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Oligosaccharide mapping of therapeutic glycoproteins by high-pH anion-exchange high-performance liquid chromatography

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ABSTRACT

The Asn-linked oligosaccharides of four different glycoproteins were cleaved by N-glycanase and analysed by high-pH anion-exchange high-performance liquid chromatography coupled with a pulsed amperometric detector. Each glycoprotein produced unique sets of oligosaccharides. Sialic acid-containing oligosaccharide peaks were readily identified by their large decrease in retention time following neuraminidase digestion. The use of this HPLC technique for tentatively identifying unknown oligosaccharide peaks by retention time comparison with reference standards and for monitoring the consistency of N-glycosylation of recombinant DNA-derived glycoprotein was demonstrated.

INTRODUCTION

Analysis of carbohydrate-mediated heterogeneity of therapeutic glycoproteins is important in clinical evaluation since oligosaccharides may influence biological activity, pharmacokinetics and immunogenicity of proteins[1]. High-performance liquid chromatography (HPLC) techniques for the separation of oligosaccharides, in general, offer speed and resolution, but sensitive detection often requires pre- or post-column derivatization[2–7]. Recently it has been shown that an accurate and sensitive analysis of monosaccharides and oligosaccharides including the glycoprotein-derived structures could be performed without derivatization using high-pH anion-exchange HPLC coupled with pulsed amperometric detection (PAD)[8–13]. N-Glycanase is known to cleave a wide range of Asn-linked oligosaccharides with bi- tri- and tetraantennary chains[7,14–16]. This enzymatic hydrolysis has been shown to be a simple and practical alternative to hydrazinolysis for obtaining N-linked oligosaccharide with an intact di-N-acetylchitobiose on the reducing end[7,14]. In this report, we investigated the utility of this HPLC technique for comparative analysis of N-glycanase-released oligosaccharides from different glycoproteins and for monitoring consistency of glycosylation of recombinant DNA (rDNA)-derived glycoprotein produced in mammalian cell culture.

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EXPERIMENTAL

Materials

The glycoproteins, human serum transferrin, human immunoglobulin G (IgG), human α_1-antitrypsin, N-acetylneuraminic acid (NeuAc), neuraminidase (type x) were obtained from Sigma (St. Louis, MO, U.S.A.). N-Glycanase was purchased from Genzyme (Boston, MA, U.S.A.). rDNA-derived glycoprotein used in this study was human interleukin-4 expressed in Chinese hamster ovary cells. NMR-verified glycoprotein-derived oligosaccharide standards, asialo diantennary, asialo triantennary, sialylated diantennary and sialylated triantennary oligosaccharides (structures I to IV in Fig. 1) were from Dionex (Sunnyvale, CA, U.S.A.) and from Biocarb (Lund, Sweden). 50% (w/w) sodium hydroxide solution was from Fisher Scientific (Rockville, MD, U.S.A.) and sodium acetate was reagent grade from J. T. Baker (Philipsburg, NJ, U.S.A.). Eluents and buffers were prepared with distilled water further purified on a Millipore water system (Milli-Q) or HPLC-grade water from J. T. Baker.

Methods

Enzymatic removal of N-linked oligosaccharides from glycoproteins. Asn-linked oligosaccharides were released by incubating the glycoproteins (100–500 μg) with 0.01–0.05 units of N-glycanase in 50–200 μl of 50 m*M* sodium phosphate buffer, pH

