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Quantitative Analysis of Oligosaccharide Structure of Glycoproteins

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Abstract A sensitive and quantitative method for the structural analysis of oligosaccharide was established for the glycoform analysis of glycoproteins. In this study, *N*-linked oligosaccharides of human IgG and bovine transferrin were analyzed for the evaluation of the method. Carbohydrate moiety of glycoprotein was released by hydrazinolysis and purified by paper chromatography. The oligosaccharides were labeled with a fluorescent dye, 2-aminobenzamide, for the enhancement of detection sensitivity. Sialylated (acidic) oligosaccharides were separated from neutral oligosaccharide by employing a strong anion-exchange column (MonoQ) followed by the treatment with sialidase. Enzymatically desialyated fractions and neutral fractions of oligosaccharides were applied to normal-phase HPLC to resolve the peaks according to glucose unit (GU). The structure of separated molecules was further determined by sequential digestion with exoglycosidases. As a result, disialylated biantennary complex-type oligosaccharide was found to be a major sugar chain in bovine transferrin (63%). In human IgG, core fucosylated asialobiantennary complex oligosaccharides were dominant. These results coincided well with reported results.

Keywords hydrazinolysis, oligosaccharide, 2-aminobenzamide, IgG, bovine transferrin

INTRODUCTION

It is well known that more than 90 percent of all proteins from animal cells are glycoproteins. Among these glycoproteins, many have been recognized for their important therapeutic uses. The carbohydrate portion of glycoproteins usually exists in *N*-linked (asparagine-linked) or *O*-linked (serine-, threonine-linked) form. It has been reported that the structure of *N*-linked glycan is crucial for the biological activity of glycoproteins. This drew much attention from many of the world's research groups and leading pharmaceutical companies that had intended to produce commercial therapeutic glycoproteins. One of the well-known cases involved the production of Erythropoietin (EPO). Many research groups had reported that the structure of *N*-linked oligosaccharide of EPO affected its biological activity and half-life in circulation according to its antennary structure and sialylation of oligosaccharide [1,2]. This became the most important reason for the researcher to pay great deal of attention to the analysis of the carbohydrate structure of EPO.

The *N*-linked glycan structure of human immunoglobulin G (IgG) was found to be important in immunopathological aspects, since the lack of terminal galactosylation might induce diseases, such as rheumatoid arthritis [3]. Therefore, the structural integrity of the glycan of IgG must be verified before applying it toward the use of pharmaceutical agents (*e.g.*, recombinant human IgG). Transferrin is also one of the common glycoproteins found in blood as an antibody, which is secreted from the liver. One of the most interesting features of the *N*-linked glycan structure of transferrin is that the unusual Lewis x structures (containing core-fucose) appear in hepatocellular carcinoma patients' transferrin [4]. This shows that the glycan analysis can also be used for diagnosing cancer. As described above, glycan analysis results can be utilized in many respects, including quality control in industry and in medical diagnosis.

We have previously reported a simple method for analyzing the glycoform of human Erythropoietin employing lectin-blotting technique [5]. Nevertheless, even though the lectin-blotting technique is simple, it still has problems with low sensitivity and quantification. Recently, we established a high-sensitive, low-cost and quantitative method for the analysis of oligosaccharide structure using HPLC technology by modifying the conventional methods [6,7]. Conventional methods of oligosaccharide analysis require large samples as well as the employment of radioisotopes and expensive equipment for analysis of structural heterogeneity (*e.g.*, HPAEC-PAD, MALDI-TOF, etc.). Our modified method is capable of resolving sub-picomolar quantities of mixtures by fluorescence-labeled glycan using an HPLC system equipped with a fluorescence detector and quantifying their molar proportion. We use human IgG and bovine transferrin as model glycoproteins in this study and demonstrate the applicability of this method for the analysis of oligosaccharide structure of glycoproteins.

