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RAT-COLONIC, MUCUS GLYCOPROTEIN*†

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ABSTRACT

A glycoprotein was isolated from rat-colonic mucosa. Analytical ultracentrifugation studies showed the glycoprotein to be homogeneous, having an apparent molecular weight of 9.0×10^5; no subunits could be detected in the presence of sodium dodecyl sulfate. It contained 14% of protein and 86% of carbohydrate. The principal sugars in the glycoprotein were galactose, fucose, sialic acid, 2-acetamido-2-deoxygalactose, and 2-acetamido-2-deoxyglucose. A small proportion of mannose was also present. The glycoprotein, apart from the usual carbohydrate constituents present in mucus glycoproteins, contained sulfate, but no uronic acid. High amounts of serine and threonine, and low contents of aromatic and traces of sulfur-containing amino acids, reflect a similarity of this glycoprotein to other mammalian mucus glycoproteins; it differs, however, by its high proportions of Asx + Glx (26 mol.%). Cleavage studies with alkaline borohydride indicated *O*-glycosidic linkages between *N*-acetylhexosamine and serine, and threonine, of the peptide core in the glycoprotein. Only about one third of the serine and threonine was linked to the carbohydrate side-chains, which averaged about 22 units in length and were apparently branched.

INTRODUCTION

In our laboratory, we have purified and characterized the following mucus glycoproteins: bovine[1], ovine[1], porcine[2], hamster[3], armadillo[4], and canine[5] submandibular glycoproteins and bovine[6], rat[7], and hamster[8] sublingual glycoproteins.

Wold *et al.*[9], from their studies on the composition of water-soluble extracts from intestinal mucus, showed that their heterogeneous material consisted of mucin-type glycoproteins. Several years ago, we started studies on rat-colonic tissue, normal and malignant. This paper describes the isolation, purification and partial characterization of rat-colonic glycoprotein. Such a study should be of interest for comparison

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of the chemical and structural composition of the purified glycoproteins from normal and tumorous colonic tissue.

EXPERIMENTAL

Preparation of the glycoprotein (Chart I). — Colons from adult male Fischer rats, immediately after sacrifice, were excised lengthwise, and the mucosal layer was removed as described elsewhere[10]. The mucosal scrapings were washed several times with cold saline containing a mixture of antibiotics. The colonic scrapings from several rats were pooled, suspended in cold, distilled water (10 ml per g of scrapings) and homogenized in a Potter–Elvejhem glass homogenizer. The homogenized slurry was kept for 5 min in boiling water, cooled in ice, sonicated for 3-sec durations, and dialyzed at 4° against several changes of distilled water for 2 days. This step removed material of low molecular weight and also eliminated the unpleasant odor of the mucosal scrapings. The dialyzed material was centrifuged at 19,000 r.p.m. for 1 h at 4°, and the clear supernatant was lyophilized. More than 90% of the sialic acid of the mucosa was recovered in the supernatant. Purification of the rat-colonic glycoprotein was performed first by using lithium diiodosalicylate[11], followed by three successive treatments with hydroxylapatite, according to the procedure of Tettamanti and Pigman[1].

