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ISOLATION OF SULFITED OLIGOSACCHARIDES FROM GLYCOPROTEINS TREATED WITH ALKALINE SULFITE*

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ABSTRACT

The oligosaccharide products resulting from treatment of mucin-type glycoproteins with alkali in the presence of the sulfite anion have been investigated. Treatment of fetuin and of tryptic glycopeptides from the human erythrocyte with this reagent resulted in the release of sulfited oligosaccharides identified as *N*-acetylsulfohexosamine ($HexNAcSO_3$), α-NeuAc-(2→6)-$HexNAcSO_3$, and α-NeuAc-(2→3)-Gal-(1→3 or 4)-[GlcNAc-(1→6)]-$HexNAcSO_3$. In addition, 2.7 moles of sialic acid were released per mole of α-NeuAc-(2→6)-$HexNAcSO_3$ from fetuin. The sulfohexosamine moiety is formed *via* unsaturated intermediates from a 3-*O*-substituted 2-acetamido-2-deoxy-D-galactosyl residue at the carbohydrate–peptide linkage site when this residue is not substituted at O-4 by another sugar residue. A reaction mechanism accounting for the release of the sulfited oligosaccharides from a 3-*O*- and 6-*O*-substituted hexosamine is proposed in which the oligosaccharide branch attached to O-6 is obtained as a specific fragment terminating in sulfohexosamine.

INTRODUCTION

The mucin-type glycoproteins are members of a major class of vertebrate glycoproteins that have carbohydrate chains attached to protein *via* an O–glycosyl bond between 2-acetamido-2-deoxy-D-galactose and L-serine or L-threonine. The determination of the amino acids involved in O–glycosyl linkage to sugars in these glycoproteins has been facilitated by the finding that the sulfite anion adds efficiently across the double bonds generated by alkali-catalyzed β-elimination of these oligosaccharides, yielding cysteic acid and 2-amino-3-sulfobutanoic acid from

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substituted serine and threonine, respectively[1]. A sulfo derivative of 2-amino-2-deoxy-D-galactose is also formed in high yield during the reaction, if the linkage hexosamine is 3-substituted, but not if it is 4-substituted[2]. Analysis for sulfohexosamine and the sulfoamino acids has been of use in structural studies of mucin-type glycoproteins[3,4], but the nature of the original oligosaccharide products released in the reaction has not been investigated, as the reaction mixture is normally hydrolyzed, for analysis of the individual derivatives therein.

It was anticipated that a 6-substituent on a 3,6-disubstituted hexosamine should be stable to the alkaline sulfite reaction-conditions, without preventing β-elimination of the 3-linked residue and formation of sulfohexosamine. By making use of model glycoproteins of known structure, we have demonstrated in this study that sulfo-oligosaccharides are, indeed, formed in this way.

EXPERIMENTAL

Materials. — Fetuin and *Clostridium perfringens* neuraminidase were obtained from Sigma Chemical Company, and Purpald® and *o*-phthalaldehyde from Aldrich Chemical Company. Tryptic peptides derived from human, MM-active erythrocytes[5] were purified by passage through Dowex-50 X-2 (H^+) resin, followed by chromatography on DEAE-cellulose. Peptides were eluted with a 0 to 1M exponential gradient of NaCl in 50mM Na formate buffer, pH 4.05, and acidic glycopeptides eluted between 0.30 and 0.56M NaCl were freed of salt by gel filtration through Sephadex G25, and then used for these studies. Sulfohexosamine was prepared as previously described[2].

Alkaline sulfite treatment. — For the alkaline sulfite treatment, glycoprotein (0.5–10 mg) was dissolved in 0.5M Na_2SO_3 in 0.1M NaOH (1 mL) and incubated at 25° for the times indicated. The reaction was monitored by removing aliquots equivalent to ~5 nmol of linkage hexosamine, and analyzing for the presence of cysteic acid and sulfohexosamine with an automated, amino acid analyzer[2]. At the completion of the reaction, as judged by no further increase in the sulfo products, the mixture was brought to ice-bath temperature, the pH was adjusted to 5.0 by the addition of 2.0M pyridine acetate buffer, pH 3.5, and cationic compounds were removed by passage through Dowex-50 X-2 (H^+) resin (1 mL of resin/mL of reaction mixture) in the cold. The column was washed with 1:1 water–methanol, and the pH of the eluate immediately adjusted to 5.0 with pyridine.

