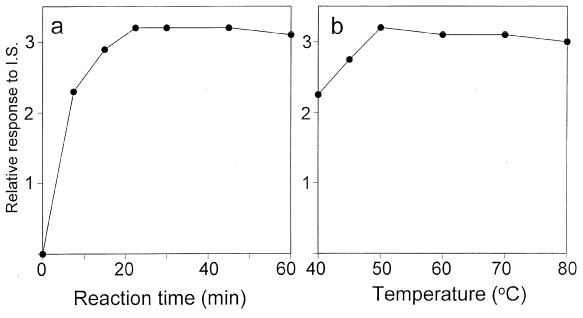

Fig. 4. Optimization studies on the labeling of maltohexaose with 3-ABA. (a) Effect of the reaction time. The reaction temperature was 50°C. (b) Effect of the reaction temperature. The reaction time was 60 min. 3-ABA Mal was used as the internal standard.

3.3. Linearity

Calibration curves obtained for the analysis of 3-ABA-maltose as a model oligosaccharide showed good linearity at least from $1 \cdot 10^{-7}$ M to $6 \cdot 10^{-4}$ M in 50 mM borate buffer (pH 9.5) containing 150 mM SDS. The amount of the injected sample in the lower range of calibration graph was calculated to be a few hundred attomols (10^{-16} mol) with LIF detection. Because a 10 μl solution (10^{-7} M) is enough for introduction of the sample to the capillary, the minimum amount of the required carbohydrate sample is about 10^{-12} mol. However, in an acidic buffer such as 50 mM phosphate buffer (pH 2.5), the detection sensitivity was ten times worse, as described later.

3.4. Application to the analysis of carbohydrate chains

3.4.1. Analysis of dextran hydrolyzates

A fused-silica capillary was used for the analysis of both 3-ABA and 3-AB derivatives. An inner surface-modified capillary with (50% phenyl)methyl polysiloxane was employed for the analysis of 3-AB derivatives. This capillary showed low electroen-

doosmosis and was not appropriate for the separation of 3-ABA derivatives having only positive charge of the imino group.

The results obtained on the analysis of dextran hydrolyzates labeled with 3-ABA or 3-AB using these capillaries are shown in Fig. 5.

Fig. 5a shows the electropherogram obtained in 50 m*M* borate buffer (pH 9.3) containing 150 m*M* SDS with a fused-silica capillary. Larger oligosaccharides were observed earlier, but their separation was not satisfactory. By employing the positive charge of the imino group of the tag as the driving force in the acidic phosphate buffer (pH 2.5) in a fused-silica capillary, smaller oligomers were observed earlier according to their charge to molecular mass ratios, and their resolution was improved, as shown in Fig. 5b. However, the sensitivity became about one-tenth of that under the alkaline conditions in Fig. 5a. Fig. 5c shows the separation of 3-AB derivatives in a fused-silica capillary. The resolution of peaks was improved in a buffer of higher pH (pH 10.5), where the carboxylate ion of the tag contributed to the separation. The use of Tris–borate buffer and a DB-17 capillary was also possible for the separation of 3-AB derivatives, and the results are shown in Fig. 5d. Because the carboxylic acid ion is the driving force in this separation, smaller oligomers were observed earlier and larger oligomers composed of more than 50 monosaccharides could be distinguished. The migration of this oligomeric ladder can be used as markers of the electrophoretic mobilities of unknown carbohydrate chains obtained from glycoconjugates.

3.4.2. Analysis of carbohydrate chains from some glycoproteins

The usefulness of LIF detection is exemplified in Fig. 6, which presents the results of analyses of sialo-oligosaccharides released from glycopeptides derived from fetuin (50 μg as protein) with PNGase F.