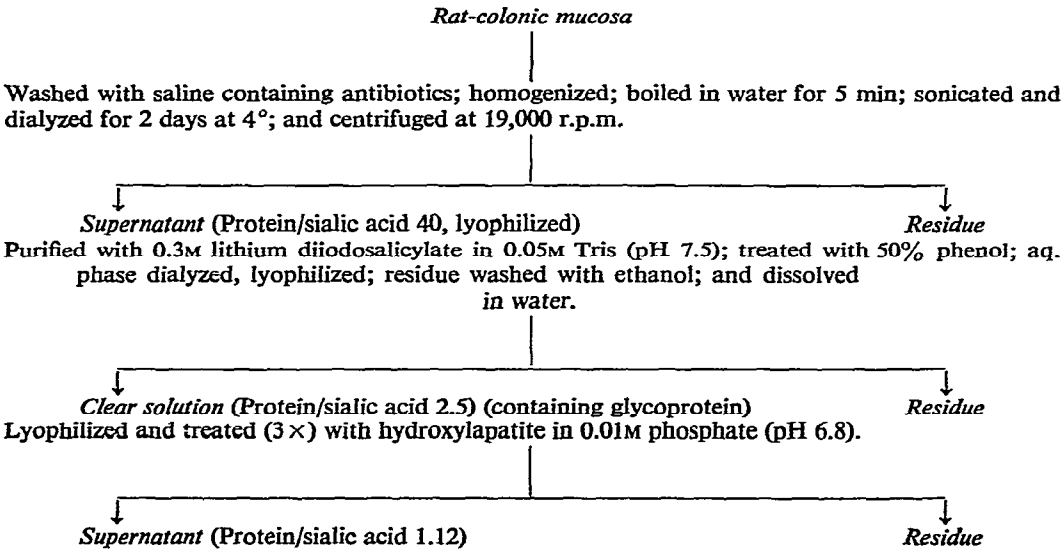

Chart I. Isolation and purification of rat-colonic glycoprotein.

Analytical methods. — The protein content of the samples was determined by the method of Lowry *et al.*[12] with crystalline bovine serum albumin as standard, and also with an amino acid analyzer. A Beckman Model 120B amino acid analyzer was used for assay of amino acids under conditions (6M hydrochloric acid, 22 h, 122°)

found suitable for mucus glycoproteins[13]. Total hexosamine was determined by the Blix modification[14] of the Elson–Morgan method, after hydrolysis in 6M hydrochloric acid for 4 h at 105°. The differential determination of 2-amino-2-deoxy-D-galactose and 2-amino-2-deoxy-D-glucose was conducted with an amino acid analyzer, as described by Downs and Pigman[15].

Sialic acids were determined by the resorcinol method of Svennerholm[16], with *N*-acetylneuraminic acid as the standard. The *N*-glycolyl content was determined by the method of Klenk and Uhlenbruck[17]. The *O*-acetyl content of the glycoprotein was measured by the alkaline hydroxylamine procedure of Hestrin[18], with ethyl acetate as the reference compound. Galactose, mannose, and fucose were assayed by the respective methods of Dische *et al.*[19,20]; uronic acid was assayed by the method of Bitter and Muir[21]; and sulfate was determined by the method of Schrager and Oates[22] using the dye Sulphonazo III, and by the procedure of Dodgson[23] using barium chloride–gelatin.

Ultracentrifugal analyses were made with a Spinco Model E analytical ultracentrifuge, using conditions of equilibrium centrifugation, as described by Payza *et al.*[24]. Glycoprotein solutions, at a concentration of 0.3 mg per ml in M sodium chloride, were centrifuged for 24 h at 20°, in a double-sector 12-mm interference centerpiece, at a speed of 5,784 r.p.m. A partial specific-volume of 0.633 was used for calculation of the molecular weight[25,26].

The alkali-catalyzed β-elimination reaction and reduction were carried out as described earlier[27,28].

Qualitative identification of neutral sugars was performed by both descending paper chromatography and by t.l.c. on microcrystalline cellulose plates ("Q-2", Quantum Industries, Fairfield, NJ 07006). For the release of the neutral sugars, the glycoprotein (3–4 mg) was hydrolyzed in 2M hydrochloric acid for 2 h at 100°, treated[7] with Bio-Rad AG1-X8 (HCO_3^-) resin, followed by AG50-X8 (H^+ form), and evaporated to dryness. The residue was dissolved in a few drops of water and chromatographed in the solvent system 4:1:5 1-butanol–acetic acid–water. The paper chromatogram was developed for 72 h, with L-fucose, D-galactose, D-mannose, and D-xylose as reference compounds. T.l.c. was performed with 2:1:2 ethyl acetate–pyridine–water. All sugars were located by the silver nitrate method[29] and by aniline phthalate[30].

For the release of sialic acids, glycoprotein samples (4–5 mg per ml) were dissolved in water, adjusted to pH 2.0 with 0.5M sulfuric acid, and heated for 90 min at 80°. The hydrolyzates were applied to a column of Bio-Rad AG1-X8 resin, 50–100 mesh (formate form) and eluted according to the method of Svennerholm[31], as described by Spiro[32]. The eluates, after drying in a rotary evaporator, were dissolved in a suitable quantity of water for the identification of sialic acids. T.l.c. on microcrystalline-cellulose plates was carried out with the following solvent systems: 1:2:1 (v/v) 1-butanol–1-propanol–0.1M hydrochloric acid, and 3:2:1 (v/v) 1-butyl acetate–acetic acid–water. Commercially available *N*-acetylneuraminic acid, and sialic acids released from purified bovine submaxillary glycoprotein under the

hydrolysis conditions, as already described, were used as reference compounds. Sialic acids on the plates were detected by the thiobarbituric acid reagent[33].

