Capillary electrophoresis-electrospray ionization mass spectrometry for rapid and sensitive N-glycan analysis of glycoproteins as 9-fluorenylmethyl derivatives

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It is well known that most protein therapeutics such as monoclonal antibody pharmaceuticals and other biopharmaceuticals including cancer biomarkers are glycoproteins, and thus the development of high-throughput and sensitive analytical methods for glycans is essential in terms of their determination and quality control. We previously reported a novel alternative labeling method for glycans involving 9-fluorenylmethyl chloroformate (Fmoc-Cl) instead of the conventional reductive amination procedure. The derivatives were analyzed by high-performance liquid chromatography (HPLC) (Kamoda S, Nakano M, Ishikawa R, Suzuki S, Kakehi K. 2005. Rapid and sensitive screening of N-glycans as 9-fluorenylmethyl derivatives by high-performance liquid chromatography: A method which can recover free oligosaccharides after analysis. \(^{*}\)Proteome Res. 4:146–152). This method was rapid and simple; however, it was time-consuming in terms of analysis by HPLC and did not provide so much information such as the detailed structures and mass numbers of glycans. Here we have developed a high-throughput and highly sensitive method. It comprises three steps, i.e., release of glycans, derivatization with Fmoc, and capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI MS) analysis. We analyzed several glycoproteins such as fetuin, alpha1 acid glycoprotein, IgG, and transferrin in order to validate this method. We were able to analyze the above glycoproteins with the three-step procedure within only 5 h, which provided detailed N-glycan patterns. Moreover, the MS/MS analysis allowed identification of the N-glycan structures. As novel applications, the method was employed for the analysis of N-glycans derived from monoclonal antibody pharmaceuticals and also from alpha-fetoprotein; the latter is known as one of the tumor markers of hepatocellular carcinomas. We were able to easily and rapidly determine the detailed structures of the N-glycans. The present method is very useful for the analysis of large numbers of samples such as a routine analysis.

Keywords: carbohydrate/CE-ESI MS/Fmoc/glycoprotein/N-glycan

Introduction

Analysis of glycans linked to proteins is important for precise understanding of the functions of glycoproteins. In contrast to profiling of total proteins (i.e., proteomics), high-throughput analysis of total glycans (i.e., glycomics) is still at a developmental stage, and various techniques for glycan mapping involving high-performance liquid chromatography (HPLC) (Hase and Ikenaka 1990; Takahashi et al. 1993, 1995; Hase 1994; Kondo et al. 1994; Anumula and Dhume 1998; Nakano et al. 2004) and capillary electrophoresis (CE) (Kinoshita et al. 2000; Kakehi et al. 2001) have been reported. High-pH anion exchange chromatography with pulsed amperometric detection is a powerful tool for the analysis of free glycans (Rice et al. 1992; Nakano et al. 2003). CE is an efficient and versatile method for the separation of complex glycan mixtures obtained from biological sources with high sensitivity. CE for glycan analysis involving various modes of sample separation such as simple zone electrophoresis (Ha et al. 2006; Kamoda and Kakehi 2006) and complex formation with borate ions (Suzuki et al. 2003; Sato et al. 2005; Dang et al. 2006) has been developed.

