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Journal of Chromatography A, 1005 (2003) 13–21
JOURNAL OF CHROMATOGRAPHY A
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Sample clean-up method for analysis of complex-type *N*-glycans released from glycopeptides

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Received 20 February 2003; received in revised form 15 May 2003; accepted 20 May 2003

Abstract

N-Glycans in glycoprotein can be liberated either from glycoproteins or from their glycopeptides with glycoamidases. The latter approach is preferable, because it requires a smaller amount of the enzyme, and yields *N*-glycans in excellent yields. Moreover it alleviates the necessity of removing from the reaction mixture the detergents needed to denature the glycoproteins. On the other hand, this approach necessitates removal of interfering peptidic materials, because some of the peptide peaks often overlap with the peaks of carbohydrate chains in high-performance anion-exchange chromatography (HPAEC). These peptidic materials also hinder labeling of *N*-glycans by reductive amination. We have tried to remove the interfering peptidic materials by several different methods—octadecyl (C_{18}) silica cartridge, cation-exchange resin column, and graphitized carbon cartridge. Unfortunately, none of these could completely remove the interfering peptidic materials. Therefore, we resorted to modify the amino groups of the peptidic materials with sodium 2,4,6-trinitro-benzene-1-sulfonate (TNBS) to render them more hydrophobic, so that they can be retained more strongly on the C_{18} or graphitized carbon cartridges. In the model study presented here, we were able to obtain *N*-glycans for HPAEC analyses without any interfering materials by a combination of TNBS reaction and graphitized carbon treatment.

Keywords: Graphitized carbon; Derivatization, LC; Glycans; Glycoamidases; Enzymes; Peptides; Oligosaccharides; Sodium trinitrobenzenesulfonate

1. Introduction

To release *N*-glycans in glycoproteins, either the whole glycoprotein or the glycopeptide thereof can be used as starting material. In the first approach, the whole glycoproteins are usually digested with a large excess of glycoamidase F (GAF, also known as PNGAse F) in the presence of detergents such as sodium dodecyl sulfate (SDS), Triton X-100 or NP-40. We used this strategy to analyze carbohydrate chains in α_1-acid glycoprotein glycoforms collected by capillary electrophoresis [1]. Another example of this approach [2] denatured fetuin by heating at 100 °C in phosphate buffer containing SDS and dithiothreitol, followed by digestion with GAF in the presence of NP-40 to release *N*-glycans. Although it is possible to obtain high quality *N*-glycans by this approach, the yields are usually not very high. Moreover, detergents must be removed from the

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00926-9

sample before high-performance liquid chromatography (HPLC) analysis.

In contrast, glycopeptides can be digested with a much smaller amount of GAF to obtain *N*-glycans in high yields. No or little detergents are required for proteolysis (e.g., with trypsin and chymotrypsin) to generate glycopeptides. However, a serious problem in the latter approach is to completely remove peptides and residual glycopeptides which interfere with analysis of *N*-glycans, either by high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC–PAD) or after pre-column labeling (e.g., by reductive amination with 2-aminopyridine) [3]. To overcome this problem, we have developed a method for removal of interfering substances before analysis of *N*-glycan.

2. Experimental

2.1. Materials

Bovine fetuin was purchased from GIBCO-BRL (Gaithersburg, MD, USA). Glycoamidase F (peptide-*N*-glycosidase F) was from Prozyme (San Leandro, CA, USA). Trypsin was from Worthington (Freehold, NJ, USA). Chymotrypsin was from Sigma (St. Louis, MO, USA). Dowex 50-X2 (50–100 mesh) was from Supelco (Bellefonte, PA, USA). The Sep-Pak C_{18} cartridge was from Waters (Milford, MA, USA). The graphitized carbon cartridge was from Alltech (Deerfield, IL, USA). Sodium 2,4,6-trinitrobenzene-1-sulfonate (TBNS) was from Tokyo Kasei (Tokyo, Japan). *N*,*N*-Bis-(2-hydroxyethyl)glycine (bicine) was from Research Organics (Cleveland, OH, USA). Tris(hydroxymethyl)-aminomethane (Tris) was from Aldrich (Milwaukee, WI, USA). Pectin-derived α(1–4)-galacturonic acid trimer $(GalA)_3$ used as internal standard for analysis by HPAEC was prepared as described [4]. Microtiter plates (96-well flat bottom) used for analysis in the TNBS reaction were from Fisher Scientific (Pittsburg, PA, USA). A microplate reader (model Benchmark) was from Bio-Rad Labs. (Hercules, CA, USA).

