The supramolecular organization of ovomucin

Biophysical and morphological studies

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Ovomucin participates in the ovomucin-gel-forming properties because of its shape and its ability to interact in a specific spatial organization. Purified from chicken egg-white by exclusion chromatography with Sephacryl S-300 and Sepharose CL-2B and analysed by light-scattering, it exhibited an M_r of about 40×10^6 . This large M_r can be explained by the aggregation of polymers that can be degraded into 3×10^6 - M_r fragments by reduction with dithiothreitol. The values for hydrodynamic parameters such as M_r , radius of gyration, hydrodynamic radius, mass per unit length and combinations of them suggested that ovomucin is a linear and highly flexible molecule conferring upon it a random-coil-like structure in 0.2 M-NaCl solution. Analysis of the ovomucin molecules by electron microscopy revealed its linear character but also indicated a lower M_r than that obtained in the light-scattering experiments. By temperature-induced non-specific aggregation of an ovomucin solution containing other globular egg proteins, an attempt was made to find out what conditions are required for gel formation and to examine the quality of aggregation that is obtained under these conditions. Results show that the viscosity of the solution did not increase after heat treatment. Apparently, in the ovomucin gel, specific spatial organization of the ovomucin molecules is required for hydrogel formation.

INTRODUCTION

Although the mucins, long highly polymerized linear glycoproteins, are present in rather low concentrations in mucous secretions, they are able to form aggregates by non-covalent interactions [1–5] and confer gel-like properties upon the mucus [6,7].

As has previously been suggested [8], ovomucin gel may be considered as a member of the family of mucus if criteria such as mode of secretion, rheological behaviour, biochemical composition and structure are considered. By using sonication, denaturing agents or high-ionic-strength treatments of the ovomucin gel, we have previously obtained results suggesting that the shape of the molecular organization determines the non-Newtonian viscous character of the gel. Indeed, theoretical studies have shown that this non-Newtonian rheological behaviour could arise from the presence of flexible and asymmetric molecules [9].

We were interested to confirm this hypothesis by investigating the supramolecular organization of the ovomucin, using light-scattering and electron microscopy before and after different physical, chemical and enzymic treatments.

In addition, the involvement of specific protein aggregation in hydrogel formation was investigated [8,10], and our results show that precise spatial organization of the ovomucin molecules together with globular chicken

proteins rather than non-specific aggregation leads to hydrogel formation, i.e. a fibrillar macromolecular network with weak and transient linkages.

MATERIALS AND METHODS

Chemicals

Trypsin (EC 3.4.21.4) from pig pancreas (type IX; 16000 units/mg), lysozyme (EC 3.2.1.17) from chicken egg-white (grade I), trypsin inhibitor from turkey egg-white (type II, T), ovalbumin from chicken egg-white (grade V), agarose (type II) and glycine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SDS, poly(ethylene glycol), DTT, Tris, *NN'*-methylene-bisacrylamide and acrylamide were purchased from Serva (Heidelberg, Germany). Sephacryl S-300 and Sepharose CL-2B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Ethanol, methanol, acetic acid and *NNN'*-tetramethylethylenediamine were purchased from Farmitalia Carlo Erba (Milan, Italy). Alcian Blue and Coomassie Blue were supplied by Fluka (Buchs, Switzerland). All reagents used were of analytical grade.

Preparation of ovomucin gel

Ovomucin gel was obtained as previously described [8]. Briefly, ovomucin fibres were precipitated by dilution of chicken egg-white in 10 vol. of distilled water, pelleted

Abbreviation used: DTT, dithiothreitol.

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by centrifugation at 2000 *g* for 15 min at 4 °C and washed three times with 0.2 M-NaCl.

Purification of ovomucin

Ovomucin was purified by the sequence shown in Scheme 1.

Elastic and quasi-elastic light-scattering

Results were evaluated as described by Muller *et al.* [11] and plotted in accordance with Zimm [12] (see the Appendix).

Measurements of scattered light intensities at angles between 20° and 150° were performed with a Macrotron spectrogoniometer (AMTEC Society, Villeneuve Loubet, France). The light source consisted of a polarized monochromatic beam emitted by an He/Ne laser ($\lambda = 632.8$ nm). The solutions were filtered through 0.45 μ m-pore-size Millex filters (Millipore) and introduced into a cylindrical measuring cell (diameter 23 mm). In practice, the intensity of scattered light beams $\Delta I(\theta)$ from the solute were compared with the intensities obtained from the reference (benzene) at 90° [11]:

$$\Delta I(\theta) = \frac{I_{\theta, \text{solution}} - I_{\theta, \text{solvent}}}{I_{B, 90^\circ}} \cdot \frac{n_0^2}{n_B^2} \cdot \frac{\Delta R(\theta)}{R_{\theta, B, 90^\circ}} \cdot \sin \theta$$

with $R_{\theta, B, 90^\circ} = 12.55 \times 10^{-6} \text{ cm}^{-1}$ and $I_{B, 90^\circ} = 3300$. $I_{\theta, \text{solution}}$ and $I_{\theta, \text{solvent}}$ are the light-scattering intensities of the solution and the solvent respectively, and n_B and n_0 are the refractive indices of benzene and the solvent respectively.