Mass spectrometry (MS) has become a powerful tool for the detection of glycans and for acquiring structural information on them. Online coupling of CE and MS has been proved to be very useful for glycan analysis (Schmitt-Kopplin and Fromberger 2003; Zamfir and Peter-Katalinic 2004; Zamfir et al. 2004). This technique allows minimum sample handling, which significantly reduces potential sample loss, and mass spectrometry is a powerful tool for the extremely selective and sensitive detection of specific structures of glycans. The application of capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI MS) and MS/MS to glycoscreening in the biomedical field has recently been highlighted (Sandra et al. 2004; Zamfir and Peter-Katalinic 2004). Bindila et al. (2005) reported the analysis of underivatized O-glycosylated sialylated amino acid derived from Schindler’s disease type I by sheathless reverse-polarity CE-ESI MS. Here, we investigated a method for N-glycans involving CE-ESI MS. N-Glycans are found in the sequence of Asn-X-Ser/Thr of a protein core, where X can be any amino acid except for proline. For the release of N-glycans attached to Asn residues through N-glycosidic linkages (structure 1 in Figure 1A), digestion with PNGase F (referred to as peptide \(N^\alpha\)(acetyl-β-D-glucosaminyl) asparagine amidase or peptide: N-glycanase F; EC 3.5.1.52) is most frequently performed, and the glycans are released as N-glycosylamine (structure 2). The N-glycosylamine is readily hydrolyzed to a free form (structure 3) under slightly acidic conditions, but is relatively stable under slightly basic conditions. The free form (structure 3) has
Fig. 1. (A) Flowchart for the preparation of Fmoc-labeled N-glycans after the release of N-glycans from protein. The N-glycan (structure 1) linked to the Asn-residue of the core protein/peptide is released with PNGase F as N-glycosylamine (structure 2). Structure 2 is hydrolyzed to yield free N-glycan (structure 3) under acidic conditions. However, at above pH 8, N-glycosylamine (structure 2) can be stabilized, therefore it can be directly reacted the amino groups with Fmoc-Cl. The Fmoc-labeled N-glycan (structure 4) was subjected to analysis by CE-ESI MS. (B) List of the structures detected in this study. The numbers in parentheses are the mass numbers represented as [M + H]+ of Fmoc-labeled N-glycans.

been widely used for the profiling of N-glycans after derivatization with fluorogenic and chromophoric reagents such as 2-aminoypyridine (PA) and 2-aminobenzamide for HPLC analysis (Hase 1994; Guile et al. 1996), and with 8-aminopyrene-1,3,6-trisulfonate (APTS) (Evangelista et al. 1996; Sei et al. 2002), 2-aminobenzoic acid (2-AA), or 3-aminobenzoic acid (Kakehi et al. 2002) for high-resolution analysis of glycans by CE.

For simpler and more rapid and sensitive analyses, we developed a method for direct labeling of N-glycosylamine-type glycans involving 9-fluorenylmethyl chloroformate (Fmoc-Cl) in our previous study (Kamoda et al. 2005) (structures 2 and 4 in Figure 1A). The Fmoc-labeling method has some strong points: (1) simple procedure; Fmoc reagent is just added to the reaction solution after digestion with PNGase F, and it is easy to remove excess reagent by extraction with chloroform, (2) high-speed analysis; within 4 h, including the enzymatic glycan releasing reaction and the labeling reaction before analysis, (3) high-sensitivity; 5 and 30 times higher sensitivities than those for 2-AA and PA-labeled N-glycans with a fluorescent detector, respectively, and (4) easy recovery of free-form glycans (structure 3 in Figure 1A); by incubation with morpholine in a dimethylformamide solution at room temperature (Kamoda et al. 2005).

Unlike peptides and proteins, most glycans do not form multiply charged ions; therefore, for the ionization and detection of glycans with large molecular masses, it is generally difficult to obtain good quality data (Macek et al. 2001; Metelmann et al. 2001; Sagi et al. 2002). The CE-ESI MS technique for glycans produces multiply charged ions due to the use of ammonium acetate as the CE buffer (Suzuki-Sawada et al. 1992). Therefore, in the present study, we developed a method for the analysis of Fmoc-labeled N-glycans released from glycoproteins involving CE-ESI MS, and the method was applied to the analysis of glycans derived from glycoproteins, such as human α1-acid glycoprotein (AGP), bovine fetuin, human transferrin, and human IgG. Also, we analyzed the N-glycans in glycoprotein bands obtained on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. As applications, this method was applied to the analysis of N-glycans derived from some antibody pharmaceuticals and a tumor marker, it being shown that the present technique is a powerful tool for the high-speed screening of N-glycans in biological samples.