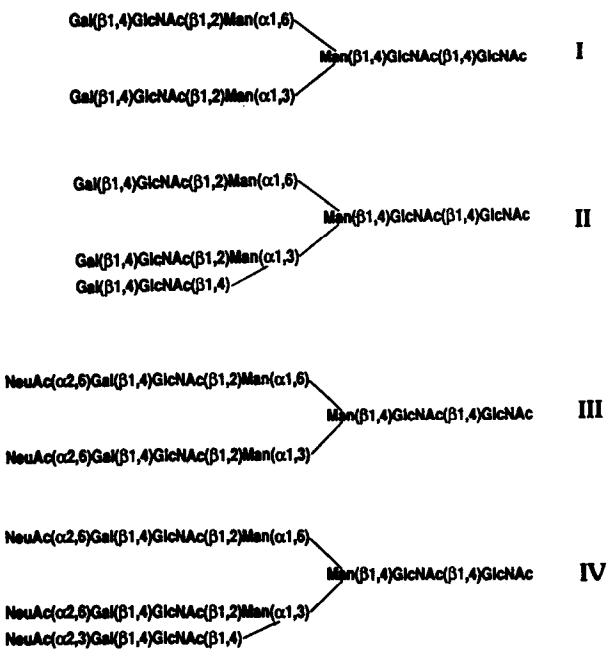

Fig. 1. Oligosaccharide standards used in HPLC analysis, NMR-verified, glycoprotein-derived oligosaccharide standards. (I) Asialo diantennary, (II) asialo triantennary, (III) sialylated diantennary, (IV) sialylated triantennary.

8.3 at 37°C for 18–36 h. In some cases, the glycoproteins were denatured by heating at 100°C for 2–5 min in the sodium phosphate buffer containing 0.1% sodium dodecyl sulfate (SDS) and 0.1% mercaptoethanol.

Deglycosylation of glycoproteins was evaluated by SDS-polyacrylamide gel electrophoresis and by binding to concanavalin A-peroxidase[14]. Following N-glycanase digestion, deglycosylated protein was removed by precipitation with three volumes of ice-cold ethanol and centrifuged at 12 000 *g* for 15 min at room temperature. The supernatant containing oligosaccharides was evaporated to dryness in a Speed-Vac and redissolved in 100–200 μl of distilled water and filtered using Millipore HV filters.

Removal of sialic acids from glycoproteins and N-glycanase-released oligosaccharides was performed by enzymatic digestion with *Clostridium perfringens* neuraminidase (0.05 units) in 50 μl of 50 m*M* sodium acetate buffer (pH 5.5) at 37°C for 4 h[17].

Chromatography. The HPLC system used for the analysis of oligosaccharides was Dionex Bio-LC set up with a pellicular anion-exchange column (Carbopac, PA-1; 250 × 4.6 mm I.D.) and a pulsed amperometric detector (Model PAD 2, Dionex). The Dionex eluent degas module was used to sparge and pressurize the eluents. For a linear gradient, eluent A was 100 m*M* sodium hydroxide and eluent B contained 100 m*M* sodium hydroxide and 200 m*M* sodium acetate. Samples were injected manually using a 50-μl-volume sample loop. Oligosaccharides were separated by a linear gradient elution with eluent A and B over the period of 40 min. A Carbopac (PA) guard column (25 × 3 mm) was used in all experiments. Sodium hydroxide (500 m*M*) was added to the post-column effluent via a mixing tee at 0.2 ml/min using a Dionex Auto-Ion reagent pump. Detection of oligosaccharides was accomplished by PAD with a gold working electrode[8,9]. The following pulse potential and durations were used E_1 = 0.05 V (t_1 = 360 ms), E_2 0.80V (t_2 = 120 ms), E_3 = −0.60V (t_3 = 420 ms). The response time of the PAD system was set to 3 s.

