Submaxillary mucins

Intermolecular interactions and gel-forming potential of concentrated solutions

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The intermolecular interactions in concentrated solutions of pig submaxillary mucin (PSM) and sheep submaxillary mucin (SSM) were studied by mechanical spectroscopy. PSM and SSM were purified from detectable protein and nucleic acid by equilibrium centrifugation in a CsCl density gradient. PSM and SSM isolated in the presence of proteinase inhibitors showed distinct differences from preparations isolated in the presence of 0.2 M-NaCl alone, the latter having a carbohydrate and amino acid analysis similar to other preparations isolated by precipitation or ion-exchange techniques. Gel-filtration studies showed that preparations isolated in the presence of 0.2 M-NaCl alone were dissociated into smaller-sized glycoprotein units by 3.5 M-CsCl or 2.0 M-NaCl (SSM), pH 2.0 (PSM) or heating at 100 °C for 10 min (PSM and SSM). Preparations isolated in the presence of proteinase inhibitors were not dissociated by these treatments. Proteolysis fragmented all submaxillary mucin preparations into small glycopeptides of M_r 13 700 for PSM and of M_r 14 000 and 15 000 for SSM. PSM preparations when concentrated formed viscoelastic gels, as determined by mechanical spectroscopy. In contrast, SSM showed characteristics of a weak viscoelastic liquid under comparable conditions (coil overlap). PSM glycoprotein isolated in proteinase inhibitors formed weak viscoelastic gels at concentrations between 5 and 15 mg/ml. Preparations of PSM glycoprotein isolated in the presence of 0.2 M-NaCl (concentration 10–97 mg/ml) had the same overall mechanical gel structure as those preparations extracted in the presence of proteinase inhibitors. This gel structure was seen to collapse following proteolysis of both preparations or after acid treatment of the glycoprotein isolated in the presence of 0.2 M-NaCl, consistent with the breakdown in size of the polymeric glycoprotein. Treatment of PSM gel with 0.2 M-2-mercaptoethanol caused a surprising increase in gel strength, which was further markedly increased on removal of the reducing agent by dialysis. An association of reduced subunits of PSM was observed by gel filtration after removal of 0.2 M-2-mercaptoethanol. These results point to intermolecular disulphide exchange occurring on reduction of these PSM glycoprotein preparations. These results demonstrate that gel formation in PSM glycoprotein is similar to that for other gastrointestinal mucus glycoproteins from stomach to colon. Gel formation in PSM, as in other mucins, depends on polymerization of subunits. From a comparison of known structures of gastrointestinal mucins it is concluded that the composition and average chain length of the carbohydrate side chains can vary substantially without qualitatively affecting their ability to form a gel.

INTRODUCTION

Salivary mucins are secreted in a viscous soluble form, and in this respect contrast with gastrointestinal mucins, which are secreted as water-insoluble gels. Mechanical spectroscopy is a widely used method for studying gel structure (Ferry, 1980; Morris & Ross-Murphy, 1981). By this method human and pig gastric mucus and pig duodenal, small-intestinal (jejunal and ileal) and colonic mucus are shown to have properties characteristic of weak viscoelastic gels (Bell *et al.*, 1985; Sellers *et al.*, 1983). The mechanical properties of the native pig gastrointestinal mucus secretions can be reproduced by the purified glycoprotein components (mucins), at a concentration equivalent to that secreted.

Mucins from different regions of the gastrointestinal tract have the same general structure of glycosylated regions and non-glycosylated regions; the latter contain

disulphide bridges that join glycoprotein subunits together to form the polymeric structure (Snary *et al.*, 1970; Allen, 1981). Proteolysis fragments the polymeric mucus glycoprotein into degraded units consisting only of the glycosylated parts of the original molecule. Mechanical spectroscopy shows that these proteolytically digested fragments retain some of the non-covalent associations seen in the intact gel. Primarily on the basis of this evidence, it has been proposed that the non-covalent gel-forming interactions of the polymeric mucus glycoproteins arise in part by interdigitation of their carbohydrate side chains (Bell *et al.*, 1984; Sellers *et al.*, 1987). The carbohydrate side chains of these gel-forming gastrointestinal mucins differ substantially in both composition and complexity. For example, pig gastric mucin has chains of up to 19 sugar residues in length (Slomiany & Meyer, 1972), whereas pig small-intestinal mucin has an average of six to eight sugar residues per chain and a

Abbreviations used: PSM, pig submaxillary mucin; SSM, sheep submaxillary mucin.

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greater content of *N*-acetylgalactosamine and sialic acid (Mantle & Allen, 1981).

In order to investigate further the relationship between glycoprotein structure and gel formation we have studied model systems of sheep and pig submaxillary mucins (SSM and PSM) at concentrations well in excess of those at which they are secreted. In this study we also prepared submaxillary mucins in the presence of proteinase inhibitors and compared these preparations with those extracted in the absence of such inhibitors but which have been well characterized by others. The reason for choosing SSM and PSM is that they have short carbohydrate side chains of up to a maximum of two and five sugar residues respectively (Gottschalk *et al.*, 1972; Carlson, 1968).

