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Short communication

Carbohydrate release from picomole quantities of glycoprotein and characterisation of glycans by high-performance liquid chromatography and mass spectrometry

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Abstract

Samples of 5 to 20 μg of human IgG were subjected to dithiothreitol treatment to reduce disulphide bridges, followed by tryptic digestion. Glycans released from the tryptic peptide mixture by PNGase F digestion were then derivatised with 2-aminoacridone. Labelled oligosaccharides were separated by normal-phase high-performance liquid chromatography and individual components were collected for matrix-assisted laser desorption ionization time-of-flight and electrospray mass spectrometric analysis. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Glycoproteins; Glycans; Carbohydrates; Immunoglobulins

1. Introduction

The presence of a number of N-linked glycans at a single asparagine residue renders characterisation of protein glycosylation a complex exercise. It is common practise to first release carbohydrate by chemical or enzymatic methods and then analyse by high-performance liquid chromatography (HPLC) [1]. As most glycans lack a suitable chromophore, direct analysis can be carried out using an ion-exchange column at alkaline pH values followed by amperometric detection [2]. Alternatively mixtures of glycans are derivatised by reductive amination, separated by reversed- or normal-phase chromatography and detected by fluorescence detection [3].

Different glycoforms of the same glycoprotein can have different biological properties [4]. Moreover, knowledge of the distribution of the glycoforms covalently attached to a particular protein recovered from a biological fluid or tissue can be important as this can be diagnostic of a disease process or of exposure to a toxin or drug substance. However, the levels of protein normally available are low, commonly between 5 to 20 μg, so that efficient methods are required both for release of glycans and detection at the picomole level.

In this study we have investigated the enzymatic release of carbohydrate from human immunoglobulin G (IgG) at levels between 5 and 20 μg. We present a detailed protocol outlining the clean-up of glycan

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samples prior to derivatisation with 2-aminoacridone [1,5,6], separation of mixtures of labelled glycans by normal-phase HPLC and collection of individual oligosaccharides for mass spectrometric analysis.

2. Materials and methods

Human IgG was purchased from Sigma (unknown purity). GlycoClean cartridges were purchased from Oxford GlycoSciences. Sep-Pak, C_{18} cartridges were obtained from Waters.

2.1. Enzymatic release of glycans

For small scale release of glycans 5 μg to 20 μg IgG was dissolved in 40 μl of 5 m*M* dithiothreitol (DTT) in 0.6 *M* Tris buffer, pH 8.5. The sample was vortexed and incubated at 37°C for 30 min. Then 1 μl of 1 *M* iodoacetic acid (IAA) was added and the sample incubated at room temperature for 30 min in the dark. The reaction was terminated by diluting the sample in 5% acetic acid. The sample was then desalted using a Sep-Pak C_{18} cartridge. The cartridge was primed with 2 ml methanol, 2 ml 5% acetic acid, 2 ml propan-1-ol and 4 ml 5% acetic acid. The sample was dissolved in 200 μl of 5% acetic acid, applied to the cartridge and washed with 2 ml of 5% acetic acid. The IgG was eluted with 2×1 ml 80% propan-1-ol in 5% acetic acid. This fraction was freeze–dried and then dissolved in 40 μl of 50 m*M* sodium phosphate buffer, pH 8.4 and 1 μl of 1 mg/ml trypsin (Sigma) added. This was incubated for 5 h at 37°C. To stop the reaction the sample was heated at 100°C for 3 min and freeze–dried. The trypsin digested IgG was dissolved in 40 μl of 50 m*M* sodium phosphate buffer, pH 8.4 and 1 μl of PNGase F (Boehringer) added. This was incubated for 18 h at 37°C followed by freeze–drying.

2.2. Clean-up protocol

Mini GlycoClean cartridges were prepared by dismantling a normal sized GlycoClean H cartridge (Oxford GlycoSciences) and resuspending the packing in 1 ml of 50% methanol. From this 10 mini-columns were made in 1-ml polypropylene filtration tubes. The GlycoClean columns were sequentially washed with 1 ml of 1 *M* sodium hydroxide, 2 ml of water, 1 ml of 30% acetic acid, 2 ml water, 1 ml 50% acetonitrile–1% trifluoroacetic acid–water and finally 2 ml 5% acetonitrile–1% trifluoroacetic acid–water. The sample was dissolved in 100 μl of water and applied to the column. The column was then washed with 1 ml of water followed by 1 ml of 5% acetonitrile–1% trifluoroacetic acid–water. The glycans were eluted by the application of 2×0.5 ml of 50% acetonitrile–1% trifluoroacetic acid–water. These fractions were collected and freeze–dried.

