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Molecular Structure of Mucus Glycoprotein in Human Colon

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Abbreviations : ERBI, endoscopic retrograde bowel insertion; Gu-HCl, guanidine hydrochloride; CsCl, cesium chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; Kav., distribution coefficient average.

SUMMARY

Mucin was collected from human colon by the endoscopic retrograde bowel insertion method; the colonic lumen was perfused by repeated instillation and ingestion of distilled water. The mucus glycoprotein was extracted from the mucin, purified by repeated gel filtration chromatography and ultracentrifugation, subjected to chemical treatment and enzymatic digestion, and its properties were examined using high-performance liquid chromatography (HPLC). Our results can be summarized as follows. 1) The molecular weight of the mucus glycoprotein distributed polydispersely (Mr=5,800 K - 1,600 K). 2) The mucus glycoprotein was not reduced by 0.5 M β-mercaptoethanol, which contained 4 M guanidine hydrochloride. 3) Amino acid analysis demonstrated a predominance of five amino acids (serine, threonine, proline, alanine and glycine), which together comprised about 65-70 mol% of the total amino acids. No significant differences between the samples taken from five individuals, with respect to the molecular weight of the mucus glycoproterin were observed. Cysteine was not detected in any samples. 4) The carbohydrate moieties were composed of N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and N-acetylneuraminic acid; the molar ratio was about 1 : 4 : 4 : 2 : 2 respectively. Despite the different molecular weight no significant differences between the carbohydrate moieties and the molar ratio were

observed. The average carbohydrate chain length was about 13 monosaccharides. 5) Actinase E digestion of the mucus glycoprotein produced glycosylated "basic units" with an average molecular weight of 260 K. These results indicate that human colonic mucus glycoprotein molecules consist of several glycosylated "basic units". Each "basic unit" possesses long carbohydrate chains which consist of an average of about 13 monosaccharides, and is polymerized by stable covalent bonds, not by less stable non-covalent or disulfide bonds. Hitherto, all mucus glycoprotein molecules have been considered to have a "subunit structure" which could be separated from the mucus glycoprotein by reduction. However, the human colonic mucus glycoprotein was not degraded by redution with β-mercaptoethanol nor did it contain cysteine. In conclusion, this study has demonstrated, for the first time, that molecules have a novel "no subunit" structure.

Introduction

Interest in colonic mucus glycoprotein has increased considerably in recent years, because of its possible relationship with several diseases, such as colonic cancer, chronic ulcerative colitis, Crohn's disease (1-3) and similar conditions. However, only limited details of the chemical nature and metabolism of the colonic mucus glycoprotein are available (4-6).

In most studies, human colonic mucus glycoprotein has been extracted from tissue specimens obtained at surgery and autopsy. Therefore, due to the limited source material available, the yields are very small and, moreover, it is very difficult to separate the secretory mucus glycoprotein from other glycoproteins present in the glycocalyx of the epithelial cell surface and proteoglycans in the submucosa.

Recently, Muramoto et al. devised a new perfusion method, which enabled large amounts of human colonic mucin to be collected (7-9), and was called the "endoscopic retrograde bowel insertion (ERBI)" method. The colonic lumen is perfused with distilled water during clinical examination with a colonofiberscope and the perfusate is recovered.

The colonic mucus glycoprotein obtained by this method is of a high degree of purity and not contaminated by other glycans. Thus, it is possible to examine the chemical fea-

tures of the native colonic mucus glycoprotein in the form in which it is secreted into the colonic lumen. This paper describes the chemical features, especially the molecular structure, of secretory human colonic mucus glycoprotein.

MATERIALS AND METHODS

Chemicals

Actinase E was purchased from Kaken Kagaku Co., Tokyo, Sepharose CL-2B and CL-4B were from Pharmacia Fine Chemicals Co., Uppsala, Sweden, dextrans (Mr=2,000 K, 400 K and 70 K) were from Seikagaku Kogyo Co., Tokyo and glycogen (Mr=400 K, from shellfish) was from Nacalai tesque Co., Kyoto. All other reagents used were of analytical grade and obtained from commercial sources.

