Partial Characterization of Neutral Oligosaccharides from a Normal Human Bronchial Secretion

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Summary: Normal human bronchial secretion was obtained by washing the lungs with normal saline. The lavage was midly centrifuged to remove the cellular debris, dialyzed and lyophilized. The residue was chromatographed on Bio-Gel P-200 and then on Sepharose 2B. The major oligosaccharides. The neutral oligosaccharides, after separation from acidic oligosaccharides, were further purified by gel filtration and ion-exchange chromatography. Five neutral oligosaccharides were characterized using enzymic and chemical procedures, and - following structures are proposed:

(i) Gaß(1→4) GlcNAc (1→3) → GalNAc-ol

(ii) Galβ (1→4) Glc NAc (1→3) → GalNAc-ol
Fucα(1→2)

iii) Glc NAc (1→3) → GalNAc-ol
Galß(1→4)GlcNAc (1→6)

iv) Galß(1→3) → GalNAc-ol
Gal (1→4)GlcNA (1→6)

v) Fucα(1→2)Gal (1→3) → GalNAc-ol
Gal (1→4)GlcNAc(1→6)

Fig.1: Proposed structure for neutral oligo- saccharides.

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Introduction

The tracheobronchial system is an important component of mucociliary function. This system provides airways with necessary protection by removing pathogenic materials that are inhaled. An important constituent of the tracheobronchial system is the epithelial secretion, mucus, that consists of glycoproteins, protein and lipids. The mucus possesses rheological properties which are important for its physiological functions. The rheological properties are necessarily imparted to the secretion by the glycoproteins and are mainly due to carbohydrates.

In pathological conditions, characterized by hypersecretion and changes in the rheological behaviour of the mucus, the mucociliary function is altered, resulting in bronchial disfunction of airways.

Human bronchial mucus glycoproteins (mucins) prepared from lavages of macroscopically healthy bronchial areas have shown to be acidic in nature, predominantly sialylated, and in which the average chain length of oligosaccharide is relatively small [1]. Acidic functions, carboxyl and sulfate groups, present in the sugar residues provide electronegative charges to the glycoproteins. In several glycoproteins changes in the quantity and position of the neuraminic acid and sulfate groups result in alteration in the behaviour of these macromolecules [2-5]. Also the asialoglycoproteins are knwon to be susceptible to proteases [2]. It would appear from these chemical modifications in the glycoproteins that acid functions in the biopolymers impart resistance to enzymic degradation that may directly or indirectly effect the efficiency of mucociliary system.

Despite various studies performed on human secretion no detailed structural characterization of carbohydrates, so necessary for mucocilary function, have been achieved; yet because of the difficulty of obtaining workable amounts of normal human bronchial mucin glycoproteins. Most studies related to human bronchial mucins have mainly concerned material isolated from sputum of patients suffering from bronchial hypersecretion [6-8].

Materials and Methods

Collection of bronchal secretion

The secretion was obtained by aspirating bronchus with normal saline. The secretion was frozen and maintained in the frozen state prior to use.

Analytical methods

The hexose content of the glycoproteins was estimated by the phenol sulphuric acid method [9] the protein content by measuring absorbance at 278 nm and the neuraminic acid content by the thiobarbituric acid procedure of Warren [10] after acid hydrolysis with 50mM sulphuric acid, or by gas liquid chromatography.

Gas-liquid chromatography (GLC)

GLC determinations of carbohydrate moiety of the glycoproteins were performed according to the procedure of Reinhold [11]. GLC-MS (mass spectrometry) of the methylated sugars was performed on a Varian MAT 731 instrument fitted with the combined EI, CI and FD ion source.

Column chromatography

Bio-Gel P-200 (Bio-Rad laboratories) and Sepharose 4B (Pharmacia fine chemicals) columns were run in 50mM

sodium phosphate (pH 7.0) containing 0.02% sodium azide. The DEAE-cellulose (Whatman) column was eluted with 0.1M NaCl followed by a gradient of 0.1M to 1M sodium chloride containing 10mM HCl. Bio-Gel P-4 and Bio-Gel P-6 (200-400 mesh) chromatography was performed in 50mM pyridine-acetic acid. The sugar containing fractions, detected by phenol sulphuric acid procedure and/or by counting tritium, were combined and lyophilized. DEAE-Sephadex A-50 columns were run with 50mM - 0.5M phosphate buffer (pH 7.0) followed by 0.1M - 0.5M LiCl, portions containing carbohydrates were combined and desalted on a column of Bio-Gel P-2. Agarose gel electrophoresis was performed in 50mM barbital buffer (pH 8.2) on glass slides containing 1% agrose. Polyacrylamide and agrose gel electrophoresis was carried out according to the method of Holden et al., [12]. The agarose and polyacrylamide slides were stained with Amido black or Coomassie blue, and with periodate-Schiff reagent. For agrose and polyacrylamide electrophoresis nearly 0.3mg per ml of each substance was used and each well had 25 µl of the solution.

