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Characterisation of oligosaccharides from a glycoprotein variant of human serum albumin (albumin Casebrook) using high-performance anion-exchange chromatography and nuclear magnetic resonance spectroscopy

Paul A. Haynes and Michael Batley

School of Chemistry, Macquarie University, North Ryde 2109, N.S.W. (Australia)

Robert J. Peach and Stephen O. Brennan

Molecular Pathology Laboratory, Department of Pathology, Christchurch School of Medicine, Christchurch (New Zealand)

John W. Redmond

School of Chemistry, Macquarie University, North Ryde 2109, N.S.W. (Australia)

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ABSTRACT

The characterisation of oligosaccharides present on albumin Casebrook, a glycoprotein variant of human serum albumin, which contains an N-linked oligosaccharide at an attachment site formed by a point mutation of 494 Asp→Asn, is described. The monosaccharide compositional analysis of purified glycopeptides suggested the presence of complex biantennary carbohydrate structures. The oligosaccharides which were released by N-glycosidase-F appeared to be a single molecular species according to their retention on high-performance anion-exchange chromatography. The structure of the oligosaccharide was suggested by sequential exoglycosidase digestions and confirmed by proton nuclear magnetic resonance spectroscopy. It was concluded that the oligosaccharides were essentially homogeneous and consisted of an α(2–6)-disialylated complex biantennary glycan.

INTRODUCTION

Serum albumin, the most abundant human plasma protein, consists of a single chain of 585 amino acids [1] and is not glycosylated. A number of genetic variants, or alloalbumins, have been identified and characterised [2–4], with two alloalbumins reported, Redhill [5] and Casebrook [6], which contain point mutations that introduce N-linked oligosaccharide attachment sites and lead to the subsequent addition of carbohydrate.

Albumin Casebrook, which has been detected in two unrelated individuals, is expressed heterozygously but constitutes only 35% of total serum albumin present with the remainder occurring as normal albumin [6]. Characterisation by electrophoresis, peptide mapping [6] and protein sequencing [7] has shown that the change in molecular mass is due to the attachment of an N-linked oligosaccharide at an Asn-Glu-Thr site intro-

Correspondence to: Paul A. Haynes, School of Chemistry, Macquarie University, North Ryde 2109, N.S.W., Australia.

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duced by a point mutation of 494 Asp → Asn. The nature and extent of glycosylation of such adventitious attachment sites may contribute to our understanding of protein processing in the liver.

The use of high-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection has been demonstrated in the separation of a wide variety of glycoconjugates [8,9]. Exoglycosidase enzymes have been used in the determination of oligosaccharide structures, but usually in conjunction with radiolabelling and large-bore gel permeation chromatography [10,11] or precolumn derivatisation and reversed-phase high-performance liquid chromatography (HPLC) [12]. In the present investigation we have been able to combine the advantages of speed and resolution of HPAEC with sequential exoglycosidase digestions by using efficient desalting procedures. Proton nuclear magnetic resonance spectroscopy (NMR) provided additional structural information in the oligosaccharide characterisation.

EXPERIMENTAL

Purification of Casebrook albumin glycopeptides

Glycopeptides from Casebrook albumin were prepared as previously described [6]. Briefly, plasma samples were applied to DEAE Sephadex and Concanavalin A-Sepharose columns to purify the intact glycoprotein. After digestion with trypsin or chymotrypsin, glycopeptides were purified using the Concanavalin A-Sepharose column. This procedure was used for purification only, and no fractionation was attempted.

Carbohydrate analysis

Analysis of monosaccharides and oligosaccharides was performed using HPAEC with pulsed amperometric detection. The system was from Waters and consisted of a quaternary M-6000 pump, a 600E gradient controller, a 464 electrochemical detector, an SSI post-column mixing pump, a Dionex Carbopac-1 250 mm × 4.6 mm I.D. column and Waters Baseline data acquisition software running on an NEC APC-IV computer. The post-column mixing pump was used to add 0.6 *M* sodium hydroxide to column eluents prior to detection.