Determination of Mucus Glycoprotein Constituents

The constituents of mucus glycoprotein were determined as follows; hexose by the phenol-H_2SO_4 method, using galactose as a standard (10); N-acetylneuraminic acid, after hydrolysis with 0.2 M H_2SO_4 at 80°C for 1 h, according to the method of Warren (11); protein by the Bio-Rad Protein Assay method (12) and absorbance at 280 nm, using bovine γ-globulin as a standard.

The amino acid content was analyzed after hydrolysis of mucus glycoprotein (500 μg) with 5.6 N HCl at 110°C for 20 h, by a JEOL amino acid analyzer (Model JLC-6AH).

The content of each monosaccharide, with the exception of N-acetylneuraminic acid, was measured by the fluorescent pyridylamination method devised by Takemoto et al. (13). In brief, mucus glycoprotein (500 µg) was hydrolyzed at 100°C with 2 N HCl for 4 h (for hexose) or 4 N HCl for 8 h (for hexosamine), then each hydrolyzed carbohydrate was coupled with 2-aminopyridine. The excess reagent (non-coupled 2-aminopyridine) was removed by gel filtration high-performance liquid chromatography (HPLC) using a column of OHpak KB-803 (0.8 x 30 cm, Showa Denko Co., Tokyo), as described by Takagaki et al. (14). The fluorescent carbohydrate pyridylamino-derivatives were separated and quantified by HPLC on a reverse-phase column using an Ultrasphere-ODS column (0.46 x 25 cm, Beckman Co., CA, U.S.A.).

Purification of Mucus Glycoprotein

The extraction and purification procedures carried out on the human colonic mucus glycoprotein are summarized in Fig. 1.

Starting material: Human colonic mucin was collected by colonic perfusion with distilled water, using the ERBI method (7-9). Five healthy subjects (all male, mean age= 37.6 years, blood group type A) all gave informed consent to participation. In brief, at clinical examination by colonofiberscopy, the colonic lumen was perfused by repeated instillation and ingestion with distilled water,

and the perfusate recovered was prepared as described below.

Solubilization of mucin: Each perfusate containing mucus glycoprotein was lyophilized separetely, and the lyophilizate was agitated in 4 M guanidine (Gu)-HCl/50 mM Tris-HCl buffer (pH 7.4), which contained protease inhibitors (10 mM ethylenediamine tetraacetate, 10 mM N-ethylmaleimide, 2 mM phenylmethane sulfonyl fluoride and 5 mM benzamidine chloride) at 4°C for 24 h. The solution was centrifuged at 10,500 x g for 90 min and the supernatant was concentrated by ultrafiltration on a Diaflo YM-10 membrane with a 10 K cut-off (Amicon Co., MA, U.S.A.). This concentrated solution designated the crude mucin fraction.

First chromatography on Sepharose CL-4B: Each crude mucin fraction was chromatographed separately at 20°C on a column (2.2 x 100 cm) of Sepharose CL-4B with 4 M Gu-HCl/50 mM Tris-HCl buffer (pH 7.4) containing the protease inhibitors as the mobile phase at a flow rate of 10 ml/h. The eluted fractions (5 ml) were analyzed for hexose and protein, and then the carbohydrate-rich fractions were pooled (Fig. 2).

Equilibrium density gradient centrifugation: The first ultracentrifugation of the fractions recovered from Sepharose CL-4B was carried out on a cesium chloride (CsCl) density gradient at an initial density of 1.38 g/ml, dissolved in 4 M Gu-HCl/50 mM Tris-HCl buffer (pH 7.4) containing the protease inhibitors, at 20°C using a Hitachi RPS 40T Rotor at 156,000 x g for 72 h. Fractions were collected from the tops of the tubes, fractionated into 25

fractions, with a Hitachi Fractionater DGF-U, and analyzed for hexose and protein. The carbohydrate-rich fractions (density=1.50 - 1.30 g/ml) were pooled (Fig. 3a).