METHODS

Preparation of glycoprotein samples

The constituted 'gels' were formed from mucins isolated from pig or sheep submaxillary glands. The glands were dissected from attached lymph nodes and from their connective capsules, finely diced and briefly homogenized (0.5 min) at low shear in 4 vol (by wt.) of one of the following ice-cold solutions: (a) 0.2 M-NaCl containing 0.02% NaN_3 or (b) a solution containing 1.0 mM-phenylmethanesulphonyl fluoride, 1.0 mM-sodium iodoacetate, 0.1 M- α -aminohexanoic acid, 5.0 mM-benzamidine hydrochloride, 1.0 mg of soya-bean trypsin inhibitor/l and 10 mM-EDTA (disodium salt) (all final concentrations) in 0.5 M-Tris buffer at pH 6.5 (Tris base adjusted to stated pH with 6.0 M-HCl). Soluble components were separated from tissue debris by centrifugation (6000 g for 1 h) at 4 °C, and the supernatant, after straining through glass-wool to remove lipid material, was a clear pink-coloured solution. Analysis of the glycoprotein content of the soluble components and the discarded pellet was performed after exhaustive digestion with papain and dialysis to remove digested peptide. Over 65% and 73% of the total glycoprotein-positive material in the sheep and pig submaxillary glands respectively was contained in the soluble fraction. The purification of glycoprotein from protein and nucleic acid in the soluble preparation was by two successive fractionations using equilibrium centrifugation in a CsCl density gradient (Creeth & Denborough, 1970).

Mechanical properties

The glycoprotein fractions after equilibrium centrifugation were pooled and dialysed against 0.2 M-NaCl containing 0.02% NaN_3 and concentrated by vacuum dialysis. For SSM the glycoprotein concentrations of the samples isolated under the two extraction conditions were between 74 and 133 mg/ml for those isolated in the presence of 0.2 M-NaCl and between 24 and 31 mg/ml for the glycoprotein extracted in the presence of proteinase inhibitors. PSM glycoprotein isolated in the presence of 0.2 M-NaCl was concentrated to between 10 and 97 mg/ml and with the preparations isolated in the presence of proteinase inhibitors to between 5 and 15 mg/ml. Dynamic oscillatory measurements on 'gels' constituted from isolated mucins were performed on a Rheometrics mechanical spectrometer (Rheometrics, Piscataway, NJ, U.S.A.) with a cone-and-plate geometry (cone diameter 25 mm, cone angle 0.1 rad). Sinusoidal deformation of the sample under test (sample volume approx. 1.5 ml) was produced by an electronically con-

trolled servo system (range 10^{-3} – 10^2 rad/s, with a resolution of better than 0.1% per decade) driving the cone. The energy transmitted to the lower plate was detected by a transducer (Rheometrics type ST; 0.01–10 g/cm) mounted on an air-bearing to minimize frictional losses. Environmental control of the sample chamber was by forced air convection, the air being supplied from a thermostatically controlled water bath and humidified at the working temperature of 25 °C. Sample temperature was regulated to ± 0.30 °C and sensed with a platinum resistance thermometer. The storage modulus G' (elastic component) and loss modulus G'' (viscous component) were measured as functions of both strain (γ range 5–100%) and frequency (ω range 10^{-2} – 10^2 rad/s). Strain sweeps were run at 10 rad/s, with measurements being taken every 5% increase in strain, and rate sweeps were run at 20% strain for all samples, with three measurements of G' and G'' being made per frequency decade.

After loading, all samples were allowed to equilibrate (15 min) to the measuring temperature and to relax from any stresses that may have been induced by the loading procedure. The strain profiles were not seen to change after a period of 3 h, showing that 15 min was long enough for stresses to relax. Three successive measurements of the mechanical spectra of the mucus glycoprotein samples (a period of 2 h) were also unchanged, showing that dehydration of the sample or other mechanical damage was not a factor in the measurements.

To examine the effects of varying the pH on the mechanical properties, the mucin glycoprotein samples were dialysed at 4 °C for 24 h against one of the following: 0.2 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer at pH 8.0 or 7.0 or 0.2 M-glycine/HCl buffer at pH 2.0; all solutions contained 0.02% NaN_3 . Mucin samples were reduced by dialysis for 24 h at 4 °C against 0.2 M-2-mercaptoethanol in 0.2 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0, containing 0.02% NaN_3 . Proteolytic digests of glycoprotein samples were obtained by using papain (20 μg of papain/mg of glycoprotein) at 37 °C. Samples of 'gel' were added with the papain to a dialysis sac immersed in digestion buffer, namely 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.5, containing 5.0 mM-cysteine and 5.0 mM-EDTA (disodium salt).

Mechanical properties of the samples were measured both before and after the respective treatment and compared with those of controls. After measurement of the mechanical spectra, all samples were stored at -20 °C for further biochemical analysis. The glycoprotein concentration within each sample was measured and analysed for the presence of included degraded glycoprotein by gel filtration on a Sepharose CL-2B column (Mantle & Allen, 1978) after first solubilizing the samples by brief homogenization.

