Macromolecular organization of saliva: identification of 'insoluble' MUC5B assemblies and non-mucin proteins in the gel phase

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Stimulated human submandibular/sublingual (HSMSL) and whole saliva were separated into sol and gel phases and mucins were isolated by density-gradient centrifugation in CsCl/4M guanidinium chloride. MUC5B and MUC7 were identified using anti-peptide antisera raised against sequences within the MUC5B and MUC7 apoproteins respectively. MUC7 was found mainly in the sol phase of both HSMSL and whole saliva, but some MUC7 was consistently present in the gel phase, suggesting that this mucin may interact with the salivary gel matrix. In HSMSL saliva, MUC5B was found in the gel phase; however, most of the material was 'insoluble' in guanidinium chloride and was only brought into solution by reduction. In whole saliva, the MUC5B mucin was present both in the sol and gel phases although some material was again 'insoluble'. Rate-zonal centrifugation of whole saliva showed that MUC5B mucins in the sol phase were smaller than those in the gel phase, suggesting differences in oligomerization and/or degradation. Antibodies against IgA, secretory component, lysozyme and lactoferrin were used to study the distribution of non-gel-forming proteins in the different phases of saliva. The majority of these proteins was found in the sol phase of both HSMSL and whole saliva. However, a significant fraction was present in the gel phase of whole saliva, suggesting a post-secretory interaction with the salivary gel matrix. A monoclonal antibody against a parotid salivary agglutinin was used to show that this protein is present mainly in the gel phase of both whole saliva and parotid secretion.

Key words: gel matrix, lactoferrin, lysozyme, MUC7, mucins.

INTRODUCTION

Saliva is a complex secretion originating from the parotid, submandibular/sublingual and so-called minor glands in the oral cavity. The major functions of saliva include lubrication of the oral mucosal surfaces, protection against micro-organisms and neutralization of acids produced by plaque bacteria. The most prominent macromolecular components of the secretion are high-molecular-mass glycoproteins, or mucins, although other proteins, such as secretory IgA (sIgA), amylase, lactoferrin and lysozyme, some of which play important roles in oral mucosal defence, are also present. In addition, a large high-molecular-mass oligomeric agglutinin originating from the parotid glands has been identified [1].

Biochemical studies have shown that two major mucin populations are present in saliva, MG1 and MG2 [2]. MG1 (> 1000 kDa) is composed of approx. 15 % protein and 78 % carbohydrate and has an oligomeric structure where mucin monomers are joined by disulphide bonds. The peptide is glycosylated, with hydrophobic domains present on the naked, non-glycosylated stretches [3]. MG2 (120–150 kDa) is composed of up to approx. 30 % protein and 70 % carbohydrate and was originally described as a single, monomeric molecule [2]. More recent studies suggest, however, that this glycoprotein may self-associate and thus form larger assemblies through non-covalent bonds [4]. Two isoforms of MG2, named MG2a and MG2b, have been identified and these differ in their sialic acid/fucose ratio [5]. MG2 has been reported to interact with oral micro-organisms [3,6] and further studies have implicated the T and sialyl-T antigens, as well as lactosamine sequences, on MG2 in such interactions [7]. Furthermore, MG2 expresses the sialyl-Le^x determinant and this glycoprotein can thus act as a ligand for L-selectin [8]. MG1 has now been shown to be the product of the *MUC5B* gene [9] and Thornton and colleagues [10] have concluded that MUC5B is the predominant MG1 mucin although it cannot be ruled out that this mucin, in fact, is a mixture of more than one gene product. For example, it has been suggested that MUC4 is also a major component of the MG1 population originating from the submandibular glands [11]. The whole genomic sequence of *MUC5B* is known [12] and the gene is clustered together with *MUC6*, *MUC2* and *MUC5AC* at chromosome 11p15.5 [13]. MUC5B is large and oligomeric and has been identified in saliva, respiratory-tract secretions and cervical mucus [14,15] as well as the colon [16] and the gall bladder [16,17]. MG2 has also been cloned and sequenced and is now referred to as MUC7 [18]. MUC5B and MUC7 are thought to originate from different cells in the secretory acini of the mucin-producing submandibular, sublingual and minor salivary glands [19].