The gradient used for separation of desialylated oligosaccharides was 0–100 m*M* sodium acetate in 30 min, with sodium hydroxide maintained at 160 m*M*. For sialylated oligosaccharides, the gradient used was 0–400 mM sodium acetate in 30 min, with sodium hydroxide maintained at 250 m*M*. Monosaccharides were separated using isocratic elution with 15 m*M* sodium hydroxide.

Hydrolysis procedures

Samples were placed in glass autosampler vials (ICI Instruments) and dissolved in 400 μl of the appropriate acid. These were placed in a hydrolysis vessel (Pierce, Rockford, IL, USA) which was then sealed under argon. After hydrolysis the samples were transferred to polypropylene tubes and lyophilised. Conditions used were: (A) 2 *M* trifluoroacetic acid at 100°C for 3 h for monosaccharides; (B) 4 *M* hydrochloric acid at 100°C for 6 h for amino sugars; and (C) 0.1 *M* trifluoroacetic acid at 80°C for 45 min for liberation of sialic acids.

Enzyme digestions

Glycopeptides were digested with N-glycosidase F from *Flavobacterium meningosepticum* (EC 3.2.2.18) using 1 U/100 μg glycopeptide in 100 m*M* ammonium bicarbonate at pH 7.9, at 37°C. The digests were acidified with acetic acid, passed through a cation-exchange column (Bio-Rad AG-50-X2, 50 mm × 5.0 mm I.D.) to remove peptide material [13], and lyophilised.

Desialylated samples were purified from liberated sialic acid by elution from an anion-exchange column (BioRad AG-1-X8, formate form, 50 mm × 5.0 mm I.D.) with 20 ml of water, and lyophilised.

Digestion with β-galactosidase from *Diplococcus pneumoniae* (EC 3.2.1.23) was performed with 5 mU/30 μg glycopeptide in 50 m*M* sodium acetate, pH 6.0, at 37°C for 16 h. Digestion with β-N-acetylglucosaminidase from *Diplococcus pneumoniae* (EC 3.2.1.30) was performed with 5

mU/20 μg glycopeptide in 50 m*M* citrate–phosphate buffer, pH 4.8, at 37°C for 16 h.

Reversed-phase HPLC and desalting

A Pharmacia Smart System HPLC apparatus fitted with a 214-nm UV detector was used for both reversed-phase HPLC to monitor the digestion of the glycopeptide and purifying and desalting oligosaccharides after enzyme digestions. Reversed-phase chromatography was performed with a C_{18}–C_2 (100 mm × 2.1 mm I.D.) column using elution with a gradient from 0.05% (v/v) trifluoroacetic acid in 10% acetonitrile to 0.05% (v/v) trifluoroacetic acid in 70% acetonitrile over 12 min at a flow-rate of 300 μl/min. Desalting was performed with a Pharmacia Fast Desalting column (100 mm × 3.2 mm I.D.) eluted with water at a flow-rate of 50 μl/min, with oligosaccharides collected in the 150–450 μl fraction of the eluent.

Nuclear magnetic resonance spectroscopy

For proton NMR analysis, oligosaccharides were exchanged twice with ^2H_2O with intermediate lyophilising. The spectra were recorded at 27°C and 400 MHz on a Varian XL-400 spectrometer. Chemical shifts were measured relative to internal acetone at 2.225 ppm.

RESULTS

Monosaccharide compositional analysis

Aliquots of the purified glycopeptides (1 nmol) were analysed for monosaccharide composition after hydrolysis with condition A (see *Hydrolysis procedures*), using HPAEC with pulsed amperometric detection. The results (Table I) were consistent with a complex biantennary carbohydrate structure and showed no trace of fucose which is often present on such structures. A second series of analyses using hydrolysis condition B (see *Hydrolysis procedures*) which is more appropriate for amino sugars gave very similar results for composition, which indicated that there were no highly acid-resistant linkages present. HPAEC of sialic acids released from the oligosaccharide using hydrolysis condition C (see *Hydrolysis procedures*) (data not shown) confirmed the presence of N-acetylneuraminic acid and the absence of N-glycolylneuraminic acid.