The fractions from the first ultracentrifugation were subjected to a second ultracentrifugation performed under the same conditions as described above. The carbohydrate -rich fractions (density=1.50 - 1.30 g/ml) were pooled and the combined fraction was dialyzed against 0.2 M Gu-HCl, to which CsCl was added to adjust the initial density to 1.38 g/ml, at 4°C for 48 h (Fig. 3b).

The third ultracentrifugation, of the fractions from the second was carried out on a CsCl density gradient, at an initial density of 1.38 g/ml with 0.2 M Gu-HCl. The carbohydrate-rich fractions (density=1.55 - 1.40 g/ml) were pooled (Fig. 3c).

Second chromatography on Sepharose CL-4B: The carbohydrate-rich fractions (density=1.30 - 1.40 g/ml), shown in Fig. 3c were concentrated by ultrafiltration on a Diaflo YM-10 membrane. The concentrated solutions were applied to a column (2.2 x 100 cm) of Sepharose CL-4B under the same conditions as described above and illustrated in Fig. 2. The carbohydrate-rich fractions were pooled and designated the mucus glycoprotein fraction (Fig. 4). The samples obtained from each of the five subjects were purified separately.

Fractionation of mucus glycoprotein by Sepharose CL-2B chromatography: In order to fractionate the purified mucus glycoprotein fraction according to molecular size, an aliquot of the mucus glycoprotein fraction (30 mg) was applied to a column (1.5 x 150 cm) of Sepharose CL-2B and eluted with 4 M Gu-HCl/50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 6 ml/h at 20°C. The eluted fractions (3 ml) were analyzed for hexose and protein. The eluted mucus glycoprotein was fractionated into five subfractions (Frs. I - V) according to the order of elution. Subfractions I, III and V were designated H-GP, M-GP and L-GP, that is high-, middle- and low-molecular-weight glycoprotein respectively (Fig. 5) and then used for experimentation as described below.

SDS-PAGE of Mucus Glycoprotein

In order to examine the purity of mucus glycoprotein at each purification step, sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) through 10% gel was carried out according to the method of Laemmli (15). The gel was stained for protein with Coomassie brilliant blue R-250 (Fig. 6).

Estimation of Mucus Glycoprotein Molecular Weight

The molecular weight of the mucus glycoprotein was estimated using gel filtration HPLC, which was performed using a column of Shodex OHpak KB-805 (0.8 x 30 cm, Showa Denko Co., Tokyo) with 0.2 M NaCl at an elution flow rate

of 0.5 ml/min at 30°C. The detector was a refractive index monitor (Hitachi L-3300 RI Monitor), and dextrans and glycogen were used as standards for calibration (Figs. 7 and 8).

Chemical Treatment

β-Mercaptoethanol treatment: Each mucus glycoprotein fraction (1 mg) was incubated in 1 ml of 0.5 M β-mercaptoethanol dissolved in 4 M Gu-HCl/50 mM Tris-HCl buffer (pH 8.0), which contained 5 mM ethylenediamine tetraacetate, at 37°C for periods of 24 h, 48 h and 120 h in order to reduce any disulfide bonds. After incubation, the samples were subjected to HPLC under the same conditions as described above, except that, samples were eluted with 0.1 M β-mercaptoethanol in 0.2 M NaCl to prevent repolymerization (Fig. 9).

Actinase E digestion: Each mucus glycoprotein (1 mg) was dissolved in 0.5 ml of distilled water, heated at 100°C in a water bath for 5 min, an equal volume of 0.1 M Tris-HCl buffer (pH 8.0) which contained 20 mM $CaCl_2$, was added and digestion was performed with 100 μg of actinase E at 37°C for 120 h. In order to ensure the glycoproteins were completely digested with actinase E, the samples were recovered by ethanol precipitation (at 24 h and 48 h of incubation), the precipitates were digested, and the digested materials were subjected to HPLC under the same conditions as described above (Fig. 10).