Chemical analyses

Amino sugars were released by acid hydrolysis (4.0 M-HCl) for 8 h (maximum release) at 100 °C and identified on a Locarte amino acid autoanalyser. Sialic acids were determined by the method of Aminoff (1961), with *N*-acetylneuraminic acid as a standard. Amino acid analyses were kindly performed by Dr. H. Gregory, Bioscience Department, ICI Pharmaceuticals Division, Alderly Park, Macclesfield, Cheshire, U.K. Determination of *N*-terminal amino acid residues was by the method of Hartley (1970).

The gel-filtration media (Sephadex G-75 and Sepha-

rose CL-2B) used for chromatographic separation were supplied by Pharmacia, Milton Keynes, Bucks., U.K. The Sepharose CL-2B columns (1.5 cm diameter, length 135–140 cm) were eluted by upward flow with 0.2 M-NaCl containing 0.02% NaN_3 (unless otherwise stated).

Polyacrylamide-gel electrophoresis in the presence of SDS at pH 6.8 was carried out by the method of Laemmli (1970). Samples were denatured by heating for 2 min at 100 °C with or without 5% (v/v) 2-mercaptoethanol. Between 400 and 500 μg of glycoprotein (sample volume less than 200 μl) was applied to the stacking gel of disc gels, and 100 μg of glycoprotein (sample volume 40 μl) was applied to individual channels on a slab gel. Running gels were 7.5% (w/v) polyacrylamide with a 3% stacking gel. Gels were stained for protein with 0.25% Coomassie Blue or with AgNO_3 by using the Bio-Rad silver stain kit supplied by Bio-Rad Laboratories, Watford, Herts., U.K. Gels were stained for glycoprotein by using the method of Zacharius *et al.* (1969).

Glycoprotein was determined by using the periodic acid/Schiff method (Mantle & Allen, 1978), with purified PSM and SSM as standards. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The enzyme papain (papainase, EC 3.4.22.2; from papaya latex, type 111) was supplied by Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS

Purification of PSM and SSM

Submaxillary glands were immersed immediately following dissection in either ice-cold 0.2 M-NaCl containing 0.02% NaN_3 or Tris buffer, pH 6.5, containing a spectrum of proteinase inhibitors. These proteinase inhibitors were included throughout the subsequent glycoprotein fractionation procedures. Soluble SSM and PSM were purified by equilibrium centrifugation in a CsCl density gradient. After fractionation of the gradient there was clear separation of the lower-density protein-positive fractions (Lowry) from the higher-density glycoprotein-positive material (periodic acid/Schiff). The percentages of glycoprotein, protein and nucleic acid in the soluble extracts of the submaxillary glands were respectively 27, 70 and 2% by wt. for SSM (eight preparations) and 26, 72 and 2% for PSM (14 preparations). The mucin-rich fractions were further purified by a second CsCl density gradient, which separated the remaining protein and nucleic acid from the glycoprotein.

Characterization of PSM and SSM

Preparations of purified SSM and PSM glycoproteins isolated in the presence of proteinase inhibitors and following fractionation in CsCl were excluded on Sepharose CL-2B and unaffected by heating for 10 min at 100 °C or dialysis against 2.0 M-NaCl or 0.2 M-glycine/HCl buffer, pH 2.0, for 24 h at 4 °C.

Purified SSM and PSM glycoproteins were also prepared in the absence of proteolytic inhibitors and showed marked differences in elution patterns on Sepharose CL-2B compared with preparations isolated in the presence of inhibitors. Following fractionation in CsCl, SSM was eluted well into the included volume (two-thirds) of a Sepharose CL-2B column. This same SSM preparation before fractionation in CsCl was eluted as a single excluded peak on Sepharose CL-2B. Thus SSM that had been prepared in the absence of proteolytic inhibitors

dissociated into smaller units during density-gradient fractionation in CsCl. This dissociation was confirmed by dialysis of the unfractionated SSM against high-salt solutions of 3.5 M-CsCl or 2.0 M-NaCl, which also resulted in a previously excluded glycoprotein peak becoming well included on Sepharose CL-2B. Heating at 100 °C for 10 min also dissociated the purified PSM isolated in the presence of 0.2 M-NaCl and the unfractionated soluble SSM (isolated in the presence of 0.2 M-NaCl), as shown by inclusion on Sepharose CL-2B.

No protein bands could be detected with either Coomassie Blue or the more sensitive AgNO_3 stain following electrophoresis of the purified SSM or PSM glycoproteins (isolated by successive CsCl-density-gradient fractionations), although a strongly staining band remained at the interface between the stacking and running gels. This band presumably represented glycoprotein that had not penetrated the higher-density polyacrylamide gel. Only the slightest traces of protein, detected by the sensitive AgNO_3 stain, were liberated following reduction of the purified glycoprotein, showing no release of a major protein component on reduction.

