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Rapid size distribution and purity analysis of gastric mucus glycoproteins by size exclusion chromatography/multi angle laser light scattering

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Abstract

The weight average molar masses and molar mass distributions of two commercial pig gastric mucins and two fresh mucin preparations were determined by the technique of size exclusion chromatography coupled on-line to multi angle laser light scattering (SEC/MALLS). Both commercial samples exhibited much lower molar mass averages than the freshly prepared material and contained more impurities. On the other hand, a fresh mucin preparation examined after being stored frozen for 18 months revealed a slight increase in molar mass.

Keywords: Mucus glycoproteins; Size exclusion chromatography/multi angle laser light scattering; Molar mass determination

1. Introduction

Mucus glycoproteins ('mucins') form part of the complex mixture of glyoprotein, water, salts, lipids and cellular materials which constitute mucus. They are the components of mucus secretions lining respiratory, urogenital and digestive tracts and they are the determining factor in their physical properties, i.e. high viscosity and viscoelasticity and gel characteristics of the mucus. These physical properties appear to be independent of source [1] leading to the assumption that their basic structures are identical and that they vary only in molecular size [2]. Mucins typically have molecular weights above 2×10^6 and have a highly swollen structure in solution [3]. They are composed of a polypeptide backbone containing highly glycosylated regions and regions of much lower glycosylation, the protein content is approximately 20%, whereas the carbohydrate content constitutes 70–80% of the molecule [3]. The depth of knowledge of these substances is increasing, and at present seven mucin genes have been characterized, six of which are secreted mucins (MUC 2–7) [4,5]. The macromolecule consists of several subunits which in turn consist of several basic units. A basic unit consists of a highly glycosylated backbone region which is resistant to proteolysis and one or two naked protein regions which are sensitive to proteolytic degradation. Subunits are obtained by thiol reduction of the native mucin and they may have molecular weights of $\sim 2 \times 10^6$, digestion with proteases will then yield the basic units (or 'T-domains') [6] which typically exhibit molecular weight values [3] of $\sim 0.5 \times 10^6$.

The determination of mucin molar mass is no easy task as these substances are strongly non-ideal in the thermodynamic sense and they are polydisperse. Unfortunately, knowledge of molar mass and molar mass distributions is crucial for the understanding of the performance of these molecules in health and disease as a physical and chemical barrier. Knowledge of molar mass/distribution is also important in phenomena like bioadhesion (polymer-mucin interactions) for controlled drug release pharmaceutical formulations [7,8].

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In the past, the three key methods for determining molar mass and molar mass distributions for mucins and related glycopolymers (such as polysaccharides — equally difficult to characterize because of their non-ideality and polydispersity) has been either size exclusion chromatography (SEC), sedimentation equilibrium in the analytical ultracentrifuge and classical or 'static' (as opposed to 'dynamic') light scattering. The highly precise method of mass spectrometry is unfortunately not applicable to intact mucins, because of their size and polydipersity. For obtaining weight and z-average molar masses, sedimentation equilibrium is highly reliable, but is relatively slow (a measurement takes up to 3–4 days) and to determine molar mass distributions requires rather complex modelling strategies [9] or measurements at several fractions obtained from preparative SEC [10]. SEC by itself is not an absolute method, requiring calibration using standards of known molar mass and the same conformation as the mucin. The latter criterium is highly restrictive and the method by itself is at best only a relative technique. Other relative techniques are dynamic light scattering, sedimentation velocity and viscometry. Static light scattering by itself gives also only molar mass averages (usually the weight average). Although it is in principle quicker than sedimentation equilibrium, sample preparation to ensure samples are completely free of dust — a necessary pre-requisite — is a laborious process, and if not done properly gives results which are not useful [11].