For vertically polarized light the relationship between the Rayleigh factor $R(\theta)$ and the physical characteristics

of the molecules under study [see eqn. (1) in the Appendix] may be written:

$$K''(\partial n / \partial C)^2 I_{B, 90^\circ} \cdot \frac{C}{\alpha \Delta I(\theta)} = \frac{1}{M_r} \cdot \frac{(1 + q^2 R_g^2 + \dots)}{3} + 2A_2 C$$

with $K'' = 2\pi^2 n_B^2 N^{-1} \lambda_0^{-4} R_{\theta, B, 90^\circ}^{-1} (1 + \rho u)$ and $\alpha = \sin \theta / (1 + \cos 2\theta)$. In our calculations $K'' = 0.79$, and thus:

$$\frac{1}{M_r} = 0.79 (\partial n / \partial C)^2 I_{B, 90^\circ} \lim_{\theta \rightarrow 0} P(\theta) \quad c \rightarrow 0$$

The apparent refractive-index increment ($\partial n / \partial c = 0.16$) was taken from Sheehan & Carlstedt [13]. Values for the hydrodynamic parameters, namely relative molecular mass (M_r), radius of gyration (R_g) and relative molecular mass per unit length (M_r/l) were obtained from the Zimm and Holtzer plots [14] as described in the Appendix. Hydrodynamic radius (R_h) was obtained from quasi-elastic light-scattering experiments.

Light-scattering experiments were performed with purified ovomucin under the following conditions. (i) Mucin solution in 0.2 M-NaCl was analysed at four different concentrations (10, 15, 20 and 30 μ g of protein/ml). (ii) A 5 ml portion of mucin (0.16 mg of protein/ml) was treated for 1 h at 90 °C with 1% (w/v) SDS. After dilution in 1% SDS, four different mucin concentrations were analysed (8, 12, 32 and 48 μ g of protein/ml). (iii) After concentration of the mucin solution [0.16–2.4 mg of protein/ml by poly(ethylene glycol) dialysis] it was treated with 4 mg of DTT (to give 1%, w/v), for 12 h at 37 °C. The mucin was analysed at five different concentrations (40, 60, 80, 120 and 240 μ g of protein/ml). After the DTT treatment, one of the samples corresponding to a protein concentration of 120 μ g/ml was digested with trypsin (10 μ g/ml; 600 units) for 1 h at 20 °C. After proteolysis, the mucin solution was diluted with 0.2 M-NaCl to several concentrations (40, 60, 80 and 120 μ g of protein/ml) and subjected to elastic light-scattering analysis.

For all experiments, mucin concentrations were calculated from the values for protein concentration [15] corrected by using a sugar percentage of 57% (w/w) as reported by Robinson & Monsey [3].

Electron microscopy

Before electron-microscopy experiments, ovomucin was dialysed extensively against 0.15 M-ammonium acetate buffer, pH 6.7, containing 25% (w/v) glycerol and was diluted in the same buffer to obtain a protein concentration of 120 μ g/ml. The macromolecular replica method was used [16,17]. Briefly, 100 μ l of aqueous ovomucin solution was sprayed with a glass nebulizer (Devillebiss, Summerset, PA, U.S.A.) on to 1 cm² of freshly cleaved mica, which after evaporation for 15 min at 0.6 mPa was rotary-shadowed at 15 °C with 0.1 nm of platinum and subsequently coated with a 2.5 nm layer of carbon. Carbon replicas were stripped from the mica and their micrographs were recorded with a Phillips 400 electron microscope.

Phase transitions

The phase-transition experiments were performed with the ovomucin preparation (2.5 ml; 2.3 mg of protein/ml of 0.2 M-NaCl), which was incubated for 1 h at each

Scheme 1. Purification of ovomucin

The Sephacryl S-300 gel and Sepharose CL-2B (2 cm $\times$ 40 cm) columns were equilibrated with 0.2 M-NaCl. Calibration of the columns was performed as previously described [8]. The flow rates were 44 ml/h and 25 ml/h, respectively. The ovomucin preparation refers to that mentioned in the phase-transition and intrinsic-viscosity experiments (Figs. 7 and 8).

of different temperatures from 10 °C to 80 °C in successive increments of 5 or 10 °C. A spectrum of the solution was recorded between 200 and 700 nm at each temperature. The absorbance at 340 nm was also measured.

Measurement of intrinsic viscosity

The viscosity of the ovomucin preparation (2.3 mg of protein/ml) was measured in a capillary viscometer

(Fica). The viscosity η was calculated as described by Tanford [9]. The solvent and solution fluid flow times, denoted t_0 and t respectively, were used to calculate relative viscosity, $\eta_{rel.} = (\eta_t - \eta_{t_0})/\eta_{t_0}$, and specific viscosity, $\eta_{sp.} (= \eta_{rel.}/c$, where c is the macromolecular concentration). Intrinsic viscosity $[\eta]$ was obtained by extrapolation of the plot of $\eta_{sp.}$ versus c to $c = 0$. The relationship between $\eta_{sp.}$ and c may be expressed as $\eta_{sp.} = [\eta] + [\eta]^2 k' c$, where k' is the Huggins constant [18].