The sialic acid, *N*-acetylgalactosamine and individual amino acid contents of purified SSM and PSM glycoproteins isolated in the presence of 0.2 M-NaCl were not significantly different from values obtained by others for SSM and PSM preparations where proteinase inhibitors had not been included in their isolation (Gottschalk *et al.*, 1972; Pigman, 1977). There was 48 and 41% by wt. total protein in the SSM and PSM glycoprotein preparations respectively. *N*-Terminal amino acid analyses by hydrolysing dansylated SSM and PSM glycoproteins (isolated in the presence of 0.2 M-NaCl) produced no detectable dansyl-amino acid residues in either case. This result for SSM agrees with that obtained by Hill *et al.* (1977), who found a blocked *N*-terminal amino acid residue in this mucin. This lack of *N*-terminal amino acids also confirmed a lack of protein contamination in both SSM and PSM glycoprotein preparations.

Mechanical spectroscopy of concentrated preparations of purified PSM and SSM

Solutions of the following glycoproteins were concentrated in 0.2 M-NaCl containing 0.02% NaN_3 by vacuum dialysis as follows: PSM (isolated in the presence of proteinase inhibitors) to 5–15 mg/ml, SSM (isolated in the presence of proteinase inhibitors) to 24–31 mg/ml, PSM (isolated in the presence of 0.2 M-NaCl) to 10–97 mg/ml, and SSM (isolated in the presence of 0.2 M-NaCl) to 74–133 mg/ml. Below these concentrations the glycoprotein solutions were not sufficiently viscous to generate torque for investigation of their mechanical properties on the rheometer.

In all frequency scans of either preparation of PSM (isolated in the presence or in the absence of proteinase inhibitors) the storage modulus G' predominated over the loss modulus G'' throughout the frequency range accessed (10^{-2} – 10^2 rad/s) (Fig. 1). The values of both moduli G' and G'' were independent of strain over the range 5–100% and showed only slight frequency-dependent behaviour, typical of a viscoelastic gel (Fig. 1). The decrease in the loss modulus G'' at the highest frequencies (Fig. 1) may be an artifact due to resonance effects. The absolute values of the moduli G' and G'' for PSM preparations isolated in the presence of proteinase inhibitors were approximately twice those of PSM prepa-

Fig. 1. Mechanical properties of concentrated samples of PSM isolated in the presence of 0.2 M-NaCl containing 0.02% NaN_3

The Figure shows plots of the storage modulus G' (—) and loss modulus G'' (----) against frequency (at 20% strain) for (curve 1) PSM (concentration 97 mg/ml), (curve 2) PSM after dialysis against 0.2 M-glycine/HCl buffer, pH 2.0, for 24 h at 4 °C and (curve 3) PSM after incubation with papain for 4 h at 37 °C.

Fig. 2. Mechanical properties of concentrated samples of SSM

The Figure shows plots of the storage modulus G' (—) and loss modulus G'' (----) against frequency (at 20% strain) for (curve 1) SSM (concentration 24 mg/ml) isolated in the presence of proteinase inhibitors, (curve 2) SSM (concentration 133 mg/ml) isolated in the presence of 0.2 M-NaCl containing 0.02% NaN_3 and (curve 3) SSM (concentration 31 mg/ml; isolated in the presence of proteinase inhibitors) incubated with papain for 1.5 h at 37 °C.

rations isolated in the presence of 0.2 M-NaCl, but the patterns of the frequency spectra were the same for both.

For SSM glycoprotein preparations extracted in the presence of proteinase inhibitors the storage modulus G' predominated over the loss modulus G'' , but only at frequencies above 0.2 rad/s. Both moduli G' and G'' increased with frequency over the range measured (10^{-2} – 10^2 rad/s), but the dependence was greater for the storage modulus G' , thus producing a cross-over of the two moduli (Fig. 2). The absolute values for both moduli G' and G'' increased as the glycoprotein concentration increased. For all SSM glycoprotein samples (concentration 74–133 mg/ml) isolated in the absence of proteinase inhibitors, the loss modulus G'' predominated over the storage modulus G' throughout the frequency range accessed (10^{-1} – 10^2 rad/s) (Fig. 2). Both moduli G' and G'' increased sharply with increasing frequency, although the frequency-dependence of the storage modulus G' was more pronounced. These mechanical properties for both types of SSM glycoprotein preparations (isolated in the presence or in the absence of proteinase inhibitors) were characteristic of viscoelastic liquids in which the only intermolecular interactions were those of entanglement.

Effect of acid on purified PSM and SSM

PSM glycoprotein isolated in the presence of proteinase inhibitors remained excluded by gel filtration on Sepharose CL-2B after dialysis against pH 2.0 for 24 h. In contrast, PSM isolated in the presence of 0.2 M-NaCl after dialysis at pH 2.0 for 24 h was over 80% included on Sepharose CL-2B provided that the eluent was maintained at pH 3.0 (Fig. 3). If the acid-treated glycoprotein was eluted from the Sepharose CL-2B column

with 0.2 M-NaCl at pH 6.5, then only an excluded periodic acid/Schiff-positive peak was obtained.