RESULTS AND DISCUSSION

Four different glycoproteins were digested with N-glycanase and the released oligosaccharides were separated by high-pH anion-exchange HPLC using 0–200 m*M* sodium acetate gradient in 100 m*M* sodium hydroxide. Fig. 2 shows the N-linked oligosaccharide profile of human IgG, human serum transferrin, human α_1-antitrypsin, and rDNA-derived glycoprotein produced in cell culture. Human IgG oligosaccharides were eluted as a cluster of multiple peaks (Fig. 2A). Both α_1-antitrypsin and transferrin produced one major peak each with identical retention time along with several minor peaks while rDNA-derived glycoprotein showed two major peaks (Fig. 2A). Under the conditions used, *ca.* 70–90% of N-linked oligosaccharides in glycoproteins were released by N-glycanase digestion as estimated by the carbohydrate analysis of deglycosylated protein and in the case of transferrin and rDNA-derived glycoprotein, by blotting with concanavalin A-peroxidase following SDS-gel electrophoresis[14]. Denaturation of glycoproteins with either SDS or mercaptoethanol did not enhance the release of oligosaccharides by N-glycanase. Comparison of elution profile of oligosaccharides in human serum transferrin and α_1-antitrypsin suggests that both may contain similar oligosaccharide structures (Fig. 2A). Both

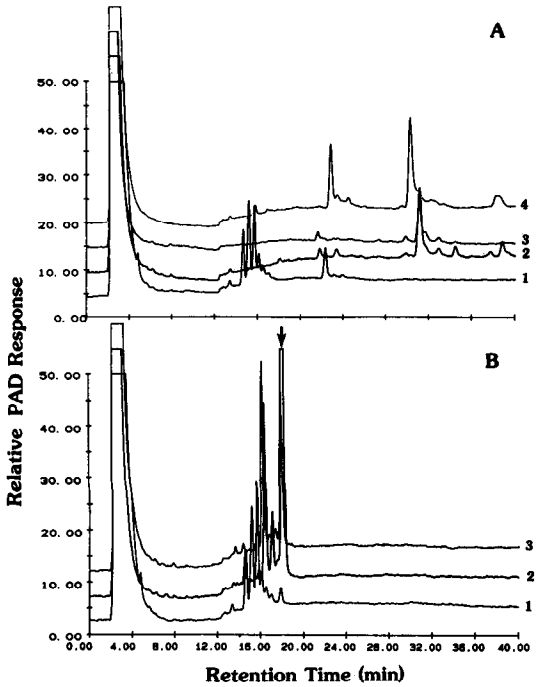

Fig. 2. HPLC separation of N-linked oligosaccharides released from glycoproteins by digestion with N-glycanase alone (A), followed by neuraminidase treatment (B). Oligosaccharide profiles were from (1) 150 μg of human IgG, (2) 100 μg of human α_1-antitrypsin, (3) 100 μg of human transferrin, (4) 25 μg of rDNA-derived glycoprotein. Oligosaccharides were separated on an anion-exchange column with a linear gradient of 0–200 m*M* sodium acetate in 100 m*M* sodium hydroxide over a period of 40 min and detected by PAD. The arrow indicates the elution position of NeuAc standard (1 nmol).

glycoproteins were shown previously by various analytical techniques[18,19] to carry two oligosaccharide chains each with common sialylated diantennary structures, and α_1-antitrypsin, in addition to two diantennary oligosaccharides, also carried an oligosaccharide with triantennary structure[18]. rDNA-derived glycoprotein contained predominantly diantennary structure, but unlike α_1-antitrypsin and serum transferrin, its oligosaccharides carried fucose residues[20].

In order to find out sialylated oligosaccharides in the chromatographic profile in Fig. 2A, N-glycanase released oligosaccharides were digested with neuraminidase and rechromatographed. Neuraminidase treatment converted most of the oligosaccharide peaks in α_1-antitrypsin, transferrin and rDNA-derived glycoprotein (data not shown) into faster eluting species while the majority of oligosaccharides in IgG remained unaffected (Fig. 2B). The release of NeuAc in the digest was confirmed by coelution experiments with authentic NeuAc standard. Under similar chromatographic conditions, glycoprotein-derived sialylated oligosaccharide standards were well separated from their asialo forms (Figs. 2 and 3). These experiments confirm the earlier finding that the oligosaccharides with sialic acid moieties interact strongly with the column and elute progressively later[12]. This HPLC technique has been shown to