RESULTS

Isolation. — The rat-colonic glycoprotein was isolated by using successively water extraction, treatment with lithium diiodosalicylate, and hydroxylapatite (Chart I). The purified glycoprotein, which accounted for 40% of the total sialic acid-containing material of the wet, homogenized colons, was recovered in a yield of 1.2% from the original scrapings.

A preliminary step added in the isolation of the glycoprotein involved heating of the colonic scrapings for 5 min in a boiling-water bath. This treatment not only inactivated the hydrolytic enzymes and bacteria, but also eliminated the unpleasant odor of the colonic homogenate, and caused the precipitation of some extraneous proteins. Mucus glycoproteins do withstand such treatment at a slightly acidic pH without loss of viscosity of the solution.

The purified rat-colonic glycoprotein consisted of 14% protein, the rest being carbohydrate. The hydroxylapatite, in 3 consecutive batchwise treatments, eliminated the extraneous protein and led to a decrease of the protein/sialic acid ratio from 40 to 1.1. Further treatment with hydroxylapatite did not lower this ratio and caused increasing losses of glycoprotein.

The apparent molecular weight, based on a partial specific-volume of 0.633,

TABLE I

CHEMICAL COMPOSITION OF RAT-COLONIC GLYCOPROTEIN[a]

Component	*g/100 g*	*Molar ratio*[b]
Protein	13.7	
Sialic acid	15.0	2.2
NANA[c]	11.7	1.8
NGLNA[c]	3.3	0.4
GalNAc[c] (Total)	10.1	2.2
GalNAc[c] (Terminal)	4.6	1.0
GalNAc[c] (Internal)	5.5	1.2
GlcNAc[c]	8.4	1.8
Galactose	37.4	10.2
Fucose	15.3	4.7
Mannose	1.3	0.4
Sulfate	5.37	2.7

[a]Yield, 40% in terms of sialic acid. Molecular weight, 9.0×10^5 (sed. equil.). Average chain-length, 22 units. [b]The figures in the second column are based on the number of GalNAc residues actually attached to Ser + Thr residues in the core protein. [c]Abbreviations: NANA, *N*-acetylneuraminic acid; NGLNA, *N*-glycolylneuraminic acid; GalNAc, 2-acetamido-2-deoxy-D-galactose; and GlcNAc, 2-acetamido-2-deoxy-D-glucose.

was 9.0×10^5. There was no change in the molecular weight when the run was carried out in 0.2% sodium dodecyl sulfate (w/v), thus indicating the absence of subunits under these conditions.

Carbohydrate and amino acid components. — The chemical composition of the rat-colonic glycoprotein is presented in Table I. The analytical data constitute the average of triplicate analyses of 3 preparations.

Based on t.l.c. and paper chromatography, the principal neutral sugars were galactose and fucose. A small amount of mannose was also detected. These results were confirmed by quantitative colorimetric analyses of the foregoing sugars.

The differential hexosamine assay made with the amino and acid analyzer

TABLE II

AMINO ACID COMPOSITION OF RAT-COLONIC GLYCOPROTEIN

Amino acid[a]	*mol/100 mol*	*Amino acid*	*mol/100 mol*
Lys	2.9	Pro	9.6
Arg	1.6	Gly	8.5
Asx[b]	10.2	Ala	6.9
Thr	18.1	Val	5.1
Ser	11.7	Ile	3.0
Glx[c]	15.7	Leu	4.6
		Phe	1.7

[a]The following amino acids constitute <1% of the glycoprotein: His, Tyr, Cys1/2, Met. [b]Aspartic acid or asparagine. [c]Glutamic acid or glutamine.