Derivatization with 3-ABA or 3-AB is highly reactive and does not require any clean-up procedure to remove the enzyme and buffer salts prior to the labeling reaction. However, photometric detection at 200 nm gave a complex electropherogram probably due to contaminating peptides, as shown in Fig. 6b, and could not confirm carbohydrate peaks. The use of LIF detection greatly improved the selectivity, and the sialo-oligosaccharides labeled with 3-AB, in a DB-17 capillary, could be determined with high sensitivity as shown in Fig. 6a. The electropherogram in this figure was obtained by a 5 s injection of the sample solution by the pressure method, which corresponded to ca. 2 ng of a glycoprotein sample. Confirmation of each peak was performed by comigration with the derivatives of standard samples. In this mode of separation, the separation was mainly achieved based on the charge/molecular mass ratios, and predicted to be similar to that observed in high-performance anion-exchange chromatography. As expected, migration order of each peak was similar to that obtained by Townsend et al. on high-performance anion-exchange chromatography with pulsed amperometric detection [25].

Analysis of high-mannose type oligosaccharides obtained from soybean agglutinin released by two different enzymes gave interesting results, as shown in Fig. 7. These oligosaccharides were labeled with 3-ABA and then analyzed in a fused-silica capillary using 50 m*M* borate buffer (pH 9.3) containing 150 m*M* SDS.

Dorland et al. reported that soybean agglutinin contains a single high-mannose type oligosaccharide, Man9GlcNAc2 [18]. Only one major peak was observed on the analysis of the carbohydrate chains released by PNGase F, as shown in Fig. 7a. However, a major peak accompanied by a few small peaks appeared at earlier migration times for the digestion mixture with *endo*-β-*N*-acetyl glucosaminidase (Fig. 7b). Larger oligosaccharides appeared earlier in this separation mode (see Fig. 5a), hence these minor peaks are probably due to larger oligosaccharides of the high-mannose type. Because this enzyme cleavages the linkage between GlcNAcβ1-4GlcNAc linkage at the reducing terminal, these results suggested that trimming of the chitobiose core to a single *N*-acetylglucosamine residue caused improvement of the resolution of high-mannose type oligosaccharides. The presence of larger oligosaccharides was also observed after the carbohydrate chains released with PNGase F was labeled with 3-AB (data not shown). Fig. 8a shows another example for the analysis of a mixture of high-mannose type oligosaccharides derived from