Purification and protease treatment of the mucus glycoprotein

The crude mucus was solubilized in 50mM sodium monophosphate (pH 7.0) containing 0.02% sodium azide by stirring for 24 hours. The cellular debris and other insoluble materials were removed by centrifugation (2500 rev./min.), the supernatant was dialyzed and the nondiffusible material was lyophilized to give the crude mucus glycoprotein. The residue (50 mg) was dissolved in 50mM sodium monophosphate (60 ml, pH 7.0) containing 0.02% sodium azide by stirring for 16 hours at 4°C. The solution was applied to a column (2.0 x 60 cm) of Bio-Gel P-200 (50-100 mesh). The carbohydrate and protein containing fractions were pooled, the pH was adjusted to 5.5 with 4M acetic acid, and the solution was dialyzed and lyophilized to give the purified mucus glycoprotein (20 mg). The purified glycoprotein (15mg) in 50mM sodium monophosphate buffer (30 ml, pH 8.0) containing 0.1% sodium azide was stirred at 22°C until solubilized and then treated with insolubilized Pronase (20mg, Enzite protease, Miles laboratories, Inc.). The mixture was incubated for one day at 37°C with stirring. Another addition of prewashed enzyme (8mg) was made and the solution was incubated for another three days. The suspension was centrifuged, and the residue was washed with buffer. The pH of the supernatant was adjusted to pH 5.0 with acetic acid and the solution dialyzed against distilled water. The nondialyzable material was lyophilized and the residue (12 mg) was applied to a column (2.5 x 60 cm) of Sepharose 2B. Fractions containing carbohydrate and protein were combined and dialyzed and the nondiffusible material was lyophilized. The Sepharose 2B purified carbohydrate containing material was further chromatographed on a column of DEAE-celluse, the carbohydrate and protein containing fractions eluted with a gradient of lithium chloride were combined and dialyzed, and the retentate was lyophilized to give the Pronase-treated glycoprotein (8mg). The glycoprotein was examined by agarose and polyacrylamide gel electrophoresis.

Sedimentation equilibrium studies

The sedimentation equilibrium study was performed on a solution of Pronase-treated glycoprotein (1.5 mg) in a ml of 6M guanidine hydrochloride -0.05M tris (pH 7.0), dialyzed for 48 hours against the same buffer, with

the meniscus - depletion sedimentation method of Yphantis [13] on a model E Ultracentrifuge. A value of 0.637 for partial specific volume was used. The molecular weight was calculated by extrapolation of the point average molecular weights of infinite dilutions.

Alkaline borohydride treatment

The Pronase-treated glycoprotein (7 mg) was treated with 2M sodium borohydride in 50mM NaOH according to the procedure of Iyer and Carlson [14].

A 0.2% solution of the glycoprotein in 2M sodium borohydride containing 5mCi of sodium borotritide in 0.05M NaOH was incubated for 18 hours at 45°C. Following alkaline borohydride treatment the mixture was adjusted with 4M acetic acid to pH 5.4. The reaction mixture was applied to a column (2.5 x 68 cm) of AG 50 W-X8 (H, 100-200 mesh) ion exchange resin containing 150 fold excess with respect to the sodium ions from NaOH and $NaHB_4$. Oligosaccharides, oligopeptides and glycopeptides were eluted with water and 50 mM acetic acid. Reduced oligosaccaride were separated into neutral and acidic oligosaccharides on a column (3.4 x 70 cm) of AG 1-X2 OAc-(200-400 mesh). The column was washed with water, 0.5mM pridine -acetic acid (pH 5.4) and then with 0.1M - 1.5M acetic acid in the cold. The acidic oligosaccharides were further chromatographed on Bio-Gel P-6 (200-400 mesh) in 10mM pyridine-acetic acid (pH 5.4) followed by paper chromatography or paper electrophoresis. Paper chromatography was performed in solvents: (A) ethyl acetate-pyridine-acetic acid-water (5:5:1:3) (B) butanol-propanol-1.10M acetic acid (1:2:1 V/V).

