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Determination of Polydispersity of a Human Colonic Mucus Glycoprotein Using Rate-Zonal Centrifugation and Laser Light-Scattering

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Abstract: The colonic epithelium is coated by a visco-elastic gel, mucus that lubricates and protects the mucosa from the external environment. Mucus glycoproteins (mucins, MUCs) are major macromolecular components with MUC2 representing the major gel-forming secretory mucin. Previously we purified MUC2 from a cell culture environment with either three rounds of isopicnic centrifugation or gel filtration chromatography followed by a single round of centrifugation. In the present work, using rate-zonal centrifugation in a gradient of guanidinium chloride we fractionated this purified MUC2 and its subunits. Analyses of the fractions showed that the unreduced MUC2 molecules are quite large and polydisperse in molecular mass. Reduction of disulphide bonds cleaved the large polydisperse molecules into smaller and more uniformly sedimenting species. Individual fractions from both the unreduced and reduced MUC2 molecules were then subjected to laser light-scattering performed as absolute intensity measurements to obtain average molecular mass (M_r) and radius of gyration (R_G). The M_r for the unreduced and reduced MUC2 was 30 x 10^6 and 16 x 10^6 and R_G was in the range 290-309 nm and 200-260 nm, respectively. We concluded that colonic MUC2 mucin can be described as polydisperse and the combination of rate-zonal centrifugation and laser light-scattering techniques could certainly be applied as an effective method to determine their polydispersity.

Key Words: Polydispersity, colonic mucins, rate-zonal centrifugation, light-scattering

Introduction

Human colonic mucosa synthesises and secretes mucins which constituent a highly-hydrated, visco-elastic gel barrier (mucus) on the luminal side of the intestine. Once secreted, mucins form this barrier, not only to protect the delicate epithelial cells against the extracellular environment, but also to select substances for binding and uptake by these epithelia (1,2). Considerable advances have been made in recent years in the understanding of the biochemical and biophysical characteristics of mucins. Their hydrodynamic properties are consistent with the macromolecules being linear flexible chains that occupy an expanded spheroidal domain in dilute solution (3). The specific gel forming ability of mucins is due to a combination of structural features of the mucin molecules, which have an oligomeric structure containing several monomeric glycoproteins (4).

In the human colon the major secretory mucin MUC2 consists of two heavily glycosylated domains: the variable number of tandem repeat (VNTR) domain and the non-tandemly repeated domain. The MUC2 gene has been mapped to chromosome 11p15 (5). Recent studies of MUC2 from cell culture suggest that the MUC2 apoprotein is N-glycosylated in the endoplasmic reticulum (ER) prior to disulphide bond formation, followed by O-glycosylation in the Golgi (4,6-9). It is also clear that MUC2 undergoes further oligomerisation within the cell to produce very high M_r mucins (9).

Using two different approaches we previously purified colonic MUC2 mucin from a cell culture environment (10). In the present study we attempted to investigate molecular mass differences of the unreduced and reduced MUC2 mucins obtained from the same cell line (PC/AA) with rate-zonal centrifugation in a gradient of guanidinium chloride. We have demonstrated the presence of a huge difference in their sedimenting rates. The individual fractions of both populations were then analysed with laser light-scattering performed as absolute-intensity measurements. The results of these two experiments suggested that colonic MUC2 mucins were quite large and polydisperse in molecular mass and

the combination of rate-zonal centrifugation and laser light-scattering techniques could be used as a suitable approach to demonstrate their polydispersity.

Materials and Methods

Chemicals

PBS tablets, EDTA, goat anti-rabbit IgG horseradish peroxidase conjugate, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, periodic acid, Schiff's reagent, dithiothreitol (DTT), iodoacetamide and guanidinium chloride (practical grade) were purchased from Sigma Chemical Co. (Poole, UK). Stock solutions of guanidinium chloride (approx. 8 M) were treated with charcoal before use.