Fig. 1. PAGE profile of the ovomucin preparation and purified ovomucin

(a) Coomassie Blue staining and (b) Alcian blue staining in a linear polyacrylamide gradient between 6 and 20%. Ovomucin preparation (100 μ l; 0.4 mg of protein/ml) was treated with 1% (w/v) SDS for 5 min at 100 °C. A 1 ml portion of purified ovomucin solution (0.16 mg of protein/ml) was concentrated 10-fold with poly(ethylene glycol). When the volume reached 100 μ l, two portions were taken and treated with 1% (w/v) SDS for 5 min at 100 °C or with 1% (w/v) DTT for 12 h at 37 °C plus 1% (w/v) SDS for 5 min at 100 °C. Standards were treated with 1% SDS for 5 min at 100 °C. Each sample loaded on the gel contained 10 μ g of protein. (a) Lane 1, high- M_r standards from Pharmacia (669 000 to 67 000); lanes 2 and 9, low- M_r standards from Pharmacia (67 to 10 500); lane 3, ovomucin preparation; lane 4, ovalbumin; lane 5, lysozyme; lane 6, trypsin inhibitor; lane 7, purified ovomucin; lane 8, DTT-treated purified ovomucin. (b) Lane 1, DTT-treated purified ovomucin; lane 2, purified ovomucin; lane 3, ovomucin preparation; lane 4, trypsin inhibitor; lane 5, lysozyme; lane 6, ovalbumin. (c) Periodic acid/Schiff staining in a linear polyacrylamide gradient between 5 and 15%. Two 2 ml portions of purified ovomucin (0.16 mg of protein/ml) were either treated with 1% (w/v) DTT for 12 h at 37 °C or not treated, and then extensively dialysed and freeze-dried. Before being loaded on the gel, 1 mg of each sample or standard was heated at 100 °C for 5 min in the presence of 1% (w/v) SDS. Each sample loaded on the gel contained 100 μ g of protein except for bronchial glycopeptides (80 μ g) and whole bronchial mucin (80 μ g). Lane 1, purified ovomucin; lane 2, DTT-treated purified ovomucin; lane 3, orosomucoid; lane 4, bronchial glycopeptides; lane 5, whole bronchial mucin.

PAGE

Page was carried out in accordance with Laemmli [19] under denaturing conditions. Proteins, carbohydrates and sialic acid were detected with Coomassie Blue, periodic acid/Schiff reagent and Alcian Blue staining respectively. For the last, the gel was washed after fixation with water and 0.1 M-acetate buffer, pH 4, stained for 1 h with an aqueous solution of Alcian Blue (0.5 % w/v) and destained with 0.1 M-acetate buffer, pH 4.

Agarose-gel electrophoresis

Electrophoresis was performed in 1 % agarose gel in the presence of Tris/HCl buffer, pH 8.8, containing 0.1 % SDS. Samples were treated with 1 % (w/w) SDS before analysis, and portions corresponding to 20 μ g of protein were loaded on to the gel. The gel was stained with Coomassie Blue.

Analytical procedures

Sialic acid was removed from mucin either by neuraminidase treatment or by hydrolysis with 0.1 M- H_2SO_4 at 80 °C [20] and assayed by the method of Warren [21].

Proteins were determined by the method of Lowry *et al.* [15] after sonication of ovomucin gel or purified ovomucin. Determination of neutral sugars was performed by the phenol/ H_2SO_4 method [22].

RESULTS

Ovomucin purification

After sonication the ovomucin gel loses its high viscosity, and analysis of this material by gel filtration on Sephacryl S-300 led to recovery of three major peaks [8]. The first, corresponding to the void volume, contains

Fig. 2. Elution profile of the ovomucin preparation on Sepharose CL-2B

The Sepharose column (1 cm $\times$ 30 cm) was equilibrated in 0.2 M-NaCl. The flow rate was 15 ml/h. Elution was carried out at 20 °C in the presence of 0.2 M-NaCl and the eluate was monitored for absorbance at 280 nm. V_0 and V_t are the excluded and total inclusion volumes, determined with heated bacteria and $\text{K}_3\text{Fe}(\text{CN})_6$, respectively. The ovomucin preparation (700 μ g of protein) was analysed before (—) and after concentration with poly(ethylene glycol) or ethanol precipitation (---).

mucin (sialic acid-positive) and is denoted here the ovomucin preparation.

When the ovomucin preparation was analysed by SDS/PAGE, a large amount of the protein did not penetrate the gel (Fig. 1a, lane 3) but was stained by Alcian Blue (Fig. 1b, lane 3), indicating the presence of sialic acid and sulphate, with both of which ovomucin is known to be richly endowed [3].

Also, an amount of polydisperse material (Coomassie Blue-positive but Alcian Blue-negative; Fig. 1b, lane 3) and one discrete band corresponding to lysozyme were observed (Fig. 1a, lane 3). In addition, some preparations showed evidence for the presence of ovalbumin and conalbumin (results not shown).

