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Intestinal mucin of germ-free rats. Biochemical and electron-microscopic characterization*

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ABSTRACT

Purified germ-free rat intestinal mucin was found by chemical analysis to contain 25% protein, enriched in serine, threonine, and proline, 75% carbohydrate, and no nucleic acid. It was analyzed by darkfield electron microscopy and found to consist of long filamentous molecules with a maximum length of ~740 nm, a mean length of 456 nm, and a mean width of 7 nm. Given reasonable assumptions derived from earlier work on other well-characterized mucins, the molecular weight of the peptide, calculated by the length from electron microscopy, was 200 000, and, given the chemical composition, the molecular weight of the entire mucin molecule was calculated to be ~800 000.

INTRODUCTION

Mucus glycoproteins, or mucins, are the most important components of the mucus layer that covers and protects the human intestinal mucosa. These macromolecules are very complex, and the determination of their molecular mass, like that of most mucins in general, and in particular respiratory mucins, is a matter of controversy[1,2]. These high-molecular-mass glycoproteins are polydisperse and contain about 80% carbohydrate in the form of O-linked oligosaccharides. Mucins are believed to be made of highly glycosylated regions and of naked regions more or less devoid of carbohydrate[3]. The peptide part of the glycosylated regions is rich in hydroxylated amino acids. When examined by electron microscopy, mucins are observed as thread-like structures ranging in length from 200 to > 1000 nm [4–10]. The reasons for such polydispersity remain obscure. Although some proteolytic degradation of the mucin peptide is always possible, recent biosynthetic evidence suggests that human bronchial mucins are inherently polydisperse[11].

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Mammalian intestinal mucins are carbohydrate-rich, high-molecular-weight, complex glycoproteins that are claimed to be produced continuously by secretory cells of the intestinal mucosa[12–15]. These glycoproteins form a viscoelastic gel serving as a protective barrier at the epithelial cell surface. The microflora of the digestive tract degrades mucin extensively[16,17], a fact that complicates the isolation and the study of the native substance. Furthermore, mucin-containing preparations from mucosal scrapings and especially from intestinal contents may be contaminated with other high-molecular-weight components from the diet. By the use of animals devoid of any microflora in the digestive tract, it is possible to isolate the native intestinal mucin in a high yield, as compared to that obtained from conventional animals[18]. In addition, by feeding the animals a defined diet, it is also possible to reduce significantly the amount of exogenous macromolecules in the intestinal contents.

We describe herein the isolation and characterization, by analytical, biochemical, and electron-microscopic examination, of intestinal mucin obtained from germ-free rats.

EXPERIMENTAL

Animals.—Adult, germ-free rats of the AGUS strain were fed a defined diet containing casein as the only high-molecular-weight component. After receiving only this diet for ten days, the animals were killed, and the contents of the cecum and colon collected.

Preparation of purified mucin fraction.— Four germ-free rats were put to death and their cecum and colon contents immediately collected. To remove lipids, the material was treated with organic solvents by stirring for 20 min at room temperature consecutively with a 20-fold excess of analytical grade acetone, 1:1 (v/v) chloroform–methanol, 4:1 (v/v) chloroform–methanol, and diethyl ether, respectively.

After each treatment, the organic solvent was removed rapidly by filtration. The residual dried product (1.74 g) was stirred with 25mM Tris·HCl buffer, pH 7.3 (40 mL) for 4 h at 5°. Undissolved material was centrifuged off, and the supernatant dialyzed with magnetic stirring for 3 days at 5° against distilled water. The clear, dialyzed solution was freeze-dried to give a crude extract (0.472 g). Intestinal mucins are considered to have been solubilized by this procedure, whereas dietary products are generally not.

Half of this material (0.24 g) was dissolved in 25mM Tris·HCl buffer, pH 7.3 (7.0 mL), applied onto a column (2.5 × 90 cm) of Sepharose CL-4B, and eluted with the same buffer. Fractions (8.0 mL) were collected and aliquots analyzed for carbohydrate and protein. Eluate fractions containing the glycoprotein were pooled, dialyzed, and lyophilized, yielding 57 mg. This product was dissolved in 50mM Tris·HCl buffer, pH 7.4 (3.5 mL), and applied onto a column (2.5 × 14 cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer. The column was eluted in 10-mL fractions with a linear gradient 0–1.0M of NaCl in the 50mM Tris·HCl buffer, in a total volume of 400 mL. Appropriate fractions (Fig. 1b) were pooled, dialyzed, and lyophilized to give a 38

mg yield. This material was subjected to a final gel filtration in the Sepharose CL-4B column, under the same conditions as for the first gel-filtration experiment. After dialysis and lyophilization, 6 mg of a purified mucin was obtained.