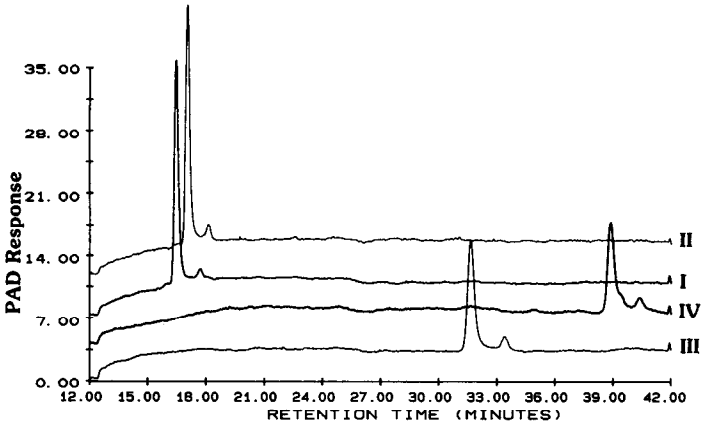

Fig. 3. Separation of NMR-verified oligosaccharide standards in Fig. 1 (5 nmol each) by anion-exchange HPLC under identical conditions as in Fig. 2.

separate positional isomers in both sialylated and neutral oligosaccharide structures[11,12]. In the case of neutral oligosaccharides, separation appears to be dependent on the relative acidity of sugar hydroxyl groups at alkaline pH and also their accessibility to the column matrix[11–13]. The molar response factor for desialylated oligosaccharides in Fig. 3 was found to be two-fold higher compared to sialylated oligosaccharides. Such differences in the electrochemical responses between synthetic neutral and sialylated oligosaccharides were observed previously[12,13]. During quantitative analysis of monosaccharides, molar response for aminosugars was found to be 20–30% higher than that obtained for neutral sugars such as glucose[10]. Hence, for

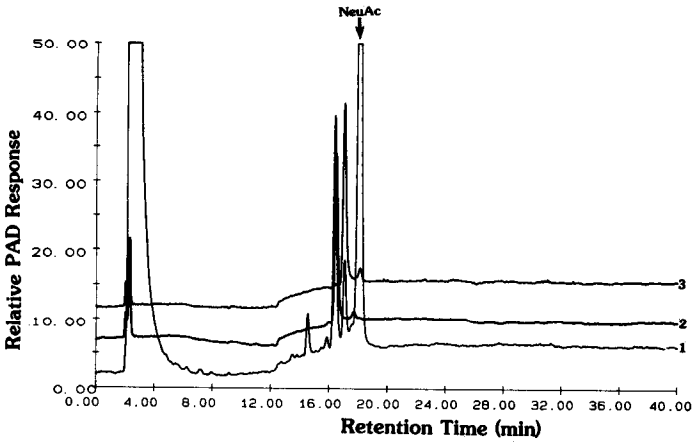

Fig. 4. HPLC analysis of oligosaccharides of α_1-antitrypsin and oligosaccharide standards. (1) Oligosaccharides from α_1-antitrypsin following digestion with neuraminidase and N-glycanase, (2) and (3) NMR-verified asialo biantennary and asialo triantennary standards (5 nmol each), respectively. Arrow indicates the elution position of NeuAc standard. Chromatographic conditions as in Fig. 2.

quantitative analysis of oligosaccharides in glycoprotein, response curves for each standard oligosaccharide must be obtained.