Enzyme degradation

Oligosaccharides were digested with some or all of the following enzyme:

i) α-L-fucosidase from beef epididymis (Sigma, 10mM sodium citrate buffer, pH 6.0 at 37°C for 50 hours) and from emulsin (50 mM sodium citrate buffer, pH 5.0, 50 hours at 37°C).

ii) β-galactosidase from Aspergillus niger (Sigma, 50mM sodium-citrate, pH 4.1, 70 hours at 37°C).

iii) β-galactosidase from Escherichia coli (Boehringer, 50mM sodium phosphate, pH 7.0, 48 hours at 37°C), and β-galactosidase form Charonia lampas (Miles, 50mM sodium citrate buffer, pH 4.0, 48 hours 37°C).

iv) β-N-acetylglucosaminidase from jack bean (Sigma, 50mM sodium citrate buffer, pH 4.5, 48 hours at 37°C.

Methylation analysis

Methylation of oligosaccharides was performed according to the procedure of Hakomori [15]. The methylated oligosaccharides were recovered by partition between chloroform and water. The methylated product was converted into monomers by treatment with 2M trifluoroacetic acid at 105°C for three hours. The solution was diluted (x10) after cooling, and freeze-dried. The residue in water-methanol (4:1) was treated with sodium borohydride (25 mg) for 12 hours at 4°C and for 3 hours at 22°C. The excess of sodium borohydride and sodium ions were removed simultaneously by treatment with AG 50W-X8 (H, 100-200 mesh) ion-exchange resin, reduced sugars were eluted with water, methanol and methanolic-

ammonia. The combined washings were evaporated, and the residue in methanol was repeatedly evaporated to remove boric acid. Finally, the residue was acetylated with pyridine (0.5 ml) and acetic anhydride (0.4 ml) for 12 hours at 22°C and the methylated alditols were examined by g.l.c. and g.l.c. - m.s.

Periodate oxidation-sodium borohydride reduction

The reduced oligosaccharides (300 µg)were oxidized with periodate (0.1M) at 4°C for 12 hours, then at room temperature for 8 hours.

The excess of periodate was destroyed by addition of 1,2-ethanediol and the oxidized material was reduced by treatment with sodium borohyride (25 mg per mg of the starting material) for 12 hours at 4°C, followed by another addition of sodium-borohydride (5 mg per mg of oligosaccharide), and the reduction was allowed to proceed at room temeprature for 6 hours. Excess borohyride and sodium ions were removed by adding an excess of AG 50W-X8 (100-200 mesh) ion-exchange resin. The resin was filtered off, and boric acid was removed by repeated evaporation with methanol. The residue was treated with 0.25M sulfuric acid for 2.5 hours, the acidic solution was treated with AG1-X8 (OAc-100-200 mesh). After methanolysis with 0.5M methanolic hydrogen chloride for 20 hours at 80°C the oxidized oligosaccharides were examined by g.l.c. for the presence of sugars. A sample of 2-acetamido-2-deoxyserinol was obtained by N-acetylation of commercially available 2-amino-2-deoxy-serinol (Sigma).

Samples of N-acetylthreosaminitol and 2-N-acetylarabinosaminitol alongwith N-acetylated serinol were obtained by mild (10mM) periodate oxidation of N-acetylgalactosaminitol for 30 minutes at 220°C.

Results

Purification and Characterization of Pronase-degraded cervical mucus glycoproteins

The bronchial glycoproteins obtained from normal human beings were purified by gel filtration on Bio-Gel P-200 followed by very mild treatment with Pronase. Degeraded normal human bronchial mucus glycoproteins.

Pronase-treated glycoproteins were fractionated on Sepharose 4 B. The main fraction (60%) showed the presence of a single glycoprtein and in gel electrophoresis barely entered the polyacrylamide gel. In agarose (1%) the glycoprotein entered the gel and exhibited a single dispersed component.

Preparation of oligosaccharide-alditols

The glycoprotein was subjected to alkaline borohydride-reductive cleavage yielding a mixture of oligosaccharide-alditols. A decrease of serine and threonine and a corresponding increase of alanine and the appearance of γ-aminobutyric acid were detected. The neutral oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into five oligosaccharide fractions (see Table-1, Fig. 1).