Progress was made with the appearance of single, fixed low angle light scattering instruments (LALLS) coupled directly on-line to a size exclusion column [12]. Although in principle this can give rapid molar mass distributions, because of the lack of an angular extrapolation, and, more seriously, because at low angles the effects at even trace amounts of dust can seriously distort the data, some doubt has to remain on the quality of the data. A recent major advance has been the appearance of a multi-angle laser light scattering (MALLS) photometer coupled to size exclusion chromatography [13]. SEC/MALLS provides a reliable and quick technique for determining molar mass and molar mass distributions of macromolecules. Molecules are separated according to size by the size exclusion columns and the light scattering signal from the eluting molecules is collected simultaneously at up to 15 angles [14]. The corresponding concentration trace is obtained downstream by a refractive index detector and molecular weight values for each fraction are calculated according to the following equation [15]:

$$\frac{Kc}{R_\Theta} = \frac{1}{M_w P(\Theta)} (1 + 2A_2c + ...) \quad (1)$$

where K is the polymer constant

$$\frac{2\pi^2 n_0 (dn/dc)^2}{\lambda^4 N_a}$$

(n_0 is the refractive index, dn/dc is the differential refractive index increment, λ is the wavelength in vacuo and N_A is Avogadro's number), c is the polymer concentration (calculated using a dn/dc value of 0.165 ml/g), R_Θ is the excess Rayleigh factor, M_w is the weight average molar mass (g/mol) and A_2 (ml·mol·g^{-1}) is the second virial coefficient which is a measure of solution non-ideality. If the concentration is sufficiently small ($\leq$0.2 mg/ml), a condition often valid after separation dilution on the SEC column, $A_2c \approx 0$. A plot of R_Θ/Kc versus $\sin^2\Theta/2$ [15] will give M_w at the intercept. Values of M_w are obtained for a large number of slices along the elution curve, at each slice correlating the calculated concentration with the excess Rayleigh ratio thus allowing M_w and other molar mass averages for the whole distribution as well as the M_w distribution itself to be calculated in the usual way [16]. In this study, we use this technique to demonstrate the ease of application of this method to mucins and, specifically, to compare the molar mass averages and molar mass distributions for a variety of PGM preparations in order to assess the quality of commercially available material compared to freshly prepared material extraced and purified according to accepted criteria [6,17].

2. Materials and methods

Commercial pig gastric mucin (PGM) samples (see Table 1) were purchased from the respective suppliers and in some cases further purified as indicated in Table

Table 1
Sample description and sample codes for pig gastric mucin preparations

Sample code	Sample
PGM 1	Sigma Cat. No. M1778, partially purified from porcine stomach
PGM 2	Sigma Cat. No. M1778, subjected to ultrafiltration prior to analysis
PGM 3	Partially pepsin-digested mucin from Orthana Kemisk Fabrik, Denmark
PGM 4A	Freshly prepared pig gastric mucin as described above, Batch 1
PGM 4B	As for PGM 4A but further purified by preparative SEC on Sepharose 2B column
PGM 4C	As for PGM 4B but additional caesium chloride density gradient ultracentrifugation
PGM 5B1	As for PGM 4B, Batch 2
PGM 5B2	As for PGM 5B1 but sample was frozen for approx. 18 months

1. Fresh pig gastric mucin was purified from fresh pig gastric mucus by preparative caesium chloride isopycnic density gradient ultracentrifugation in an enzyme inhibitor cocktail according to a modified procedure described by Hutton et al. [17]. This was followed by preparative size exclusion chromatography of the glycoprotein fraction on a Sepharose CL-2B column. The totally excluded volume fractions were pooled and concentrated by ultrafiltration, dialysed against distilled water and 1-ml aliquots were kept frozen at −20°C or freeze-dried. The mucin preparations were gently defrosted and dialysed into the buffer prior to use. Purity of the mucin preparations was checked by analytical isopycnic density gradient ultracentrifugation as described by the procedures of Creeth et al. [18].

Freshly prepared and commercial samples are identified according to the nomenclature indicated in Table 1. Samples were diluted in a phosphate/chloride buffer (ionic strength = 0.1, pH 6.8) containing 0.04% diaminotetraacetic acid-disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.45 μm filter membranes (Millex HV-type, Millipore, Watford, UK) prior to injection into the SEC/MALLS system.