Sep-Pak C_{18} (100 mg) cartridges were activated with acetonitrile (2 ml) and equilibrated with 0.05% trifluoroacetic acid (TFA) (4 ml). Sep-Pak C_{18} (1 g) cartridges were activated with acetonitrile (6 ml) and equilibrated with 0.05% TFA (12 ml). Graphitized carbon (25 mg) cartridges were activated with 80% acetonitrile containing 0.1% TFA (1 ml) and equilibrated with 0.05% TFA (2 ml). Graphitized carbon (150 mg) cartridges were activated with 80% acetonitrile containing 0.1% TFA (3 ml) and equilibrated with water (6 ml).

2.2. Analytical instrumentation

2.2.1. High performance anion-exchange chromatography

HPAEC analysis was carried out at ambient temperature on a DX-600 system (Dionex, Sunnyvale, CA, USA) using a CarboPac PA-100 column (250× 4 mm) with a guard column (50×4 mm) and a PAD system from Dionex. The elution for sialyl oligosaccharides was performed with segmented linear gradients: 0–10 min, 5–50 m*M* CH_3COONa in 100 m*M* NaOH; 10–50 min, 50–225 m*M* CH_3COONa in 100 m*M* NaOH; 50–60 min, 225–275 m*M* CH_3COONa in 100 m*M* NaOH; and then stepping up to 500 m*M* CH_3COONa in 100 m*M* NaOH which was kept for 10 min (to cleanse the column) and back to 5 m*M* CH_3COONa in 100 m*M* NaOH for 15 min (to re-equilibrate) [5]. The injection volume was typically 20 μl and the flow-rate was 0.8 ml/min.

2.2.2. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS experiments were carried out on a Voyager DE PRO system (PE Biosystems, Framingham, MA, USA) according to the method reported previously [1]. A nitrogen laser was used to irradiate (337 nm) samples, and an average of 50 shots was taken. The instrument was operated in a linear mode using positive polarity, using an accelerating voltage of 20 kV. The sample solution (0.5 μl) was applied to a polished stainless steel target, to which was added a solution (0.5 μl) of 1% 2,5-dihydroxybenzoic acid (DHB) in a solution of methanol–water (1:1). The mixture was dried at room temperature in atmosphere before MS measurement.

2.3. Sample preparation

2.3.1. Preparation of trypsin–chymotrypsin digestion mixture of bovine fetuin (t/c-fetuin)

Fetuin (10 mg) was dissolved in 500 μl of a 20 m*M* TBS [Tris buffer (pH 8.0) with 150 m*M* NaCl] or a 20 m*M* bicine buffer (pH 8.0), and kept in a boiling water bath for 10 min for denaturation. Trypsin and chymotrypsin (100 μg each) were added, and the mixture was kept at 37 °C overnight, and boiled in a water bath for 10 min. This digest is designated ''t/c-fetuin'' hereafter.

2.3.2. Direct release of N-glycans from fetuin (fetuin N-glycans)

Fetuin (10 mg) in 500 μl of 20 m*M* bicine (pH 8.0) was heated in a boiling water bath for 10 min for denaturation. After cooling, the denatured fetuin solution was mixed with GAF (20 U in 4 μl), and incubated at 37 °C overnight, then boiled in a water bath for 10 min. Finally, the solution was made to 78% (v/v) by adding the appropriate volume of 95% ethanol, and the mixture was kept at 4 °C for 1 h to precipitate proteins. The mixture was centrifuged at 10 000 rpm, 4 °C, and a portion of the supernatant (containing *N*-glycans) was evaporated in small vials. This sample is designated ''fetuin *N*-glycans''. To each of the vials (each containing 0.16 mg fetuin equivalent) was added 100 μl of 50 μ*M* of $(GalA)_3$ as internal standard (I.S.), and a portion (20 μl) of the mixture was analyzed by HPAEC.