2.3. Derivatisation of glycans with 2-amino-acridone (2-AMAC) and HPLC analysis

Derivatisation was as described previously [1,5,6] except that the sodium cyanoborohydride was prepared in dimethyl sulphoxide–acetic acid (17:3) and the reaction was carried out at 70°C for 2 h and the reaction volume was kept at 10 μl. Analysis by hydrophilic interaction liquid chromatography was as described previously [7].

2.4. Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the derivatised glycans was carried out using a TofSpec SE mass spectrometer operated in the reflectron mode. Photon irradiation from a 337 nm pulsed nitrogen laser and 25 kV accelerating voltage was used. The instrument was externally calibrated using the $[M+H]^+$ peaks of the peptides Substance P (M_r 1347) and adrenocorticotropic hormone fragment 18–39 (ACTH, M_r 2436) using an α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in acetonitrile–water, 1:1).

For the 2-AMAC-derivatised glycan fractions, a different matrix solution was prepared consisting of a mass (7.5 mg) of 2,5-dihydroxybenzoic acid and (2.5 mg) of 1-hydroxyisoquinoline dissolved in 1 ml of acetonitrile–water (1:1). The lyophilised glycan fractions were reconstituted in 2 μl of water and an aliquot (1 μl) of these solutions were spotted along with the matrix (1 μl) onto a stainless steel target and allowed to dry at room temperature. Derivatised

glycan fractions were detected as their $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ adducts.

Electrospray MS and MS–MS spectra were acquired on a Micromass Q-Tof hybrid quadrupole orthogonal TOF instrument (Manchester, UK) fitted with a nanoflow Z-spray ion source. Collected fractions from the normal-phase HPLC were redissolved in 5 μl of methanol–0.1% formic acid (1:1, v/v) and loaded into borosilicate nanoflow tips. In MS mode the source temperature was set to 50°C, the capillary voltage was 1200 V, the cone voltage was 48 V and the collision energy was set to 4 V. For MS–MS acquisitions, nitrogen was introduced into the collision cell (pressure $6 \cdot 10^{-5}$ Torr; 1 Torr= 133.322 Pa) and the collision energy was adjusted to produce the desired degree of fragmentation, typically 20 V.

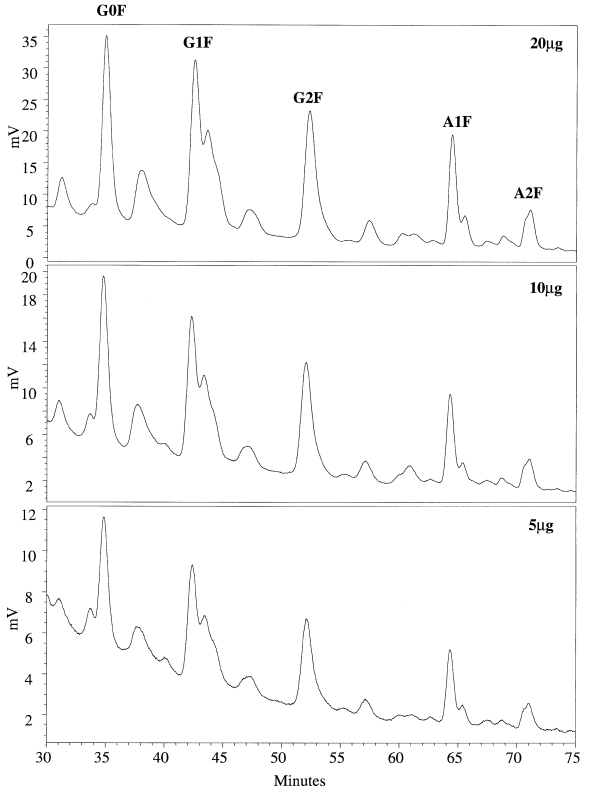

Fig. 1. Normal phase chromatographic profiles for 2-AMAC derivatised glycans released from (a, upper chromatogram) 20 μg, (b, centre chromatogram) 10 μg and (c, lower chromatogram) 5 μg IgG. See Fig. 2 for explanation of glycan structures.