2.1. Chromatographic system

The chromatographic system (for a schematic diagram see Fig. 1) consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a high performance pump (Model 590 Programmable Solvent Delivery Module, Waters, Millipore, Watford, UK), an injection valve (Rheodyne Inc., Cotati, CA, USA) fitted with a 100-μl loop, and two analytical SEC columns — one PSS HemaBio Linear and one PSS HemaBio 40 (Polymer Standards Service GmbH, Mainz, Germany) connected in series in that order; the analytical columns were protected by a guard column. Analytical column dimensions were 300 × 7.5 mm, with the first (PSS HemaBio Linear) having an exclusion limit for dextrans of $7 \times 10^6$and the second column (PSS HemaBio 40) an exclusion limit of 40 000 for dextrans. Exclusion volume and total permeation volume for the column system were found to be 9.8 and 19.8 ml respectively. The flow rate was 0.8 ml/min and the injection volume was 100 μl.

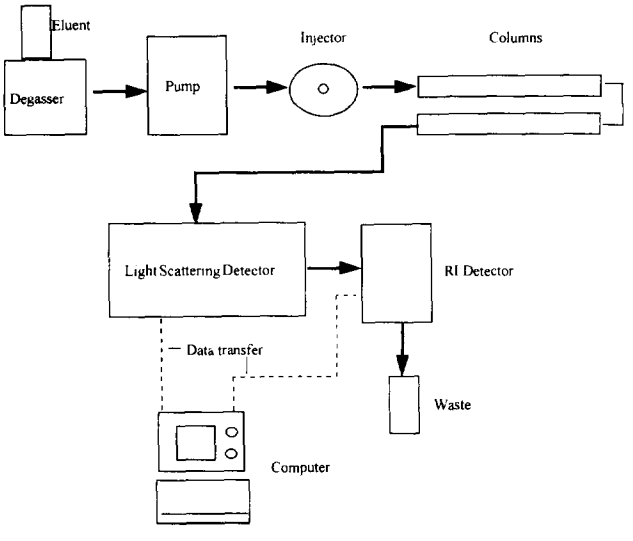

Fig. 1. Schematic description of SEC/MALLS system.

Column effluent was monitored using a Dawn F laser light scattering photometer (Wyatt Technology, Santa Barbara, CA, USA) and a differential refractive index detector (Model 410, Waters, Millipore, Watford, UK).

3. Results and discussion

Weight average molar masses for commercial and fresh mucin preparations are shown in Table 2. Due to lack of material some of the fresh mucin samples were only run once, however, reproducibility studies of the technique have shown it to be reliable to within ± 10%. Table 2 reveals that the investigated commercial preparations are of much lower molar mass than the freshly prepared material. The molar mass of PGM 1 was found to be lower than would be expected from a subunit and purification by ultrafiltration did not result in a significant increase in molar mass. The elution profiles (see Fig. 2a–c) also indicated that these materials contained low molar mass components which could not be removed (in the case of PGM 1) by extensive ultrafiltration. It may be possible that these low molar mass components are due to the results of any protease activity still present in the preparation or could be proteins themselves. Indeed, the molar mass values for PGM 3 confirmed that this sample was at least partially digested as they were only slightly higher than would be expected

Table 2
Weight average molecular weight values for pig gastric mucins

Sample	$10^{-6} \times M_w$
Commercial samples	
PGM 1	1.25 ± 0.01[a]
PGM 2	1.33 ± 0.01[a]
PGM 3	0.73 ± 0.05[a]
Freshly prepared samples	
PGM 4A	4.72
PGM 4B	8.20
PGM 4C	9.09
PGM 5B1	11.8 ± 0.1[a]
PGM 5B2	14.7

[a]Results are means of duplicate runs.