2.4. Sample clean-up method using solid-phase extraction (SPE) cartridges

2.4.1. Sample clean-up with a cation-exchange column

In order to test the efficiency of removal of the contaminating peptides with a cation-exchange column, time course of the release of *N*-glycans from t/c-fetuin with GAF was followed over 15 h. The samples containing t/c-fetuin (100 μl) and GAF (2U in 0.4 μl) were prepared and each mixture was incubated at 37 °C for specified time periods (a sample without addition of GAF was used as a 0-h sample). After each sample was boiled in a water bath for 10 min, an aqueous solution (100 μl) of 1 m*M* $(GalA)_3$ as internal standard was added, and the mixture was passed through a column (1 cm× 1 cm I.D.) of Dowex 50 (500 mg). The column was washed with water (5 ml), and the effluent was neutralized immediately with 3 ml of 100 m*M* ammonium hydrogencarbonate to prevent possible desialylation of glycans, and was evaporated to dryness. The residue was dissolved with 400 μl of water, and a portion (20 μl) was used for HPAEC analysis.

2.4.2. Sample clean-up with an octadecyl silica cartridge

Sep-Pak C_{18} cartridges were tested for removal of the interfering peptides. To a dried sample of fetuin *N*-glycans (prepared as described above, equivalent to 0.5 mg of fetuin) was added 25 μl of t/c-fetuin solution (corresponding to 0.5 mg of fetuin). The mixture was applied to a Sep-Pak C_{18} (100 mg) cartridge, and the cartridge was eluted with 2 ml of 0.05% TFA and then with 2 ml of 25% acetonitrile containing 0.05% TFA. Both eluates were individually evaporated to dryness, and dissolved in 300 μl of 50 μ*M* $(GalA)_3$, from which a 20-μl portion was analyzed by HPAEC.

2.4.3. Sample clean-up with a graphitized carbon cartridge

We treated two different samples with graphitized carbon cartridges according to the method by Packer et al. [2].

Sample 1: A t/c-fetuin sample (100 μl, equivalent to 2.0 mg fetuin) was mixed with GAF (2 U in 0.4 μl) and incubated at 37 °C overnight. A portion (10 μl) of the mixture was diluted 10-fold with water. Sample 2: This was prepared as above but without the GAF digestion.

A diluted solution (100 μl) from each of these two samples was applied to a graphitized carbon (150 mg) cartridge. The cartridge was washed with water (2 ml), eluted with 25% acetonitrile (2 ml) followed by 25% acetonitrile containing 0.05% TFA (2 ml). Each eluate was evaporated, and dissolved in

200 μl of 60 μ*M* of $(GalA)_3$ and a portion (20 μl) thereof was analyzed by HPAEC.

2.5. TNBS reaction followed by graphitized carbon treatment

To a dried sample of fetuin *N*-glycans (equivalent to 1.0 mg of fetuin) was added 50 μl of t/c-fetuin solution (corresponding to 1.0 mg of fetuin). After boiling the mixture, it was mixed with water (250 μl), 0.25 *M* $NaHCO_3$ (300 μl), and 0.5% TNBS (300 μl), and the mixture was kept at 55 °C for 30 min. To the mixture was added 100 μl of 0.3 *M* NH_4HCO_3, and the mixture was further kept at 55 °C for 30 min. The TNBS-reacted sample was applied to a graphitized carbon (150 mg) cartridge, and the cartridge was eluted with water (2 ml) followed by 25% acetonitrile containing 0.05% TFA (2 ml). The eluate with the latter solvent was evaporated to dryness, and dissolved in 500 μl of 50 μ*M* of $(GalA)_3$, and a portion (20 μl) thereof was used for HPAEC analysis.

2.6. Proposed procedure for the analysis of N-glycans of a fetuin sample after derivatization of contaminating peptides with TNBS (TNBS-graphitized carbon method)

Fetuin (1.0 mg) in 50 μl of 20 m*M* bicine buffer (pH 8.0) was boiled in a water bath for 10 min. Trypsin and chymotrypsin (10 μg each) were added, and the mixture was kept at 37 °C overnight. After boiling the mixture for 10 min, GAF (1 U in 0.25 μl) was added, and the mixture was incubated at 37 °C overnight to release *N*-glycans. After boiling the mixture for 10 min, a portion (8 μl) thereof was mixed with water (37 μl), 0.25 *M* $NaHCO_3$ (45 μl),and 0.5% TNBS (45 μl), and the mixture was kept at 55 °C for 30 min. To the mixture was added 15 μl of 0.3 *M* NH_4HCO_3, and the mixture was kept at 55 °C for 30 min. The mixture was applied to a graphitized carbon (25 mg) cartridge. The cartridge was washed with 0.5 ml of 0.05% TFA to remove salts (ammonium hydrogencarbonate, carbonate and sulfite), and the *N*-glycans were eluted with 0.5 ml of 25% acetonitrile containing 0.05% TFA. The eluate was evaporated to dryness, and dissolved in 100 μl of 50 μ*M* of $(GalA)_3$, and a portion (20 μl) from it was analyzed by HPAEC.