Oligosaccharide N-1

The oligosaccharide purified on Bio-Gel-P-6 was identified as a single component in solvent systems A and B. Methylation of the oligosaccharide and identification of the products showed

Table-1: Carbohydrate composition of purified oligosaccharides

Oligosaccharides	Fucose		Galactose		N-Acetyl-Glucosamine		N-Acetyl-Galactosaminitol	
	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]
N-1			26	0.94	30	0.88	34	1.0
N-2	15	0.67	20	0.86	26	0.86	30	1.00
N-3			21	0.99	49	0.94	26	1.00
N-4			38	1.60	24	0.90	29	
N-5	14	0.75	34	1.82	19	0.83	23	

[a]MR : Molar ratio is releative to N-acetylgalactosaminitol

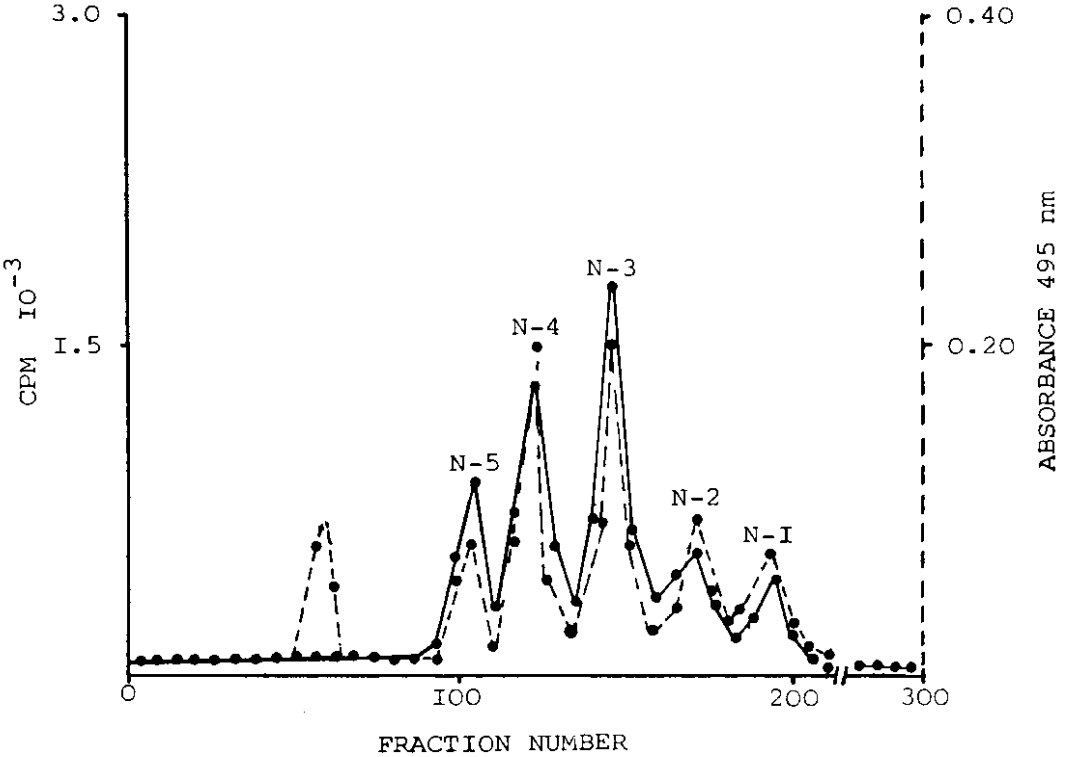

Fig.2: Separation of oligosaccharides on a column of Bio-Gel P-6. Five fractions (N-1, 1.2 mg;N-2, 1.4 mg; N-3, 1.6 mg; N-4, 1.7 mg; N-5, 1.2 mg) were obtained.

a terminal galactose, 4-linked N-acetylglucosamine and a 3-linked N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction and identification of the resistant products showed the presence of N-acetylglucosamine and N-acetylthereosaminitol. Treatment of the oligosaccharide with β-galactosidase removed a galactose residue. These results suggest the structure N-1 as shown in figure 2.

Oligosaccharide N-2

The oligosaccharide N-2 was purified by chromatography on DEAE Sephadex A-50 and was homogeneous in solvents A and B. The oligosaccharide on methylation showed the presence of terminal fucose, 2-linked galactose, 4-linked N-acetylglucosamine and 3-linked N-acetyl galactosaminitol. Sequential treatment with α-fucosidase, β-galactosidase, and subsequent methylation of the remaining oligosaccharide showed the presence of terminal N-acetyl glucosamine and 3-linked N-acetylgalactosaminitol. Based on these results structure N-2 is proposed for this oligosaccharide (Fig. 2).

Oligosaccharide fraction N-3

This fraction was further purified by chromatography on Bio-Gel P-4 and was homogeneous in paper chromatography in solvents A and B.