Accordingly, further purification of the ovomucin preparation by gel filtration on Sepharose CL-2B yielded two peaks (Fig. 2). The first peak, which was sialic acid-positive, was eluted in the void volume ($M_r > 40 \times 10^6$), whereas the second one, which was sialic acid-negative, was eluted near the total inclusion volume. Measurement of the relative areas of the two peaks indicated that the first peak represented 65 % of the ovomucin preparation, and this material is here referred to as purified ovomucin. When analysed by SDS/PAGE, it did not penetrate the gel (Fig. 1a, lane 7). This material was periodic acid/Schiff-positive (Fig. 1c, lane 1) and Alcian Blue-positive (Fig. 1b, lane 2). Its purity was estimated to be

Fig. 3. Agarose-gel-electrophoresis profile of purified ovomucin: effect of DTT treatment

A 1 ml portion of purified ovomucin solution (0.16 mg of protein/ml) was concentrated 10-fold with poly(ethylene glycol). When the volume reached 100 μ l, two portions were taken and treated with 1 % (w/v) SDS for 5 min at 100 °C or with 1 % (w/v) DTT for 12 h at 37 °C plus 1 % (w/v) SDS for 5 min at 100 °C. Each sample loaded on the gel contained 20 μ g of protein (see the Materials and methods section). Lane 1, purified ovomucin; lane 2, DTT-treated purified ovomucin.

Fig. 4. Zimm plot of light-scattering data for purified ovomucin

All measurements were performed in 0.2 M-NaCl. The following concentrations were used: 30, 25, 15 and 10 μg of protein/ml. The scattered light intensity (I_θ) was measured at various angles between 20 and 150° ($k = 1 \times 10^5$). Values of $1/M_r$ and R_g were obtained by extrapolating to $\theta = 0^\circ$ and $c = 0$. M_r/l was obtained from the plot $q\Delta I\theta/K''c\pi$ versus q^2 , with $q = 4\pi n_0/\lambda_0 \cdot \sin \theta/2$ (Holtzer plot; see the Appendix).

about 90%, and it was found to be only slightly contaminated with lysozyme. The purified ovomucin enters agarose gels only after DTT treatment, at which time its polydisperse nature becomes apparent (Fig. 3, lane 2).

Unlike the results reported by Robinson & Monsey [3] and Hayakawa & Sato [4], no molecular species with M_r around 0.2×10^6 , corresponding to α -ovomucin in their nomenclature, appear in PAGE after purified ovomucin was treated with SDS or SDS plus DTT (Fig. 1a, lanes 7 and 8). It appears, then, that our purified ovomucin corresponds to the previously named β -ovomucin [3,4].

Purified ovomucin and its molecular organization

Light-scattering. Purified ovomucin was investigated by means of elastic and quasi-elastic light scattering before and after physical, chemical and enzymic treatments. Details of molecular configuration and subunit organization may be deduced from these types of studies.

Fig. 4 shows a Zimm plot [12] representing angular and concentration extrapolation of the scattered-light intensities from the purified ovomucin in 0.2 M-NaCl.

Measurements of M_r and R_g were obtained from plots of this type, and M_r/l was obtained from Holtzer plots [14] (see the Appendix). Values for such hydrodynamic parameters (M_r , R_g and M_r/l), the R_h and the ratio R_g/R_h were obtained both before and after treatment of the purified mucin with SDS, DTT and DTT plus trypsin and are presented in Table 1.

SDS treatment caused a decrease in M_r from 43×10^6 to 21.5×10^6 , but no significant changes in other hydrodynamic parameters were observed. However, after treatment with DTT, which is known to break disulphide bridges, dramatic changes in the hydrodynamic parameters were observed. The M_r and M_r/l decreased approximately 10-fold, the R_g increased 1.5-fold and the R_h increased 2-fold. After treatment with DTT plus trypsin, M_r , R_g and R_h decreased a further 2-fold but without a concomitant change in M_r/l . These results suggest that liberation of the fragments by DTT treatment of the purified mucin is concomitant with rigidification (see the Discussion section). The decrease in M_r of purified ovomucin after DTT treatment was confirmed by agarose-gel electrophoresis (Fig. 3, lane 2).

Electron microscopy. Results obtained from light-scattering studies were correlated to data derived from electron-microscopy experiments. In fact, ovomucin appears to consist of a long linear molecule with more-coiled regions at its extremities (a in Fig. 5). By statistical analysis of the molecules observed in electron micrographs (similar to that shown in Fig. 5) a polydisperse distribution of molecular lengths is observed showing a peak around 600 nm (Fig. 6). High- M_r ovomucin molecules (2000–2500 nm), which may correspond to aggregates, were also observed.

In addition, careful inspection of molecules a in Fig. 5 reveals that the width of the molecules at their rigid linear portions is not the same as in the randomly coiled regions. It appears, then, that the linear and more rigid portions of the molecule correspond to the association of two or more molecular strands that are separated at the end of the molecule, so forming the randomly coiled structure (b in Fig. 5). However, it is also possible to find double-stranded molecules without coiled regions at the end (c in Fig. 5) and single-stranded molecules corresponding probably to degradation states. Fragments obtained from sonicated purified molecules show lengths varying between 50 and 100 nm (d in Fig. 5).

Another series of experiments was performed with

Table 1. Hydrodynamic parameters of purified mucin obtained from elastic and quasi-elastic light-scattering after different physical, chemical and enzymic treatments

Purified mucin was subjected to the following treatments (see also the Materials and methods section): 0.2 M-NaCl; 1% SDS; sonication; 1% DTT; 1% DTT plus trypsin. All measurements were performed as described in Fig. 4 legend. Abbreviation: N.D., not determined.