2.7. Linearity of HPAEC analysis using the TNBS-graphitized carbon method

Linearity of HPAEC analysis of *N*-glycans after the proposed clean-up procedure was examined. To each of the vials containing different amounts of fetuin *N*-glycans (equivalent to 0, 0.04, 0.08, 0.15, 0.3 and 0.6 mg of fetuin) were added 8 μl of the t/c-fetuin solution (equivalent to 0.16 mg fetuin) and water (37 μl). TNBS reaction was performed to remove the contaminating peptidic materials, and then excess TNBS was decomposed with ammonium hydrogencarbonate. Each of the mixtures was treated with a graphitized carbon cartridge, and analyzed by HPAEC.

2.8. Repeatability

Repeatability of HPAEC analysis of *N*-glycans after TNBS-graphitized carbon method was examined. Six samples of a mixture of t/c-fetuin and fetuin *N*-glycans (both equivalent to 0.16 mg of fetuin) were reacted with TNBS, followed by treatment of the reaction mixture with ammonium hydrogencarbonate, and passed through a graphitized carbon cartridge, and the carbohydrate chains were analyzed by HPAEC.

2.9. Efficacy of removal of peptides using the TNBS-graphitized carbon method for the determination of N-glycans released from fetuin by MALDI-TOF-MS

Two samples containing *N*-glycans released from fetuin were prepared for analysis by MALDI-TOF-MS. The one was obtained from t/c-fetuin according to the proposed method. The other was the digest (8 μl) of t/c-fetuin (in 20 m*M* bicine buffer, pH 8.0) with GAF but not treated with a graphitized carbon cartridge. A portion (0.5 μl) was analyzed by MALDI-TOF-MS.

3. Results

3.1. Analyses of N-glycans released from fetuin and those from t/c-fetuin

We prepared *N*-glycans from fetuin and t/c-fetuin according to the method described in the Experimental section. As shown in Fig. 1a, *N*-glycans from fetuin showed all di-, tri- and tetra-sialo carbohydrate chains definitively.

The results are in good agreement with those reported previously [2,6,7].

Recoveries of *N*-glycans were determined based on the monosaccharide composition [8], and estimated to be 70–80%. On the contrary, *N*-glycans released from t/c-fetuin without the clean-up procedure overlap with many unidentified peaks probably due to peptides (Fig. 1b).

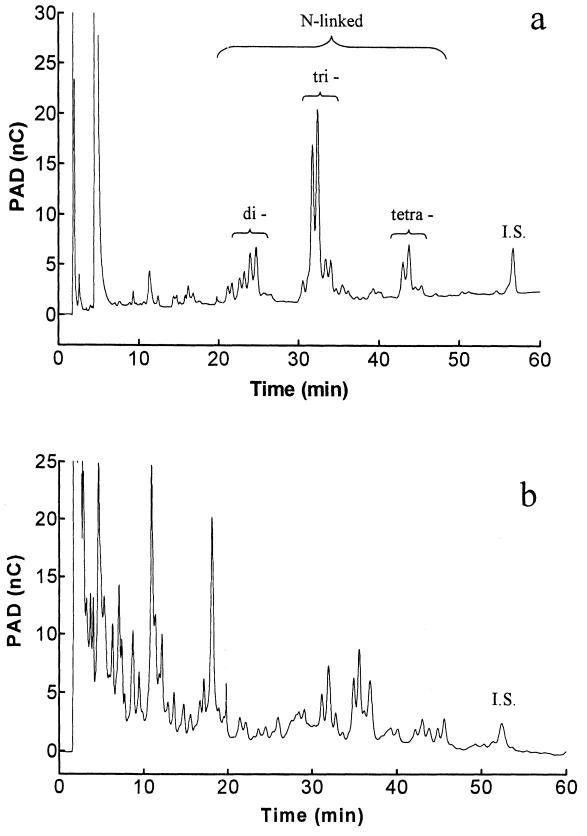

Fig. 1. HPAEC analyses of *N*-glycans released from fetuin (a) or t/c-fetuin (b). Procedures for preparing *N*-glycans from fetuin or t/c-fetuin and the conditions for analysis of *N*-glycans are described in the Experimental section.