The effect of this reversible acid dissociation of PSM was investigated on concentrated solutions (72–80 mg/ml) by mechanical spectroscopy. At pH 2.0 there was a dramatic collapse of gel structure. The storage modulus G' , although greater in value at higher frequencies than the loss modulus G'' , showed more frequency-dependence than the latter, and this resulted in a cross-over of the moduli G' and G'' below frequency levels of 10 rad/s (Fig. 1). The absolute values of the moduli G' and G'' were both much lower than those measured for PSM glycoprotein at pH 7.0 or 8.0, which showed the same frequency profiles as controls at pH 6.5. Both the mechanical and gel-filtration profiles of SSM prepared in the presence of proteinase inhibitors were unaffected by exposure to acid (pH 2.0).

Effect of disulphide-bond reduction on purified PSM and SSM

A surprising and large increase in the absolute values of the storage modulus G' and loss modulus G'' occurred after reduction (0.2 M-2-mercaptoethanol) of concentrated solutions of PSM (isolated in the presence of 0.2 M-NaCl), although the pattern of the moduli with respect to frequency was identical with that for non-reduced glycoprotein. Thus at constant concentration the storage modulus G' taken at 1 rad/s rose 13-fold and the loss modulus G'' increased 10-fold on reduction of the glycoprotein preparation, suggesting that elastically effective cross-links had been enhanced in the reduced sample. If the reduced glycoprotein preparations (after treatment with 0.2 M-2-mercaptoethanol) were dialysed against

Fig. 3. Gel-filtration analysis of PSM

PSM glycoprotein extracted in the presence of 0.2 M-NaCl containing 0.02% NaN_3 was purified by equilibrium centrifugation (at 40000 rev./min in a preparative ultracentrifuge for 48 h at 4 °C) in two successive CsCl density gradients. The following samples (1.0 ml) were applied to the Sepharose CL-2B column: sample 1, isolated glycoprotein (concentration 2.6 mg/ml), eluted by 0.2 M-NaCl containing 0.2% NaN_3 ; sample 2, isolated glycoprotein (concentration 2.8 mg/ml) dialysed against 0.2 M-glycine/HCl buffer, pH 2.0, for 24 h at 4 °C, eluted by 0.2 M-NaCl/HCl adjusted to pH 3.0; sample 3, isolated glycoprotein (concentration 2.2 mg/ml) dialysed against 0.2 M-2-mercaptoethanol in 0.2 M-phosphate buffer, pH 7.0, for 24 h at 4 °C, eluted by 0.2 M-2-mercaptoethanol in 0.2 M-NaCl; sample 4, isolated glycoprotein (concentration 2.0 mg/ml) heated at 100 °C for 10 min, eluted by 0.2 M-NaCl containing 0.02% NaN_3 ; sample 5, isolated glycoprotein (concentration 1.2 mg/ml) incubated with papain for 24 h at 60 °C, eluted by 0.2 M-NaCl containing 0.02% NaN_3 . Fractions (3.0 ml) of the column eluate were collected in each case and analysed for glycoprotein (periodic acid/Schiff method).

0.2 M-NaCl for 24 h at 4 °C the samples became so rigid that it was difficult to load them on to the rheometer, and the torque produced overloaded the transducer, making measurement impossible.

PSM glycoprotein from the reduced preparations,

when fractionated on Sepharose CL-2B in 0.2 M-2-mercaptoethanol, was surprisingly over 80 % included (Fig. 3). However, if the reduced PSM glycoprotein was first dialysed against and eluted by 0.2 M-NaCl it became excluded on Sepharose CL-2B with a profile the same as that of the non-reduced glycoprotein. This strongly suggests that reassociation of the 2-mercaptoethanol-dissociated PSM glycoprotein was occurring after removal of the reducing conditions.

Both the mechanical and gel-filtration profiles of SSM were unchanged by exposure to 0.2 M-2-mercaptoethanol.

Effect of papain digestion on PSM and SSM

There were marked changes in the mechanical properties of both preparations of concentrated SSM and PSM glycoproteins on papain digestion. The mechanical spectra indicated a rapid loss during digestion of intermolecular structure for all SSM and PSM glycoprotein preparations. A relationship was observed between the percentage of total undigested PSM glycoproteins excluded on Sepharose CL-2B and the value of the storage modulus G' at a given time of digestion (Fig. 4). After 4 h the loss modulus G'' predominated over the storage modulus G' throughout the entire frequency range (10^{-1} – 10^2 rad/s) (Figs. 1 and 2). Both moduli G' and G'' showed frequency-dependence, although the storage modulus G' was slightly more dependent than the loss modulus G'' . The absolute values of both moduli G' and G'' were very low.