3. Results and discussion

For this study we used human IgG as a model glycoprotein to optimise the yield of glycans released by enzyme digestion with PNGase F. Unlike our previous studies [1,6] we carried out the trypsin cleavage and deglycosylation steps in smaller volumes and samples were desalted using Sep-Pak columns rather than dialysis. The reason for these changes were to minimise losses in yield.

In order to determine the minimum amount of glycoprotein that was required for efficient release of glycans and sufficient characterisation by mass spectrometric methods, glycans were released from 5 μg, 10 μg and 20 μg of IgG. Fig. 1 shows the normal-phase chromatograms obtained after release of glycans from 5, 10 and 20 μg of IgG (see Fig. 2 for glycan structures). In each case the pattern obtained from separation of the glycan pool is similar. In an attempt to quantitate the relative yield for each of the different release experiments, the areas of each of the

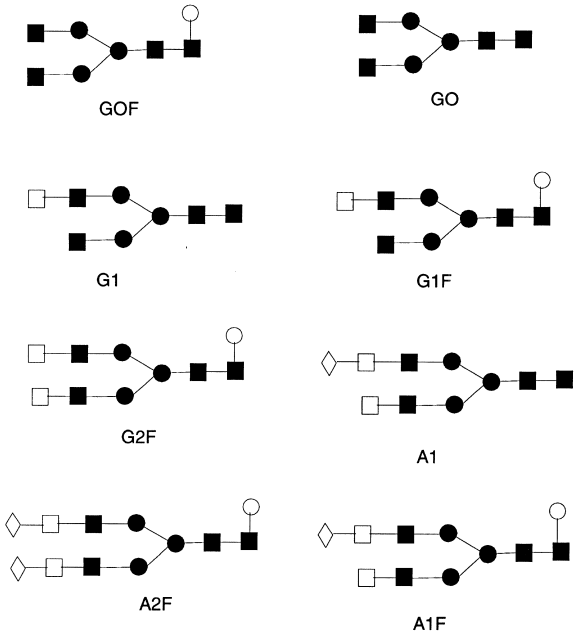

Fig. 2. Structures of glycans referred to in Fig. 1. The following symbols have been used to identify the various sugar residues: (■) *N*-acetyl glucosamine; (●) mannose; (□) galactose; (○) fucose; (◇) sialic acid. Linkages between sugar residues have been omitted for simplicity.