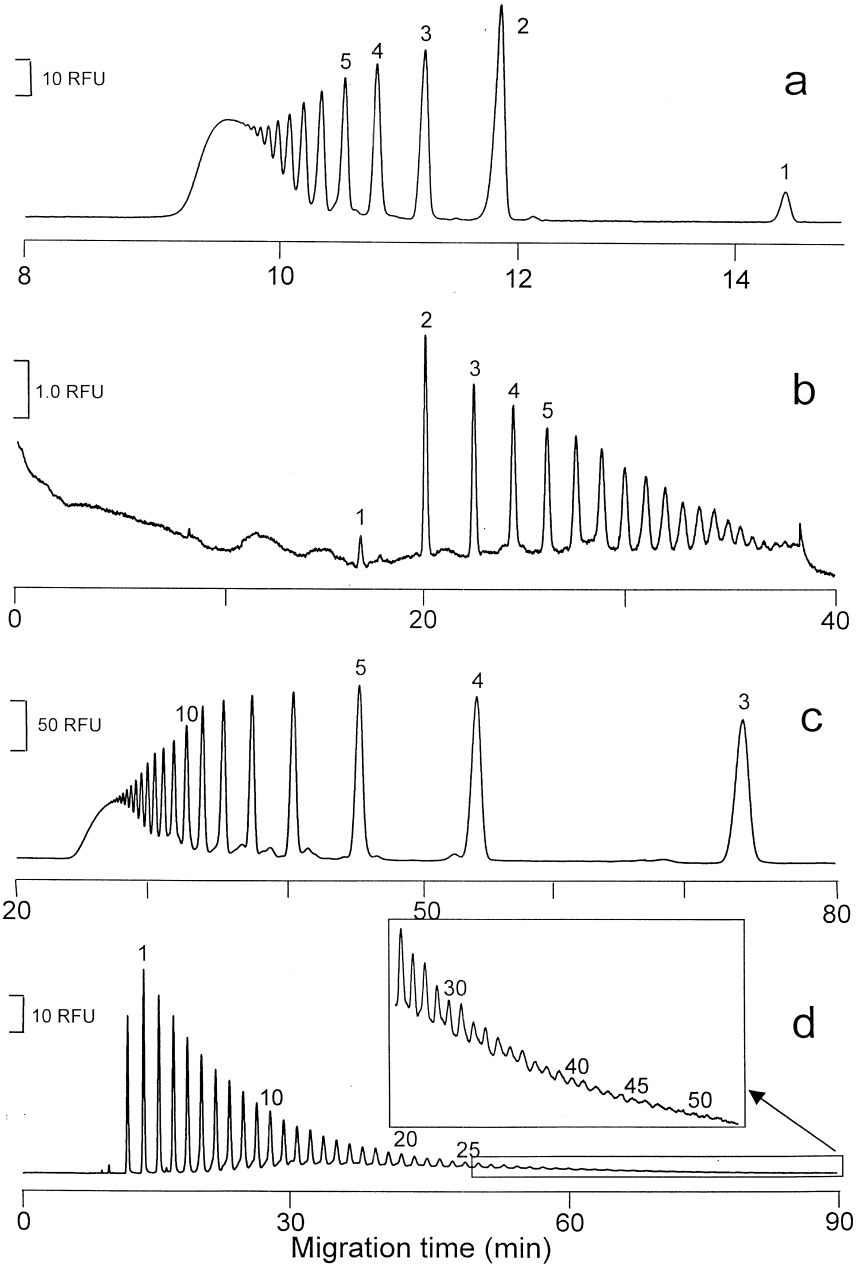

Fig. 5. Analysis of 3-ABA (a and b) and 3-AB (c and d) derivatives of dextran hydrolyzates. Analytical conditions: (a) 50 m*M* borate buffer (pH 9.3) containing 150 m*M* SDS with a fused-silica capillary [57 cm (50 cm effective length)×75 μm I.D.], (b) 50 m*M* phosphate buffer (pH 2.5) with a fused-silica capillary [57 cm (50 cm effective length)×75 μm I.D.]; (c) 200 m*M* borate buffer (pH 10.5) with a fused-silica capillary [57 cm (50 cm effective length)×75 μm I.D.]; (d) 50 m*M* Tris–borate buffer (pH 8.5) containing 10% PEG 70 000 with a DB-17 capillary [27 cm (20 cm effective length)×100 μm I.D.]. Fluorometric detection was performed with a 450 nm light filter with excitation by light through a 325 nm light filter. The numbers on the peaks indicate the degree of polymerization of the oligomers.