Sequential treatment of the oligosaccharide with β-galactosidase and βN-acetylglucosaminidase (96 hours) removed a galactose and a N-acetyglucosamine residue. Methylation of the residual oligosaccharide showed the presence of a terminal N-acetylglucosamine, and 3,6-linked N-acetylgalactosaninitol residues. Methylation of the oligosaccharide showed the presence of a terminal N-acetylglucosamine, a terminal galactose, a 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. The results of these experiment showed that oligosaccharide N-3 is a tetrasaccharide with sequence, linkage and anomery as shown in Figure 2.

Oligosaccharide N-4

Oligosaccharide N-4 was purified on DEAE Sephadex A-50, and was homogeneous in paper chromatography (solvents A and B) and electrophoresis. Periodate oxidation-borohydride reduction completely removed galactose residues, and converted N-acetylgalactosaminitol to 2-acetamido-2-deoxythreitol. Methylation of the oligosaccharide showed the presence of terminal galactose, 4-linked N-acetylglucosamne and 3- and 6-linked N-acetylgalactosaminitol. Sequential treatment of oligosac-charide with β-galactosidase (C.Lampas) and β-N-acetylglucosaminidase showed the loss of galactose residues. Subsequent methylation of the enzyme-treated oligosaccharide showed the presence of terminal N-acetylglucosamine and 3-linked N-acetyl-thresaminitol. These results suggest that oligosaccharide is a tetrasaccharide with structure given in Figure 2.

Oligosaccharide N-5

Methylation of the oligosaccharide showed the presence of terminal fucose, terminal galactose, 2-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Periodate oxidation-borohydride reduction resulted in destruction of fucose and galactose and residues of N-acetylgalactosamine to N-acetylthreosaminitol. N-acetylglucosamine was recovered unchanged. Treatment of the oligosaccharide with α-fucosidase and subsequent methylation of

the residual oligosaccharide showed that the fucose is linked to C-2 of galactose. Sequential treatment of the oligosaccharide with α-fucosidase and βgalactosidase and subsequent methylation of the residual oligosaccharide showed the presence of terminal N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. A small amount of terminal galactose was also identified. These results suggest the structure of oligosaccharide A-5 as shown in Figure 2.

Discussion

The separation of bronchial glycoprotein from the remaining polymeric materials i.e., protein and lipids was readily accomplished by gel filtration on Bio-Gel P-200. The glycoprotein component from the Bio-Gel P-200 column was treated with Pronase to remove any contaminating proteins and glycoproteins. it is known that proteolytic enzymes degrade a minor glycoprotein component of cervical mucus glycoproteins [2]. Secreted glycoproteins are known to undergo proteolysis when treated with Pronase, particularly in the hydrophobic regions of the protein moiety [16]. Fractionation of Pronase treated glycoprotein on Sepharose 4B resulted in two fractions. The main fraction in ion-exchange chromatography resulted in a single high molecular weight component. Despite the fact that glycoproteins are known to be degraded [16] by Pronase, a high molecular weight ($1x10^5$ to $1x10^6$) glycoprotein homogeneous, albeit polydisperse, in agarose electrophoresis and free of contaminating proteins was obtained. Sialic acid in the glycoprotein was present as N-acetylneuraminic acid. This is similar to human mucus [18] and different from bovine which contains N-glycolyneuraminic acid. The glycoprotein was free of cross-linked protein fraction containing cystine, as is observed in the case of bovine mucus [19,20].

Alkaline-borohydride treatment of the glycoprotein resulted in a mixture of oligosaccharide alditols that were fractionated on Bio-Gel P-6 and further purified by ion-exchange and paper chromatography affording five neutral oligosaccharides. The oligosaccharides structures characterized can be divided into three groups on the basis of the core structures:

i) Galβ(1→3)Gal-NAc-ol
ii) GlcNAcβ(1→3)Gal-NAc-ol
iii) HlcNacβ(1→6)Gal-Nac-ol

It appears that the biosynthesis or the chain elongation of the oligosaccharides occurs in core structures resulting in the structure that has been characterized and shown in Figure 2. The glycoprotein obtained from the normal bronchial secretion was isolated in very small quantity and a large number of secretions from different secretor types were combined. The heterogeneity observed in the carbohydrate chains perhaps, arises, due to mixing of secretions. A variety of carbohydrate chain lengths in mucins [6,8,21] and in blood group active glycoproteins [22-24] is known, and the heterogeneity of bronchial mucus glycoproteins could be even wider.

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