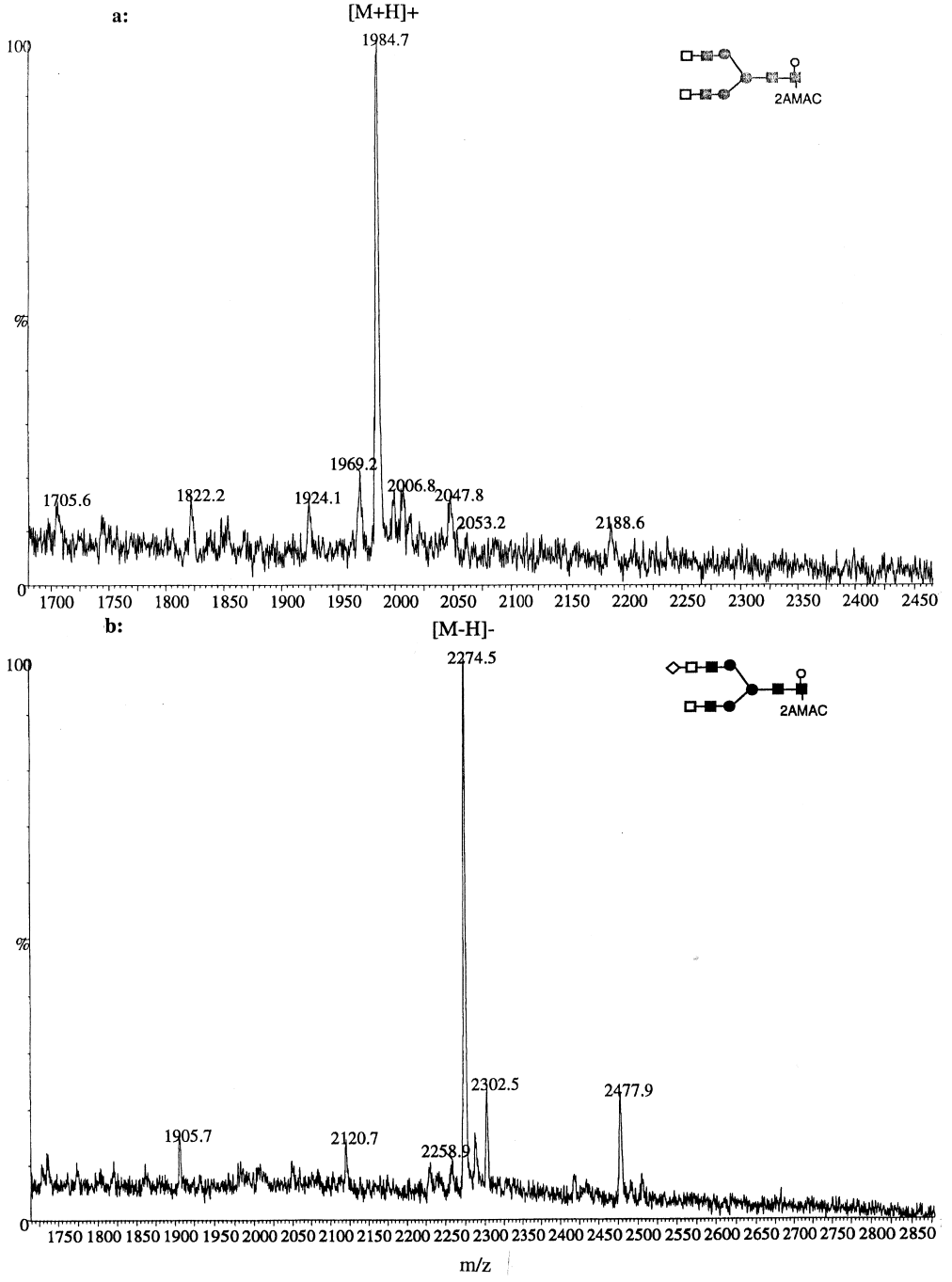

Fig. 3. MALDI-TOF data obtained for collected fractions from normal-phase chromatography of glycans released from 10 μg of IgG. (a) G2F shown as $[M+H]^+$, and (b) A1F acquired in negative mode shown as $[M-H]^-$.