3.2. Sample clean-up method using SPE cartridges

3.2.1. Sample clean-up with a cation-exchange column

We reasoned that a strong and porous cation-exchange resin such as Dowex 50-X2 can capture most peptidic materials via their amino groups, while free glycans would not be adsorbed. Time course of release of *N*-glycans from t/c-fetuin with GAF was followed over 15 h. After enzymic reaction for specified periods, each reaction mixture was applied to a Dowex 50 column, and analyzed by HPAEC. The results are shown in Fig. 2.

Although it was clear that the *N*-glycan peak areas (indicated by arrows) increased as a function of incubation time, there were large overlapping peaks around 32 and 42 min. These interfering peaks made accurate quantification of *N*-glycans difficult. Even when the samples were treated with Dowex 50 again, the contaminant peaks could not be removed (data not shown).

3.2.2. Sample clean-up with an octadecyl silica cartridge

We examined a clean-up method using Sep-Pak C_{18} cartridge to remove the interfering peptides. *N*-Glycans were observed in all fractions with inter-

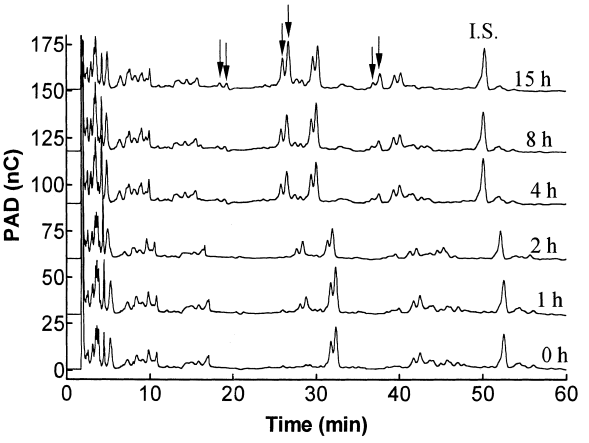

Fig. 2. Time–course of *N*-glycan release from t/c-fetuin. The samples digested with GAF were analyzed after passing through a cation-exchange cartridge.

fering peptidic materials, even when as much as 1 g of C_{18} packing material was used (data not shown). This means that *N*-glycans were not efficiently collected using Sep-Pak C_{18} cartridges. Therefore, we did not pursue testing of the Sep-Pak C_{18} cartridge for this purpose.

3.2.3. Sample clean-up with a graphitized carbon cartridge

After digestion of t/c-fetuin with GAF (sample 1 in the Experimental section), the reaction mixture was passed through a graphitized carbon cartridge. The results are shown in Fig. 3. The glycans were eluted with 25% acetonitrile containing 0.05% TFA (Fig. 3a), but not eluted with 25% acetonitrile without TFA (Fig. 3c). Result on the reference sample (sample 2) without GAF digestion is shown in Fig. 3b and d.

From these results, we concluded that *N*-glycans could be collected in the fractions eluted with 25% acetonitrile containing 0.05% TFA after removing contaminating peptides by the TNBS labeling.

3.3. Sample clean-up with SPE cartridges after TNBS derivatization of existing peptides

TNBS was developed for determination of amino

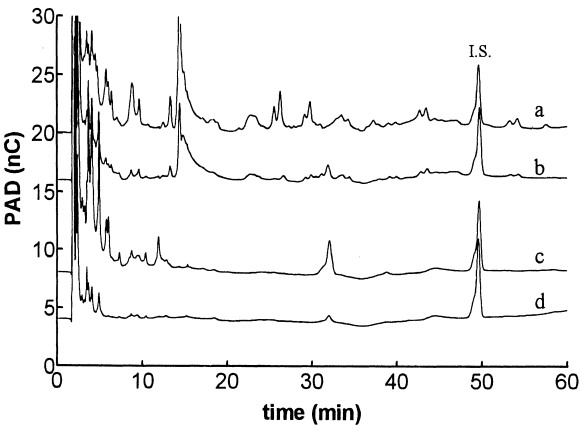

Fig. 3. Analysis of *N*-glycans released from t/c-fetuin after digestion with GAF. The digestion mixture was applied to a graphitized carbon cartridge, and eluted with an aqueous 25% acetonitrile (c), and then eluted with the same solvent containing 0.05% TFA (a). t/c-fetuin without digestion with GAF was also passed through a graphitized carbon cartridge with the same combination of the solvent; (d) with an aqueous acetonitrile and (b) with the same solvent containing 0.05% TFA.