Results

Release of N-glycans with PNGase F and in situ derivatization with Fmoc

A sample (10 µg) of each of bovine fetuin, human AGP, human transferrin, and human IgG was digested with PNGase F, and the released glycans were derivatized with Fmoc. Bovine fetuin contains monosialo-, disialo-, trisialo-, and tetrasiol-glycans (1SA, 2SA, 3SA, and 4SA, respectively) (Townsend et al. 1986;
Green et al. 1988). The results of analysis of Fmoc-labeled N-glycans are shown in Figure 2. In a base peak chromatogram (BPC) on MS (Figure 2A), sialylated N-glycans are eluted according to the number of sialic acid residues because of the electrospraying due to the combination of a bare fused silica capillary with an electrolyte at pH 6.8. Thus, 0SA were observed earliest at 12 min, and 1SA, 2SA, and 3SA were observed at 14.5, 16, and 18 min, respectively. At about 20 min, 4SA migrated the slowest. It should be noted that CE also facilitated the separation of Fmoc-labeled N-glycans from contaminants such as a polymer (the difference between each peak was 135.9 m/z) which was detected at around 5 min as a huge peak. The averaged mass spectra of 0SA, 1SA, 2SA, 3SA, and 4SA are shown in Figure 2B–F, respectively. A list of the N-glycans observed in the present study is given in Figure 1. The numbers in parentheses under the observed in the present study is given in Figure 1. The numbers in parentheses are the state of charge ((2) means [M + 2H]$^{2+}$, (3) means [M + 3H]$^{3+}$). The averaged mass spectra of 0SA, 1SA, 2SA, 3SA, and 4SA sections shown in Figure 1. The numbers in parentheses are the state of charge ((2) means [M + 2H]$^{2+}$, (3) means [M + 3H]$^{3+}$). In Figure 2E and F, 3SA and 4SA comprised trisialo-triantennary glycan ([19 [M + 3H]$^{3+}$ = 1034.8 m/z] and tetrasiolato-triantennary glycan (21 [M + 3H]$^{3+}$ = 1131.7 m/z), respectively. From the results of analysis of N-glycans derived from bovine fetuin, the peak areas on BPC of the 0SA–4SA N-glycans, as shown in Figure 2A, were calculated, and the results were compared with those for the peak areas of the fluorescence intensities determined on HPLC analysis in the previous study (Kamoda et al. 2005). The percentages of the peak areas in the HPLC chromatograms of the 0SA–4SA were 1.1, 3.2, 24.8, 59.4, and 11.5%, respectively, on the other hand, the percentages of peak areas on BPC in the present study were 2.9, 3.5, 14.6, 67.4, and 11.7%. The percentages were, thus, demonstrated to show a similar pattern, therefore, it is possible to determine the amounts of N-glycans using this method.

Typical MS/MS spectra of the trisialo-triantennary glycan (19) are shown in Figure 3. The fragment ions that released N-acetyllactosaminic acid (NeuAc), galactose (Gal), N-acetylglucosamine (GlcNAc), and mannose (Man) from the nonreducing end, which had Fmoc residues, were observed as singly or doubly charged ones. The ions which did not contain Fmoc residues were observed in the low mass region as singly charged ones. It should be noted that the fragment ion at m/z 366 due to lactosamine (Gal-GlcNAc) and that at m/z 657 due to sialo-lactosamine (NeuAc-Gal-GlcNAc) were characteristic of complex-type N-glycans. We detected all N-glycans derived from bovine fetuin that were reported previously (Townsend et al. 1986; Green et al. 1988). We next analyzed N-glycans derived from human AGP (Nakano et al. 2004), transferrin (Spik et al. 1975; Satomi et al. 2004), and IgG (Tai et al. 1975; Mizuochi et al. 1982; Takahashi et al. 1987), their N-glycans being observed in the order of the number of sialic acid residues (shown in supplementary Figures S1 and S2). We also performed MS/MS analysis, and two typical MS/MS results for N-glycans of AGP are shown in supplementary Figure S1. We could identify all the structures that some groups had reported previously.

Analysis of N-glycans in glycoprotein bands obtained on a SDS–PAGE gel

We next examined whether the Fmoc-labeling method was applicable to the direct derivatization of the N-glycans released from proteins on SDS–PAGE. Ten micrograms of each of bovine fetuin, human AGP, human transferrin, and human IgG was subjected to SDS–PAGE, followed by staining with Coomassie blue R-250 (CBB). The bands stained with CBB were cut out and destained, and then the materials were digested with PNGase F, derivatized with Fmoc, and then analyzed by CE-ESI MS. The results for bovine fetuin are shown in Figure 4. Fmoc-labeled N-glycans were not separated based on the number of sialic acids, and a clear peak of Fmoc-labeled N-glycans was not observed on BPC MS (Figure 4A). The reasons were that the yield of N-glycans released from the gel was not high, and the contaminating polymer was increased compared with analysis involving in situ digestion. Therefore, the N-glycans gave a diffused pattern on electrophoresis on a capillary, and all N-glycans were detected widely. However, the averaged mass spectra from 12 to 17 min revealed all N-glycan peaks derived
The presence of bisecting GlcNAc has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC), which is a major function of some therapeutic antibodies (Kumpel et al. 1994, 1995). The presence of Gal residues in the nonreducing terminals affects antibody-pharmaceuticals has been extensively studied. Kumpel et al. (2002) reported that the lactosamine structure (i.e., the presence of Gal residues in the nonreducing terminals) affects antibody-

