Isolation, Purification and Partial Characterization of Neutral oligosaccharides from Bovine Gallbladder Mucin Glycoprotein

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Summary: A glycoprotein was isolated from the bovine gallbladder secretion. The glycoprotein was purified on Bio-Gel P-200 and by subsequent mild treatment with Pronase. The Pronase treated glycoprotein was further purified by gel chromarography on Sepharose 4B and by ion-exchange chromatography. The purified glycoprotein was subjected to alkaline degradation-borohydride reduction. The liberated oligosaccharide alditols were purified by gel filtration, and separated into neutral and acidic oligosaccharides. Four neutral oligosaccharides, after purification, were characterized by chemical and enzymatic studies and were assigned the following structures and partial structure.

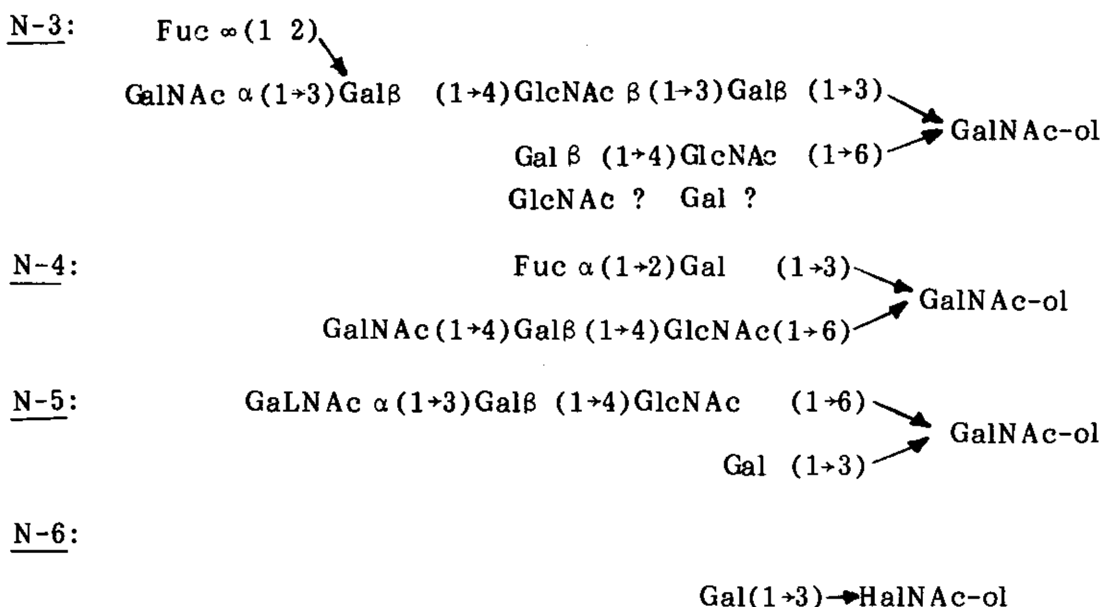

Fig.1: Proposed structures for neutral oligosaccharides.

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Introduction

The gallbladder secretion, mucus, is of vital importance in the pathogenesis of gallstones. It has been observed that gallbladder hypersecretion preceeded gallstone formation [1]. It was further observed that cholesterol crystals first formed in aggregates of mucus rather than in the liquid phase of gallbladder bile and thereby it was implied that mucus was essential not only for development of cholestrol type gallstones but important in the pathophysiology of other types of gallstones [1]. Purified human gallbladder mucin glycoproteins have been shown to induce nucleation of lecithin-cholesterol crytals from supersaturated hepatic bile. These in turn resulted in formation of cholesterol monohydrate crystals within short period. In addition, it has been shown that gastric [2,3] and bronchial [4] mucins exhibit lipid-binding properties.

Mucins are highly glycosylated proteins, which are secreted by a variety of epithelial cells lining the trachaeobronchial, genitourinary and gastrointestinal tracts. These glycosylated proteins are composed of nearly 75% carbohydrates arranged as branching side chains varying from two to twelve sugar residues. The hydroxyl functions in carbohydrate moiety of the glycoproteins in solution are extensively hydrated and contribute to the rheologic, viscoelastic and gel-forming properties of the macromolecule.