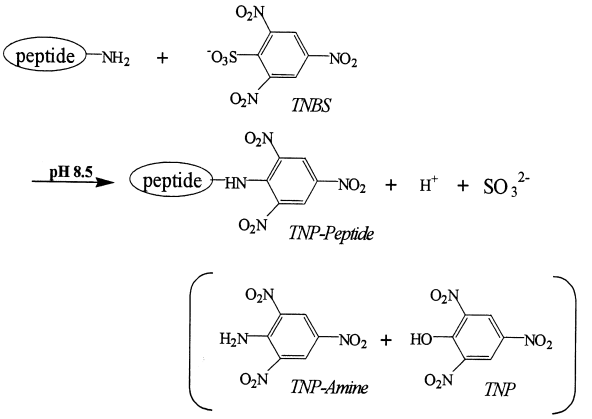

Fig. 4. Reaction scheme of TNBS with amino compounds.

compounds and is in wide use [9–11]. The reaction scheme between TNBS and amino compounds is shown in Fig. 4.

TNBS modifies amino groups of peptidic materials with trinitrophenyl group. The derivatized peptides consequently become more hydrophobic, and are likely to be retained on the graphitized carbon cartridge more strongly, while the *N*-glycans should remain unaffected by this treatment. However, it should be noted that the sulfite ion (SO_3^{2-}) is also formed during the course of derivatization of peptides. Trinitrophenyl (TNP) peptides manifest absorption maxima of 350 and 420 nm, but TNP-NH_2 shows maxima of 330 and 420 nm. In contrast, TNBS reagent shows absorption in the 300–400 nm range, but hardly any absorption above 400 nm. Therefore the total TNBS derivatives (TNP-NH_2 and TNP-peptide) can be assessed by $A_{415\ nm}$ to find the optimum quantity required for TNP derivatization (shown in Fig. 5). $A_{415\ nm}$ of the TNBS derivatives reached a plateau at 0.5% TNBS.

We also searched for the most suitable buffer for digestion of fetuin with trypsin, chymotrypsin and GAF, which does not introduce additional complication during TNBS reaction (Tris has a primary amino group, and does react with TNBS). We selected bicine that has tertiary amino groups, and impervious to the TNBS reaction.

3.3.1. TNBS reaction followed by graphitized carbon treatment

Although most interfering peaks due to peptidic materials could be nearly completely removed by

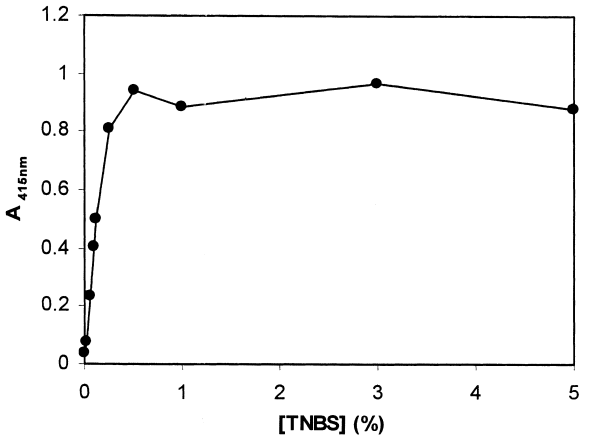

Fig. 5. Optimization for TNP derivatization of t/c-fetuin. The amount of t/c-fetuin was 0.16 mg.

this treatment, the presence of a negative peak of sulfite during HPAEC (data not shown) is undesirable. After TNBS reaction, the mixture still contains a large quantity of unreacted TNBS, in addition to TNP-peptides, sulfite, and some decomposition products from TNBS. Although sulfite generated during the intended labeling of peptidic materials can be removed with the water wash from the graphitized carbon cartridge, the excess TNBS in the reaction mixture eluted with the free glycans rapidly decomposes when exposed to the strongly alkaline eluent of HPAEC, generating sulfite which results in a negative peak. Therefore, the excess reagent was transformed into TNP-amine by addition of ammonium hydrogencarbonate prior to application to the graphitized carbon cartridge.