from bovine fetuin, as shown in Figure 4B. Monosialo-, disialo-, trisialo-, and tetrasialo-triantennary glycans (16, 18, 19, and 21) were observed as doubly or triply charged ions. Monosialo- and disialo-biantennary glycans (13 and 15) were also observed as doubly charged ions. Regarding the results for human AGP, transferrin and IgG, all N-glycans observed on solution phase digestion were observed. Therefore, our results show that the present method is useful for evaluation of the N-glycan profiles of glycoproteins after SDS–PAGE separation.

Analysis of N-glycans derived from antibody pharmaceutical preparations

N-Glycans in glycoprotein pharmaceuticals play important roles in the expression of their biological activities. Therefore, the development of an assessment method for these glycans is important for the quality control of glycoprotein pharmaceuticals such as newly developed therapeutic antibodies. The relationship between the biological functions and glycans of antibody pharmaceuticals has been extensively studied. Kumpel et al. reported that the lactosamine structure (i.e., the presence of Gal residues in the nonreducing terminals) affects antibody-dependent cellular cytotoxicity (ADCC), which is a major function of some therapeutic antibodies (Kumpel et al. 1994, 1995). The presence of bisecting GlcNAc has also been reported to improve ADCC (Umana et al. 1999; Davies et al. 2001). Furthermore, recent reports indicated that the absence of a fucose (Fuc) residue at the innermost GlcNAc of reducing ends caused more obvious ADCC than that caused by the presence of bisecting GlcNAc (Shields et al. 2002; Shinkawa et al. 2003). The conditions for the industrial production of glycoprotein pharmaceuticals are quite important for maintaining the consistency of glycans (Parekh et al. 1989; Gawlitzek et al. 1995). Therefore, assessment studies on glycans in glycoprotein pharmaceuticals are crucial for quality assurance. Regulatory agencies are increasingly requiring the determination of glycan distributions quantitatively.

In this study, we applied the combination of Fmoc-labeling and the CE-ESI MS method for the glycan mapping of commercial therapeutic antibody pharmaceuticals: a humanized monoclonal antibody for treating metastatic breast cancer (trastuzumab), a chimeric monoclonal antibody for treating non-Hodgkin’s lymphoma (rituximab), and a humanized monoclonal antibody against a respiratory syncytial virus (palivizumab).

The results of analysis of trastuzumab are shown in Figure 5A and B. N-Glycans were observed at 8–10 min on BPC MS, as shown in Figure 5A. The result of averaging of the mass spectra (8–10 min) in Figure 5A is shown in Figure 5B. Alpha 1-6 fucosylated monogalactosyl biantennary glycans (9), α1-6 fucosylated agalactosyl biantennary glycans (8), and α1-6 fucosylated monoglcNAc biantennary glycans (5) were observed as major peaks. Alpha 1-6 fucosylated biantennary glycans (11), α1-6 fucosylated mono GlcNAc monogalactosyl biantennary glycans (7), and α1-6 fucosylated core five-structure glycans (2) were detected as minor peaks. These results were consistent with a previous report of Kamoda et al., who analyzed N-glycans in pharmaceutical preparations by CE (Kamoda et al. 2004, 2005). Moreover, we found N-glycans without α1-6 Fuc (1, 4, 6, 10) and a linear structure glycan without a six-branch Man chain (3). The analysis of N-glycans derived from rituximab and palivizumab was performed in the same manner, and the relative abundance of each N-glycan structure in each antibody pharmaceutical was calculated based on the signal intensities of the corresponding Fmoc-labeled N-glycans obtained on CE-ESI MS analysis. Briefly, the total detected signal intensities of Fmoc-labeled N-glycans were set at 100% for each antibody pharmaceutical was calculated based on the signal intensities of Fmoc-labeled N-glycans without α1-6 Fuc (1, 4, 6, 10) and a linear structure glycan without a six-branch Man chain (3). The analysis of N-glycans derived from rituximab and palivizumab was performed in the same manner, and the relative abundance of each N-glycan structure in each antibody pharmaceutical was calculated based on the signal intensities of the corresponding Fmoc-labeled N-glycans obtained on CE-ESI MS analysis. Briefly, the total detected signal intensities of Fmoc-labeled N-glycans were set at 100% for each antibody pharmaceutical, and the percentage of each Fmoc-labeled N-glycan was calculated. The results are summarized in Figure 5C. The relative abundances of N-glycan structures in the
three kinds of antibody pharmaceuticals were basically similar; however, the imperfect structures of N-glycans such as (1, 2, 4) were slightly increased in palivizumab. Trastuzumab and rituximab are produced from CHO cells, and palivizumab is produced from mouse myeloma cells. From these results, we found that the antibody pharmaceuticals from different cells did not show characteristic carbohydrate patterns. This present method is a suitable one for their quality control.