The mechanism by which glycoproteins participate in the process of gallstone formation is still unclear. In order to understand the role of the carbohydrates a glycoprotein was isolated and structures of neutral oligosaccharides have been partially characterized to translate the biochemical structure into the physiological functions.

Material and Methods

Isolation of gallbladder mucus

Bovine gallbladders were obtained at a local abatoir, drained of bile, and transported to the laboratory on ice. The gallbladders were opened and the mucosal surface washed with iced saline to remove blood and debris. The mucosal surface was then gently scraped with a glass slide to remove the adherent mucus gel, after which the mucosa was removed by sharp dissection. The mucosa was minced with a scalpel, combined with the mucus gel in 0.2 m NaCl, 0.04% Na_3 azine (10 ml/g of tissue) and homogenized. The bile and mucosal gel were processed for isolation of glycoproteins in the same manner but separately.

Analytical Methods

The hexose content of glycoprotein was estimated by the phenol sulphuric acid method [5]. The protein content by measuring absorbance at 278 nm and the neuraminic acid content by the thiobarbituric acid procedure of Warren [6] after acid hydrolysis with 50 mM sulphuric acid, or by gas-liquid chromatography (GLC).

Gas Liquid Chromatography (GLC)

GLC determinations of the carbohydrate moiety of the glycoprotein were performed according to the procedure of Reinhold [7]. GLC-MS (mass spectrometry) of the methylated sugars was performed on a varian MAT 731 instrument fitted with the combined source EI, CI/FD ion source.

Column chromatography

Bio-Gel P-200 (Bio-Rad Laboratories) and Sepharose 4B (Pharmacia Fine Chemicals) column 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-sulfuric acid procedure 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.

Gel Electrophoresis

Agarose gel electrophorjesis was performed in 50 mM barbital buffer (pH 8.2) on glass slides containing 1% agarose. Polyacrylamide and agarose gel electrophoresis was carried out according to the method of Holden et al. [8]. The agarose and polyacrylamide slides were stained with Amido black or Coomassie blue, and with periodate-Schiff reagent. For agarose and polyacrylamide electrophoresis nearly 0.1 mg of each substance per ml was used and each well had 25 ul 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 centrifugration (2500 rev./min), the supernatant was dialyzed, and the retentate was lyophilized to give the crude mucus glycoprotein. The residue (80 mg) was dissolved in 50mM sodium monophosphate (10 ml, pH 7.0) containing 0.02% sodium azide by stirring for 16 hours at 4°. 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 then lyophilized to give the purified mucus glycoprotein (20 mg). The purified glycoprotein (19 mg) was treated with insolubilized Pronase (10 mg, Enzite protease, Miles Laboratories, Inc.) in 50mM sodium monophosphate buffer 30 ml, pH 8.0) containing 0.1% sodium azide. The mixture was stirred at 22° until solubilized and then incubated for 48 hours at 37° with stirring. Another addition of prewashed enzyme (2 mg) was made and the solution incubated for another 8 hours. 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 non-dialyzable material was lyophilized and the residue (8 mg) was applied to a column (1.5 x 48 cm) of Sepharose 4B. Fractions containing carbohydrate and protein were combined and dialyzed, and the non diffusible material was lyophilized. The sepharose 4B purified carbohydrate containing material was further chromatographed on a column of DEAE-cellulose, the carbohydrate and protein containing fractions eluted with a gradient of lithium chloride were combined and dialyzed. The retentate was lyphilized to give the Pronase-treated glycoprotein (5 mg). The glycoprotein was examined by agarose and polyacrylamide electrophoresis.

Alkaline Borohydride Treatment

The Pronase-treated glycoprotein (4 mg) was treated with 2M sodium borohydride in 50mM sodium hydroxide according to the procedure of Ilyer and Carlson [9]. A 0.2% solution of the glycoprotein in 2M sodium borohydride in 0.05M sodium hydroxide was incubated for 18 hours at 45°. Following alkaline-borohydride treatment, mixture was adjusted with 4M acetic acid to pH 5.4.