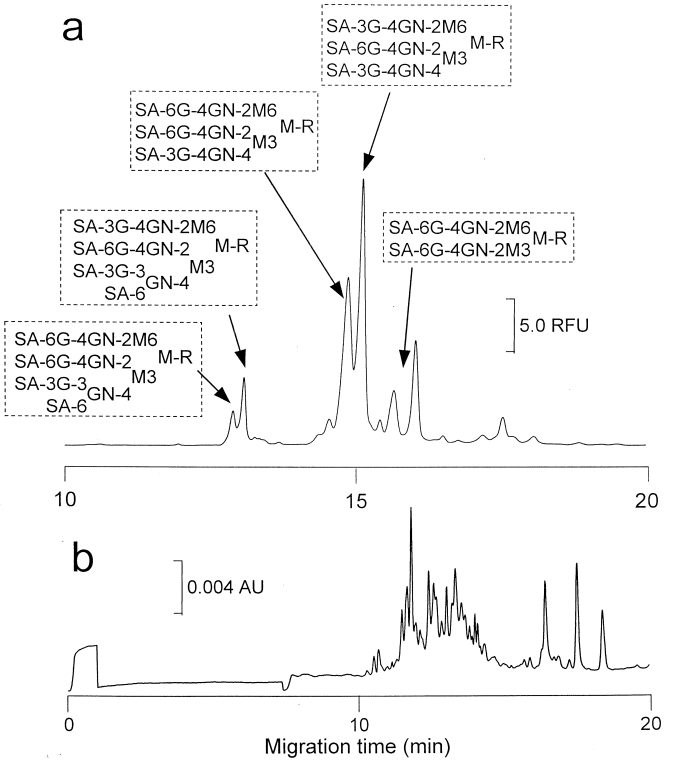

Fig. 6. Analysis of sialooligosaccharides labeled with 3-AB released on digestion of a fetuin glycopeptide mixture with PNGase F. (a) Fluorescence detection was performed as in Fig. 5d. (b) UV absorption detection was performed at 200 nm. Analytical conditions: (a) the same conditions as in Fig. 5d; applied potential, 15 kV; operation temperature, 25°C; injection, pressure method (5 s); (b) the same conditions as in Fig. 5a with a fused-silica capillary [57 cm (50 cm effective length)×50 μm I.D.]; applied potential, 20 kV.

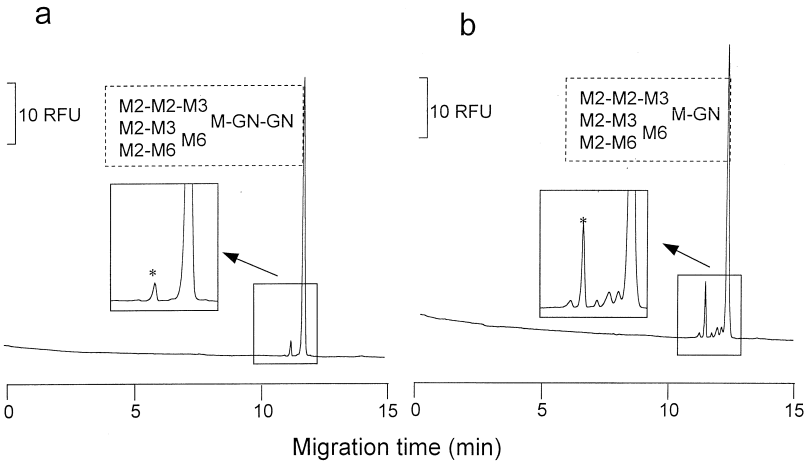

Fig. 7. Analysis of 3-ABA labeled carbohydrate chains released from a glycopeptide derived from soybean agglutinin with (a) PNGase F or (b) *endo-N*-acetyl-β-glucosaminidase. The analytical conditions were the same as in Fig. 5a. The asterisks in both figures indicate a peak due to a contaminant in the reagents.