RESULTS

Purification of Mucus Glycoprotein

The results obtained at each of the purification stages performed on the crude mucin fractions are illustrated as follows: First Sepharose CL-4B column chromatograms (Fig. 2); three ultracentrifugations on a CsCl equillibrium density gradient (Fig. 3) and second Sepharose CL-4B column chromatograms (Fig. 4). The molecular sizes, determined by gel filtration HPLC, of each mucus glycoprotein fraction obtained from the five subjects, did not differ. The total purified mucus glycoprotein yield from the five colonic perfusates was 73 mg (dry weight).

Fractionation of Mucus Glycoprotein by Sepharose CL-2B Chromatography

The Sepharose CL-2B fractionation chromatograms of the purified mucus glycoprotein fraction are shown in Fig. 5. The sample eluted as a broad area and Kav. ranged from 0 (the exclude fraction) to 0.52. The eluate separated into five subfractions designated as Fr. I (tube numbers, 35-39), Fr. II (40-44), Fr. III (45-49), Fr. IV (50-54) and Fr. V (55-64) in order of elution.

SDS-PAGE

The results of SDS-PAGE at each purification step are illustrated in Fig. 6. The mucus glycoprotein itself did not penetrate the gel but innumerable proteins were present

in the crude mucin fraction (Lane 2). As the purification process progressed, fewer contaminating proteins were present at each step (Lanes 3 - 6). As shown in Lane 7 there were no contaminating proteins in the mucus glycoprotein fraction.

Mucus Glycoprotein Constituents

The purified mucus glycoprotein consisted of 18% protein and 82% carbohydrate moieties.

Amino acid composition: Considerable amounts of serine threonine, proline, glycine and alanine were present; their sum total comprised about 65-70% of the total amino acid residues (Table 1). The amino acid profiles of all the fractions were remarkably similar. No cysteine was detected in the mucus glycoprotein which indicates there were no disulfide bonds present.

Carbohydrate composition: The carbohydrate moieties consisted of N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and N-acetylneuraminic acid, and their molar ratios were about 1 : 4 : 4 : 2 : 2 respectively. No glucose, mannose, glucuronic acid or iduronic acid were detected (Table 2). Therefore, the mucus glycoproctein carbohydrate chains consisted of O-glycosidically linked oligosaccharides and contained no N-glycosidically linked oligosaccharides or glycosaminoglycans. The average carbohydrate chain length was estimated to be about than 13 monosaccharide residues.

Molecular Weight of Mucus Glycoprotein

The estimated molecular weights of the mucus glycoprotein subfractions are shown in Fig. 7. Each subfraction (Frs. I - V) showed successively different retention times, in order of their Sepharose CL-2B chromatographic elution positions. The molecular weight of human colonic mucus glycoprotein ranged from 5,800 K to 1,600 K, which indicates that the molecular weight of human colonic mucus glycoprotein is distributed polydispersely.

Three subfractions (Frs. I, III and V) were detected which were designated high-, middle- and low-molecular-weight mucus glycoprotein (H-GP, M-GP and L-GP) respectively, with molecular weights of 5,800 K, 4,200 K and 1,600 K respectively (Fig. 8). The amino acid and carbohydrate constituents of the three mucus glycoprotein fractions were the same, despite their markedly different molecular weights (Tables 1 and 2).

Molecular Structure of Mucus Glycoprotein

Dissociation of non-covalent bonds: In order to investigate the molecular structure of mucus glycoprotein, the samples were subjected to the following chemical treatments, which dissociate non-covalent bonds. Aliquots (200 µg) of the mucus glycoprotein fractions were dissolved in 200 µl of 1.0 M NaCl, 7 M urea, 4 M Gu-HCl or 2% EDTA, and then incubated in each solution at 37°C for 24 h. The reference solution was a control preparation of 200 µg crude mucus glycoprotein

fraction incubated in 0.2 M NaCl. Each sample was subjected to HPLC and eluted with the same solution that was used for incubation. The HPLC elution profiles of the samples after chemical treatment to dissociate non-covalent bonds were the same as that of the control preparation in 0.2 M NaCl.