	Aggregates	SDS-treated aggregates	DTT-treated aggregates	DTT + trypsin-treated aggregates
M_r	43	21.5	3.3	1.75
R_g (nm)	172	192	265	140
M_r/l (nm^{-1})	24500	25000	2000	1700
R_h (nm)	72	80	150	N.D.
R_g/R_h	2.38	2.4	1.76	N.D.

Fig. 5. Electron micrographs of high- M_r ovomucin

Purified ovomucin obtained as described in Scheme 1 was subjected to the macromolecular replica method [16,17]. Key to molecules: a, ovomucin molecule with double- and single-stranded regions; b, double-stranded ovomucin molecule; c, single-stranded molecule; d, sonicated ovomucin; e, mucin purified from ovomucin gel without sonication.

purified ovomucin obtained from the ovomucin gel without a sonication step. In this case the molecules exhibit a length of 2000–3000 nm (e in Fig. 5), suggesting that the previous value for the mean length (600 nm) may not represent the actual length of the ovomucin molecule.

Ability of proteins to interact and their gel-forming capability

After demonstrating the linear character of the supra-molecular organization of the ovomucin, which is in agreement with the non-Newtonian rheological behav-

iour of the ovomucin gel, we wanted to determine what kind of aggregation of ovomucin molecules together with other globular egg proteins naturally present in the gel (ovomucin preparation, Fig. 2) leads to the formation of this gel (first characterized by an increase in the value of viscosity).

We induced non-specific interactions by a heat treatment of the ovomucin preparation, and protein turbidity recorded at 340 nm was used to monitor aggregation [23–25].

Fig. 7 shows a plot of absorbance values at 340 nm

Fig. 6. Length distribution of purified ovomucin

Ovomucin was purified according to the procedure described in Scheme 1. A sample of molecules ($n = 96$) was taken from micrographs and projected through a camera on to the screen of a computer. The length was automatically registered in nm. The error of the method was estimated to be 7%.

versus temperature. An abrupt increase in absorbance at 340 nm, which according to Williams [26] may be described as a transition phase, appeared between 60 °C and 70 °C. In 0.2 M-NaCl, the ovomucin preparation exhibited a transition at 65 °C and a maximal absorbance of 0.95. The observed protein aggregation was irreversible.

By analysing intrinsic and specific viscosities, a correlation between aggregation and gelation could be obtained. The intrinsic viscosity of the ovomucin preparation was measured before and after heating for 1 h at 80 °C. Fig. 8 shows that the specific and intrinsic viscosities of the ovomucin preparation decreased by 25% after heating (29 ml/g compared with 22 ml/g). Therefore protein aggregation did not necessarily lead to an

Fig. 7. Absorbance changes at 340 nm of the ovomucin preparation incubated at different temperatures

A 2.5 ml portion of ovomucin preparation in 0.2 M-NaCl (2.3 mg of protein/ml) was heated for 1 h at different temperatures between 10 °C to 80 °C in increments of 5 or 10 °C and the spectra were recorded as described in the Materials and methods section.

Fig. 8. Plot of specific viscosity (η_{sp}) versus concentration (c) of the ovomucin preparation

Different concentrations of ovomucin preparation (between 2.3 and 0.83 mg of protein/ml) were obtained by dilution in 0.2 M-NaCl (■). A 10 ml portion of ovomucin preparation (2.3 mg of protein/ml) was heated for 1 h at 80 °C and diluted with 0.2 M-NaCl (●). All measurements were performed at 20 °C. In each case the flow times t of the sample and t_0 of the solvent were measured and η_{sp} . $\{ = [(\eta_t - \eta_{t_0})/\eta_{t_0}]/c \}$ was calculated (see the Materials and methods section). Extrapolations to $c = 0$ were performed by linear regression, with resulting correlation coefficients of 0.95.

increase in viscosity, suggesting that the gelling involves qualitative changes other than pure aggregation, such as changes in molecular shape and/or supramolecular organization.

DISCUSSION

The aim of this work was, first, to investigate the shape and subunit architecture of ovomucin after purification from the egg-white, and, secondly, to emphasize the involvement of specific protein aggregation in gel formation together with the influence of the linear nature of ovomucin on non-Newtonian properties of the ovomucin gel.

By using light-scattering studies, we have shown that organization of purified ovomucin corresponds to aggregated and highly polymerized states. After SDS treatment a 50% decrease in the M_r of ovomucin was observed with respect to the untreated material (Table 1). It is known that SDS treatment at high temperature (80 °C) breaks non-covalent interactions, which can explain the decrease in M_r . But, according to Silberberg & Meyer

[27], it is not possible to rule out the hypothesis of thermal degradation of polypeptide chains due to the presence of heat-labile amino acids.

Previous results had shown that the ovomucin gel lost its non-Newtonian properties after SDS treatment [8]. By correlating light-scattering results with rheological studies of the ovomucin gel, it appears that the shift from non-Newtonian to Newtonian behaviour is associated with ovomucin polymer disaggregation or rupture.