Mucins have been reported to interact with non-gel-forming proteins and it has been suggested that the glycoprotein layer on the oral surfaces forms a matrix for the recruitment of other protective proteins such as sIgA, lactoferrin and lysozyme to the teeth and mucosal surfaces [20]. Heterotypic complexes may occur between MG1 and salivary proteins [21] and it has also been shown that MG2 and sIgA form an agglutinating complex [22]. In human bronchial secretion, lysozyme shows a strong ionic interaction with mucins [23,24] and lactoferrin has been identified in bronchial mucus [25].

Abbreviations used: AP, alkaline phosphatase; DTT, dithiothreitol; HSMSL saliva, stimulated human submandibular/sublingual saliva; sIgA, secretory IgA; PAS, periodic acid/Schiff.

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In order to identify the major gel-forming mucins as well as components associated with the salivary gel, we have separated both human whole and stimulated human submandibular/sublingual (HSMSL) saliva into gel and sol phases. In HSMSL saliva, MUC5B was found in the gel phase and the major part was 'insoluble' in guanidinium chloride. In whole saliva, MUC5B was found in both the sol and gel phases and a significant portion of the mucins was, again, 'insoluble'. The MUC5B mucins in the sol phase of whole saliva were smaller than those of the gel, suggesting differences in oligomerization and/or that degradation had occurred. Significant amounts of non-gel-forming components such as MUC7, sIgA, lactoferrin and lysozyme were found in the gel phase, suggesting that they may interact with components of the gel.

EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) and iodoacetamide were from ICN Biochemicals. Stock solutions of guanidinium chloride (approx. 8 M) were treated with activated charcoal and filtered through a PM10 filter (Amicon) before use. Nitrophenyl phosphate and BSA (fraction V, pH 7.0) were purchased from Serva; diaminobenzidine, *N*-ethylmaleimide and alkaline phosphatase (AP)-conjugated anti-mouse IgG were from Sigma; dithiothreitol (DTT) was from Merck and di-isopropyl phosphofluoridate was from Fluka. Polyclonal antibodies recognizing human IgA, secretory component, lysozyme and lactoferrin, as well as AP-conjugated swine anti-rabbit serum, were bought from Dakopatts. A monoclonal antibody (mAb 143) against the parotid agglutinin was a kind gift from Professor Niclas Strömberg, University of Umeå, Umeå, Sweden. The LUM5B-2 antibody reacting with MUC5B has been described previously [15]. The Superose-6HR column was from Amersham Pharmacia Biotech. All other reagents were of AnalaR or equivalent quality.

Preparation of antisera against MUC7

Synthetic peptides with sequences EGRERDHELRHRRHHHQC (LUM7-1) and NKPPKFPNPHQPPKHPDC (LUM7-2) were conjugated to keyhole limpet haemocyanin. An initial injection of 100 μg of peptide in Freund's complete adjuvant was given intracutaneously to rabbits and was followed 4 weeks later by a booster injection of 100 μg of peptide in Freund's incomplete adjuvant. The animals were bled 2–3 weeks later and the antisera named LUM7-1 and LUM7-2, respectively.

Immunohistochemistry

Macroscopically normal human submandibular and sublingual salivary-gland tissue obtained from specimens resected for tumour was fixed in 10 % neutral buffered formal saline overnight, dehydrated, embedded in paraffin and 4-μm sections cut. Sections were dewaxed, rehydrated and treated with 10 mM sodium citrate buffer, pH 6, at 100 °C in a microwave oven for 10 min. Sections stained with the LUM5B-2 antiserum were reduced with 10 mM DTT in 0.1 M Tris/HCl buffer, pH 8.0, at room temperature for 30 min and alkylated with 25 mM iodoacetamide in 0.1 M Tris/HCl buffer, pH 8.0, for 30 min. Endogenous peroxidase activity was quenched by immersion in 3 % H_2O_2 for 30 min. Sections were blocked with goat serum for 1 h and endogenous biotin was blocked by treatment with the Dako biotin blocking kit (avidin for 10 min followed by biotin for 10 min). Sections were incubated with the LUM7-1, LUM7-2 or LUM5B-2 antisera [diluted 1:1000 in Tris-buffered saline (0.15 M NaCl in 0.05M Tris/HCl, pH 7.6)] for 1 h in Coverplate immunostaining chambers (Shandon). Antibody binding was detected using the Dako StreptABComplex/HRP kit with 3,3′-diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer's haematoxylin.