The ultility of the HPLC method for identifying unknown peaks in the sample was investigated using α_1-antitrypsin as a model glycoprotein with NMR-verified oligosaccharide standards. α_1-Antitrypsin contains three N-glycosylation sites carrying mostly two sialylated biantennary and one sialylated triantennary chains[18]. α_1-Antitrypsin was digested with neuraminidase and N-glycanase, sequentially and chromatographed. Fig. 4 shows the elution profile of N-glycanase-released, desialylated oligosaccharides of α_1-antitrypsin. Four major chromatographic peaks were seen in the enzyme digest (Fig. 4); three of them coeluted with asialo biantennary, asialo triantennary and NeuAc standards, respectively (Fig. 4). The identity of the fastest eluting peak was not known (Fig. 4). The relative proportion of biantennary and triantennary chains in α_1-antitrypsin as calculated from peak areas in Fig. 4. corresponded to the relative number of these structures present in the molecule[18], suggesting that this technique can be used to tentatively identify unknown oligosaccharides in the glycoproteins with previously characterized authentic standards. However, post-column analysis of oligosaccharide peaks by techniques such as fast atom bombardment mass spectrometry and high-field NMR spectroscopy may be needed to confirm unknown oligosaccharide structure since structural isomers and other unrelated oligosaccharides might have identical chromatographic retention time.

The elution time characteristics of N-glycanase released oligosaccharides from glycoproteins in Fig. 2 was highly reproducible. This led us to investigate whether this HPLC technique could be used to monitor the consistency of glycosylation of rDNA-derived therapeutic glycoprotein produced in cell culture. This glycoprotein was shown previously to carry Asn-linked oligosaccharides at a single glycosylation site in the molecule and contained predominantly fucosylated, mono/disialylated, biantennary structures[20]. Fig. 5 shows N-glycanase-released oligosaccharide profile of five

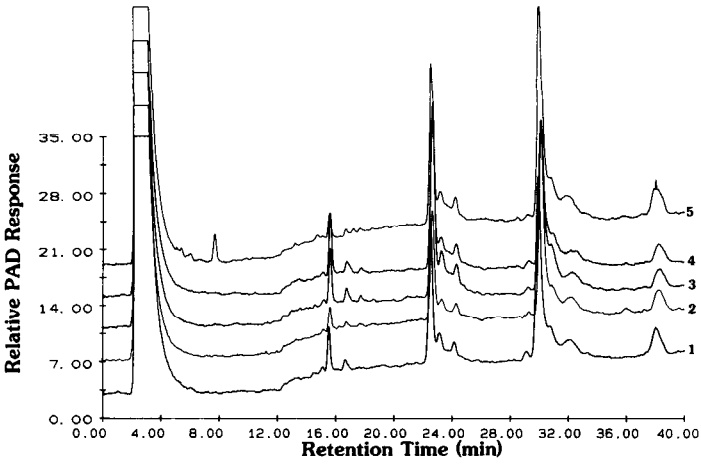

Fig. 5. Anion-exchange HPLC analysis of N-glycanase-released oligosaccharides from rDNA-derived glycoprotein batches. Approximately 25 μg of glycoprotein from each batch (1–5) following N-glycanase digestion was analysed under identical conditions as in Fig. 2.

different batches of glycoproteins. The enzymatic digestion released more than 90% of carbohydrate in each of five glycoprotein batches as estimated by SDS-gel electrophoresis. There are two oligosaccharide peaks and several minor species (Fig. 5). Qualitatively, the chromatographic profiles of five batches are similar (Fig. 5) except for batch number five which showed an additional peak (Fig. 5). Once the structure of the oligosaccharide in the glycoprotein is established, the consistency and identity of N-linked oligosaccharides in the glycoprotein can be monitored by comparing the chromatographic profile of N-glycanase digest of production batches *versus* the reference standard chromatographed in parallel. The structures of oligosaccharides are heterogenous even at single glycosylation sites on the polypeptide chain. HPLC techniques are increasingly being used to analyse the microheterogeneity of oligosaccharides in glycoproteins[3,7,13,15]. The chromatographic selectivity and specificity of the high-pH anion-exchange HPLC with pulsed amperometry offer a rapid and sensitive analysis of oligosaccharide distribution in glycoproteins.

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