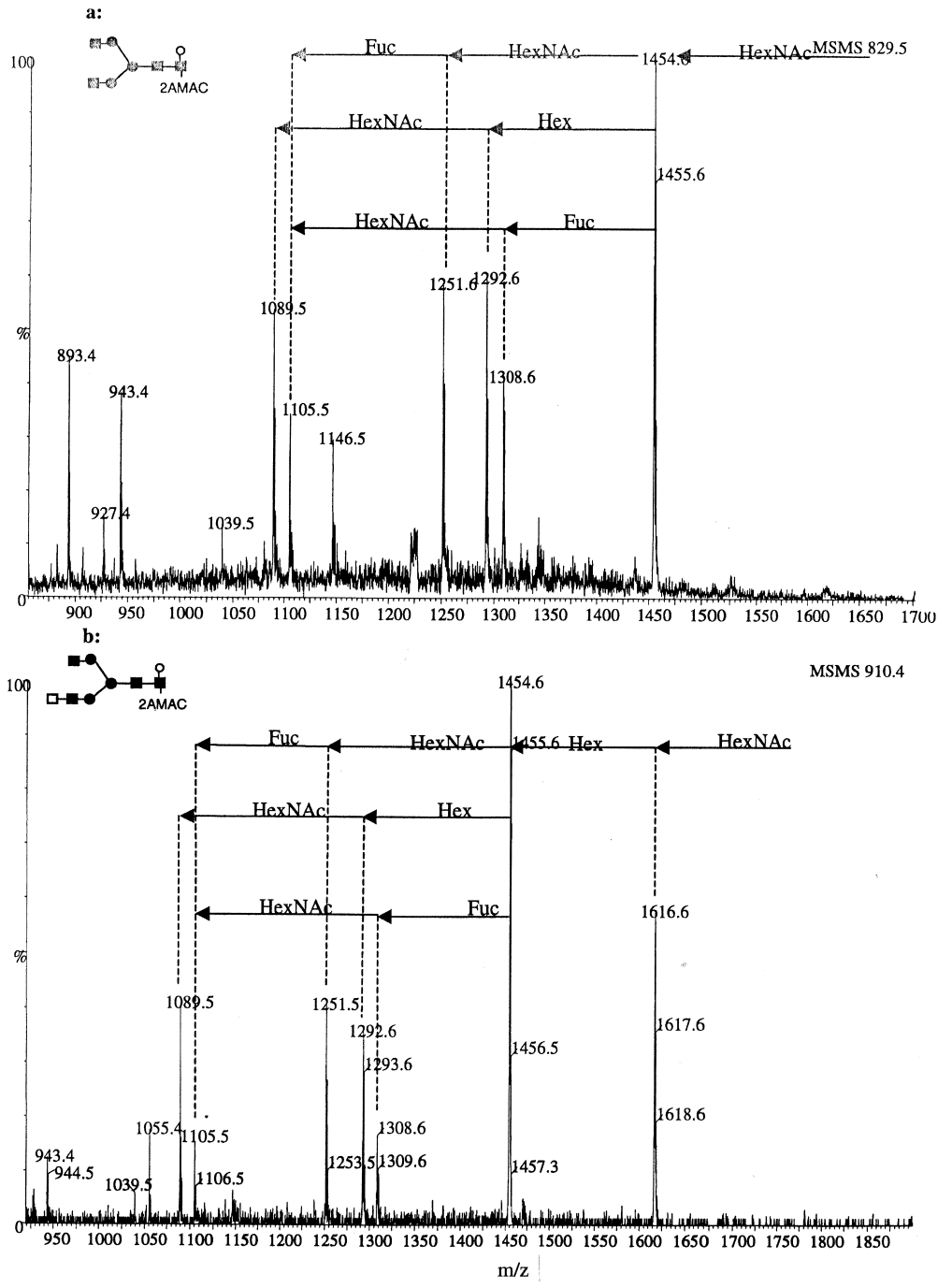

Fig. 4. Q-Tof MS–MS sequencing data obtained for collected fractions from normal-phase chromatography of glycans released from 10 μg of IgG. (a) G0F and (b) G1F.

peaks for the glycans were calculated. The total yield was estimated by calibration of the system by injection of a glycan standard at 0.2 μg/μl. It was found that 20 μg of IgG yielded 0.10±0.015 μg of the major glycans (G0F, G1F, G2F A1F and A2F), 10 μg yielded 0.05±0.010 μg and 5 μg yielded 0.02±0.002 μg. From this it is quite clear that the percentage yield is reproducible down to as low as 5 μg. Although the human IgG used was of unknown purity, the low levels of protein used in the present study relate to low picomole quantities of protein and subsequently picomoles of glycan.

Similar experiments were also carried out on 2.5 μg of IgG, but at such low concentrations artefacts (system peaks) were found to interfere with the glycan peaks of interest so that it was not possible to estimate accurately the yield of the derivatised glycans. From these experiments it was determined that the minimum amount of IgG that was required for HPLC analysis was of the order of 5 μg. From the chromatographic characteristics of the glycans it was also possible to determine likely structure by assigning each of the peaks with a glucose unit equivalent value (GU) value [6].

Although GU values are a convenient and quick method of assigning a glycan structure, the assignment has to be considered preliminary, and it is often necessary to confirm a glycan structure by mass spectrometric analysis. Thus each of the major glycans were also collected manually as fractions and subjected to mass spectrometric analysis either by MALDI or MS–MS sequencing on the Q-Tof [8,9].

MALDI analysis was only possible on fractions of glycans released from 10 or 20 μg of IgG. Fig. 3 shows typical MALDI data obtained for two of the glycan fractions, G2F and A1F released from 10 μg of IgG. G2F was analysed by positive ion MALDI and identified as the protonated species $[M+H]^+$ (Fig. 3a). The sialylated species A1F was analysed by negative ion MALDI as the $[M-H]^-$ ion (Fig. 3b).

For the two major fractions G1F and G0F, again released from 10 μg of IgG, it was possible to obtain MS–MS sequencing data on the Q-Tof, allowing determination of the complete sequence of these 2-AMAC labelled glycans as shown in Fig. 4. It was not possible to sequence any of the less abundant components by electrospray ionisation mass spectrometry due to low signal-to-noise ratios.

In conclusion, methods have been developed allowing the release of complex glycans from quantities of IgG as low as 5 μg and analysis by HPLC. For sufficient mass spectrometric data to be acquired, 10 μg of IgG were necessary. However, IgG is estimated to have only about 3% of its molecular mass as glycans. As a large number of glycoproteins contain a much higher percentage of oligosaccharides, the methodology described is expected to be applicable for the characterisation of glycans released from low μg quantities of a wide range of glycoproteins.

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