TABLE III

ALKALINE β-ELIMINATION AND REDUCTION OF RAT-COLONIC GLYCOPROTEIN

Amino acid	*Moles/100 moles*			
	0	*5 h*	*10 h*	*15 h*
Threonine	18.1	15.3	14.6	12.5
Loss of threonine		2.8	3.5	5.6
Recovered as ABA[a]		2.8	3.4	5.6
Recovery (%)		100%	97%	100%
Serine	11.7	9.4	8.6	8.2
Loss of serine		2.3	3.1	3.5
Recovery of alanine		2.2	3.1	3.5
Recovery (%)		95%	100%	100%
Ser + Thr	29.8	24.7	22.2	20.7
Loss of Ser + Thr		5.1	6.6	9.1
Gal-NH_3	23.0	18.0	16.1	13.8
Loss of Gal-NH_3		5.0	6.8	9.2

[a]2-Aminobutanoic acid.

showed a slightly higher amount of 2-amino-2-deoxygalactose than of 2-amino-2-deoxyglucose. The molar ratio of these two amino sugars was found to be 1.2:1.0. As the amount of fucose was relatively high (Table I), this glycoprotein could be classified as a fucomucin. The sialic acid component of the glycoprotein consisted of *N*-acetyl- and *N*-glycolyl-neuraminic acids. The ratio of the former to the latter was found to be 4:1. The glycoprotein contained 1 mol of *O*-acetyl group per mol of sialic acid.

Table II gives the amino acid composition of this rat-colonic glycoprotein. Serine and threonine made up 30% of the protein core. Basic, aromatic, and sulfur-containing amino acids constituted only a small proportion. An unusually high percentage of Glx + Asx was found (25.9 mol. %).

Protein–carbohydrate linkage. — The alkali-catalyzed β-elimination reaction demonstrated an *O*-glycosyl linkage between the hydroxyamino acids and the carbohydrate chains in the glycoprotein. Table III shows the result of treatment of the glycoprotein with alkaline sodium borohydride and colloidal palladium for periods ranging from 0 to 15 h. A 100% recovery of 2-aminobutanoic acid and alanine, after 10 h, is evident from these data. Complete conversion of 2-amino-2-deoxygalactose into the alditol was observed.

DISCUSSION

The purified, rat-colonic mucus glycoprotein exhibited one symmetrical peak in the ultracentrifugal Schlieren pattern, and gave a linear Yphantis plot for log-$(c - c_0)$ *vs* x^2 by high-speed equilibrium sedimentation. Such a plot indicates apparent homogeneity with respect to molecular weight. Under the experimental conditions used, the molecular weight of this glycoprotein is 9×10^5, a value similar to that reported for the other mucus glycoproteins that we have studied, with the exception of the armadillo submandibular glycoprotein[4]. The low content of basic and aromatic amino acids (Table II) also supports the absence of extraneous protein in the isolated glycoprotein. Although the occurrence of sulfated glycoproteins in rat[9] and sheep[34] colons was previously reported, the purified, rat-colonic mucus glycoprotein reported here contains a higher amount of ester-sulfate groups than the former glycoproteins. The relatively high proportion of galactose of this glycoprotein (37%) is a characteristic shared by other sulfated glycoproteins[35,36] in contrast to unsulfated, secretory, mucus glycoproteins, which contain considerably lesser proportions of this sugar. Sialic acid accounts for 15% of the dry weight of the purified glycoprotein; a similar value was reported by Reid *et al.*[38] for rat-colonic glycoproteins. The molar ratio of the sum of fucose and sialic acid to 2-acetamido-2-deoxygalactose is 3.1:1.0. This ratio is even greater, if only 2-acetamido-2-deoxygalactose involved in an *O*-glycosyl linkage is taken into account. As calculated from Table III, only 33% of the 2-acetamido-2-deoxygalactose residues are involved in *O*-glycosyl linkages. Hence, this ratio is 9.1:1.0. As 2-acetamido-2-deoxygalactose is the sugar residue linking the oligosaccharide side-chains to peptidyl serine and threonine, and

as fucose and sialic acid are usually terminal groups, the side chains must be branched. The unsubstituted 2-acetamido-2-deoxygalactose and all of the 2-acetamido-2-deoxyglucose residues have to be internally linked in the carbohydrate side-chains. The average length of 22 sugar residues, based on the glycosidically linked 2-acetamido-2-deoxygalactose, is unusually long, compared to that of mucus glycoproteins; only the side chains of human ovarian-cyst glycoproteins are as long or longer[37].

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