3.4. Optimization of graphitized carbon quantity for clean-up of N-glycan samples

In order to determine the minimum quantity of graphitized carbon required for clean-up of a sample of 0.16 mg t/c-fetuin, graphitized carbon cartridges packed with 25 mg and 150 mg of graphitized carbon were compared. There was no difference between these two cases in the recovery of *N*-glycans, nor in the removal of the interfering materials. We also examined capacity of graphitized carbon (25 mg) cartridge. After passage of 200 μl of the reaction mixture (equivalent to ca. 0.2 mg of fetuin), the TNBS-related compounds were no longer effectively adsorbed by the cartridge. The mixture after the TNBS reaction gave $A_{340\ nm}$ of 2.48 on the microtiter plate reader (100 μl/well), but after passing through the graphitized carbon cartridge, $A_{340\ nm}$ of the eluate with 25% acetonitrile with 0.05% TFA was 0.005 (after proper correction for volume change), indicating that only about 0.2% of the TNBS used was eluted, which was negligible.

3.5. Linearity of analyses

A typical chromatogram for the analysis of *N*-glycans is shown in Fig. 6 after mixing fetuin *N*-glycans followed by clean-up using the proposed procedure described in the Experimental section.

All the *N*-glycan chains derived from di-, tri- and tetra-sialo carbohydrate chains were clearly observed, and the data were comparable to those in Fig. 1a.

All *N*-glycan peaks observed by HPAEC analysis showed good linearity at least from 10 to 1000 μg as fetuin. Table 1 shows the data on linearity for observed peaks. As indicated in the previous paper [8], it should be noticed that the releasing reaction of carbohydrate chains is about 70–80%, although the recoveries of each *N*-glycan after TNBS reaction and graphitized carbon cartridge treatment were 92–100%.

3.6. Repeatability

Repeatability in the analysis of *N*-glycans was also

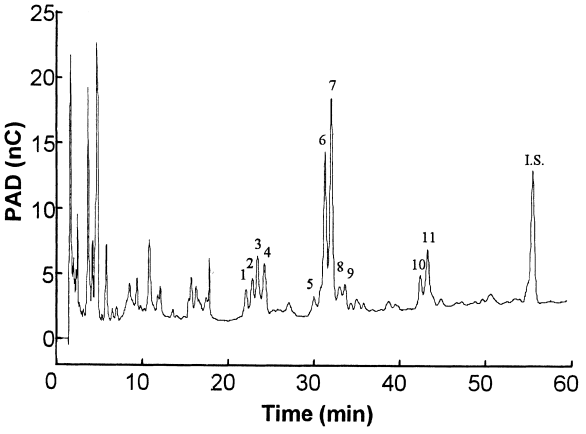

Fig. 6. Analysis of *N*-glycans for calibration studies (equivalent to 150 μg as fetuin).

Table 1
Linearity for calibration of *N*-glycans

Peak No.	Regression equation	Correlation coefficient
1	$y=0.6637x-0.0034$	0.999
2	$y=0.6837x-0.0076$	0.998
3	$y=1.5012x-0.0099$	0.998
4	$y=1.0943x+0.0030$	0.999
5	$y=0.2975x+0.0008$	0.999
6	$y=2.6705x+0.0458$	0.999
7	$y=2.7247x+0.0602$	0.999
8	$y=0.4099x+0.0233$	0.995
9	$y=0.3358x+0.0103$	0.999
10	$y=0.2627x+0.0094$	0.998
11	$y=0.3973x+0.0336$	0.999

The peak numbers are those shown in Fig. 6. The regression equations were calculated using the relative peak intensities to that of the I.S.

examined. The relative standard deviation (RSD) showed the values within 7.5–10.8%. However, peaks 8 and 9 in Fig. 6 gave RSD values of 32.9 and 33.6%, respectively. The reason for poor repeatability for peaks 8 and 9 is because these peaks were very small, and the peak areas could not be accurately measured.