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MATERIALS AND METHODS

Chemicals, Reagents, and Enzymes

Human IgG and bovine transferrin were purchased from Sigma and used for the analysis. Anhydrous hydrazine was obtained from Oxford Glycosystems (Oxford, United Kingdom), 2-aminobenzamide (2-AB) from Fluka Chemie (Steinehem, Switzerland) and *Arthrobacter ureafaciens* sialidase, β-galactosidase, N-acetylglucosaminidase, α-mannosidase, α-fucosidase from Seikagaku corp. (Tokyo, Japan). A cation-exchange resin, AG50-X12, was purchased from Bio-Rad (Hercules, CA) MonoQ was obtained from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden) and an amide column, GlycoSep-N, from Oxford Glycosystems (Oxford, United Kingdom). 3MM CHR for paper chromatography was purchased from Whatman International Ltd (Maidstone, United Kingdom) All the other chemicals, reagents, and solvents were obtained from available commercial sources and were of ACS grade or higher purity

Preparation of Oligosaccharide Samples

The overall procedure for the preparation of the oligosaccharide samples is schematically described in Fig. 1. *N*-linked sugar chains of the glycoprotein were converted to oligosaccharide derivatives by chemical reaction. Samples were thoroughly dried and subjected to hydrazinolysis for 10 h at 100°C [8,9] Afterwards, the reaction mixture was evaporated to dryness under reduced pressure over concentrated H_2SO_4 at room temperature The sample was subjected to N-acetylation followed by paper chromatography using 1-butanol: ethanol:water (4:1.1, v/v) for 18 hrs. The area of the paper from the origin to the position of authentic lactose was extracted with water, and the extracted oligosaccharides were labeled with 2-aminobenzamide (2-AB) [6] As for the 2-AB-labeled oligosaccharides, fluorescence was monitored at 430 nm (excitation, 330 nm).

Anion-Exchange Chromatography

A strong anion-exchange column (MonoQ) was used to separate neutral oligosaccharides from acidic oligosaccharides The oligoaccharide samples dissolved in distilled water were applied to the MonoQ HR 5/5 column. After the elution of neutral oligosaccharides with 10 mL of water, acidic oligosaccharides were eluted with a 0-1 M gradient of ammonium acetate, pH 4.0, at a flow rate of 1 mL/min at room temperature.

Sialidase Treatment and Amide-Column Chromatography

The acidic (sialylated) oligosaccharides were further treated with *Arthrobacter ureafaciens* sialidase for neutralization. Desialylated oligosaccharides and neutral oligosaccharides were applied to amide column HPLC (Glycosep-N) to resolve according to glucose unit (GU) [6]. The ratio of acetonitrile and 250 mM ammonium acetate (pH 4 0) was changed linearly from 80:20 to 47:53 (v/v) over 132 min after the injection at a flow rate of 1.0 mL/min at 30°C. The structural assignment of separated peaks was further determined by sequential digestion with four kinds of exoglycosidases (β-galactosidase, N-acetylglucosaminidase, α-mannosidase, and α-fucosidase)