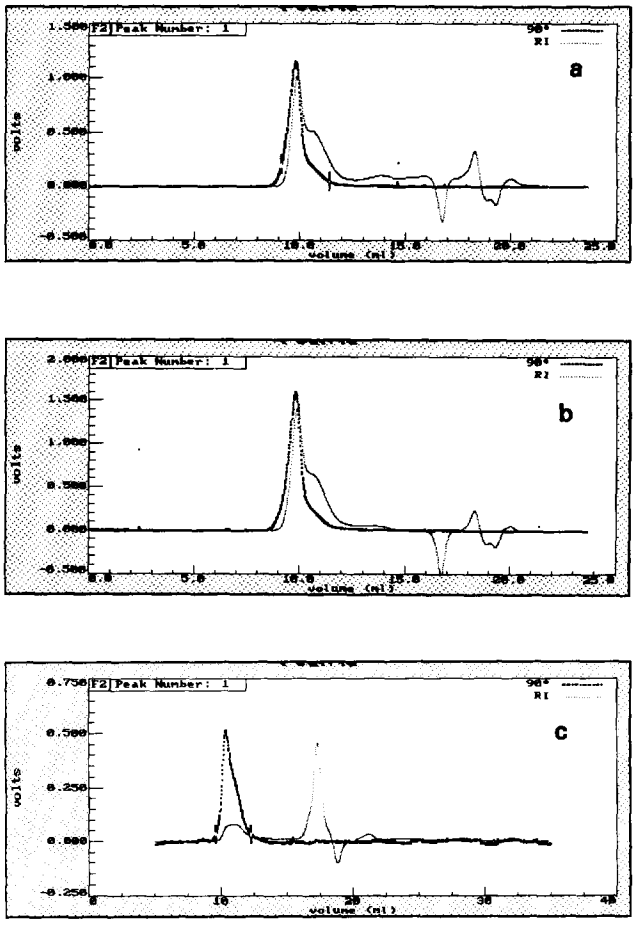

Fig. 2. Elution profiles from (a) PGM 1, (b) PGM 2, (c) PGM 3. For chromatographic conditions see Section 2.1.

for basic mucin units. The use of either of these commercial mucins as model compounds for the study of bioadhesive agents would therefore not be recommended.

Size exclusion chromatography is an excellent technique for separating molecules of different sizes and the addition of on-line light scattering allows the elucidation of absolute molecular weight averages and distributions. However, the separation range of the SEC columns which to our knowledge cannot exceed 10 million daltons represents a limiting factor for the technique, especially for large molecules such as mucins. If a large proportion of the sample elutes at the void volume, fractionation is incomplete and whilst the weight average molecular weight obtained from light scattering will still be correct, the molecular weight distribution of the sample is no longer valid. Care must also be taken in the Debye plot extrapolation to zero angle for each fraction. For large molecules, significant angular dependence expressed by an upward curvature of the Debye plot at low angles would be expected. However, this curvature may also be due to, and/or exaggerated by a wide molecular weight distribution within the sample, the presence of dust, or microgels and branching [19]. This therefore introduces some degree of uncertainty into the final molecular weight values and will also increase the difficulty in obtaining correct radius of gyration (R_G) values. R_G values are obtained form the initial slope of the Debye plot and are therefore extremely sensitive to even slight