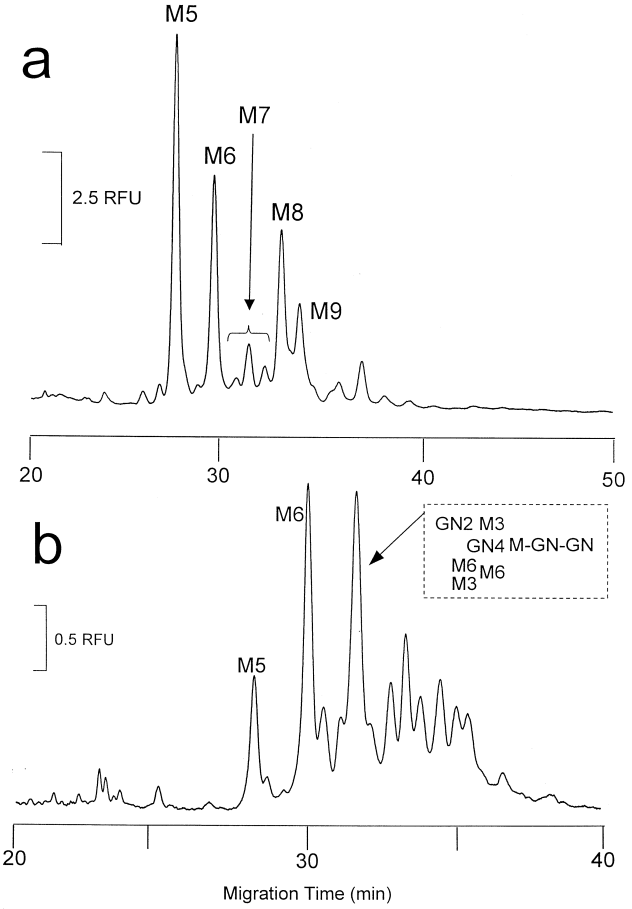

Fig. 8. Analysis of carbohydrate chains derived from ribonuclease B (Fig. 8a) and ovalbumin (Fig. 8b) after labeling with 3-AB. The analytical conditions were the same as in Fig. 5d.

ribonuclease B (bovine pancreas) as their 3-AB derivatives.

With a DB-17 capillary and Tris–borate buffer (pH 8.5) containing 10% PEG 70 000, the resolution of peaks was excellent, although these oligosaccharides retained the chitobiose core. The earliest peak was due to Man5GlcNAc2 (M5) which was confirmed by comigration with the 3-AB derivative of the standard sample obtained from ribonuclease B [24], and the other peaks were those of larger carbohydrate chains of the high mannose-type. It is noted that M7 was resolved into three peaks [24]. Their separation was much better than that reported in the previous paper [6]. Improvement of the resolution among 3-AB labeled carbohydrate chains was easily achieved by controlling the pH of the running buffer, because the 3-AB residue in a carbohydrate chain has both positive and negative charges due to the imino and carboxylic acid ions. The borate ions in Tris–borate buffer also affect the mobility of carbohydrate chains through the formation of borate complexes with the hydroxyl groups of the carbohydrates. Guttman and Pritchett also reported the excellent resolution of high-mannose oligosaccharides derived from ribonuclease B, after labeling with 8-aminopyrene-1,3,6-trisulphonate (APTS) by reductive amination [26]. The present results can be comparable with theirs in the resolution ability of labeled carbohydrate chains. Fig. 8b shows the separation of 3-AB derivatives of a mixture of carbohydrate chains derived from hen ovalbumin under the same analytical conditions as in

Fig. 8a. Two of the high-mannose oligosaccharides found abundantly in ovalbumin were assigned by co-electrophoresis with standard samples obtained from ribonuclease B. The large peak observed after two manno-oligosaccharides was probably due to a hybrid-type oligosaccharide by comparing the report by Takahashi et al. [7]. In the previous paper, we reported capillary electrophoresis of oligosaccharides obtained from ovalbumin after labeling with 2-aminopyridine [6]. By using 3-AB as the labeling reagent, the resolution of the peaks has been much improved.

The results of analyses of sialo- and asialo-oligosaccharides derived from transferrin and fetuin are shown in Fig. 9.