TABLE I

COMPOSITIONAL ANALYSIS OF CASEBROOK ALBUMIN GLYCOPEPTIDE

Values presented are from triplicate analyses.

Monosaccharide	Glycopeptide content (mol/mol)
Glucosamine	2.2 ± 0.11
Galactose	0.9 ± 0.01
Mannose	1.3 ± 0.04
Galactosamine	Not detected
Fucose	Not detected
Glucose	0.2 ± 0.01

Release of oligosaccharides by N-glycosidase F

Glycopeptides were digested with N-glycosidase F to release oligosaccharides. This reaction was followed by analysing aliquots on reversed-phase HPLC and monitoring the disappearance of the starting material and the appearance of the deglycosylated peptide. The time course (Fig. 1) indicates that the digestion is rapid and quantitative, and shows there is no subpopulation of the glycopeptide that is resistant to digestion.

High-performance anion-exchange chromatography of released oligosaccharides

The oligosaccharides released by N-glycosidase F were characterised by their chromatographic behaviour. A gradient elution system was used which separates standard oligosaccharides from bovine fetuin into well defined groups of compounds containing different numbers of sialic acid residues. The results (Fig. 2) show that the oligosaccharide elutes at a position corresponding to a disialylated oligosaccharide. There was only one major compound detected, with a minor component (less than 10% by peak area) eluting slightly later. This minor component appears to be isomeric in the attachment of sialic acids, as it is no longer present in the desialylated oligosaccharides (Fig. 3A). Similar chromatographic conditions have been used previously to distinguish

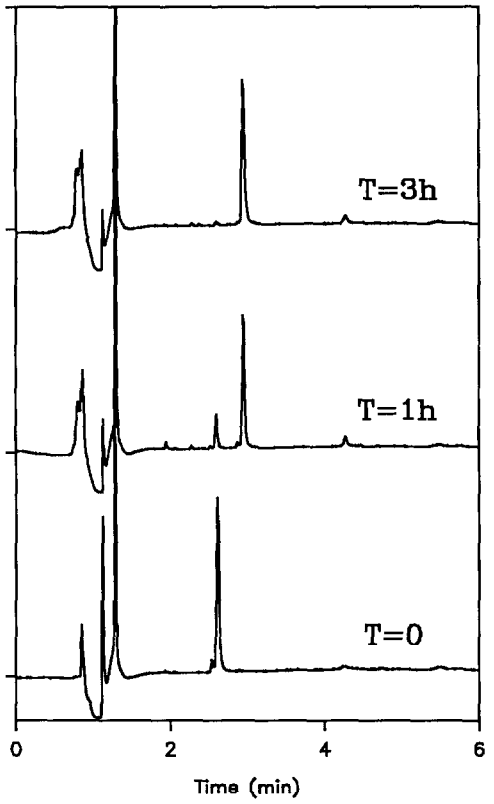

Fig. 1. Time course of digestion of Casebrook albumin glycopeptide with N-glycosidase F. Aliquots were analysed by reversed-phase HPLC as described in Experimental.

