Structure of the Sulfated Oligosaccharide Chain of Ovomucin†

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The structure of sulfated oligosaccharide II, one of the main sulfated oligosaccharide chains of ovomucin, has been determined. This oligosaccharide was found to compose of each 1 mole of N-acetylgalactosaminitol, galactose, N-acetylneuraminic acid and sulfate. The sequential periodate oxidation, borohydride reduction and acid hydrolysis of N-acetylneuraminic acid-free oligosaccharide resulted in the formation of glycerol and N-acetylthreosaminitol.

From these results, the following structure was proposed for sulfated oligosaccharide II: N-acetylneuraminyl-(2→3)-galactosyl-(1→3)-N-acetylgalactosaminitol-6-sulfate.

Little is hitherto known about the structure of sulfated carbohydrate units in the glycoproteins. In the previous paper,¹⁾ the O-glycosidically linked carbohydrate units of ovomucin were shown to be released from serine and threonine in peptide as oligosaccharide chains by alkali treatment in the presence of borohydride and to be consisted of two sulfated oligosaccharide chains, an oligosaccharide (named sulfated oligosaccharide I) composed of N-acetylgalactosaminitol, galactose, N-acetylneuraminic acid and sulfate in a molar ratio of about 1.0:1.0:0.6:0.5 and another oligosaccharide (named sulfated oligosaccharide II) in a molar ratio of about 1:1:1:1. The sulfated oligosaccharide I seems either to lack partially in N-acetylneuraminic acid and sulfate or to have a complex structure. On the other hand, the sulfated oligosaccharide II contains both N-acetylneuraminic acid and sulfate as acidic residues in an equimolar ratio in one oligosaccharide chain. Therefore, the structure of sulfated oligosaccharide II is very interesting.

This paper describes the structure of sulfated oligosaccharide II.

MATERIALS AND METHODS

Materials. Ovomucin was prepared by the method

† Nature of the Carbohydrate Side Chains and Their Linkage to the Protein in Chicken Egg White Ovomucin. Part III. For Part II, see reference 1).

described in the previous paper.²⁾ N-Acetylgalactosaminitol was prepared by NaBH₄ reduction of N-acetylgalactosamine.³⁾ N-Acetylthreosaminitol was prepared by the sequential periodate oxidation, borohydride reduction and acid hydrolysis of N-acetylchondrosinitol, which was prepared by N-acetylation⁴⁾ and NaBH₄ reduction of chondrosine (Seikagaku Kogyo Co.). Neuraminidase (Type VI, 2 units/mg) from *Cl. perfringens* was a product of Sigma Chemical Co. All reagents employed were analytical grade.

Preparation of sulfated oligosaccharide II. Sulfated oligosaccharide II was prepared by the method described in the previous paper.¹⁾ Sulfated glycopeptides were purified from the proteolytic digests of ovomucin by gel filtration on a column of Bio-Gel P-100 and ion-exchange chromatography on a column of DEAE-Sephadex A-25. Sulfated glycopeptides thus obtained were treated with 0.1 N NaOH in 0.5 M NaBH₄ for 12 hr at 45°C. Sulfated oligosaccharides liberated by β -elimination were purified by gel filtration on a column (1.8 $\times$ 100 cm) of Bio-Gel P-4 and ion-exchange chromatography on a column (1.4 $\times$ 17 cm) of DEAE-Sephadex A-25 eluting with a linear gradient of 0.1 M ~ 1.0 M pyridine-acetate buffer, pH 5.0. Thus two fractions (Fraction I and II) containing N-acetylgalactosaminitol were obtained, as described in the previous paper.¹⁾ Fraction II was pooled and named sulfated oligosaccharide II.

Removal of N-acetylneuraminic acid of sulfated oligosaccharide II. N-Acetylneuraminic acid was removed from sulfated oligosaccharide II by neuraminidase or mild acid hydrolysis. Five μ g/ml of neuraminidase was added to a solution of sulfated oligosaccharide II. Enzymatic action was carried out at 30°C for 24 hr in 1/15 M phosphate buffer, pH 5.4. Mild acid hydrolysis for the removal of N-acetylneuraminic acid was carried out in 0.05 N H₂SO₄ at 75°C for 1 hr.