The major peak observed on the analysis of sialooligosaccharides derived from transferrin is due to a biantennary complex-type oligosaccharide (Fig. 9a). After digestion of the labeled mixture with neuraminidase, a large peak was observed at 33 min. With this separation mode involving a DB-17 capillary and Tris–borate buffer, the negative charges due to sialic acids and the carboxylic acid group of 3-AB are the major driving force in electrophoresis. Hence, the biantennary sialooligosaccharide is observed earlier and its asialo-oligosaccharide migrates at a slower velocity. It is important that there are no detectable peaks in Fig. 9a in the region where the asialooligosaccharides are observed [24]. These results indicate that no sialic acids were released in the labeling procedure. Similar results were also obtained on the analysis of sialo- and asialo-oligosaccharides derived from fetuin. No sialic acids were released either in this analysis (Fig. 9c). Although we could not confirm the correlation between individual peaks and the reported structures, the earliest peaks are probably due to tetrasialo-triantennary oligosaccharides, the second peaks to trisialo-triantennary oligosaccharides, and the last peaks to disialo-biantennary oligosaccharides as discussed in Fig. 6a. In the analysis of asialooligosaccharides derived from fetuin (Fig. 9d), the peak observed at 33 min is due to a biantennary asialooligosaccharide, as judged on comparison of the migration time with those on the

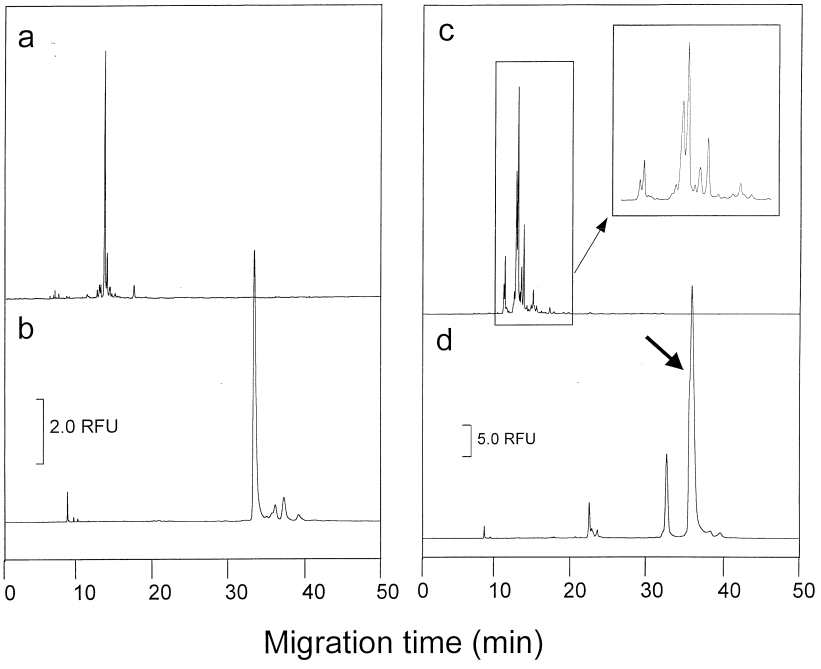

Fig. 9. Analysis of 3-AB labeled sialo- and asialo-oligosaccharides derived from transferrin and fetuin. (a) Sialooligosaccharides derived from transferrin; (b) asialooligosaccharides of the sample in Fig. 9a obtained by digestion with neuraminidase; (c) sialooligosaccharides derived from fetuin; (d) asialooligosaccharides of the sample in Fig. 9c obtained by digestion with neuraminidase. The analytical conditions were the same as in Fig. 5d. The inset in Fig. 9d is an expansion along the *x*-axis.

analysis of transferrin (Fig. 9b and 9d). The largest peak observed at 37 min has a shoulder indicated with bold arrow, which is probably due to an asialo-triantennary oligosaccharide with a Galβ1-3GlcNAc-branch [24].

Fig. 10 shows the results of analysis of carbohydrate chains derived from porcine thyroglobulin after labeling with 3-AB. The separation was also performed using a DB-1 capillary in 50 m*M* Tris–borate buffer (pH 8.5) containing 10% PEG 70 000.