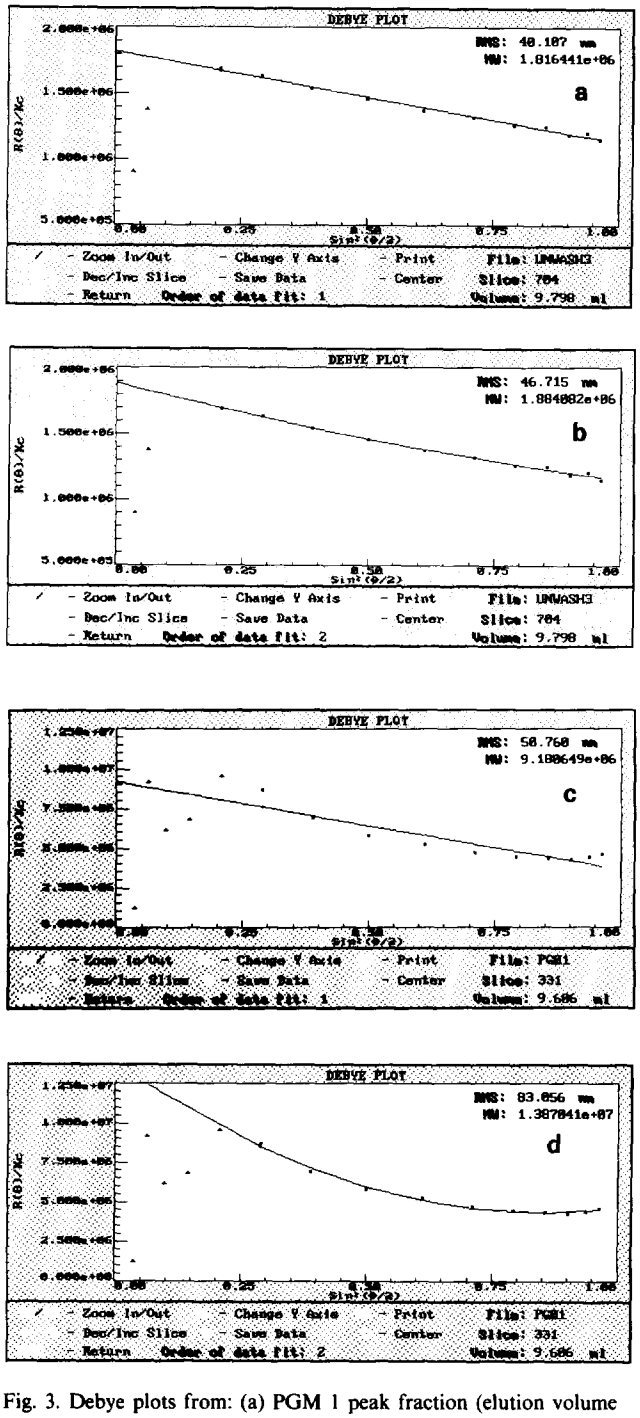

Fig. 3. Debye plots from: (a) PGM 1 peak fraction (elution volume 9.798 ml) first order data fit, (b) as in (a) but second order data fit, (c) PGM 5B1 peak fraction (elution volume 9.606 ml) first order data fit, (d) as in (c) but second order data fit.

errors, for this reason they have not been included in our results. Fig. 3a–d shows Debye plots using first and second order fits for sample PGM 1 (a–b) and PGM 5B1 (c–d) illustrating the problem with extrapolation. On the evidence of these plots it was decided that for the commercial samples a first order fit was appropriate whereas a second order fit was used for all fresh PGMs.

The preparation of fresh mucin from starting material (in this case pig gastric mucus) is very time consuming, laborious and yields are low; one can therefore see the temptation in using commercial material in bioadhesive studies etc. The freshly extracted materials were monitored after each purification stage in order to find the optimum number of purification steps, i.e. the point after which further purification no longer significantly increases molar mass or purity. As Table 2 and the shoulder on the main peak in Fig. 4a shows, the freshly prepared mucin (PGM 4A) still contained some impurities and low molecular weight material. However, the molecular weight obtained was already far higher than that of either of the commercial samples. Purification of the fresh PGM by preparative size exclusion chromatography led to a significant increase in weight average molecular weight (cf. PGM 4B in Table 2) and a highly pure mucin as is indicated in Fig. 4b by the highly symmetrical peak. Further purification of this sample by caesium chloride isopycnic density gradient ultracentrifugation (PGM 4C) led to a slight 'improvement' in molar mass (see Table 2) but no further improvment in purity as demonstrated by Fig. 4c. This stage was therefore not carried out in later preparations. In Fig. 5 the molecular weight versus elution volume plots of the freshly prepared PGM at the various purification stages are shown. These reveal that the plots of the partially purified and pure PGM are virtually identical and it was therefore decided that the final isopycnic density gradient ultracentrifugation stage would not be required in subsequent preparations. In order to check the reproducibility of the preparation, a second batch of mucin was prepared. The weight average molecular weight of the purified material was found to be much higher than that of the previous preparation. Whether this may be due to slight differences in the actual preparation procedure or in the original mucus (i.e. variability due to different animals) is not quite clear at this time and should possibly be investigated.