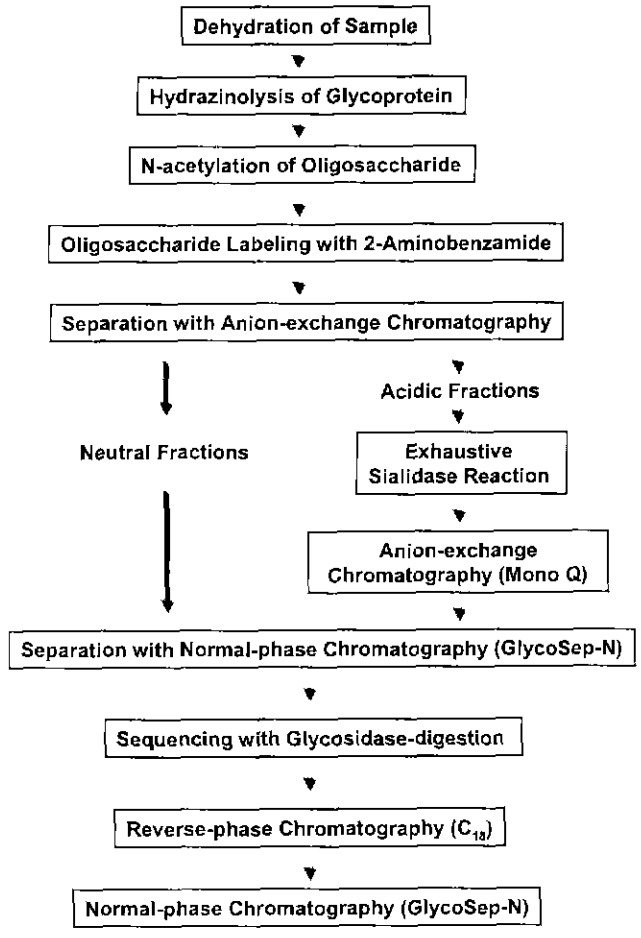

Fig 1. Schematic diagram of structural analysis of N-linked oligosaccharide from glycoproteins.

RESULTS

Hydrazinolysis and 2-Aminobenzamide Labeling

Two mg of bovine transferrin and human IgG were subjected to hydrazinolysis. Next, the released oligosaccharides were purified by a paper chromatography. After 2 h of labeling reaction with a fluorescent tag, 2-amino-benzamide, labeled oligosaccharides were purified with paper chromatography. Oligosaccharides were extracted from paper using centrifugation, then aspirated for HPLC analysis.

Separation of Oligosaccharide by MonoQ

The quantity of sialylated oligosaccharides and the number of sialylation were determined by an anion-

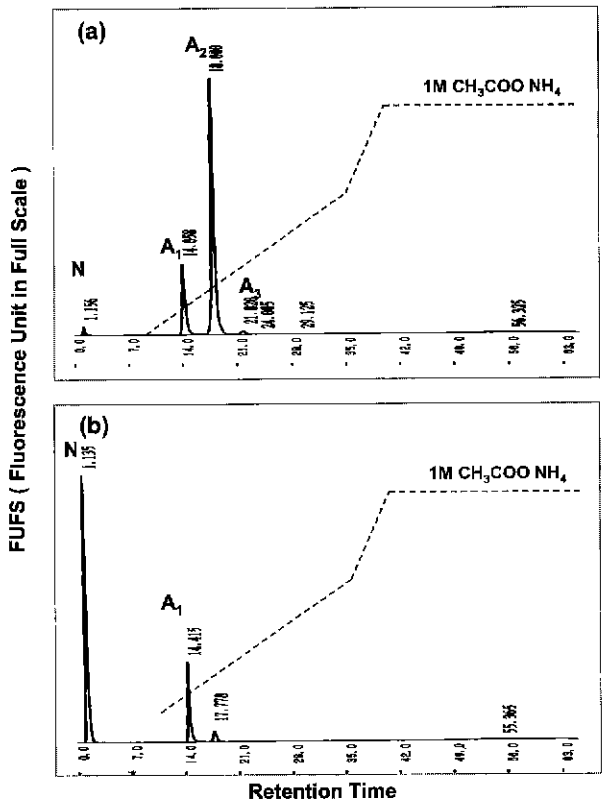

Fig 2 Mono Q column chromatography of the 2-AB labeled oligosaccharides from bovine transferrin (a) and human IgG (b) The column was eluted with a 0-1 M gradient of ammonium acetate as indicated by a broken line, pH 4.0, at a flow rate of 1 0 mL/min at room temperature The chromatogram shows neutral (N), monosialylated (A_1), disialylated (A_2), and trisialylated (A_3) fractions.

exchange column, Mono Q, at pH 4 0. Neutral oligosaccharide was eluted in 10 min. Next, the sialylated oligosaccharide was eluted according to the number of linked sialic acid The HPLC profile of transferrin glycans (Fig. 2(a)) showed that the majority of the N-linked oligosaccharides were sialylated in mono-, di-, and trisialylated forms. Neutral oligosaccharides were found to be dominant in human IgG glycans pool (Fig. 2(b)).