**Western blotting and lectin-affinity electrophoresis for α-fetoprotein (AFP), and CE-ESI MS analysis of N-glycans of AFP**

*Lens culinaris* agglutinin (LCA) is a lectin which exhibits affinity to biantennary glycans with α1-6 Fuc (Kaifu et al. 1975). An-reactive AFP with LCA (AFP-L3) has been used as an effective marker for earlier diagnosis, for the assessment of therapeutic effects, and for predicting the prognosis of hepatocellular carcinoma (HCC). Using LCA, AFP is divided into three types by HCC; therefore, AFP-L3 is highly specific to HCC (Sato et al. 1993; Taketa et al. 1993; Shiraki et al. 1995). In this study, we analyzed N-glycans in AFP derived from human normal placenta and human hepatoma cells (Huh-7) in order to determine any differences in N-glycan structures for an application for this CE-ESI MS technique. A portion of them was subjected to SDS–PAGE, and only one band was stained with CBB (Figure 6A). The molecular weight corresponding to the band (ca. 75 kDa) was the expected molecular weight of AFP; furthermore, Western blot analysis with anti-AFP antibodies showed that the band was AFP (Figure 6B). Although there was no difference between AFP derived from normal human placenta and Huh-7 cells on both SDS–PAGE analysis and Western blot analysis, there was a big difference on electrophoresis with LCA (Figure 6C). AFP derived from normal human placenta gave one band at the L1 position. AFP derived from Huh-7 cells gave two bands at the L1 and L3 positions. The L3 band means AFP bound to LCA strongly; in other words, AFP at the L3 position has an N-glycan containing α1-6 Fuc at the reducing terminus of GlcNAc. We could confirm that nonfucosylated N-glycans were major in AFP from normal human placenta, but α1-6 fucosylated N-glycans were major in AFP from Huh-7 cells. We next tried to analyze N-glycans in these AFPs by means of our CE-ESI MS technique. The results are shown in Figure 6D–J. The N-glycans in AFP derived from human normal placenta were observed at 17–19 min (1SA) and 20–21 min (2SA), as shown in Figure 6D (BPC MS). On the other hand, N-glycans in AFP derived from Huh-7 cells were observed at 19–20 min (1SA), 21–23 min (2SA), and 24–26 min (3SA), as shown in Figure 6E (BPC MS). N-Glycans of 1SA and 2SA from AFP derived from human normal placenta were monosialo biantennary ones (13 in Figure 6F) and disialo-biantennary ones (15 in Figure 6G), respectively. We could slightly detect α1-6 fucosylated monosialo- and disialo-biantennary glycans (14 in Figure 6F and 17 in Figure 6G). These fucosylated N-glycans must be included in the L3 fraction in lane p, Figure 6C. On the other hand, the N-glycans of 1SA, 2SA, and 3SA from AFP derived from Huh-7 cells were α1-6 fucosylated monosialo-biantennary ones (14 in Figure 6H), α1-6 fucosylated disialo-biantennary ones (17 in Figure 6I), and α1-6 fucosylated trisialo-triantennary ones (20 in Figure 6J), respectively. Disialo-biantennary glycan without Fuc (15 in Figure 6I) was also slightly detected. This N-glycan (15) is located at the L1 position in lane h, Figure 6C. From these results, although the electrophoresis with LCA in Figure 6C could reveal differences in the patterns of N-glycans in AFP derived from human placenta and Huh-7 cells, our CE-ESI MS technique could reveal the differences based on the detailed N-glycan structures.