Controlled Smith degradation. The sequential periodate oxidation, borohydride reduction and acid hydrolysis of N-acetylneuraminic acid-free oligosaccharide was carried out as described below. The N-acetylneuraminic acid-free oligosaccharide was dissolved in 0.5 ml of 0.1 M acetate buffer, pH 4.4 and oxidized by 0.1 M sodium metaperiodate at 5°C for 24 hr in the dark. Excess sodium metaperiodates were destroyed by the addition of 0.1 ml of 10% ethylene-glycol at 20°C for 1 hr. The oxidized product was reduced in 0.5 M NaBH₄ at 20°C for 2 hr. Excess sodium borohydrides were destroyed by the addition of 0.6 ml of 1 N acetic acid. The reduced product was hydrolyzed with 0.1 N H₂SO₄ at 30°C for 20 hr. The hydrolysates were passed through the column of Dowex 50 to be desalted, and were evaporated to dryness.

Analytical methods. Hexose was determined by the orcinol methods.⁵⁾ Galactose was determined by gas-liquid chromatography as described in the previous paper.¹⁾ N-Acetylgalactosaminitol was determined by gas-liquid chromatography as described in the previous paper.¹⁾ Sulfate was determined by the benzidine method⁶⁾ after hydrolysis with 6 N HCl at 100°C for 9 hr in sealed ampoules. The trimethylsilyl derivatives of glycerol and N-acetylthreosaminitol were identified by gas-liquid chromatography and mass spectral analysis. Gas-liquid chromatography was carried out on a glass column (3 mm × 2 m) packed with 2% OV-1 on Gas Chrom Q (60~80 mesh). The column was scanned by temperature gradient from 80°C to 170°C (3°C/min). The mass spectra were recorded with a gas chromatograph-mass spectrometer (JEOL, Model D-100). The column (2 mm × 1 m) packed with 5% OV-1 on Gas Chrom Q (100~120 mesh) was maintained at 170°C. The ionization voltage was 25 eV.

RESULTS AND DISCUSSION

Linkage between N-acetylneuraminic acid and galactose

In glycoproteins, N-acetylneuraminic acids are known to be ketosidically bound to either galactose or N-acetylgalactosamine in a non-reducing terminal position.⁷⁾ The N-acetylneuraminic acids were liberated selectively from ovomucin by 0.05 N H₂SO₄ at 75°C for 1 hr or by neuraminidase from *Cl. perfringens*.⁸⁾ As shown in Table I, the galactose of sulfated oligosaccharide II were not at all destroyed by periodate oxidation, but most of galactoses were destroyed by periodate oxida-

TABLE I. DESTRUCTION OF GALACTOSE IN SULFATED OLIGOSACCHARIDE II BY PERIODATE OXIDATION

Galactose content is expressed as the ratio of that of control sulfated oligosaccharide II. Sulfated oligosaccharides were oxidized in 0.1 M acetate buffer pH 4.4, containing 0.1 M sodium metaperiodate at 5°C for 24 hr in the dark.

Sulfated oligosaccharide	Galactose content
Control	1.00
Oxidized	1.04
Oxidized after treatment with neuraminidase	0.28
Oxidized after treatment with 0.05 N H ₂ SO ₄	0.13

tion after the N-acetylneuraminic acid of sulfated oligosaccharide II were liberated by mild acid hydrolysis or by neuraminidase. This result indicates that the N-acetylneuraminic acid of sulfated oligosaccharide II links to C-3 of galactose because otherwise the destruction of galactose should result by periodate oxidation in the same manner before and after the liberation of N-acetylneuraminic acid. Neuraminidase are able to split α -ketosidically bound neuraminic acid. Therefore, N-acetylneuraminic acid residue at the non-reducing end of sulfated oligosaccharide II seems to be linked to galactose by α -(2→3) linkages. Sulfated oligosaccharide II was composed of N-acetylgalactosaminitol, galactose, N-acetylneuraminic acid and sulfate in an equimolar ratio. Further, an almost total destruction of galactose by periodate oxidation after removal of N-acetylneuraminic acids indicates that sulfated oligosaccharide II is composed of each 1 molar residue. Thus, sulfated oligosaccharide II seemed to be trisaccharide, N-acetylneuraminyl-galactosyl-N-acetylgalactosaminitol, containing 1 residue of sulfate.

Linkage between galactose and N-acetylgalactosaminitol

It was shown in the previous paper¹⁾ that β -elimination with alkali of sulfated glycopeptide of ovomucin resulted in the formation of chromogen from N-acetylgalactosamine at