Cell culture and collection of mucins

The PC/AA adenoma cell line was derived from a single, large, colonic tubular adenoma of 3-4 cm diameter that exhibited only mild dysplasia. The cells were continuously passaged *in vitro* at 37 °C in 5% CO_2 in air and 3T3 feeder cells on collagen type IV-coated T25 flasks. The cell layers were solubilised with 6 M GuHCl containing proteinase inhibitors.

Antibodies

A polyclonal antiserum, LUM2-3, was a kind gift from Dr. I. Carlstedt (University of Lund, Sweden). This antiserum was raised against a synthetic peptide NGLQPVRVEDPDGC in the non-TR of the molecule towards the C-terminus (11).

Preparation of reduced mucin subunits

Reduced mucin subunits were obtained following dialysis of the purified whole mucins into GuHCl reduction buffer (6 M guanidinium chloride/0.1 M Tris/5 mM EDTA, pH 8.0) by treatment with 10 mM dithiothreitol for 5 h at 37 °C. Iodoacetamide was then added to a final concentration of 25 mM and the mixture left in the dark overnight at room temperature. Reduced mucin subunits were dialysed into 6 M guanidinium chloride and stored at 4 °C.

Alternatively the mucins may be reduced on the membrane after slot blotting using DTT and then iodoacetamide in reduction buffer. In brief, the blotted membrane was washed in distilled water for a few minutes and incubated in 6 M GuHCl reduction buffer containing 10 mM DTT at room temperature for 15 min. After pouring DTT off, the membrane was incubated in 6 M GuHCl reduction buffer containing 25 mM iodoacetamide at room temperature for 10 min and washed twice with distilled water (5 min each wash) and blocked in TBST buffer containing 1% (w/v) skimmed milk powder for immunoblotting assay.

Rate-zonal centrifugation

Rate zonal centrifugation was carried out as previously described by Sheehan and Carlstedt (12). The purified intact MUC2 mucin and its reduced subunits were dialysed into 4 M GuHCl. The samples in 4 M GuHCl were layered onto the top of the preformed GuHCl gradients (6-8 M), produced by using a linear gradient maker (MSC), with a Hamilton syringe and centrifuged in a Beckman SW40 (13 ml gradient tube containing 500 µl of sample, at 40,000 rpm, at 20 °C) swing out rotor for 2.5 h. The tubes were unloaded from the top into twenty-four 500 µl fractions. Fractions were analysed by the LUM2-3 antibody and PAS (Periodic Acid Schiff's). The molarity of GuHCl was determined by measuring the refractive index with an Abbe refrectometer.

Laser light-scattering

The fractions obtained from rate-zonal centrifugation performed on the purified intact and reduced MUC2 mucin molecules were passed through an in-line Dawn® DSP laser photometer and a Wyatt/Optilab 903 interferometric refractometer (Optichem, Clywd, UK) to measure light scattering and sample concentration respectively. Light scattering measurements were taken continuously at 18 angles between 15° and 151°.

Determination of molecular mass and radius of gyration

Unreduced and reduced mucin subunits in 4 M GuHCl were chromatographed on a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min and the fractions passed through an on-line Dawn DSP laser photometer and a Wyatt/Optilab 903 interferometric refractometer (Optichem, Clywd, UK) to measure light scattering and sample concentration respectively. Light-scattering measurements were taken continuously at 18 angles between 15° and 151° and the data analysed using the following equation:

$$\frac{R_\theta}{K_C} = M_r P(\theta) - 2A_2 c M_r^2 P^2(\theta)$$

where R_θ is the excess Rayleigh ratio, C is the concentration of the solute molecules in the solvent, M_r is the average molecular mass, A_2 is the second virial coefficient, K* is an optical constant = $4\pi^2 n_0^2 (dn/dc)^2 \lambda_0^{-4} N_A^{-1}$, where n_0 is the refractive index of the solvent at the incident radiation (vacuum) wavelength, λ_0 is the incident radiation (vacuum) wavelength, expressed in nanometers, N_A is Avogadro's number, equal to 6.022×10^{23}, and dn/dc is the differential refractive index increment of the solvent-solute solution with respect to a change in solute concentration (this factor must be measured independently using an refractive index detector), $P(\theta)$ is a theoretically-derived form factor. It can be related to the distance between scattering centres in the molecule by

$$P(\theta) = 1 \frac{2\mu^2 \langle r^2 \rangle}{3!} + \ldots.$$

where $\mu = (4\pi / \lambda) \sin(\theta/2)$, r^2 is the mean square radius of a molecule with respect to its centre of gravity. The square root of the mean square radius is also called the R_G (radius of gyration).