Fig. 10a is an electropherogram of carbohydrate chains released from thyroglobulin glycopeptides with PNGase F. There have been many reports on the analysis of carbohydrate chains of porcine thyroglobulin, that contains both complex-type and high-mannose type carbohydrate chains [27]. To determine which of the peaks are due to high-mannose and complex-type oligosaccharides, the mixture of the labeled oligosaccharides was separated on a small Con A column as described in the Experimental section. Fig. 10b shows results of analysis of the fractions not retained on the Con A column. Kamerling et al. reported the presence of sulfated sialo-oligosaccharides that have no terminal GlcNAc or

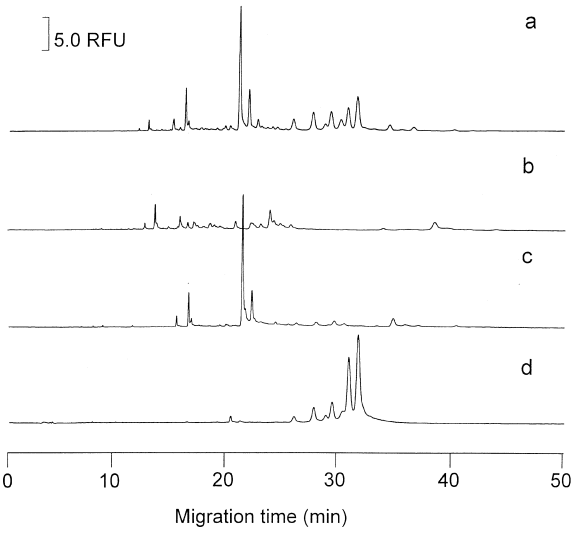

Fig. 10. Analysis of carbohydrate chains derived from porcine thyroglobulin. (a) 3-AB labeled carbohydrate chains. (b) 3-AB labeled carbohydrate chains not retained on a Con A column. (c) 3-AB labeled carbohydrate chains eluted with 10 m*M* Me α-Glc from a Con A column. (d) 3-AB labeled carbohydrate chains eluted with 100 m*M* Me α-Man from a Con A column. The analytical conditions are the same as in Fig. 5d.

Man residues [28]. The total negative charges of the carbohydrate chains bearing both sialic acids and sulfate groups become large and their mobilities will also have large values. Therefore, the small peaks observed from 15 to 20 min are probably due to such highly sialylated or sulfated oligosaccharides. This was confirmed by digestion with neuraminidase or solvolysis in methanol-dimethylsulfoxide of this fraction (data not shown). The results obtained on the analysis of the fractions weakly retained on the Con A column are shown in Fig. 10c. The peaks observed around 22 min may be due to biantennary oligosaccharides not substituted with sulfate groups. The migration times of these peaks were larger than those derived from transferrin (Fig. 9a). Biantennary oligosaccharides derived from thyroglobulin contain a fucose residue and their molecular masses are larger than those derived from transferrin. Therefore, these oligosaccharides were observed later than those observed in Fig. 9a. High-mannose type oligosaccharides were observed in the fractions eluted with 100 m*M* Me α-Man from the Con A column, as shown in Fig. 10d. Large mannooligosaccharides, M8 and M9, observed around 32 min were predominant in this product, but Tsuji et al. reported that M5 was predominant in their thyroglobulin preparation [27].

4. Conclusion

The reaction efficiencies of nine monosubstituted amino benzene derivatives to reducing carbohydrates were compared in competitive mode using equimolar mixtures of the reagents and maltose as a model oligosaccharide. Of the reagents examined in the present study, 3-aminobenzamide showed the highest reactivity. However, 2-aminobenzamide was the most intensely fluorescent and 4-aminobenzoic acid ethyl ester was the most effectively chromogenic. Therefore, we would like to propose that 3-aminobenzamide is the first choice for the analysis of an unknown mixture of carbohydrate chains. The present method seems especially useful for the analysis of acid-labile carbohydrate chains such as those of sialic acid-containing oligosaccharides. The weakness in sensitivity is overcome by employing an He–Cd laser-induced fluorometric detector. An anal-

ogous compound, 3-aminobenzoic acid, showed similar reactivity to 3-aminobenzamide in the labeling reaction and the resolution of the carbohydrate chains labeled with this reagent was excellent. The present labeling method involving both reagents is also useful for analysis by high-performance liquid chromatography, as will be reported elsewhere.