the reducing end. Under such conditions, chromogens are formed only from 3- and 6-monosubstituted and 3,6-disubstituted N-acetylhexosamines.⁹⁾ Therefore, the galactose of sulfated oligosaccharide II seems to be linked to N-acetylglucosaminitol by (1→3) or (1→6) linkages. Glick *et al.*¹⁰⁾ indicated that the disaccharide galactose-N-acetylglucosamine was alkali-sensitive when it was linked by β -(1→3) linkage; but was stable in alkali when it was linked by β -(1→4) or β -(1→6) linkage. Sulfated oligosaccharide II was subjected to a peeling reaction by alkali treatment without borohydride.¹⁾ Therefore, it seems likely that galactose should be linked to N-acetylglucosaminitol by β -(1→3) linkage. Such a sequence and linkage of sulfated oligosaccharide II was also determined by the controlled Smith degradation of N-acetylneuraminic acid-free oligosaccharide, as shown in Fig. 1. The N-acetylneuraminic acid of sulfated oligosaccharide II was removed by mild acid hydrolysis. Then the sequential periodate oxidation, borohydride reduction

and acid hydrolysis of N-acetylneuraminic acid-free oligosaccharide were carried out. If sulfated oligosaccharide II has such a structure as shown in Fig. 1, the formation of glycerol and N-acetylthreosaminitol should result by the controlled Smith degradation. The trimethylsilyl derivatives of the products formed by the controlled Smith degradation were identified by gas-liquid chromatography and mass spectral analysis. Gas-liquid chromatogram revealed the presence of two main peaks (peak 1, 2), as shown in Fig. 2. Peak 1 and 2 were found to have identical retention time with authentic samples of glycerol and N-acetylthreosaminitol, respectively. Peak 2 was also identified by mass spectral analysis. The mass spectrum of the compound is shown in Fig. 3. Its characteristic peaks were compatible with the structure of fragmentation, as

FIG. 1. Reaction Schemes for the Controlled Smith Degradation of Sulfated Oligosaccharide II.

(1) glycerol, (2) N-acetylthreosaminitol, (3) glycolaldehyde, NeuAc: N-acetylneuraminic acid.

FIG. 2. Gas-liquid Chromatogram of the Trimethylsilyl Derivatives of the Products Formed by the Controlled Smith Degradation.

Peaks: 1, glycerol; 2, N-acetylthreosaminitol

FIG. 3. Mass Spectrum for Peak 2 in the Gas-liquid Chromatogram.

TABLE II. EFFECT OF CONTROLLED SMITH DEGRADATION OF CHEMICAL COMPOSITION OF SULFATED GLYCOPEPTIDE

The content of each component is expressed as the ratio of that of intact sulfated glycopeptide. Sulfated glycopeptide was hydrolyzed with 0.05 N H₂SO₄ at 75°C for 1 hr, subjected to controlled Smith degradation, and dialyzed against water.

Sulfated glycopeptide	Content			
	N-Acetyl galactosamine	Galactose	Sulfate	N-Acetyl neuraminic acid
Intact	1.00	1.00	1.00	1.00
Degradated	1.06	0.55	1.04	0

m/e 364, M-15
 m/e 289, M-TMSOH
 m/e 217, 276-Ac
 m/e 186, 276-TMSOH

FIG. 4. Fragmentation Routes for the Trimethylsilyl Derivative of N-Acetylthreosaminitol.

shown in Fig. 4.

From these results, it was confirmed that N-acetylneuraminic acid was linked to galactose by α -(2 $\rightarrow$ 3) linkage and galactose was linked to N-acetylgalactosaminitol by β -(1 $\rightarrow$ 3) linkage in sulfated oligosaccharide II.

Linkage between oligosaccharide and sulfate

It was shown in the previous paper²⁾ that the sulfate residues of ovomucin existed as ester sulfate at equatorial position of carbohydrate. Therefore, the sulfate residues of sulfated oligosaccharide II are possible to link at C-3 or C-6 of galactose or N-acetylgalactosaminitol. As noted above, C-3 of galactose and N-acetylgalactosaminitol were linked to N-acetylneuraminic acid and galactose, respectively. As shown in Fig. 2, the production of glycerol by the controlled Smith degradation indicates that C-6 of galactose is unsubstituted. Therefore, it is probable only that the sulfate residue is linked to C-6 of N-acetylgalactosaminitol. This is also supported from the result that all sulfate residues remained to link to the sulfated glycopeptide of ovomucin though the N-acetylneuraminic

acid and galactose were removed by mild acid hydrolysis and subsequent controlled Smith degradation, as shown in Table II.

In conclusion, the following structure can be proposed for the sulfated oligosaccharide II:

N-acetylneuraminyl-(2 $\rightarrow$ 3)-galactosyl-(1 $\rightarrow$ 3)-N-acetylgalactosaminitol-6-sulfate.

The possible structure of a sulfated carbohydrate chain linked to the protein portion of ovomucin is shown in Fig. 5.

Further studies on the structure of sulfated oligosaccharide I are in progress.

FIG. 5. Possible Structure of Sulfated Carbohydrate Side Chain Linked to the Protein Moiety of Ovomucin.

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