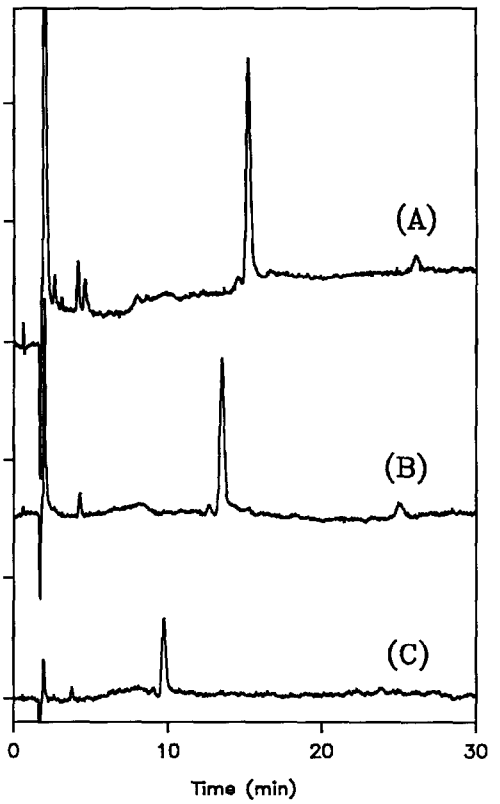

Fig. 3. HPAEC of Casebrook albumin oligosaccharides at different stages of digestion: (A) after 0.1 *M* trifluoroacetic acid hydrolysis and anion-exchange clean-up to remove liberated sialic acid; (B) after digestion with *Diplococcus pneumoniae* β-galactosidase; (C) after digestion with *Diplococcus pneumoniae* β-N-acetylglucosaminisase.

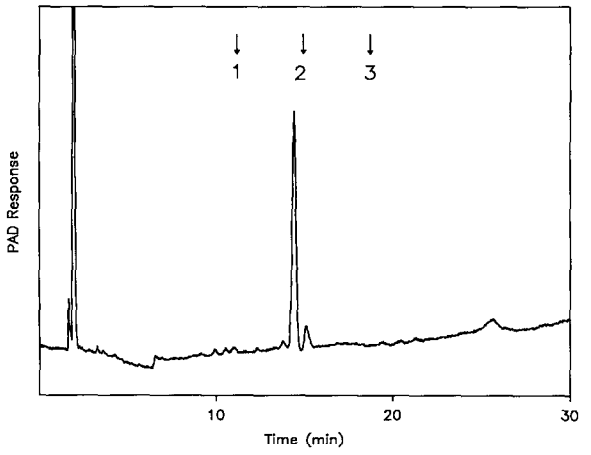

Fig. 2. HPAEC of oligosaccharides released from Casebrook albumin glycopeptide. Arrows indicate the elution position of (1) mono-, (2) di-, and (3) trisialylated oligosaccharides from bovine fetuin.

heterogeneity in the attachment of sialic acids [14]. Thus, the chromatographic profile suggested that the oligosaccharides were essentially homogeneous.

Sequential exoglycosidase digestion of released oligosaccharides

Chromatography of the desialylated oligosaccharides showed only one major constituent (Fig. 3A), which was sequentially digested with two exoglycosidases and monitored by HPAEC at each step. The enzymes used were β-galactosidase from *Diplococcus pneumoniae*, which is specific for β(1–4)-linked galactose [15] and N-acetyl-β-D-glucosaminidase from *Diplococcus pneu-*

moniae, which is specific for GlcNAc in β(1–2) linkage to mannose, or β(1–3) or β(1–6) linkage to galactose [16]. In each case the starting material was completely converted to an earlier-eluting product (Fig. 3B and 3C).

These results are consistent with the core structure expected of a classical biantennary complex carbohydrate [17], and the complete digestion at each step indicates there is no heterogeneity in the oligosaccharide core structure. To exclude other possibilities such as a disialylated triantennary structure or a bisected biantennary structure [17], and to establish the nature of the attachment of the sialic acids, the oligosaccharide was analysed by proton NMR spectroscopy.

Proton nuclear magnetic resonance spectroscopy

The positions of NMR signals from structural reporter groups in the oligosaccharide are presented in Table II, along with chemical shift values for α(2-6)-disialylated biantennary complex oligosaccharides that have been previously reported from three different sources: human serum α1-antichymotrypsin [18], with the first N-acetylglucosaminyl residue present in the reduced form; rat serotransferrin [19]; and human serotransferrin [20], both of which have the glycan attached to a peptide. The positions of the NMR signals are in excellent agreement with the previously reported values, with the only significant differences consistent with the differences in the