**Discussion**

We have developed a simple, rapid and sensitive method involving CE-ESI MS with Fmoc-labeling of N-glycans derived from several glycoproteins. We could also identify the N-glycans, especially N-glycans containing sialic acids, based on the mass number and the fragment pattern on MS/MS analysis with appropriate separation on CE. The advantages of the present CE/MS approach are as follows: (1) N-glycans derivatized with fluorogenic or chromophoric reagents such as Fmoc are suitable for detailed MS/MS analysis to investigate the linkage patterns of N-glycans. (2) Also, they have a single configuration (i.e., β-form) at the reducing end and have no isomers which...
complicate the chromatography. (3) The Fmoc-labeling method is very simple because the releasing of $N$-linked oligosaccharides from a glycoprotein sample followed by labeling with Fmoc is performed in a one-pot reaction. After PNGase F digestion and then Fmoc derivatization, the proteins devoid of $N$-glycans are easily removed as fluffy material during extraction of the excess Fmoc reagent with chloroform (Kamoda et al. 2005). Most recently, Ruhaak et al. (2008) reported a high-throughput method with which $N$-glycans derived from total human plasma glycoproteins were derivatized with 2-AA in a 96-well plate followed by analysis by CE-ESI-Q-TOF-MS. Gennaro et al. also reported a method with which $N$-glycans derived from therapeutic antibodies were derivatized with APTS followed by analysis involving CE-LIF (laser-induced fluorescence)-TOF-MS (Gennaro and Salas-Solano 2008). In both of them, solid-phase extraction is performed to remove excess labeling reagent. In our present method, liquid-phase extraction is performed for this. It is much simpler. (4) Therefore, the method allows rapid mapping of $N$-glycans within 5 h, including an enzymatic glycan releasing reaction, Fmoc derivatization, analysis by CE-ESI MS, and data analysis. (5) The CE separation of $N$-glycans before subjecting them to MS is helpful for identifying the $N$-glycans because $N$-glycans are separated from contaminants such as polymers on a fused silica capillary on CE and then sialo-$N$-glycans are eluted in the order of the number of sialic acids in glycans. (6) The ammonium acetate used as the CE buffer facilitates the production of multiple charged ions of labeled $N$-glycans (Suzuki-Sawada et al. 1992), and therefore for the ionization and detection of glycans with large molecular masses, it is easy to obtain good quality data.

We applied this simple, rapid and sensitive method to identify the $N$-glycans released from the bands observed on SDS–PAGE after in-gel digestion. We could directly release $N$-glycans from glycoproteins in a gel without extracting them from the gel. The released $N$-glycans were derivatized with Fmoc followed by CE-ESI MS analysis. The present method is applicable for the analysis of crude samples after separation on a 2D-gel. Hereafter, the rapid analysis of $N$-glycans will be useful for routine work for validation of glycoprotein pharmaceuticals and disease biomarkers. We applied the method to the analysis of $N$-glycans derived from some antibody pharmaceuticals prepared from different kinds of cells and showed the similar characteristic patterns of glycans. We were also able to easily and rapidly determine the $N$-glycan structures of AFP of different sources with various sialylation and fucosylation patterns. In conclusion, this method is a powerful tool for high-speed screening of $N$-glycans in biological samples.

Material and methods

Reagents and materials
PNGase F (EC 3.5.1.52, recombinant) was obtained from Roche Diagnostics (Mannheim, Germany). Fmoc-Cl was from Tokyo Kasei (Tokyo, Japan). Human AGP, bovine fetuin, human transferrin, human IgG, and mouse anti-human AFP monoclonal antibodies were purchased from Sigma (St. Louis, MO). The recombinant antibody pharmaceutical preparations (rIgG), trastuzumab, rituximab, and palivizumab were kindly donated by Ms. Nishiura of Kinki University Nara Hospital. AFP derived from human placenta was purchased from Cosmobio (Tokyo, Japan). Another type of AFP was purified from the conditioned medium of Huh-7 cells (Japanese Collection of Research Bioresources) using an antibody column of polyclonal rabbit
anti-human AFP antibodies. Other reagents and solvents were of HPLC or LC/MS grade.