When the data obtained from our light-scattering experiments are compared with those of Shogren *et al.* [28] (their Fig. 3: plot of $\ln R_g$ versus $\ln M_{r,p}$ where $M_{r,p}$ is the relative molecular mass of the protein backbone), we observed that the ovomucin configuration was intermediate between linear mucin and non-glycosylated randomly coiled proteins. In fact, if we consider that 1 nm is occupied by 2.7 amino acid residues in a fully extended amino acid chain [29] and one amino acid residue may be linked to an oligosaccharide chain of eight to 12 sugar residues (M_r 200) [22], then an M_r/l value of about 2000 is expected for a linear chain. Since the observed value of M_r/l (Table 1) is an order of magnitude higher than that calculated for a linear chain, a randomly coiled character is suggested for the ovomucin polymer, probably induced by water structure and the pH to which ovomucin was subjected after isolation and purification. Furthermore, the experimental value of R_g/R_h was approximately 2, which is representative of polydispersed flexible coils in poor solvents such as water [28]. The small value of the intrinsic viscosity obtained for the ovomucin preparation supports the hypothesis of a randomly coiled structure (Fig. 8) [9,18]. An M_r/l value of 2000 was obtained after DTT or DTT plus trypsin treatments (Table 1), which is the value expected for a linear chain. Moreover, several authors have already proposed a linear and flexible character of mucins that is associated with the random-coil character [28,30–32].

Morphological evidence for the linear character of ovomucin has been obtained by electron microscopy (molecules a and e in Fig. 5), and our results are consistent with those previously published [17,29,33]. It must also be pointed out that the longitudinal association of ovomucin molecules in at least a double-stranded configuration has not been previously reported, although there are several examples of this kind of organization, such as in DNA and triple-helix collagen [34].

According to the hypothesis of a fully extended chain (with 2.7 amino acid residues/nm) [29] and taking into account the mass of one amino acid residue and the sugar percentage of the ovomucin, M_r values of 0.4×10^6 and 1.4×10^6 are calculated for 600 nm and 2000 nm molecular lengths (Fig. 6). Nevertheless, since macromolecules may be multiple-stranded, as it appears in molecules a in Fig. 5, the M_r values should be at least doubled.

These calculated estimates of M_r are still lower than all the values obtained by light-scattering experiments (Table 1). It is important to stress that a difference of 2–10-fold in the M_r of mucins has previously been reported between the results obtained by light-scattering and equilibrium sedimentation [35]. One possible explanation of these contradictory values is that elastic light-scattering is a dynamic method by which one measures a weight-averaged M_r in which small numbers of high- M_r species exert a disproportionally large influence, whereas in other methods the value obtained is

a number-averaged M_r , which is more insensitive to high- M_r species [9,18]. In addition, the value of M_r obtained from electron microscopy may also be artificially lowered by fracture of the fragile mucin molecules during the spray step in preparation of the sample.

The type of linkages involved in mucin polymerization were investigated by DTT and trypsin treatments. A dramatic increase in M_r of purified mucin after DTT treatment was observed (Table 1, Fig. 3, lane 2, and Fig. 2c, lane 2). This result shows that disulphide bridges are clearly involved in ovomucin polymerization. Since the treatment we used is exhaustive (1% DTT for 12 h at 37 °C) [30], it appears then that 3.3×10^6 may be the subunit M_r . However, because of the restrictions concerning the M_r obtained by light-scattering, it is possible that 3.3×10^6 is an overestimated value. Fig. 3 (lane 2) and Fig. 2(c) (lane 2) also show that DTT-depolymerized ovomucin corresponds to a polydispersed material. Thus ovomucin appears to consist of highly polymerized macromolecules, as is the case for higher-mammal mucins [1], in agreement with the Carlstedt model [30], which postulates that mucin subunits, which consist of alternate glycosylated and non-glycosylated regions, are linked via end-to-end disulphide bridges, forming long polymers.

The increase in R_g and R_h together with a decrease in M_r/l (Table 1) may result from the linear and rigid structure of the liberated ovomucin subunit after DTT treatment, whereas the polymer appears to be more flexible and occupies a smaller hydrodynamic volume, as suggested by the values shown in Table 1. Furthermore, the calculation of $\ln R_g$ versus $\ln M_{r,p}$ agrees with the plot by Shogren *et al.* ([28], their Fig. 3) obtained from data for linear mucins.

The decrease in M_r , R_g and R_h of about 2-fold without change in M_r/l after trypsin digestion of the DTT-treated material may be explained by hydrolysis of the linear subunit in the non-glycosylated regions, which are more susceptible to proteolysis [30].

Results obtained by Carlstedt *et al.* ([30], see their Table 3) for cervical mucin correspond to similar values of M_r and R_g to those reported here (under 'Aggregates' in Table 1). Furthermore, our M_r and R_g values for ovomucin polymer reasonably agree with the plot by Silberberg (his Fig. 3: plot of $\log R_g$ versus $\log M_r$), which was obtained from data from higher-mammal mucins [36].

After defining the spatial configuration of ovomucin polymers, it was of interest to determine the nature of the supramolecular organization involved in gel formation. It is known that the glycoprotein solid phase of mucus may be organized as a fibrillar macromolecular network appearing as a hydrogel structure with transient weak linkages [10,37,38]. This hydrogel structure would confer the non-Newtonian and even viscoelastic properties to mucus [39]. On the other hand, another model was proposed by Lee *et al.* [40] according to which mucus is composed of entangled randomly coiled molecules. Thus, is the appearance of gel properties due to entangled macromolecular assembly or to a specific spatial organization of ovomucin polymers that form a network and a hydrogel?