References

[1] A. Varki, Glycobiology 3 (1993) 97–130.
[2] M.C. Borys, D.I. Linzer, E.T. Papoutsakis, Biotechnol. 11 (1993) 720–724.
[3] Z. El Rassi, Y. Mechref, Electrophoresis 17 (1996) 275–301.
[4] S. Hase, Glycoprotein analysis in biomedicine, in: E.F. Hounsell (Ed.), Methods in Molecular Biology, Vol. 14, Humana Press, Totowa, 1993, pp. 55–68.
[5] P. Jackson, in: P. Jackson (Ed.), BioMethods: A Laboratory Guide to Glycoconjugate Analysis, Vol. 9, Birkhäuser Verlag, Basel, 1997, pp. 113–139.
[6] S. Suzuki, K. Kakehi, S. Honda, Anal. Biochem. 205 (1992) 227–236.
[7] N. Takahashi, Y. Wada, J. Awaya, M. Kurono, N. Tomiya, Anal. Biochem. 208 (1993) 96–109.
[8] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Anal. Biochem. 230 (1995) 229–238.
[9] F. Matsuura, M. Ohta, Y.A. Ioannou, R.J. Desnick, Glycobiology 8 (1998) 329–341.
[10] J. Plocek, M.V. Novotny, J. Chromatogr. A 357 (1997) 215–223.
[11] P. Jackson, Biochem. J. 270 (1990) 705–713.
[12] C. Chiesa, Cs. Horváth, J. Chromatogr. 645 (1993) 337–352.
[13] M. Stefansson, M.V. Novotny, Anal. Chem. 66 (1994) 3466–3471.
[14] F.T. Chen, R.A. Evangelista, Anal. Biochem. 230 (1995) 273–280.
[15] K.B. Lee, A. Al-Hakim, D. Logananthan, R.J. Linhardt, Carbohydr. Res. 214 (1991) 155–168.
[16] R.A. Keckwick, R.K. Cannan, J. Biochem. 30 (1936) 227–235.
[17] N. Ui, O. Tarutani, J. Biochem. (Tokyo) 50 (1961) 508–518.
[18] L. Dorland, H.V. Halbeek, J.F.G. Vliegenthardt, H. Lis, N. Sharon, J. Biol. Chem. 256 (1981) 7708–7711.
[19] G. Okafo, J. Langridge, S. North, A. Organ, A. West, M. Morris, P. Camilleri, Anal. Chem. 69 (1997) 4985–4993.
[20] K. Kakehi, A. Hirose, T. Tamai, A. Taga, S. Honda, Anal. Sci. 12 (1996) 171–176.
[21] Y. Ito, N. Seno, I. Matsumoto, J. Biochem. 97 (1985) 1689–1694.
[22] W.T. Wang, N.C. LeDonne Jr., B. Ackerman, C.C. Sweeley, Anal. Biochem. 141 (1984) 366–381.
[23] R.J. Linhardt, A. Pervin, J. Chromatogr. A 720 (1996) 323–335.
[24] K. Kakehi, S. Suzuki, S. Honda, Y.C. Lee, Anal. Biochem. 199 (1991) 256–268.
[25] R.R. Townsend, M.M. Hardy, D.A. Cumming, J.P. Carver, B. Bendiak, Anal. Biochem. 182 (1989) 1–8.
[26] A. Guttman, T. Pritchett, Electrophoresis 16 (1995) 1906–1911.
[27] T. Tsuji, K. Yamamoto, T. Irimura, T. Osawa, J. Biochem. 195 (1981) 691–699.
[28] J.P. Kamerling, I. Rijkse, A.A. Maas, J.A. van Kuik, J.F.G. Vliegenthardt, FEBS Lett. 241 (1988) 246–250.