**Release of N-glycans with PNGase F and in situ derivatization with Fmoc**

The release of N-glycans from several glycoprotein samples and labeling of the N-glycosylamine-type glycans with Fmoc were performed according to the procedures reported previously (Kamoda et al. 2005). Glycoprotein samples (10 µg each) were each dissolved in a 20 mM phosphate buffer (pH 8.5, 100 µL) in a sample tube (1.5 mL). PNGase F (1 unit, 1 µL) was added, and the mixture was incubated at 37°C for 2 h. After dilution of the mixture with water (300 µL), a freshly prepared solution (200 µL) of Fmoc-Cl in acetone (50 mg/mL) was added and the mixture was incubated at 37°C for 1 h. Chloroform (300 µL) was added to the mixture, and the mixture was shaken vigorously. After centrifugation, the aqueous layer was transferred to a new sample tube (1.5 mL). Care should be taken not to transfer the fluffy material over the organic layer. Another portion of chloroform (300 µL) was added to the aqueous layer, and the mixture was shaken vigorously to remove the excess reagent. The same procedures for washing the aqueous layer with chloroform were repeated five times. Finally, a portion (5 µL) of the aqueous layer containing N-glycans derivatized with Fmoc was transferred to a sample vial for injection into the CE-ESI MS. The workflow for this procedure is shown by a run in supplementary Figure S3.

**Analysis of N-glycans in glycoprotein bands obtained on a SDS–PAGE gel**

A 10 µg aliquot of each of bovine fetuin, human AGP, transferrin, and IgG was subjected to SDS–PAGE (acylamide 12%) followed by staining with CBB. The glycoprotein band visualized with CBB was rinsed with distilled water in a sample tube followed by staining with CBB. The glycoprotein band visualized with CBB was rinsed with distilled water in a sample tube and then cut into small pieces (1 mm²). The gel pieces were rinsed with an aqueous solution (500 µL) of 50 mM ammonium bicarbonate in 30% acetonitrile for 10 min to remove the dye reagent. This procedure was repeated three times. The gel pieces were kept in acetonitrile (500 µL) for 10 min for dehydration. The acetonitrile was removed and the gel pieces were dried for 15 min under reduced pressure. PNGase F (1 U) in the 20 mM phosphate buffer (pH 8.5, 5 µL) was added to the dried gel pieces. After the gel pieces had been swollen, the 20 mM phosphate buffer (pH 8.5, 95 µL) was added and the mixture was incubated at 37°C overnight. After dilution of the mixture with water (300 µL), a freshly prepared solution of Fmoc-Cl in acetone (200 µL, 50 mg/mL) was added, and then the mixture was incubated at 37°C for 1 h. After removing the excess Fmoc reagent with chloroform as described above, the aqueous layer was evaporated to dryness with a centrifugal evaporator. The residue was dissolved in water (20 µL), and a portion (5 µL) was employed for CE-ESI MS analysis. The workflow for this procedure is shown by a run in supplementary Figure S3.

**Analysis of N-glycans derived from antibody pharmaceutical preparations**

Aqueous solutions of pharmaceutical preparations (trastuzumab, rituximab, and palivizumab) were dialyzed against distilled water for 3 days, the water being changed several times at 4°C using cellulose membrane tubing (Sanko Junyaku, Tokyo, Japan), and then freeze-dried. Each freeze-dried pharmaceutical preparation (10 µg) was dissolved in the 20 mM phosphate buffer (pH 8.5, 100 µL). The following procedure was the same as that described above.