Carbohydrate analysis.— Carbohydrate analysis was performed by g.l.c. of *O*-trimethylsilylated methyl glycosides[19] in a Carlo Erba 4200 gas chromatograph, fitted with a flame-ionization detector. The sugars were separated on a SE-30 glass capillary column (30 m × 0.2 mm) with H_2 as the carrier gas; mannitol was the internal standard.

Amino acid analysis.— Amino acid analysis was performed with a Biotronik LC 5000 automatic amino acid analyzer after hydrolysis of the sample (0.50 mg) under vacuum in 6M HCl at 110° for 20 h. The elution of chromatographic columns was monitored by the phenol–H_2SO_4 test[20] for carbohydrate and by the method of Lowry *et al.*[21] for protein.

Gel electrophoresis.— Gel electrophoresis was carried out in a Protean 2 Slab Cell (Bio-Rad) under nondenaturing conditions, as described by Laemmli[22], using a 5% polyacrylamide slab gel; sample loads up to 150 μg were applied. Gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate with the periodic acid–Schiff (PAS) reagent as described by Zacharius *et al.*[23]

Electron microscopy.— Samples of mucin, dialyzed against 0.15M ammonium acetate, pH 6.8, and at a concentration of 0.1 mg/mL protein and 25% glycerol, were sprayed onto freshly cleaved mica, dried *in vacuo* by a 20-h outgassing at 1.3 μPa, and rotary shadowed with W by means of electron-bombardment heating according to a procedure described elsewhere[11,24]. The estimated average, metal-film mass thickness was 10^{-7} g/cm^2. Specimens were examined in darkfield in a JEM 100CX electron microscope using a top-entry stage with a 40-μm objective aperture, a matched annular condenser aperture, and 100-kV accelerating voltage. Micrographs were recorded at a magnification of 40 000 × and enlarged photographically to 128 000 ×. Length measurements were made on a Microplan electronic data pad and measurements plotted as histograms.

RESULTS AND DISCUSSION

The combination of gel filtration and ion-exchange chromatography was found sufficient to give a mucin preparation of satisfactory purity; thus, no use was made of enzymes or reducing agents. The fractionation steps are shown in Figs. 1a-c. Elution in the void volume from the Sepharose CL-4B column indicated a high mol. wt. characteristic of intestinal mucins. The material would be expected to be well separated from any undigested casein (mol. wt. 2–3 × 10^4) present in the rat diet. An ultraviolet spectrum of the mucin, shown in Fig. 2, indicated that no significant amount of nucleic acid contaminates the preparation.

The germ-free rat mucin was clearly polydisperse, in similarity to conventional rat-intestinal mucin preparations obtained by others[12–14,25]. This polydispersity is known to represent a mol.-wt. distribution in the range 4–5 × 10^5 to 1–2 × 10^6, as reported previously[18]. To test for purity, the mucin fraction was subjected to gel electrophoresis in