After exhaustive digestion with papain for 24 h at 37 °C both preparations of SSM and PSM glycoproteins were fragmented into smaller-sized material, all of which was eluted from Sepharose CL-2B in the total volume (Fig. 3). Further characterization of the size of the material was carried out by gel filtration on Sephadex G-75. For both digested SSM and PSM a single glycoprotein-positive (periodic acid/Schiff method) peak was eluted 40 % into the partially included volume of the column shortly ahead of myoglobin (M_r 17500). This glycoprotein (periodic acid/Schiff-positive) peak was protein-positive, with a further protein-positive peak being eluted in the total volume, which disappeared on dialysis of the digested product. Papain at the concentrations used for digestion, in the absence of glycoprotein, could not be identified on the column by either the periodic acid/Schiff or the Lowry assay.

The papain-digested SSM and PSM glycoproteins were purified by equilibrium centrifugation in a CsCl density gradient and characterized by polyacrylamide-gel electrophoresis in the presence of SDS. Half the gel was stained with AgNO_3 for protein and the remaining half stained with periodic acid/Schiff reagents for glycoprotein. Two strongly staining bands of M_r 14000 and 15000 (both protein- and glycoprotein-positive) were observed with papain-digested SSM. Only one strongly protein- and glycoprotein-positive band of M_r 13700 was observed on electrophoresis of digested PSM. In contrast with undigested SSM and PSM glycoproteins, no material was observed at the interface between the 3 % and 7.5 % gels or at the origin. No protein band at M_r 20000 (i.e. papain) was observed on electrophoresis of either the papain-digested SSM or the papain-digested PSM glycoproteins. Analysis by dansylation and hydrolysis showed the presence of several *N*-terminal amino acids in both papain-digested SSM and papain-digested PSM glycoproteins. *N*-Dansyl-serine and -glutamic acid

Fig. 4. (a) Value of the storage modulus G' and (b) percentage of the total glycoprotein remaining in polymeric form after digestion of PSM gels with papain

(a) Value of the storage modulus G' for PSM gels after digestion with papain. PSM glycoprotein gels (concentration 80 mg/ml) isolated in the presence of 0.2 M-NaCl containing 0.02% NaN_3 were digested with papain at 37 °C. The storage modulus G' (measured at 1 rad/s from frequency profiles) was plotted against the respective time of digestion. (b) Percentage of the total glycoprotein remaining in polymeric form after digestion of PSM gels with papain. The percentage of the total glycoprotein excluded on Sepharose CL-2B of gel (concentration 80 mg/ml) constituted from PSM glycoprotein isolated in the presence of 0.2 M-NaCl containing 0.02% NaN_3 was plotted against the time of incubation with papain at 37 °C.

showed the strongest fluorescence for digested SSM glycoproteins and *N*-dansyl-alanine, -glutamic acid, -glycine, -valine and in particular -serine gave the strongest fluorescence for digested PSM glycoprotein.

DISCUSSION

The aim of this study was to investigate the rheology and gel-forming potential of previously well-characterized submaxillary mucin preparations. SSM and PSM, with oligosaccharide chains of a maximum of two and five sugar residues respectively (Gottschalk *et al.*, 1972; Carlson, 1968), provided a simple model for comparison with the more complex gastrointestinal mucins. These

latter mucins have large complex sugar side chains; for example, pig gastric and rat colonic mucins have a maximum of 19 and 12 sugar residues per chain respectively (Slomiany & Meyer, 1972; Slomiany *et al.*, 1980). To study the gel-forming potential of submaxillary mucins it was necessary to concentrate them to levels well in excess of those secreted *in vivo*. Although the priority of this study was a rheology investigation of a simple well-characterized mucin system, a further comparison was made with gels from mucins prepared in as near a native state as possible by extraction in the presence of a mixture of proteinase inhibitors (Carlstedt *et al.*, 1983).

Equilibrium centrifugation in a CsCl density gradient has been successfully used for the purification of other mucins (Creeth & Denborough, 1970; Starkey *et al.*, 1974), and this method was employed here to purify the submaxillary mucins from soluble extracts of the glands. This is an alternative approach to other methods used, namely precipitation and ion-exchange (Gottschalk *et al.*, 1972; Hill *et al.*, 1977). After density-gradient centrifugation the glycoprotein from both submaxillary preparations came to equilibrium at a density range (equivalent to 1.46 g/ml for PSM and 1.43 g/ml for SSM) in keeping with other mucins (Creeth & Denborough, 1970; Starkey *et al.*, 1974). No dissociation in high salt concentrations was observed for SSM isolated in the presence of proteinase inhibitors or for either preparation of PSM when fractionated in CsCl. However, fractionation of SSM glycoprotein (isolated in the absence of inhibitors) in a CsCl density gradient was shown to be accompanied by a dissociation into lower- M_r subunits as judged by gel filtration. This dissociation in the high-salt conditions of 3.5 M-CsCl or 2.0 M-NaCl has previously been observed by sedimentation analysis in 2.0 M-NaCl (Hill *et al.*, 1977).