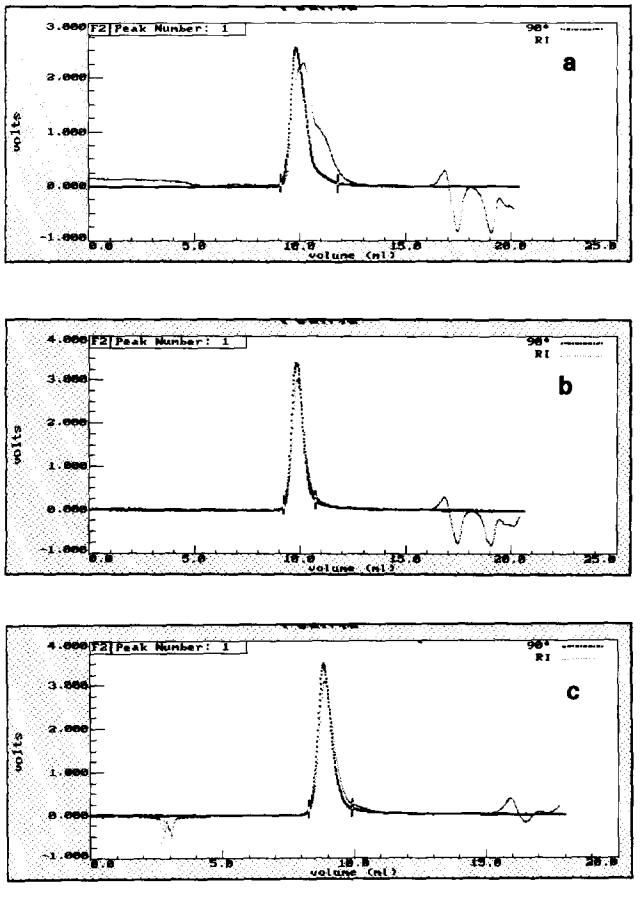

Fig. 4. Elution profiles from (a) PGM 4A, (b) PGM 4B, (c) PGM 4C. For chromatographic conditions see Section 2.1.

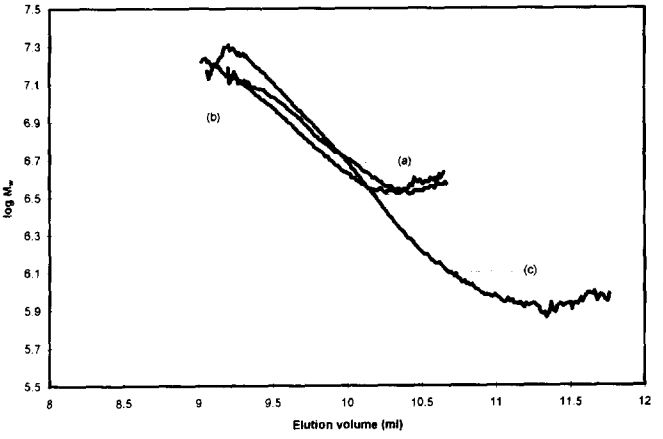

Fig. 5. Log M_w versus elution volume plot for (a) PGM 4B, (b) PGM 4C, (c) PGM 4A.

Frequently, biological materials have to be stored (usually by freezing) prior to use and to our knowledge, the effect of a freeze/thaw cycle on the molecular integrity of mucin has not been reported previously. The M_w value shown in Table 2 was somewhat surprising, as the molar mass of the mucin had increased after freezing — storage of biological materials is usually expected to cause degradation. However, it is possible that some self-association had been induced by the freeze-thaw process.

The chromatograms of the fresh and frozen mucin (see Fig. 6a and b, respectively) appear to be identical, however, the log M_w versus elution volume plots of the fresh and frozen samples (Fig. 7) are totally different.