Results

The molecular mass distribution of MUC2 macromolecules was determined by rate-zonal centrifugation using gradients of guanidinium chloride as described by Sheehan and Carlstedt (3). The distribution of intact MUC2 mucins, as monitored by using PAS and anti-MUC2 antibody LUM2-3 measurements, show at least three peaks of increasing height from the top of the gradient (Fig. 1). The PAS profile of the intact mucins was quite similar to the antibody profile (Fig. 1). The polydispersity of the MUC2 mucins obtained from the same cell line obscured when they were reduced to the subunits (Fig. 2). The distribution of the reduced MUC2 subunits showed a more uniform profile with the antibody and PAS.

The M_r and R_G of the intact and reduced MUC2 mucins in fractions across the LUM2-3 distribution were determined by in-line laser light scattering after rate-zonal centrifugation and the values are summarised in Tables 1 and 2. The M_r distribution of the MUC2 molecules was detected using a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min. The effluent was monitored by a multi-angle light scattering detector (Dawn® DSP system) and the molecular weight distribution of intact MUC2 molecules (Fig. 3) and the reduced subunits (Fig. 4) were determined. The data show that the reduced MUC2 mucin subunits are of similar molecular mass (Mm) and size (R_G). However, there was a slight increase in the size (R_G) of the polydisperse intact MUC2 species.

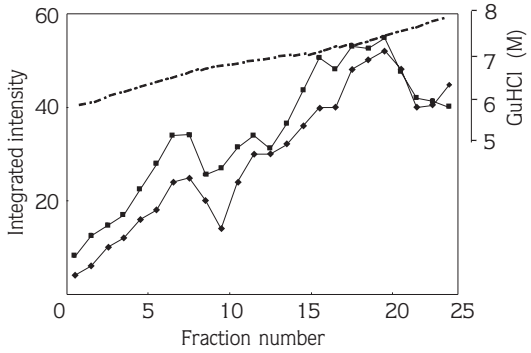

Figure 1. Rate-zonal GuHCl gradient centrifugation of the intact MUC2 mucins
The intact MUC2 mucins were dialysed against 4 M GuHCl and 500 µl of the samples were layered onto preformed GuHCl gradients (6-8 M) and thereafter subjected to centrifugation in a Beckman SW 40 swing-out rotor at 40,000 rpm for 2.5 h at 20 °C. After centrifugation the tubes were emptied from the top and the fractions (0.5 ml) were analysed with anti-MUC2 antibody LUM2-3 (-■-) and PAS (-◆-). Refractive index was measured to determine the molarity of GuHCl (—·—·—·—·).

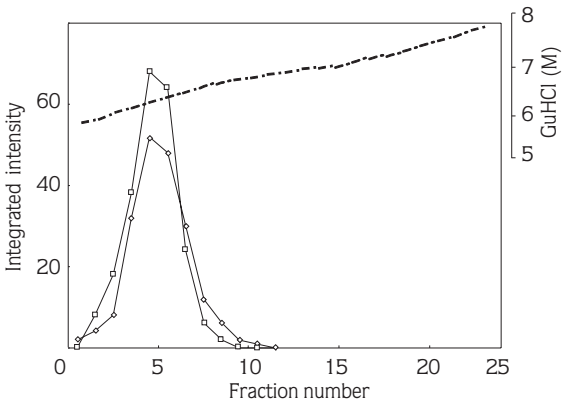

Figure 2. Rate-zonal GuHCl gradient centrifugation of the reduced MUC2 subunits
The MUC2 subunits were dialysed against 4 M GuHCl and 500 µl of the samples were layered onto preformed GuHCl gradients (6-8 M) and thereafter subjected to centrifugation in a Beckman SW 40 swing-out rotor at 40,000 rpm for 2.5 h at 20 °C. After centrifugation the tubes were emptied from the top and the fractions (0.5 ml) were analysed with anti-MUC2 antibody LUM2-3 (-⊟-) and PAS (-◇-). Refractive index was measured to determine the molarity of GuHCl (—·—·—·—·).