Both SSM and PSM mucins isolated in the presence of a mixture of proteinase inhibitors were clearly different in structure from preparations extracted in the absence of the inhibitors, in this case in the presence of 0.2 M-NaCl. Preparations of SSM and PSM prepared in the presence of proteinase inhibitors were not dissociated, as judged by inclusion on Sepharose CL-2B, by agents that dissociated preparations isolated in the presence of 0.2 M-NaCl alone, e.g. high-salt conditions (SSM), low pH (PSM) or heating at 100 °C for 10 min (SSM and PSM). Differences in structure between salivary mucins isolated in the presence and in the absence of proteinase inhibitors have previously been reported (Sellers & Allen, 1984; Shogren *et al.*, 1986, 1987). A more detailed analysis of the different preparations of the submaxillary mucins is required to clarify the extent of proteolysis during all previous extraction procedures used for salivary mucins where proteinase inhibitors were not included. Mucins of substantially larger size result when proteinase inhibitors with guanidinium chloride are used in their preparation from gastrointestinal and other mucus secretions (Carlstedt & Sheehan, 1984). Also, when extracted in the presence of proteinase inhibitors, PSM has been shown by light-scattering to have a larger molecular size than preparations isolated in the presence of 0.1 M-NaCl (M_r 13×10^6 and 8×10^6 respectively) (Shogren *et al.*, 1986).

Concentrated samples of PSM glycoprotein isolated in the presence of proteinase inhibitors or 0.2 M-NaCl alone showed mechanical behaviour typical of weak viscoelastic gels. The storage modulus G' predominated over the loss

modulus G'' throughout the frequency range accessed (Fig. 1). This showed that PSM glycoprotein gels were qualitatively the same as those from other gastrointestinal mucus secretions (Sellers *et al.*, 1987). The frequency-dependence of both moduli G' and G'' for PSM glycoprotein was greater and the ratio of the moduli G''/G' ($\tan \delta$) nearer to 1 than observed for other gastrointestinal mucus gels (Sellers *et al.*, 1987). This suggested that although the overall mechanical properties of PSM glycoprotein gels were the same as those of the other gastrointestinal mucus secretions the former was not such a good-quality gel as those from further down the gastrointestinal tract.

Concentrated samples of SSM glycoprotein preparations isolated either in the presence of proteinase inhibitors or in the presence of 0.2 M-NaCl alone gave spectra characteristic of an entangled polymer system and not that of a viscoelastic gel. The loss modulus G'' was seen to dominate the storage modulus G' throughout much of the frequency range accessed for the SSM glycoprotein preparations (Fig. 2). In other words, for both preparations of SSM the mechanical spectroscopy patterns were characteristic of a viscoelastic liquid and contrasted with the patterns characteristic of a gel system as obtained for both PSM preparations.

In contrast with the other gastrointestinal gels (e.g. gastric mucus; Bell *et al.*, 1985), PSM glycoprotein gels were not collapsed by exposure to 2-mercaptoethanol for 24 h. Indeed, although the frequency patterns remained the same after reduction, large increases were observed in the values of the storage modulus G' in the presence of 2-mercaptoethanol, with an associated decrease in the ratio G''/G' ($\tan \delta$) (from 0.68 to 0.49), indicative of a better-quality gel (Sellers *et al.*, 1987). Removal of the 2-mercaptoethanol after reduction produced a glycoprotein gel that was too strong for rheological examination. By analogy with other gastrointestinal gels where the polymeric glycoprotein is split into subunits (Allen, 1981), reduction would have been expected to collapse the gel rather than strengthen it. Evidence for such a decrease in size on reduction was seen by the decrease in mucin size observed on gel filtration of the reduced PSM mucin when eluted with 2-mercaptoethanol (included on Sepharose CL-2B), but aggregation occurred if the reduced PSM was eluted with 0.2 M-NaCl (excluded on Sepharose CL-2B). The increase in PSM gel strength in the presence of 2-mercaptoethanol and more markedly when it was dialysed away signifies an increase in intermolecular interactions associated with reduction and in the latter case intermolecular disulphide-bridge formation (previously not present). These disulphide bonds would substantially increase the cross-linking within the gel matrix, thus producing stronger gels. These results show that under certain reducing/reoxidizing conditions PSM mucin units can aggregate into larger units by disulphide-bridge formation.

Digestion with papain caused a complete collapse in the gel structure of PSM and a further collapse in the viscoelastic liquid properties of SSM to give mechanical spectroscopy profiles similar to those characteristic of a liquid system. This collapse was associated with the marked decrease in size of the glycoproteins on proteolysis to give fragmented glycosylated units of M_r 13 700–15 000. In the case of PSM glycoprotein, proteolysis was accompanied by at least a 150-fold decrease in size (M_r greater than 2×10^6 ; Shogren *et al.*, 1986), and

Fig. 5. Comparison of shear-rate ($\dot{\gamma}$)-dependence of viscosity (η) for hyaluronate (----) with frequency-dependence at constant strain (20%) of dynamic viscosity (η^*) for PSM glycoprotein and SSM glycoprotein isolated in the presence (—) or in the absence (---) of proteinase inhibitors

The coil overlap value for PSM was comparable with that of hyaluronate (20.1 and 19.5 respectively). SSM showed Newtonian behaviour at low frequencies despite having a coil overlap value greater than that of hyaluronate (45.2).

this correlated with falling values for the storage modulus G' (Fig. 4). The sizes of the digested glycopeptides from both SSM and PSM are considerably smaller than the exhaustively digested glycoproteins from other gastrointestinal mucus secretions (Scawen & Allen, 1977). This difference may reflect the lower degree of glycosylation around the protein cores of the submaxillary mucins, making them more accessible to proteolytic digestion. The proteolytic fragmentation of the submaxillary mucins by papain to glycopeptide units of uniform size suggests the protein core contains a repeating pattern of several similar-sized glycosylated segments alternating with regions that are naked or poorly glycosylated.