These results indicate that non-covalent bonds, such as ionic, hydrogen and hydrophobic bonds, were not involved in the molecular polymeric structure.

Reduction of disulfide bonds: Despite the prolonged incubation with β-mercaptoethanol, no change of the HPLC elution profiles of the H-GP, M-GP and L-GP subfractions, compared with control was observed (Fig. 9), which indicates that no disulfide bonds were involved in the molecular polymeric structure.

Actinase E digestion: After actinase E digestion (for 12 h) the three subfractions, which originally were of different molecular sizes were degraded to fractions of a similar size, with an average molecular weight of 780 K. After more prolonged digestion (for 48 h), part of the mucus glycoprotein subfractions were degraded into even smaller-sized fractions with an average molecular weight of 260 K (Fig. 10). Therefore, these results show that the mucus glycoprotein subfraction molecules possessced sites which were susceptible to actinase E degradation and those which were not.

In conclusion, it appears that the colonic mucus glycoprotein molecules are composed of common "basic

units", which can be obtained by actinase E digestion and have the same amino acid and carbohydrate composition.

DISCUSSION

In recent years, many investigators have shown a lot of interest in human colonic mucus glycoprotein, in particular from the point of view of the pathophysiology of colonic cancer and inflammatory bowel disease (1-3). However, the details of its chemical structure and metabolism have yet to be elucidated. Investigation of colonic mucus glycoprotein is difficult due to its high molecular weight, the microheterogeneity of the carbohydrate chains and sampling is not an easy procedure.

Hitherto, colonic mucus glycoprotein has been collectd by scraping it from specimens of colonic mucosa, which have been obtained at surgical operation or autopsy. It has been difficult therefore, to obtain large quantities of colonic mucus glycoprotein and impossible to obtain repeated samples from the same individual. Moreover, samples obtained by the scraping method contain not only secretory mucus glycoprotein but also cell surface glycocalyx and submucosal proteoglycans, which are difficult to separate from each other. Previous studies by Muramoto et al.(9) indicated that the mucus glycoprotein obtained by the ERBI method contained little proteoglycan contamination.

The Fluorescent pyridylamination method was used for the determination of mucus glycoprotein carbohydrate composition for the first time in this study. This technique enabled hexose, hexosamine and hexuronic acid to be assayed simultaneously, the carbohydrate composition to be determined accurately and demonstrated that there was no contamination with proteoglycans (Table 2).

Since it was first proposed by Allen and his co-workers (16) the mucus glycoproteins have been considered to have a "subunit" structure, and the subunits can be separated from the native macromolecules by reduction with -mercaptoethanol or dithiothreitol (17-29).

However, the colonic mucus glycoprotein obtained by the ERBI method was not reduced by 0.5 M β-mercaptoethanol in 4 M Gu-HCl, nor was cysteine detected by amino acid analysis. These results indicate that human colonic mucus glycoprotein does not have a "subunit" structure nor does it contain disulfiide bonds.

As shown by the Sepharose CL-2B column chromatogram (Fig. 5), the molecular weights of human colonic mucus glycoprotein distributed polydispersely, but despite this the carbohydrate and amino acid compositions of the various fractoions were very similar (Tables 1 and 2). After actinase E digestion, the human colonic mucus glycoprotein fractions, which initially had various molecular weights, were degraded into similar "basic units", with an average molecular weight of 260 K.

The polydispersity of molecular weight, therefore, appeared to be due to differing numbers of "basic units" in the various subfractions. Therefore, we hypothesize, based on this model of colonic mucus glycoprotein structure, that biosynthesis of human colonic mucus glycoprotein proceeds as follows. First, the "basic units", of average molecular weight of 260 K , are synthesized in the epithelial cells, and then are polymerized through formation of stable covalent bonds. It appears likely that the protease-stable colonic mucus glycoprotein macromolecules may be synthesized in this manner.

In conclusion, our results indicate that human colonic mucus glycoprotein does not have a "subunit" structure. Thus, the mucus glycoproteins may not be homogeneous substances, as has been believed hitherto, and there may be sub-types of these substances.

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