By heating the ovomucin preparation, we induced aggregation, which may be interpreted as an entangled macromolecular assembly sustained by non-specific interactions between hydrophobic regions newly exposed during heating denaturation [41,42]. However, specific

($\eta_{sp.}$) and intrinsic $[\eta]$ viscosities decrease by 25 % after heating the ovomucin preparation (Fig. 8), and the absorbance at 340 nm increased and reached a maximal value (Fig. 7). These results suggest that temperature-induced aggregation did not lead to a viscosity increase, thus supporting the notion that a macromolecular entanglement is not the only event that leads to ovomucin hydrogel formation. Therefore hydrogel formation would require a peculiar kind of aggregate with a definite molecular shape and solvent properties such as water-holding to occur. The latter property was absent in our experiments because temperature-triggered protein aggregation would favour interactions between hydrophobic regions, newly exposed as a result of denaturation, which exclude water [41,42].

Taken together, our results suggest that the association of two or more ovomucin polymers (2000–3000 nm), which behave in dilute solutions as a long randomly coiled and flexible structure, leads to a linear spatial organization involved in the hydrogel nature of ovomucin gel. At present the interactions of globular proteins with the linear structures of ovomucin cannot be ruled out [8]. The loss of the fibrillar nature by sonication was concomitant with the observed loss of non-Newtonian properties of the ovomucin gel and egg-white following sonication [8]. In a more general way, our results show that spatial macromolecular organization and its state of aggregation are closely related to gel-forming capability.

These studies may help us to understand the complex rheology and organization of macromolecular assemblies of cellular cytoplasm [43], which appears as a non-Newtonian fluid and a non-covalent gel network [44].

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REFERENCES

- Gallagher, J. T. & Corfield, A. P. (1978) *Trends Biochem. Sci.* **3**, 38–41
- Grassé, P. (1950) in *Traité de Zoologie* (Masson, P., ed.), vol. 15, pp. 355–377, Masson, Paris
- Robinson, D. S. & Monsey, J. B. (1971) *Biochem. J.* **121**, 537–547
- Hayakawa, S. & Sato, Y. (1976) *Agric. Biol. Chem.* **40**, 2397–2404
- Vadhera, D. V. & Nath, K. R. (1973) *Crit. Rev. Food Technol.* **4**, 193–309
- List, S. J., Findlay, B. P., Forstner, G. G. & Forstner, J. F. (1978) *Biochem. J.* **175**, 565–571
- Smith, B. F. & LaMont, J. T. (1984) *J. Biol. Chem.* **259**, 12170–12177
- Rabouille, C., Aon, M. A. & Thomas, D. (1989) *Arch. Biochem. Biophys.* **270**, 495–503
- Tanford, C. (1961) *Physical Chemistry of Macromolecules* pp. 317–456, John Wiley and Sons, New York
- Volochine, B., Cazabat, A. M., Chretien, F. C. & Kuntzmann, J. M. (1988) *Hum. Reprod.* **3**, 577–582
- Muller, G., Wehrhahn, A. K., Zentz, F. & Albagnac, G. (1988) *Spectra 2000 (Deux Mille)* **131**, 55–59
- Zimm, B. H. (1948) *J. Chem. Phys.* **16**, 1099–1104
- Sheehan, J. K. & Carlstedt, I. (1984) *Biochem. J.* **221**, 499–504
- Mueller, M. & Burchard, W. (1978) *Biochim. Biophys. Acta* **537**, 208–225
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Shotton, D. M., Burke, B. E. & Branton, D. (1979) *J. Mol. Biol.* **131**, 303–329
- Slayter, H. S., Lamblin, G., Le Treut, A., Galabert, T., Houdret, N., Degand, P. & Roussel, P. (1984) *Eur. J. Biochem.* **142**, 209–218
- Champetier, G. & Monnery, L. (1969) *Introduction à la Chimie Macromoléculaire*, pp. 252–301, Masson, Paris
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Schauer, R. (1978) *Methods Enzymol.* **50**, 64–89
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
- Ashwell, G. (1966) *Methods Enzymol.* **8**, 85–95
- Gaskin, F., Cantor, C. R. & Shelanski, M. L. (1974) *J. Mol. Biol.* **89**, 737–758
- Samejima, K., Ishioroshi, M. & Yasui, T. (1981) *J. Food Sci.* **46**, 1412–1418
- Hermansson, A. M., Harbitz, O. & Langton, M. (1986) *J. Sci. Food. Agric.* **37**, 69–84
- Williams, R. J. P. (1979) *Biol. Rev. Cambridge Philos. Soc.* **54**, 389–437
- Silberberg, A. & Meyer, F. A. (1982) in *Mucus in Health and Disease* (Chantler, E. W., Elder, J. B. & Elstein, M., eds.), vol. 2, pp. 53–74, Plenum Press, New York
- Shogren, R. L., Jentoft, N., Gerken, T. A., Jamieson, A. M. & Blackwell, J. (1987) *Carbohydr. Res.* **160**, 317–327
- Marriane, T., Perini, J. M., Laffitte, J. J., Houdret, N., Pruvot, F. R., Lamblin, G., Slayter, H. S. & Roussel, P. (1987) *Biochem. J.* **248**, 189–195
- Carlstedt, I., Lindgren, H. & Sheehan, J. K. (1983) *Biochem. J.* **213**, 427–435
- Carlstedt, I. & Sheehan, J. K. (1983) *Ciba Found Symp.* **109**, 152–172
- Shogren, R. L., Jamieson, A. M., Blackwell, J. & Jentoft, N. (1984) *J. Biol. Chem.* **259**, 14657–14662
- Rose, M. J., Voter, W. A., Brown, C. F. & Kaufman, B. (1984) *Biochem. J.* **222**, 371–377
- Na, G. C., Butz, L. J. & Carroll, R. J. (1986) *J. Biol. Chem.* **261**, 12290–12299
- Roussel, P., Lamblin, G., Lhermitte, M., Houdret, N., Laffitte, J. J., Perini, J. M., Klein, A. & Scharfman, A. (1988) *Biochimie* **70**, 1471–1482
- Silberberg, A. (1987) *Biorheology* **24**, 605–614
- Odeblad, E. (1962) *Int. J. Fertil.* **7**, 313–319
- Chretien, F. C. (1974) *J. Gynecol. Obstet. Biol. Reprod.* **3**, 711–744
- Puchelle, E., Zahm, J. M. & Quemada, D. (1985) *Biorheology* **24**, 557–563
- Lee, W., Blandau, R. J. & Verdugo, P. (1977) in *Workshop Conference on the Uterine Cervix in Reproduction* (Insler, V. & Bettendorf, G., eds.), pp. 12–13, Stuttgart
- Oosawa, F. & Asakura, S. (1975) *Thermodynamics of the Protein Polymerization*, pp. 57–69, Academic Press, London
- Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem.* **16**, 115–137
- Fulton, A. B. (1982) *Cell* **30**, 345–347
- Luby-Phelps, K., Lanni, F., Taylor, D. L. (1988) *Annu. Rev. Biophys. Biophys. Chem.* **17**, 369–396