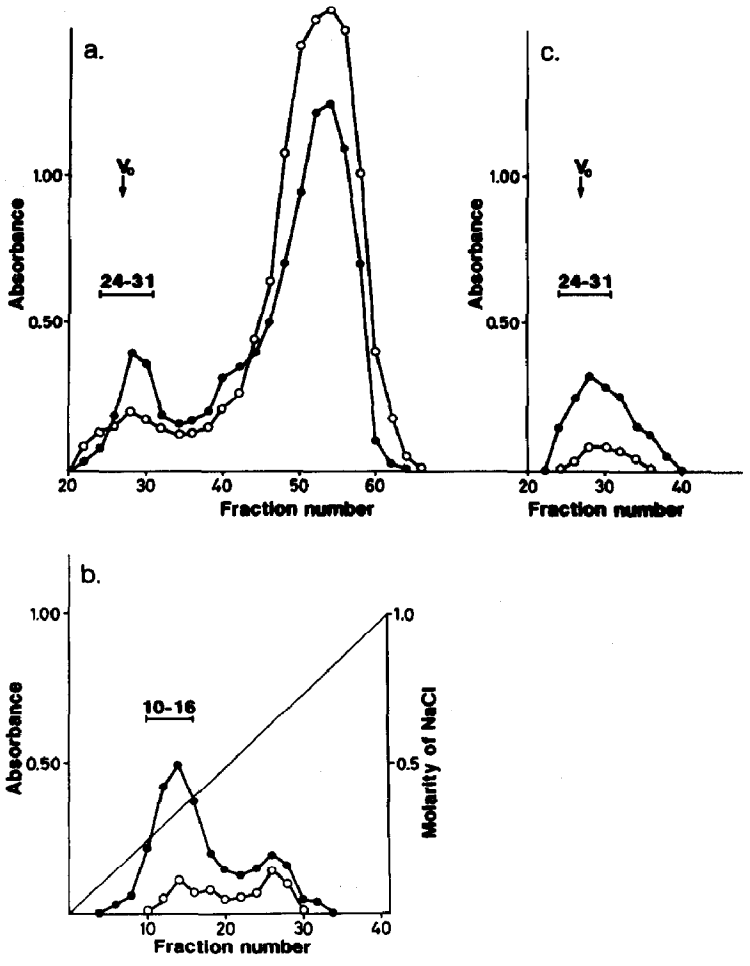

Fig. 1. Fractionation of the germ-free rat intestinal crude extract; (a.) Gel filtration in a Sepharose CL-4B column. Fractions (8.0 mL) were collected and analyzed for carbohydrate (●—●) and protein (○—○). (b.) Ion-exchange chromatography in a DEAE-Sepharose CL-6B column of the material in Fractions 24–31 from the Sepharose column. Eluate fractions (10.0 mL) were analyzed for carbohydrate (●—●) and protein (○—○). (c.) Gel filtration in the Sepharose CL-4B column of the material in Fractions 10–16 from the DEAE-Sepharose column, under the same conditions as in Fig. 1a. The fractions were analyzed for carbohydrate (●—●) and protein (○—○).

a 5% polyacrylamide gel (data not shown). Staining for carbohydrate revealed that the sample remained near the origin with a slight penetration (~1 cm) into the gel. Protein staining gave a very faint color coinciding with the much stronger carbohydrate staining. Low affinity of carbohydrate-rich glycoproteins for Coomassie Brilliant Blue in polyacrylamide or agarose has been observed previously[26]. No other bands were visible on the gels, even when, in some experiments, as much as 150 μg of sample was applied, indicating absence of contaminating proteins or glycoproteins.

The chemical composition of the mucin is shown in Table I. It has been demon-

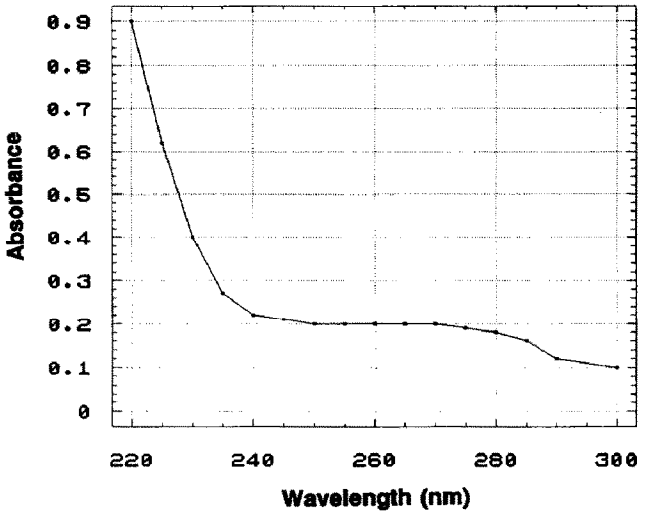

Fig. 2. U.v. absorption spectrum of 0.04% purified rat intestinal mucin in water, recorded manually with a CECIL CE-292 Digital Ultraviolet spectrometer, and showing absence of possible contaminating nucleic acid. Further experimental details are given in the text.

strated previously that part of the sialic acid occurs as *N*-glycolylneuraminic acid[18], but in the present investigation employing the standard analytical procedure, the sugar was estimated as *N*-acetylneuraminic acid. Apart from the trace of mannose, which may possibly originate from an insignificant amount of *N*-glycosidically-linked glycoprotein, the carbohydrate composition of the mucin material is quite characteristic of rat intestinal mucin, as is the proportion of amino acids, with the high values for threonine, serine, and proline (see Table II).