3.7. MALDI-TOF-MS of N-glycans in t/c-fetuin

The sample containing *N*-glycans from t/c-fetuin was prepared for MALDI-TOF-MS analysis according to the optimized TNBS-graphitized carbon method. The reference sample without the TNBS-graphitized carbon treatment was also prepared. The results are shown in Fig. 7. We could clearly observe peaks attributable to *N*-glycans from fetuin by MALDI-TOF-MS using the TNBS-graphitized carbon method (Fig. 7b). On the contrary, the reference sample without the clean-up procedure did not show molecular ions of *N*-glycans (Fig. 7a). This is probably due to the easier ionization of the peptidic materials compared to the *N*-glycans.

Peaks observed around m/z 2000 are due to diantennary carbohydrate chains. Peak around m/z 2600 were due to disialo-triantennary carbohydrate chains, and probably are fragmentation products of trisialo-triantennary carbohydrate chains observed around m/z 2900 [12]. Interestingly, we could confirm tetrasialo-triantennary carbohydrate chains around m/z 3180, although in low abundance.

4. Discussion

Although enzymatic release of *N*-glycans from glycopeptides is more effective than from glycoprotein, it is necessary to remove peptides, because they interfere with the analysis, especially when HPAEC is used. We examined possibilities of removing such impurities, using cation-exchange resin, octadecyl silica, or graphitized carbon. Of these, only a combination of TNP modification of amino groups of the

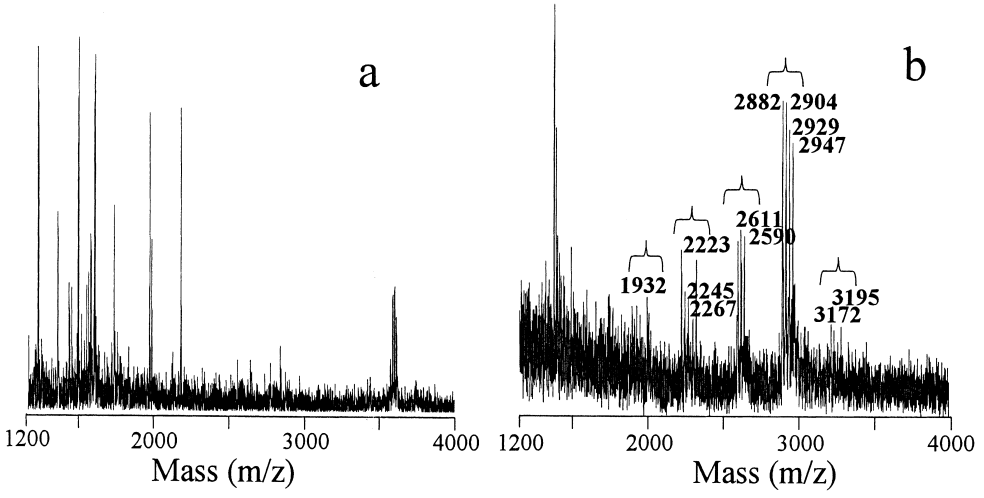

Fig. 7. MALDI-TOF-MS of *N*-glycans released from t/c-fetuin. (a) Without removal of peptides. (b) After removal of peptides using the TNBS-graphitized carbon method.

peptidic materials followed by treatment with graphitized carbon yielded satisfactory results. Originally, a problem encountered in the TNBS method, which was a negative peak due to sulfite ion generated from the excess TNBS as the sample is exposed to high pH. However, deliberate decomposition of the remaining TNBS with ammonium hydrogencarbonate prior to the graphitized carbon treatment solved this problem satisfactorily. The additional advantages of the TNBS approach are: (a) salts are also removed, thus making HPAEC analysis less troublesome; (b) in contrast to the cation-exchange method, which requires immediate neutralization of the effluent, the graphitized carbon treatment does not require neutralization. It is clear that the TNBS-graphitized carbon method is useful as a pretreatment of *N*-glycan samples to be analyzed with HPAEC–PAD method, and most likely with other methods. Other amino-modifying reagents such as fluorescamine [13] presumably may be equally effective, but was not investigated. Furthermore, we showed that a minimum amount of *N*-glycans [80 pmol (4 μg) as fetuin] could be detected by MALDI-TOF-MS after using the clean-up method thus improving de facto sensitivity of analyses.

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