APPENDIX

Theory of elastic light-scattering

The fluctuation theory shows that the relationship between the Rayleigh factor ΔR_θ and physical characteristics of scattering molecules is expressed by:

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_r \cdot P(\theta)} + 2A_2 \cdot \frac{c}{P(\theta)} + \dots \quad (1)$$

In this relation

$$K = 2\pi^2 n_0 N^{-1} \lambda_0^{-4} (dn/dc)^2 (1 + \cos^2 \theta)$$

$$\Delta R_\theta = R_{\theta, \text{solution}} - R_{\theta, \text{solvent}}$$

and

$$R_\theta = \frac{I_\theta}{I_0} \cdot r^2 \cdot \frac{1}{(1 + \cos^2 \theta)} \quad (1')$$

M_r is the relative molecular mass of the macromolecule, A_2 is the second virial coefficient, c is the macromolecule concentration (g/ml), n_0 is the solvent refractive index, λ_0 is the incident wavelength, N is Avogadro's number and dn/dc is the refractive-index increment. I_0 and I_θ are the incident and scattered light intensities (at one observation angle θ) and r is the distance between the sample and the detector. The function $P(\theta)$ is a shape factor depending only on the particle geometry (shape and size) and takes into account the angular dependence of the scattered light. With small values of θ , $P(\theta)$ versus $\sin^2 \theta/2$ is a linear function.

$$\lim P(\theta) = 1 - \frac{q^2 R_g^2}{3} = 1 - \frac{(16n_0^2 \pi^2 R_g^2 \cdot \sin^2 \theta/2)}{3\lambda_0^2} \quad (2)$$

where $q = 4\pi n_0/\lambda_0 \cdot \sin \theta/2$ (scattering factor) and R_g is the radius of gyration. Eqn. (2) shows that $P(\theta) \rightarrow 1$ when $\theta \rightarrow 0$ regardless of the size or shape of the scattering molecular species, thus:

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_r} + 2A_2 c \quad \text{when } \theta \rightarrow 0 \quad (3)$$

In view of the fact that the equation for $P(\theta)$ is itself only valid at high dilution, it is necessary to extrapolate the data at each angle to zero concentration before we can perform the extrapolation indicated by eqn. (2). At the same time, it is desirable to extrapolate the data at each concentration to zero angle, so that M_r and A_2 can be determined according to eqn. (3), which, for large molecules, is valid only at zero angle. Both these objectives can be achieved by the method of Zimm [1], which consists of plotting $Kc/\Delta R_\theta$ versus $\sin^2 \theta/2 + kc$, where k is an arbitrary constant chosen so as to provide a convenient spread of the data on the graph.

Holtzer plot

According to Mueller & Burchard [2]:

$$P(\theta) = \frac{\pi}{q \cdot l} - \frac{2}{q^2 \cdot l^2} \quad (4)$$

where l is the rod length and q is the scattering factor. For small θ values, eqn. (1) becomes:

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_r \cdot P(\theta)} \quad (5)$$

Insertion of eqn. (4) into eqn. (5) gives:

$$\frac{q \cdot \Delta R_\theta}{\pi Kc} = \frac{M_r}{l} - \frac{2M_r}{q\pi l^2}$$

From this equation, the mass per unit length can be obtained from the value of the asymptote in the plot of $q \cdot \Delta R_\theta/Kc$ versus q^2 , a Holtzer plot.

REFERENCES

1. Zimm, B. H. (1948) *J. Chem. Phys.* **16**, 1099–1104
2. Mueller, M. & Burchard, W. (1978) *Biochim. Biophys. Acta* **537**, 208–225

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