Electron micrographs showed that these mucins are filamentous (Fig. 3) with a length distribution extending to somewhat higher values than those found previously with standard, nongerm-free rats (data not shown). The mean length was 456 $\pm$188 nm (standard deviation), and the most frequent was 425 nm. The mean width was 7 $\pm$2 nm.

TABLE I

Carbohydrate composition and amino acid content of the purified mucin fraction

Sugar	*Percent*
Fucose	6.4
Mannose	Trace
Galactose	20.5
N-Acetylgalactosamine	19.5
N-Acetylglucosamine	17.8
Sialic acid[a]	9.8
Amino acids[b]	24.8

[a] Estimated as *N*-acetylneuraminic acid. [b] From the amino acid analysis

TABLE II

Amino acid composition of the purified mucin fraction

Amino acid	*Residues (per 100 residues)*	*Amino acid*	*Residues (per 100 residues)*
Asp	3.6	Val	3.8
Thr	30.4	Ile	2.3
Ser	17.0	Leu	2.1
Glu	9.4	Tyr	1.0
Pro	12.1	Phe	1.0
Gly	6.1	His	3.8
Ala	4.8	Lys	1.0
½Cy	0.6	Arg	0.7

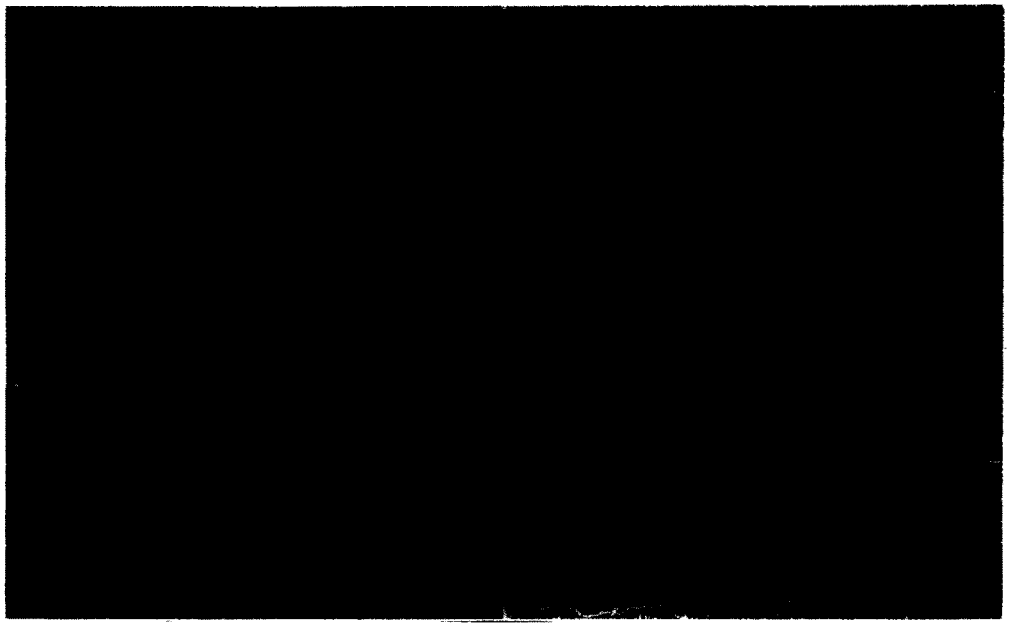

Fig. 3. Montage of darkfield electron micrographs of high-mol.-wt. rat intestinal mucins obtained from lightly tungsten-shadowed specimens (96 000 ×).