Oligosaccharide characterization. — The reaction mixture was concentrated *in vacuo* at 30°, and the concentrate applied to a column (2 × 100 cm) of Bio-Gel P2 (400 mesh; BioRad) which was eluted at 21 mL/h with 0.1M pyridine acetate buffer, pH 5.5. An aliquot (14%) of the effluent stream was monitored for formaldehyde with Purpald® after periodate oxidation in the manifold of a Technicon multi-channel, autoanalyzer pump[6]; the rest was collected in 10-min fractions. The column was calibrated with fetuin (void volume); alkali-labile oligosaccharides from fetuin[4]: α-NeuAc-(2→3)-β-Gal-(1→3)-α-NeuAc-(2→6)-GalNAc-ol; α-

NeuAc-(2→3)-β-Gal-(1→3)-GalNAc-ol; β-Gal-(1→3)-GalNAc-ol; NeuAc; GalNAc-ol; and Gal-ol.

Digestions with neuraminidase were conducted for 18 h at 37° with ~10 nmol of oligosaccharide and 20 mU of enzyme in 0.1M sodium acetate buffer, pH 5.0. Release of sialic acid was monitored by the thiobarbituric acid assay[7].

Periodate oxidation was conducted for 18 h at room temperature in the dark with oligosaccharide (30 nmol) and 50mM sodium periodate (0.5 mL) in 50mM sodium acetate buffer, pH 4.5. To terminate the reaction, 1:1 glycerol–water (50 μL) was added, following which, the pH was raised to 7–8 with sodium borate buffer, and the oligosaccharides were reduced with sodium borohydride. After addition of acetic acid, the sample was passed through a column of Dowex-50 X-8 (H^+) resin and then applied to a column of Dowex-1 X-10 ($HCOO^-$) resin, from which it was eluted with 2.0M pyridine acetate, pH 3.5, followed by lyophilization, and hydrolysis for sugar analysis.

Analytical procedures. — After hydrolysis with M HCl for 4 h at 100°, neutral sugars were reductively aminated to glycamines with ammonium sulfate and sodium cyanoborohydride, and these were analyzed with an automated, amino acid analyzer by cation-exchange chromatography with borate buffers[8], using an *o*-phthalaldehyde system[9] for detection. Hexosamines were quantitated after acid hydrolysis and cation-exchange chromatography, as previously described[10,11]. *N*-Acetylneuraminic acid was quantitated by anion-exchange chromatography, with reducing sugar detection[12,13] and by the thiobarbituric acid assay after hydrolysis of the oligosaccharides with 25mM H_2SO_4 for 1 h at 80°. Sulfohexosamine and amino acids were determined with an automated, amino acid analyzer after hydrolysis[2] with 6M HCl for 22 h at 110°.

RESULTS AND DISCUSSION

Isolation and analysis of sulfo-oligosaccharides from fetuin. — Fetuin was treated with alkaline sulfite, and the expected conversion of 2-amino-2-deoxygalactose into sulfohexosamine was observed[2,4], whereas the 2-amino-2-deoxyglucose value was unaffected. When the reaction mixture was acidified, passed through Dowex 50 X-2 (H^+) resin and applied to a column of Bio-Gel P2, the chromatogram shown in Fig. 1 was obtained. The peaks, detected by their sialic acid content, were subsequently analyzed for amino acid and carbohydrate compositions. The peak at the void volume of the column (4.5 h) was the only peptidic fraction obtained (based on amino acid analysis) and consisted of sialic acid-containing glycopeptides having N-linked oligosaccharides that remained peptide-bound during degradation of the O-linked oligosaccharides. Peaks I–IV contained only carbohydrate, peak III consisting of free sialic acid, as identified by its elution position on Bio-Gel P2 (identical to that of a reference sample) and its reactivity in the thiobarbituric acid assay without prior hydrolysis, whereas peak IV contained a mixture of monosaccharides and monomeric *N*-acetylsulfohexosamine.

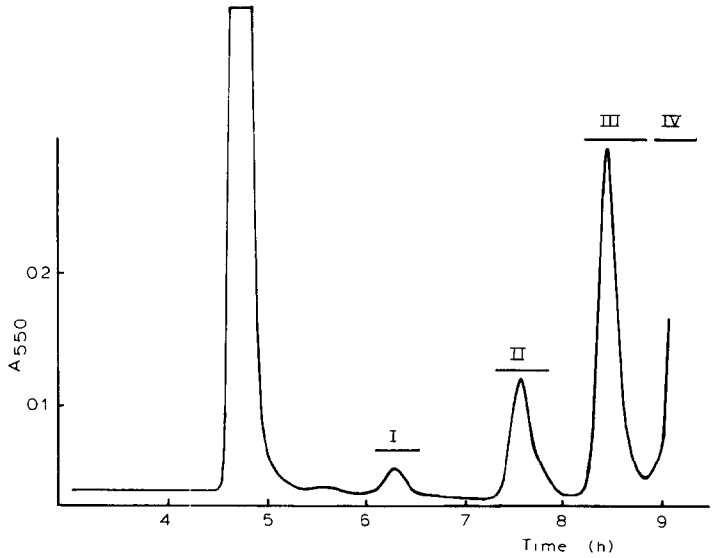


Fig. 1. Chromatography on Bio-Gel P2 of products of treatment of fetuin with alkaline sulfite. [The reaction was conducted for 144 h at room temperature, and, after passage of the reaction mixture through Dowex-50 X-2 (H^+) resin, the products were applied to a column (2 × 100 cm) of Bio-Gel P2 (400 mesh). Reaction for 72 h at 37° gave the same ratios of products. A_{550} = formaldehyde detection with Purpald® after periodate oxidation of the effluent stream.]