The reaction mixture was applied to a colunm (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 $NaBH_4$. Oligosaccharides, oligopeptides and glycopeptides were eluted with water and 50mM acetic acid. Reduced oligosaccharides 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 0.5M pyridine-acetic acid (pH 5.4) and then with 0.1M-1.5M acetic acid at 4°. 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-acidic acid-water (5:5:1:3) (B) butanol-propanol-0.1M acetic acid (1:2:1,v/v).

Enzyme Degradation

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

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

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

iii) β-galactosidase from Escherichia coli (Boehringer, 50mM sodium phosphate, pH 7.0, 48 hrs at 37°) and -galactosidase from Charonia lamps (Miles, 50 mM sodium cirate buffer, pH 4.0, 48 hrs 37°).

iv) β-N-acetylglucosaminidase from Jack Bean (Sigma, 5mM sodium citrate buffer, pH 4.5, 40 hrs).

v) α-N-acetylgalactosaminidase from Charonia lampas (Miles, 50mM sodium citrate-phosphate buffer, pH 4.1, 42 hrs at 37°).

Methylation Analysis

Methylation of oligosaccharides was carried out according to the procedure of Hakomori [10]. The methylation was achieved with iodomethane in the presence of methylsulphinyl carbanion. The methylated oligosaccharides were recovered by dissolution in chloroform. The methylated product was converted into monomers by treatment with 2M trifluoroacetic acid at 105° for 3 hrs. The solution was diluted (x10), and freeze-dried. The residue in water-methanol (4:1) was treated with $NaBH_4$ (25 mg) for 12 hrs at 4° and for 4 hrs at 22°. The excess of $NaBH_4$ and sodium ions was removed with water, methanol and methanolic-NH_3. 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 (0.4ml) for 12 hrs at 22° and the methylated alditols were examined by g.l.c. and g.l.c. -m.s.

Periodate Oxidation-Sodium Borohydride Reduction

In order to establish the sequence and linkages of the sugar residues in oligosaccharides, reduced oligosaccharide (0.1 to 0.3 mg) were oxidized with periodate (0.1M) at 4° for 12 hrs, then at room temperature for 8 hrs. The excess of periodate was destroyed by addition of 1,2-ethandiol and the oxidized material was reduced with $NaBH_4$ (5 mg per mg of the starting material) for 12 hrs at 4°, followed by another addition of $NaBH_4$ (2 mg per mg of oligosaccharide), and the reduction was allowed to proceed at room temperature for 6 hrs. Excess borohydride 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 H_2SO_4 for 2.5 hrs, the acidic solution was treated with 0.25M AG1-X8 (OAc^-, 100-200 mesh). After methanolysis with 0.5M methanolic hydrogen chloride for 20 hrs at 80°, the oxidized oligosaccharides were examined by g.l.c. for the presence of sugars. A sample of N-acetylgalactosaminitol was obtained by reduction of N-acetylgalactosamine; 2-acetamido-2-deoxyserinol was obtained by N-acetylation of commercially available 2-amino-2-deoxy-serinol (Sigma). Samples of N-acetylthreosaminitol and N-acetylarabinosaminitol alongwith N-acetylated serinol were obtained by mild (10mM) periodate oxidation of N-acetyl-galactosaminitol for 30 min at 220°.

Results

Purification and characterization of the Pronase-degraded mucus and the gallbladder glycoproteins

The mucus glycoproteins obtained from the bile were purified by gel filtration on Bio-Gel P-200 followed by treatment with the Pronase. The Pronase-treated glycoproteins were fractionated on Sepharose 4B. The major fraction (70%) showed the presence of a single glycoprotein in DEAE-cellulose chromatography. The glycoprotein in gel electrophoresis barely entered the gel and exhibited a single component.