The loss of gel structure in PSM glycoprotein preparations after dialysis against pH 2.0 or following proteolysis leads to the conclusion that, to form a gel, pig submaxillary mucus glycoprotein must be in a larger polymeric form. It is well established that the glycoprotein subunits of other gastrointestinal mucus secretions must also be polymerized (in these cases through inter-subunit disulphide bridges) in order to form a gel matrix (Sellers *et al.*, 1983, 1987).

The behaviour of the storage modulus G' and loss modulus G'' at low oscillation frequencies provides information on the nature of non-covalent interactions between the mucin units in the gel network and can be

represented by the frequency variation of dynamic viscosity (Bell *et al.*, 1984; Sellers *et al.*, 1987). Such data are shown in Fig. 5 and can be compared, at the same degree of coil overlap ($c[\eta]$), with a known entangled system such as hyaluronate. The dynamic viscosity of SSM, isolated in the presence or in the absence of proteinase inhibitors, even at a substantially greater degree of coil overlap, closely follows the viscosity of hyaluronate; both show a plateau at low frequency/shear-rate values, which is evidence for the short-time-scale interactions of a purely entangled system (Sellers *et al.* 1988). In contrast, the dynamic viscosity of PSM glycoprotein decreased steadily throughout the frequency range, indicating that the interactions between the molecules must be of a longer time-scale than would be expected for entanglement alone and compatible with a viscoelastic gel structure. Similar profiles to that of PSM showing a steady decrease in dynamic viscosity throughout the frequency range (10^{-2} – 10^2 rad/s) are observed for native and reconstituted gels from pig gastric, duodenal, small-intestinal (jejunal and ileal) and colonic mucus glycoproteins at similar degrees of coil overlap (Sellers *et al.*, 1983, 1988).

These results show that PSM glycoprotein can form a gel of similar mechanical structure to that of gastric, duodenal, small-intestinal (jejunal and ileal) and colonic mucus, and like these gels PSM shows evidence for positive interactions on gel formation (Sellers *et al.*, 1986, 1988). However, there are wide differences in the carbohydrate composition and sugar-chain length of the constituent mucins. Pig gastric mucin has the largest oligosaccharide chains, up to 19 sugar residues (Slomiany & Meyer, 1972), small-intestinal mucin has about six to eight residues per chain (Mantle & Allen, 1981) and PSM has a maximum of five sugar residues per chain (Carlson, 1968). The above evidence therefore suggests that the carbohydrate side chains of the mucin components of mucus secretions can vary considerably without qualitative changes in overall mucus gel structure.

Studies on gastric, duodenal and colonic mucins suggest that the carbohydrate chains are involved in gel formation (Bell *et al.*, 1984; Sellers *et al.*, 1988). This is based on evidence of interactions occurring between the glycosylated components of their glycoprotein units following exhaustive proteolytic digestion. The dynamic viscosity of these proteolytically digested mucins shows a continual dependence on frequency similar to that for the native gels discussed above. This demonstrates that there are interactions between these proteolytically digested glycoprotein units over and above those expected for a simple entangled system. Since these proteolytically digested glycosylated units are over 90% by weight carbohydrate and all the protein accessible to digestion (papain) has been removed, it is reasonable to presume that the observed intermolecular interactions involve the carbohydrate side chains. On the basis of these studies a model has been proposed that the gel-forming interactions in gastrointestinal mucin glycoproteins result at least in part from interdigitation of the carbohydrate side chains of adjacent molecules (Bell *et al.*, 1984; Sellers *et al.*, 1988). A model where gel-forming interactions between mucins are analogous to those seen with lectins has also been proposed (Silberberg, 1987).

The overall conclusion from these results is that gastrointestinal mucus gels and high concentrations of PSM have the same structural characteristics. Further,

differences do exist in the carbohydrate composition and chain structure of these mucins, but they do not appear to affect significantly their overall gel-forming abilities as measured by mechanical spectroscopy. At the same time evidence supports the oligosaccharide side chains participating in the gel-forming interactions of the native gastrointestinal secretions. The gel-forming ability of the mucin glycoproteins would appear to be an essential requirement for gastric, duodenal and colonic mucus to maintain a protective layer across the mucosa. PSM and SSM, in contrast with mucins of the gastrointestinal mucus gels, are secreted as viscous solutions. In the case of PSM the absence of a gel may be purely a function of the concentration of the secreted mucin.

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