**Western blotting and lectin-affinity electrophoresis for AFP, and CE-ESI MS analysis of N-glycans of AFP**

AFP derived from human placenta and Huh-7 cells (100 ng each) was subjected to SDS–PAGE (10% w/v) polyacrylamide) followed by staining with Coomassie blue R-250. For Western blotting, AFP derived from human placenta and Huh-7 (1 ng each) was subjected to SDS–PAGE (10% w/v) polyacrylamide). We also performed lectin-affinity electrophoresis to examine the N-glycan structures of AFP using an AFP differentiation kit L (Wako Pure Chemical Industries, Osaka, Japan). For better understanding of the results of lectin-affinity electrophoresis, the N-glycan profile of AFP on CE-ESI MS was examined as follows. N-Glycans were released with PNGase F as described above after denaturing the protein with RapiGest SF (Waters, Milford, MA). Briefly, a sample of AFP (10 µg) was dissolved in the 20 mM phosphate buffer (pH 8.5, 90 µL) in a sample tube (1.5 mL). RapiGest SF (10 µL) in the 20 mM phosphate buffer (pH 8.5, 1%) was added, and the mixture was incubated at 60°C for 1 h. After the sample had been cooled to room temperature, PNGase F (1 unit, 1 µL) was added, and then the mixture was incubated at 37°C for 2 h. The derivatization of the released N-glycans with Fmoc and removal of the excess Fmoc reagent with chloroform were performed in the same manners as described above. Also, the aqueous layer was evaporated to dryness in a centrifugal evaporator. The residue was dissolved in water (20 µL), and a portion was employed for CE-ESI MS analysis.

**CE-ESI MS**

CE was performed using a Beckman P/ACE MDQ system (Beckman, Fullerton, CA). The system was online coupled to an Esquire HCT ion trap mass spectrometer (Bruker Daltonics, Germany) via a capillary and a sprayer device (Bruker Daltonics). The capillary was positioned at an orthogonal position as to the ion source. The capillary inlet was placed in a vial containing the CE buffer (50 mM ammonium acetate) and a platinum electrode connected to a high-voltage power supply. All experiments were performed using an eCAP fused silica capillary (Beckman, 100 cm x 50 µm i.D., 360 µm O.D.) installed in an eCAP user-assembled capillary cartridge EDA (Beckman). The capillary had previously been washed with methanol (3 min, 50 psi) and water (2 min, 50 psi), followed by reconditioning with 0.1 M NaOH (2 min, 50 psi) and water (2 min, 50 psi) by means of the pressure method. Finally, the CE buffer was introduced into the capillary (4 min, 50 psi). The capillary was rinsed with the CE buffer (5 min, 50 psi) after each run. A sample solution (Fmoc-labeled N-glycans in water) was introduced into the capillary by means of the pressure method (15 s, 3 psi), and then analyzed at the applied potential of 30 kV at 20°C. The sample solution reaching the outlet of the capillary was sprayed with nebulizing gas (N₂, 8 psi). The sheath liquid comprising 50/49/0.1 (v/v/v) MeOH/water/formic acid was delivered to the probe tip at the rate of 2 µL/min. No electrical contact was applied on the outlet of the capillary. The sheath liquid was supplied to maintain a constant flow by means of a syringe pump.
(Cole-Parmer, Vernon Hills, IL). In the MS device, the voltage of the capillary outlet was set at −4 kV, and the temperature of the transfer capillary was maintained at 300°C. The flow rate of nitrogen gas for drying was 4 L/min. The MS spectra were obtained in the positive ion mode. The mass range covered was between m/z 300 and m/z 2000. The scan rates were 8100 amu/s for the MS mode and 26,000 amu/s for the MS/MS mode. The analytical conditions and analytical devices used for the present study are illustrated in supplementary Figure S4.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/

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Conflict of interest statement
None declared.

Abbreviations
2-AA, 2-aminobenzoic acid; ADCC, antibody-dependent cellular cytotoxicity; AFP, α-fetoprotein; AGP, α1-acid glycoprotein; BPC, base peak chromatogram; CE-ESI MS, capillary electrophoresis-electrospray ionization mass spectrometry; Fmoc-Cl, 9-fluorenylmethyl chloroformate; Fuc, fucose; Gal, lary electrophoresis-electrospray ionization mass spectrometry; Fuc, fucose; Gal, lactose; GlcNAc, N-acetylglucosamine; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; LCA, Lens culinaris agglutinin; Man, mannoside; NeuAc, N-acetylneuraminic acid; PA, pyridylamino; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Bindila L, Peter-Katalinic J, Zamfir A. 2005. Sheathless reverse-polarity capillary electrophoresis-electrospray ionization mass spectrometry; Fmoc-Cl, 9-fluorenylmethyl chloroformate; Fuc, fucose; Gal, lactose; GlcNAc, N-acetylglucosamine; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; LCA, Lens culinaris agglutinin; Man, mannoside; NeuAc, N-acetylneuraminic acid; PA, pyridylamino; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.