In studies with epiglycanin, Wold *et al.*[27] have found that its best-representative, extended length is that obtained by monitoring the length distribution up to the point where it essentially cuts off. In the present study, the maximum length observed with significant frequency is in the range of 740 nm (Fig. 4). Assuming that the mucin peptide chain is fully extended (0.364 nm per fully extended amino acid residue), a mucin species of 740 nm would correspond to a peptide having a mol. wt. of ~203 300. Given a mucin molecule consisting of 25% protein and 75% carbohydrate, the total calculated mucin mol. wt. would be ~813 000. The relatively broad length distribution is typical of

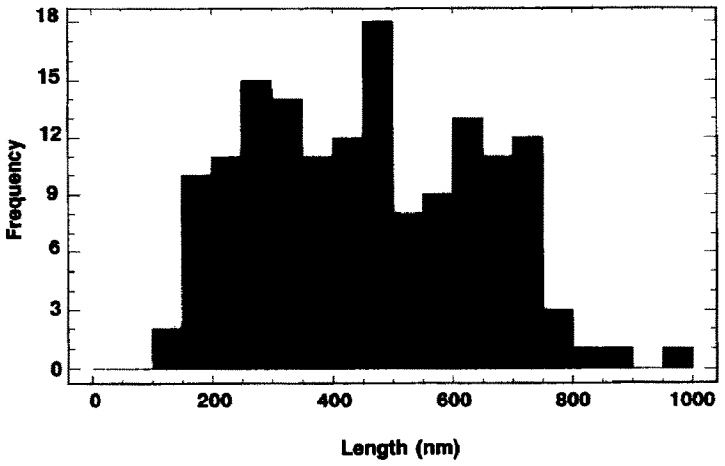

Fig. 4. Size of peptides of intestinal mucus glycoproteins. Length distribution of purified rat intestinal mucins obtained from electron micrographs. Number of measurements, 152; mean 456 nm; most frequent 425 nm.

mucins and is considered to be related to several factors: variable extension of the peptide bond; variable content of sialic acid; method of specimen preparation; frequency of carbohydrate side-chains; and secondary and tertiary structure, and diversity of mucin peptides. All of these factors have previously been implicated in polydispersity observed in mucin preparations[11].

On the other hand, our previous work with mucins indicated that even relatively homogeneous preparations of mucin molecules (determined in the ultracentrifuge) may be observed by electron microscopy to be heterogeneous in length[4,11]. Pauling *et al.*[28] have set limits on the variable extension of the peptide bond from a lower limit of 0.15 nm, equivalent to the α-helical configuration, to ~0.364 nm per residue, when fully extended. Thus, there is a range of more than two-fold over which the peptide bond can extend, and shorter lengths observed might also be associated either to this "accordion" effect on the peptide backbone or to proteolysis.

In the micrographs in Fig. 3, some regions of molecules appear rather straight, whereas others tend to be kinked, sometimes throughout their length. Moreover, in view of the observed 7-nm width, there may be unresolved microkinks related to the "accordioning" of the peptide bond. The breadth of the histogram in Fig. 4 may generally be related to the molecular mass of the polypeptide backbone by the following analysis: The peak on the low side, at ~310 nm, would correspond to a ~200 000 mol.-wt. peptide, assuming an extension per peptide bond of 0.15 nm. On the other hand, the upper range limit of 740 nm may represent a ~200 000 mol. -wt. polypeptide backbone but with a 0.364-nm extension per peptide bond; the small number of particles with a length >740 nm are considered to represent aggregates, and the small number with a length <310 nm may be due either to a low-mol.-wt. mucin species or to proteolysis.

In a recent study, Marianne *et al.*[11] collected human bronchial mucins directly

after secretion from macroscopically healthy bronchial mucosa. The samples were prepared in the presence of six proteinase inhibitors and analyzed by electron microscopy. The mucin molecules were found to be similar, in length distribution, to molecules prepared from sputum[7], although they were a little longer, their lengths being ≤ 1650 nm. This length corresponds to an extended mucin peptide of mol. wt. ~450 000. These peptide lengths were compared with the molecular size of biosynthetic precursors, and the results indicated that the 200 000–400 000 -mol.-wt. species are peptide precursors of these mucins, whose size is comparable with that obtained by electron microscopy for respiratory mucins collected directly from the macroscopically healthy bronchial mucosa. Again, in a study of pig gastric mucins, the correlation of mucin lengths observed by electron microscopy with the molecular size of the mucin precursors is relatively good[29]. By analogy with studies of human airway mucins, the maximum length obtained in significant number in the present study is considered likely to correspond to the polypeptide backbone produced biosynthetically, which would then correspond to a glycoprotein mol. wt. of the order of 800 000.

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