Table-1: Carbohydrate Composition of Purified Oligosaccharide Fractions

Oligosaccharides	Fucose		Galactose		N-Acetyl-glucosamine		N-Acetyl-galactosamine		N-Acetyl-galactos aminitol	
	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]	%	MR[a]
N-3	8	0.90	29	2.90	31	2.60	10	0.84	12	1.00
N-4	12	0.90	28	0.96	17	0.94	16	0.89	18	1.00
N-5			31	1.74	20	0.91	19	0.86	22	1.00
N-6			40	0.96					51	1.00

[a]Molar ratio relative to N-acetylgalactosaminitol.

Preparation of oligosaccharide-alditols

The glycoprotein was subjected to alkaline borohydride-reductive cleavage yielding a mixture of oligosaccharidealditols. A decrease of serine and threoine and a corresponding increase of alanine and appearance of -aminobutyric acid were dectected. The neutral oligosaccharides eluted from the column of AG 1-X2 were separated on a column of Bio-Gel P-6 into six fractions (see Table 1 and Fig. 1). Fractions N-1 and N-2 represent mainly glycopeptides as indicated by low percentage of N-acetylgalactosaminitol and the presence of hydroxylated amino acids.

Oligosaccharide fraction N-3.

This fraction was further purified by chromatography on DEAE-Sephadex A-50 and was homogeneous in paper chromatography in solvents A and B. Carbohydrate analysis of the oligosaccharide suggested this fraction to be a decasacharide. Sequential treatment of the oligosaccharide with -fucosidase and with β-galactosidase removed the fucose and a galactose residue. Methylation of the residual oligosaccharide showed the presence of a terminal N-acetylglucosamine and a N-acetylgalactosamine 2,3- and 3-linked galactose, 4-linked N-acetylglucosamine, and 3,6-linked N-acetylgalactosaminitol.

Periodate oxidation-borohydride reduction followed by methylation of the degraded oligosaccharide showed the presence of a terminal and 3-linked galactose, 4-linked N-acetylgluco, samine and 3-linked N-acetylthreosaminitol

Methylation of the oligosaccharide showed the presence of a terminal N-acetylglucosamine, N-acetylgalactosamine, galactose, and a fucose residue; 3-linked and 2,3-linked galactose; a 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. The results of these experiments showed that oligosaccharide N-3 is a decasaccharide with the possible sequence, linkage and anomery as shown in Fig. 2.

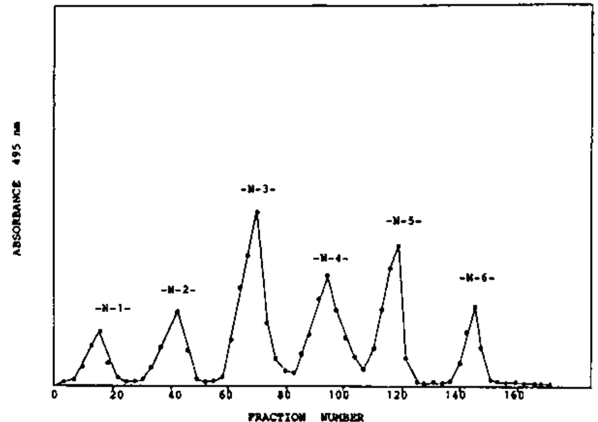

Fig. 2: Separation of oligosaccharides on a column of Bio-Gel P-6. Six fractions (N-1, 0.2 mg; N-2, 0.3 mg; N-3, 1.2 mg; N-4, 1.4mg; N-5, 1.8 mg; N-6, 0.6 mg) were obtained.

Oligosaccharide N-4

Oligosaccharide N-4 was purified on DEAE-Sephadex A-50 and was homogeneous in paper chromatography (solvent A and B).

Periodate oxidation-borohydride reduction completely removed fucose, N-acetylgalactosamine and galactose residues, and converted N-acetylgalactosaminitol to 2-acetyamido-2-deoxythreitol and N-acetylglucosamine was receoved unchanged. Sequential treatment of the oligosaccharide with α-fucosidase and β-galactosidase (C. lampas) showed the loss of only fucose.