Peaks I and II contained oligosaccharides having molecular weights of ~800 and 520, respectively and both yielded sulfohexosamine following hydrolysis, suggesting that sulfo-oligosaccharides were, indeed, formed during alkaline sulfite treatment. Peak II contained equimolar amounts of sialic acid and sulfohexosamine (see Table I), indicating that it was a disaccharide (theoretical M_r = 534). The disaccharide was unreactive in the thiobarbituric acid assay prior to acid hydrolysis. Treatment with *C. perfringens* neuraminidase released sialic acid from the disaccharide quantitatively, demonstrating that its structure was NeuAc-

TABLE I

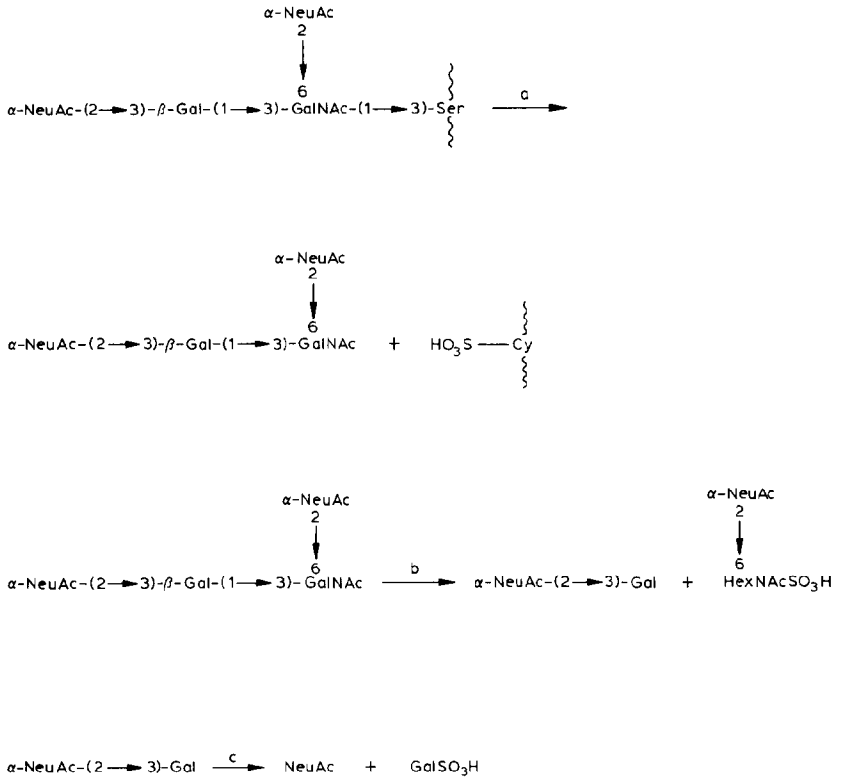
COMPOSITION OF SULFO-OLIGOSACCHARIDES ISOLATED ON BIO-GEL P2

Source	*P2 fraction*	*HexNAcSO$_3$*[a]	*NeuAc*	*Gal*	*GlcNAc*
Fetuin	I	**1.0**	1.21	1.07	0.89
	II	**1.0**	1.03	—	—
	III	—	2.78	—	—
Human erythrocyte glycopeptides	IIa[b]	**1.0**	0.96	—	—
	IIb[c]	**1.0**	1.43	0.57	—

[a]Set at 1.0 for molar ratios. [b]IIa: leading edge of rechromatographed peak II. [c]IIb: trailing edge of rechromatographed peak II.

(2→?)-HexNAcSO$_3$. Because the sulfohexosamine was derived from 2-acetamido-2-deoxygalactose, and the only NeuAc → GalNAc sequence in fetuin is that occurring in the O-linked tetrasaccharide α-NeuAc-(2→3)-β-Gal-(1→3)-[α-NeuAc-(2→6)]-GalNAc, the disaccharide must have the structure NeuAc-(2→6)-HexNAcSO$_3$. This conclusion is consistent with the known formation of sulfohexosamine from 3-substituted 2-acetamido-2-deoxygalactose. It is further confirmed by the observed recovery of 2.7 residues of free sialic acid per sulfo disaccharide molecule, as the 3-linked sialic acid should be released by a peeling reaction[14] from both the fetuin trisaccharide and tetrasaccharide, which are present[4] in the ratio of 3:1 (see Scheme 1), whereas the sulfo disaccharide should be formed only from the tetrasaccharide.