TABLE II

PROTON NMR CHEMICAL SHIFT VALUES OF REPORTER GROUPS OF OLIGOSACCHARIDES ISOLATED FROM CASEBROOK ALBUMIN

Reporter group	Residue	Chemical shift (ppm)			
		Casebrook	Literature values		
			ref. 18	ref. 19	ref. 20
H-1	1α	5.188	–	5.088	5.068
	1β	4.694	–	–	
	2	4.614	4.640	4.616	4.617
	3	4.803	4.781	4.773	–
	4	5.133	5.133	5.133	5.130
	4′	4.945	4.946	4.949	4.946
	5	4.604	4.604	4.603	4.604
	5′	4.604	4.604	4.603	4.604
	6	4.445	4.443	4.442	4.442
	6′	4.449	4.443	4.447	4.444
H-2	3	4.253	4.255	4.254	4.253
	4	4.195	4.195	4.195	4.196
	4′	4.114	4.116	4.116	4.112
H-3a	NeuAc(α(2–6))	1.754	1.716	1.716	1.718
H-3e	NeuAc(α(2–6))	2.662	2.672	2.666	2.670
		2.667		2.672	2.670
NAc	1	2.037	2.057	2.002	2.008
		2.035			
	2	2.093	2.082	2.081	2.080
		2.091			
	5	2.068	2.070	2.067	2.069
	5′	2.065	2.064	2.063	2.066
	NeuAc	2.030	2.030	2.029	2.030

structure of the first sugar residue. The positions and integrated intensities of the NMR signals provide details of the complete structure of the oligosaccharide, as explained below.

The ratios of the areas of the sialic acid H-3e and H-3a signals to the sum of the areas of the N-acetyl peaks was 2.07:2.23:18.00, with the H-3e signal resolved into two components of equal intensity. This confirms that exactly two sialic residues were present and both were α(2–6)-linked. The absence of a characteristic signal corresponding to the methyl group of fucose at 1.2 ppm confirms the absence of any fucose substituents. The relative areas for the individual N-acetyl signals were in the ratio 2.92:6.05:3.15:5.87 for the acetates on residues 2:5 and 5′:1:sialic acid. This indicates only six acetates are present, and that there is no bisecting N-acetylglucosaminyl residue. There were some impurity peaks, of peak heights less than 10% of the main signals, observed in the N-acetyl resonance region of the spectrum. Finally, using the areas Man4 H-1, Man4 H-2 and Man3 H-2 as references, the sum of the areas of the Gal6 and Gal6′ H-1 peaks confirmed that only two galactose residues were present and the chemical shift of the reporter groups confirmed that both galactoses were present in β(1–4) linkage.

CONCLUSION

The results obtained from HPAEC, sequential exoglycosidase digestion and NMR spectroscopy have enabled us to determine the structure of oligosaccharides attached to Casebrook albumin. Structural information was rapidly obtained using HPAEC profiling, and the use of a microscale HPLC system for desalting after exoglycosidase digestions minimised possible contamination due to sample handling. The oligosaccharides were found to be essentially homogeneous, consisting of a single α(2–6)-disialylated complex biantennary glycan as shown in Fig. 4.

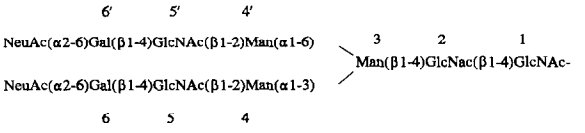

Fig. 4. Proposed structure of the oligosaccharide released from Casebrook albumin glycopeptide. Numbers given for sugar residues correspond to the assignments used in Table II.

Complex oligosaccharides purified from glycoproteins usually consist of mixtures of glycoforms [21,22], with plasma glycoproteins synthesised in the liver having been shown to contain bi-, tri- and tetraantennary complex glycans [23]. The essentially homogeneous nature of the Casebrook albumin oligosaccharides is unusual, although an identical glycan has been found as the only oligosaccharide at the four attachment sites in human antithrombin-III [24]. It is interesting to note that the addition of oligosaccharides at carbohydrate attachment sites formed by protein sequence mutations may interfere with protein function, as is the case with an antithrombin-III mutant carrying an additional oligosaccharide which displays decreased heparin affinity [25]. Casebrook albumin, along with other reported mutant albumins, has yet to be shown to have any adverse physiological effects, although it has been suggested that the carbohydrate moiety may interfere with a ligand-binding domain [6].

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