Assignment of Oligosaccharide Structures from Human IgG

Sialylated peaks were collected for sialidase treatments, then the neutralized (desialylated) fractions were recovered by a MonoQ column. Each fraction was analyzed by using a Glycosep-N column for the determination of the GU (glucose unit) value. A Standard glucose oligomer was injected primarily to form a dextran ladder for the calculation of GU. The structural heterogeneity of oligosaccharides was assigned based on the data concerned in the retention time of various standard oligosaccharides, chromatographic profile after exoglycosidase digestion, and relationship between GU

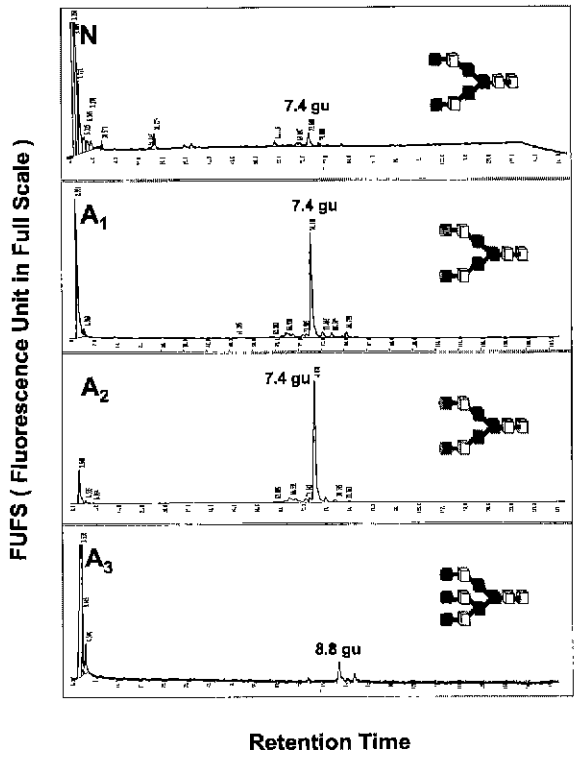

Fig. 3. Amide column chromatography of 2-AB labeled oligosaccharide from bovine transferrin after the fractionation with a Mono Q column The neutral oligosaccharide fraction obtained by exhaustive *A. ureafaciens* sialidase digestion of the sialylated fractions (A_1-A_3) was then subjected to amide column Amide column HPLC was carried out with GlycoSep-N, and the acetonitrile-250 mM ammonium acetate (pH 4.0) ratio was changed linearly from 80 20 to 47:53 (v/v) over 132 min after injection at a flow rate of 1.0 min/mL at 30°C The block models on the chromatogram show the anticipated structure of sugar chains. The gray cylinder indicates galactose residue, the white cube indicates N-acetylglucosamine residue, and the black cube indicates mannose residue

value and oligosaccharide structure as previously mentioned by Guile *et al.* [7]. As a result, four kinds of oligosaccharide structures were found in transferrin (Fig. 3, 5). Among these, disialylated biantennary complex-type oligosaccharide was found to be a major type of sugar chain (63%). In human IgG, eight types of major sugar chain structures were assigned (Fig. 4) and core fucosylated asialobiantennary complex oligosaccharides were dominant. Oligosaccharides with bisecting N-acetylglucosamine residues that are known to be present only in human IgG were also detected (Fig. 6). (The data for the assignment of the oligosaccharide sequence using four kinds of exoglycosidases are not shown in this article.) All the results were qualitatively comparable to those reported in the literature [10-12] Since the quantitative results of the oligosaccharide studies of IgG and bovine transferrin were not reported in the literature, it was not possible to compare the quantitative data.