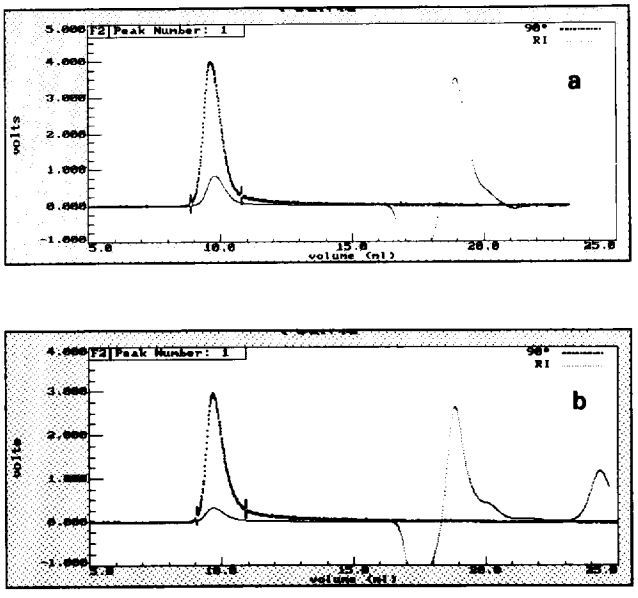

Fig. 6. Elution profiles from (a) PGM 5B1 and (b) PGM 5B2

The molar mass of the fresh mucin decreased over the elution range as expected, whilst the molar mass of the frozen mucin appeared to remain virtually stable over the elution range. This would suggest that the freeze-thaw process has had a significant effect, which in turn may have caused a breakdown of the separation mechanism. There may be various reasons for this; self-association may have resulted in a quasi-monodisperse distribution, some portions of the glycoprotein molecule may have changed in such a way that they are now interacting with the column matrix, or there may be a change in the molecular conformation for example, to a more rod-like conformation which would elute over a much larger range even if their size was fairly similar due to the difference in orientation these molecules may adopt [20].

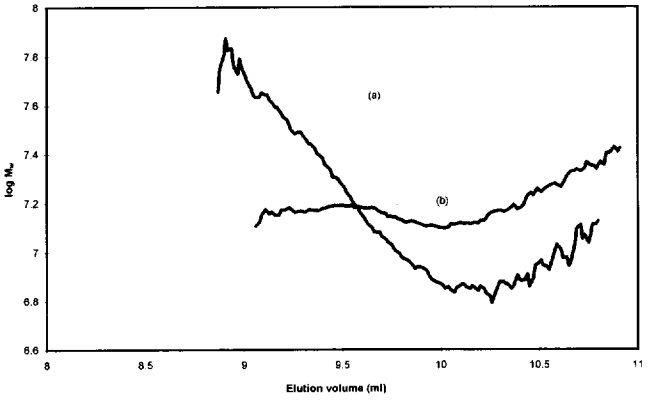

Fig. 7. Log M_w versus elution volume plots for (a) PGM 5B1 and (b) PGM 5B2.

From Table 2 it is clear that the commercial samples are considerably degraded compared to their freshly prepared counterparts. The values for the commercial samples correspond to between those expected from a basic unit (or 'T-domain') of $\sim 0.5 \times 10^6$ and a subunit (~ 2–2.5×10^6) [6]. The freshly prepared samples appear to be as expected. Creeth and Cooper [21] reported a value of 9×10^6 from sedimentation equilibrium on material which had been prepared under a similar procedure. Sheehan and Carlstedt [6] report higher values obtained by static light scattering ($\sim 32 \times 10^6$) but this latter value might have been influenced by the presence of trace amounts of supra-molecular aggregates. Sedimentation equilibrium experiments performed on PGMs 4B and 5A gave similar results to the SEC/MALLS although they took considerably longer to obtain. It is worth pointing out that we have assumed the differential refractive index increment (dn/dc) as constant (0.165 ml/g). The precise value will depend to some extent on the proportion of carbohydrate:protein in the mucin since dn/dc for pure protein ≈ 0.19 ml/g and for pure saccharide ≈ 0.15 ml/g. If there was a variation in the protein content even up to a value of $\pm 25\%$, this would lead to a variation in dn/dc of only $\approx \pm 2.5\%$ and hence an error in M_w (via errors in $(dn/dc)^2$ and c) of $\approx 5\%$ and hence would not account for the differences between the values in Table 2.

However, it should be stressed that above molar masses of $\approx 10^7$ stable column matrices are still not available which limits the technique to weight average molecular weights and molar mass distributions. It would therefore be advantageous in future to consider the use of a separation technique like, for example, field flow fractionation where a column matrix is no longer required to facilitate separation for very large molecules. Finally, above $M_w \approx 50 \times 10^6$, the limits of the Rayleigh-Gans-Debye approximation are reached and Eq. 1 is no longer valid.

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