Table 1. Molecular mass and radius of gyration data of the intact MUC2 mucins
The fractions across the LUM2-3 distribution of the intact MUC2 mucins on the rate-zonal guanidinium gradient were chromatographed at room temperature on a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min. The effluent was monitored by a multi-angle light scattering detector (Dawn® DSP system) and a Wyatt/Optilab interferometric refractometer.

Fraction number	Molecular mass (Da X 10^7)	Radius of gyration (nm)
5	2.7	292
8	2.9	294
12	3.2	295
18	3.8	304
20	4.1	307

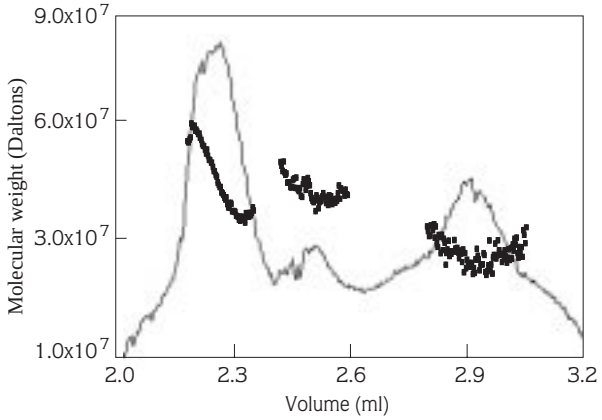

Figure 3. Molecular weight distribution of the intact MUC2 mucins
The purified intact MUC2 mucins were dialysed against 4 M GuHCl and subsequently chromatographed at room temperature on a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min. The effluent was monitored by a multi-angle light scattering detector (Dawn® DSP system) and molecular weight distribution of intact MUC2 molecules was shown.

Table 2. Molecular mass and radius of gyration data of the MUC2 subunits
The fractions across the LUM2-3 distribution of reduced MUC2 subunits on the rate-zonal guanidinium gradient were chromatographed at room temperature on a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min. The effluent was monitored by a multi-angle light scattering detector (Dawn® DSP system) and a Wyatt/Optilab interferometric refractometer.

Fraction number	Molecular mass (Da X 10^7)	Radius of gyration (nm)
4	1.5	202
6	1.6	203
8	1.6	206

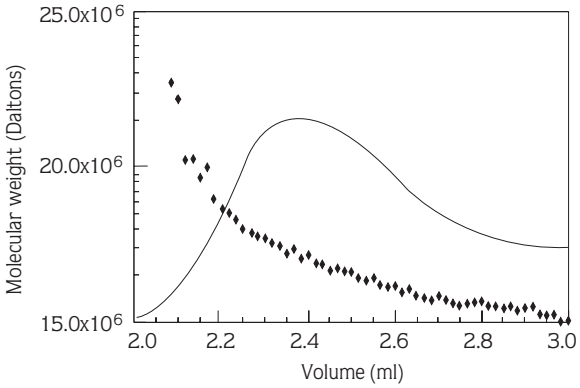

Figure 4. Molecular weight distribution of the reduced MUC2 subunits
The purified intact MUC2 mucins were reduced and dialysed against 4 M GuHCl, and subsequently chromatographed at room temperature on a Sephacryl S300 column (10 x 2 cm) eluted with 4 M GuHCl at a flow rate of 0.5 ml/min. The effluent was monitored by a multi-angle light scattering detector (Dawn® DSP system) and molecular weight distribution of reduced MUC2 subunits was shown.