Methylation of the oligosaccharide after α-fucosidase treatment showed the presence of terminal galactose and

N-acetylgalactosamine, 4-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Methylation of the sequentially α-fucosidase and -N-acetylgalactosaminidase treated oligosaccharide showed the presence of terminal galactose, terminal N-acetylgalactosamine, 4-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosaminitol. Methylation of the native oligosaccharide showed the presence of a terminal fucose and N-acetylgalactosamine, 2-linked and linked galactose, 4-linked N-acetylglucosamine, and 3,6-linked N-acetylgalatosaminitol. These results suggest that oligosaccharide N-4 is a hexasaccharide with the structure given in Fig.2. It appeared that terminal N-acetylgalactosamine is linked β or was resistant to -N-acetylgalactosaminidase due to conformational reasons.

Oligosaccharide N-5

Methylation of the oligosaccharide showed the presence of terminal galactose and N-acetylgalactosamine, 3-linked galactose, 4-linked N-acetylglucosamine and 3,6-linked N-acetylgalactosamine. Periodate oxidation-borohydride reduction resulted in total destruction of N-acetylgalactosamine and a residue of galactose and conversion of N-acetylgalactosaminitol to N-acetylthreosaminitol. N-acetylglucosamine and a galactose residue were recovered unchanged. Methylation of the residual oligosaccharide showed the presence of terminal galactose and 4-linked N-acetylglucosamine, suggesting that a galactose in the parent oligosaccaride is linked at C-3 to N-acetylgalactosamine. These results suggest the structure oligosaccharide N-5 as shown in Fig. 2.

Oligosaccharide N-6

The oligosaccharide N-6 was purified by paper chromatography in solvent A followed by chromatography on DEAE-Sephadex A-50, and was homogenous in solvents A and B. Methylation of the oligosaccharide showed the presence of a terminal galactose and 3-linked N-acetylgalactosaminitol. Treatment of the oligosaccharide with β-galactosidase (C.lampas) removed galactose partially. These results provide evidence for oligosaccharide N-6 to be a disaccharide.

Discussion

The separation of secreted glycoproteins from the remaining polymeric materials i.e., proteins, enzymes and lipids was 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, glycoprotein and bilirubin. It is known that proteolytic enzymes degrade a minor glycoprotein component of mucus glycoprotein [11]. Secreted glycoproteins are known to undergo proteolysis when treated with the Pronase, particularly in the hydrophobic region of the protein moiety [12]. A major breakdown of the glycoprotein is unlikely with the mild Pronase treatment that was given in this investigation, however. Fractionation of the Pronase-treated glcoprotein on the Sepharose 4B resulted in two fractions. The major fraction in ion-exchange chromatography resulted in a single high molecular weight component. Despite the fact that the glycoprotein are known to be degraded [12] by the Pronase, a high molecular weight glycoprotein, homogeneous, albeit polydisperse, in agarose electro-

phoresis and free of contaminting proteins was obtained. A glycoprotein, free of cross-linked protein fraction containing cystine, as is observed in the case of bovine cervical mucus [13,14], was obtained.

Treatment of the glycoprotein with alkaline borohydride resulted in a mixture of oligosaccharide alditols which was fractionated on Bio-Gel P-6 and was further purified by ion-exchange and paper chromatography affording four neutral oligosaccharides. The oligosaccharides structures characterized can be divided into two groups on the basis of the known core structures:

(i) Galβ (1→3) Gal NAc-ol
(ii) Gal (1→3) Glc NAcβ(1→6)Gal NAc-ol

The chain elongation of carbohydrate occurs on these two structures resulting in structures that has been characterized and is shown in Fig. 2. For gallabladder glycoproteins the structure of carbohydrates chains has not been completely identified and these pontial structures bear similarly to the secreted glycoprotein oligosaccharides core structures.

A variety of carbohydrate chain lengths in mucins [15] and in blood group active glycoproteins [16-18] are known, and the heterogeneity of gallabladder glycoprotein could be even complex because of the changing physicochemical behaviour.

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