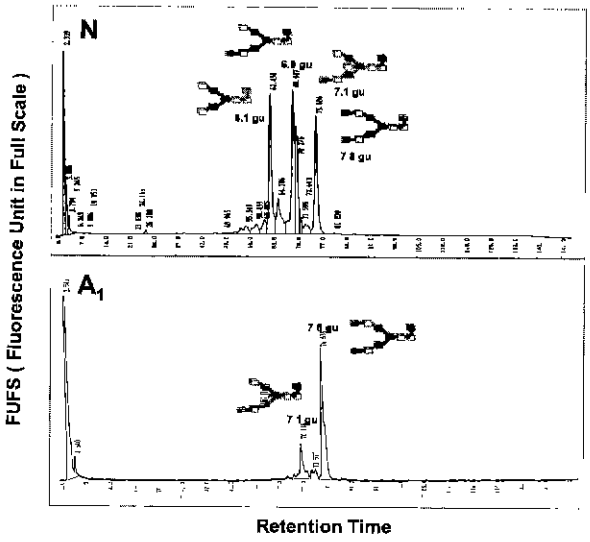

Fig. 4 Amide column chromatography of 2-AB labeled oligosaccharide from human IgG after fractionation with a Mono Q column. The neutral oligosaccharide fraction obtained by exhaustive *A. ureafaciens* sialidase digestion of the sialylated fractions (A_1, A_2) was then subjected to an amide column Amide column HPLC was carried out using GlycoSep N, and the acetonitrile-250 mM ammonium acetate (pH 4 0) ratio was changed linearly from 80 20 to 47:53 (v/v) over 132 min after being injected at a flow rate of 1.0 min/mL at 30°C The block models on the chromatogram show the anticipated structure of sugar chains The gray cylinder indicates galactose residue, the white cube indicates N-acetylglucosamine residue, the black cube indicates mannose residue, and the black cylinder indicates fucose residue.

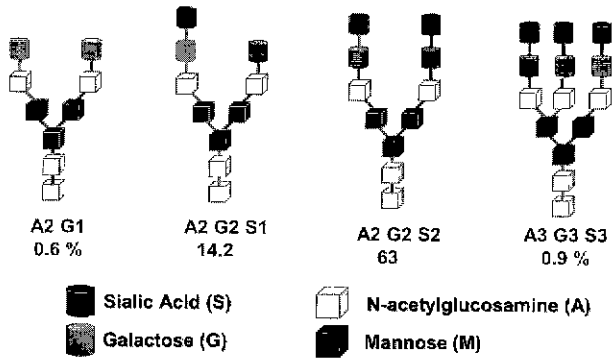

Fig. 5. Identified structure of *N*-linked oligosaccharides released from bovine transferrin

DISCUSSION

In establishing a method for the analyzing the oligosaccharide structure, we were able to find three significant advantages First, hydrazinolysis was utilized to cleave glycan completely. There are several other methods of releasing glycan from proteins, such as glycosidase treatment or protease treatment, but these methods cannot promise the high yield and complete cleavage as seen in hydrazinolysis. Second, is the employment of ultra-sensitive probe, 2-aminobenzamide, enables the detection of sub-picomolar amounts of oligosaccharide. Furthermore, accurate quantification of each peak was possible due to the high sensitivity of the fluorescence detector This is a much more sensitive method than employing radioisotopes or using a pulse-amperometric detector that also enables the use of mass-spectrometry for molecular mass determination. Oxford Glycosystem has recently commercialized a 2-AB labeling kit, but in our work, labeling was performed without using a commercial kit. In addition, we also employed a glycan purification method using a paper chromatography method specially developed for the high purity of the sample. Fractionation of the molecules according to the charge was also useful for the structural determination of sialylation. Here, we showed that the commonly used anion-exchange column, MonoQ, could be useful not only for protein but also for oligosaccharide resolution. Finally, this method does not require any particular expensive instrument and can be proceeded by any ordinary HPLC system with a fluorescent detector These results prove that our method is low-cost, highly sensitive, and accurate enough to be applied not only to the analysis of glycan structure in glycoproteins, but also to the quality control of glycoproteins in animal cell culture processes.

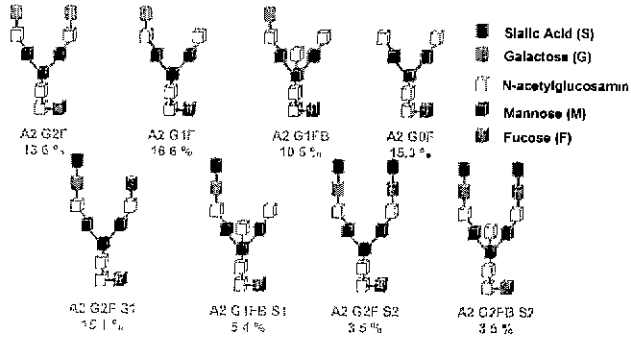

Fig. 6. Identified structure of *N*-linked oligosaccharides released from human IgG.

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