The sugar composition of peak I (see Table I), in conjunction with its molecular weight determined on Bio-Gel P2 (M_r = 800), indicated that it was a tetrasaccharide composed of sialic acid, galactose, 2-amino-2-deoxyglucose, and sulfohexosamine. Neuraminidase released all of the sialic acid from the oligosaccharide, showing that this sugar was at the nonreducing terminus. Periodate oxida-



Scheme 1. Formation of α-NeuAc-(2→6)-HexNAcSO$_3$. [A reaction mechanism is shown that accounts for the formation of the products described in the text from the erythrocyte glycopeptide or fetuin tetrasaccharide.]

tion of the resulting, sialic acid-free oligosaccharide decomposed the galactosyl residue without affecting the 2-amino-2-deoxyglucose. In contrast, periodate oxidation of the intact tetrasaccharide had no effect on the galactosyl or 2-amino-2-deoxyglucosyl residues, allowing its sequence to be written as α-NeuAc-(2→3)-Gal-(1→3 or 4)-GlcNAc-(1→6)-HexNAcSO$_3$. The tetrasaccharide was obtained in the ratio of 1 mole per 4 moles of the disaccharide, and its structure is not related to the reported structures of the fetuin O-linked oligosaccharides[4,15]. It must, therefore, be derived from a larger, O-linked unit that is present in submolar proportions in the glycoprotein and has not been detected in earlier studies, although the possibility of a contaminant present in the commercial preparation of fetuin cannot be precluded at this time.

Sulfo-oligosaccharides from glycoproteins of erythrocyte membranes. — The glycopeptides from human erythrocytes yielded a similar elution pattern from Bio-Gel P2 after treatment with sulfite for 72 h at room temperature. Presumably owing to the shorter reaction-time, a small amount of galactose, present as a disaccharide with sialic acid, was found in the peak containing α-NeuAc-(2→6)-HexNAcSO$_3$, but the latter disaccharide could be resolved into the leading edge of the peak by rechromatography (peak IIa, Table I). The trailing edge of the peak (IIb) contained a mixture of the two disaccharides. Because the structure of the O-linked saccharide from human glycophorin is identical to that from fetuin[16], the isolation of the α-NeuAc-(2→6)-HexNAcSO$_3$ disaccharide from this source provides further confirmation for the fragmentation scheme proposed.

Proposed reaction mechanism. — A reaction mechanism which would account for the observed products is shown in Scheme 1. Under the alkaline conditions, the oligosaccharide would be released from the peptide with addition of sulfite to the unsaturated amino acid (step a). The reducing terminus of the oligosaccharide is thus freed, rendering any 3-linked substituents alkali-labile by a peeling reaction[14] and, upon their elimination, sulfite adds to the resulting, unsaturated, 2-amino-2-deoxygalactosyl derivative (step b). Subsequently, in the case of the tetrasaccharide, sialic acid can be eliminated from the α-NeuAc-(2→3)-Gal disaccharide (step c), whereas the 6-linked sialic acid in the sulfo disaccharide would be stable to the alkaline conditions.

This specific pattern of fragmentation can be used to advantage both for the isolation of oligosaccharide fragments for analysis, and to assign these sequences to known sites within the oligosaccharide in a manner operationally similar to the analysis of manno-oligosaccharide branching by acetolysis[17]. For example, demonstration of the α-NeuAc-(2→6)-HexNAcSO$_3$ derivative provides evidence for a 3,6-disubstituted, core 2-acetamido-2-deoxygalactosyl residue having a sialic acid group on O-6. Periodate oxidation is not useful in this regard, as both 3-linked and 3,6-linked 2-acetamido-2-deoxy-D-galactitol yield 2-amino-2-deoxy-L-threitol on oxidation, borohydride reduction, and hydrolysis. The sulfohexosamine-containing

tetrasaccharide found in this study must, therefore, have originated from an oligosaccharide having the structure

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\quad R \\ \qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\quad 3 \\ \alpha\text{-NeuAc-}(2\rightarrow 3)\text{-Gal-}(1\rightarrow 3 \text{ or } 4)\text{-GlcNAc-}(1\rightarrow 6)\text{-GalNAc.} \end{array}$$

The structural prerequisites for formation of sulfo-oligosaccharides from O-linked glycoproteins have been described herein. Similar, susceptible oligosaccharides in which a branched, core, 2-acetamido-2-deoxygalactosyl residue is disubstituted at O-3 and O-6 have been described in a growing number of cases[18,19]. The approach described here should be quite useful for the specific fragmentation of such oligosaccharides.

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