Discussion

It has been demonstrated previously that mucins synthesised by the colonic cell line PC/AA are predominantly products of the MUC2 gene (4,9,10,13). In the present study we determined a polydisperse distribution of these MUC2 gene products by exploiting their large molecular mass with rate-zonal centrifugation followed by laser-light scattering techniques. In our previous paper, using isopicnic density-gradient centrifugation we defined polydispersity and heterogeneity of colonic MUC2 mucins on the basis of their buoyant densities (4). We characterised three MUC2 populations in different densities that were unglycosylated, partially glycosylated and fully glycosylated MUC2 species. However, in that investigation the presence of three populations with different buoyant densities shows that heterogeneity could be superimposed on polydispersity. Thus, here we worked on the purified MUC2 molecules in which we do not expect to find unglycosylated and/or partially glycosylated species (immature MUC2 molecules) but fully glycosylated (mature molecules) MUC2 molecules.

Unfortunately, isopicnic density-gradient centrifugation was not effective in demonstrating the polydispersity of the purified mature MUC2 molecules, because all polymeric forms of MUC2 were fully glycosylated and detected almost at the same buoyant densities. We therefore tried to show the polydispersity by using rate-zonal centrifugation that separates particles according to their sedimentation coefficiencies. By this method, we detected that the purified intact MUC2 mucin preparations contain at least three populations of molecules that differ with respect to their sedimentation coefficients. The LUM2-3 reactivity of these three populations increased parallel to their sedimentation rates. A similar distribution was observed with PAS indicating the presence of the increasing density of glycosylation on the MUC2 mucin polypeptide. This is in agreement with Sheehan and Carlstedt, who described a linear relationship between the sedimentation coefficient and M_r for cervical mucins (3). The polydisperse distribution of MUC2 mucins is very similar to those previously described for cervical (3) and respiratory (14) mucus glycoproteins with respect to both molecular size and buoyant density. It is therefore possible to assume that polydispersity is a characteristic feature of mucins.

The polydisperse structure of MUC2 mucins was also supported by the measurement of M_r and R_G with an in-line Dawn® DSP laser photometer and a Wyatt/Optilab 903 interferometric refractometer (Optichem, Clywd, UK). The refractive index measurement profile demonstrated that the populations of MUC2 molecules showed a continuous increase in molecular mass towards the bottom fractions of the gradient. The intact MUC2 mucins isolated from a colonic cell layer extract were polydisperse in size as seen by the measurement of R_G. This must arise from a variable number of subunits in the intact oligomeric mucins. In common with the gel-forming mucins isolated from respiratory (14) and cervical tracts (15) that had oligomeric structure they can be fragmented into their constituent subunits by reduction of disulphide bonds.

Reduction of disulphide bonds cleaved the large polydisperse molecules into smaller and more uniformly sedimenting species on the rate-zonal guanidinium gradient, and supported the finding that the mucins are assembled from subunits joined end-to-end (3,4,9,13). We previously demonstrated that MUC2 reduced mucin subunits are heterogeneous in their charge distribution and two major populations of molecules can be observed (4). We have also noted differently glycosylated populations of this mucin in the cell layer extract. The two populations of MUC2 mucin subunits can be distinguished on the basis of glycan-epitope specific monoclonal antibodies and there is evidence that the lower charged form has a higher mannose content (4). However, carbohydrate compositional data indicates the two populations may have similar glycan structures (data not shown). The heterogeneity in charge density of the MUC2 mucin subunits is not accompanied by any significant change in their molecular mass, approximately 16×10^6.

In light of this investigation it is possible to conclude that the colonic MUC2 mucins have a common macromolecular architecture with the other gel-forming secretory mucus glycoproteins such as cervical, respiratory and salivary mucins (15-16) and thus that all secretory mucins are well described as linear polymeric chains. It is also concluded that the application of rate-zonal centrifugation and laser light-scattering techniques could certainly become very useful for the investigation of the structural features of macromolecules, which will give us an opportunity to go one step further in early diagnosis and following of the treatment and prognosis for several serious diseases such as cancer, cystic fibrosis and colitis ulcerosa at the molecular level.

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