Collection and handling of saliva samples

HSMSL saliva was collected from five individuals (approx. vols: two individuals, 30 ml/sample; three individuals, 2.5 ml/sample) with a customized device [26] modified as described in [27]. Secretion was stimulated with a solution of 2 % citric acid in water applied to the anterior-lateral part of the tongue. Stimulated whole saliva was collected from three individuals (approx. 80 ml for all individuals) and was induced by chewing a piece of Parafilm after rinsing with a solution of 0.9 % NaCl. In both cases, saliva was collected in tubes on ice for 10 min and the part of the secretion collected during the first 30 s was discarded. Samples were subjected immediately to ultracentrifugation (Beckman 70.1 Ti rotor, 231000 ***g***, 4 °C, 45 min) and supernatants (sol phase) were dialysed into 6 M guanidinium chloride/5 mM sodium EDTA/10 mM sodium phosphate buffer, pH 6.5. Pellets (gel phase) were gently washed twice with PBS (4 °C) for 1 h, recovered by ultracentrifugation as above, suspended in 5 ml of 6 M guanidinium chloride/5 mM sodium EDTA/10 mM sodium phosphate buffer, pH 6.5 (extraction buffer) containing 5 mM *N*-ethylmaleimide and 1 mM di-isopropyl phosphofluoridate, and shaken overnight at 4 °C. Following centrifugation in a Beckman JA-20 rotor for 1 h at 31000 ***g*** and 4 °C, supernatants were collected and the pellets re-suspended in 5 ml of extraction buffer and shaken overnight at 4 °C. The procedure was repeated three times and the four supernatants were pooled (guanidinium chloride-soluble fraction of the gel phase). Remaining pellets were solubilized by reduction with 5 ml of 6 M guanidinium chloride/0.1 M Tris/HCl buffer, pH 8.0, containing 5 mM sodium EDTA and 10 mM DTT for 5 h at 37 °C and alkylated by the addition of iodoacetamide (2.5 molar excess over DTT) for 15 h in the dark [28]. This fraction represents the guanidinium chloride-insoluble part of the gel and is also referred to as the reduced residue. Finally, all samples were dialysed into extraction buffer. In order to isolate the mucins, samples were submitted to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride using a Beckman 70.1 Ti rotor (257000 ***g***, 15 °C, 75 h initial density 1.40 g/ml in a Beckman L-70 Optima centrifuge). Fractions were collected from the bottoms of the tubes and analysed for sialic acid, carbohydrate [periodic acid/Schiff (PAS) staining], A_{280}, density and antibody reactivity.

Parotid-gland saliva was collected from six individuals using a modified Carlsson–Crittenden cup and again 2 % citric acid was used to stimulate secretion [29]. Samples were separated into sol and gel phases prior to extraction and subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride as described above. Fractions were analysed for density and antibody reactivity.

Rate-zonal centrifugation

Rate-zonal centrifugation of whole mucins and reduced subunits was performed as described in [30]. Guanidinium chloride gradients (6–8 M) were formed in 14 × 89 mm centrifuge tubes using a Hoefer gradient maker connected to an LKB 2232 Microperpex pump at a flow rate of 50 ml/h. Samples were layered on to the tops of the gradients and tubes spun in a Beckman L-70 Optima centrifuge (Beckman SW41 Ti rotor, 2 h 45 min, 287000 ***g***, 20 °C). Fractions (300 μl) were collected from

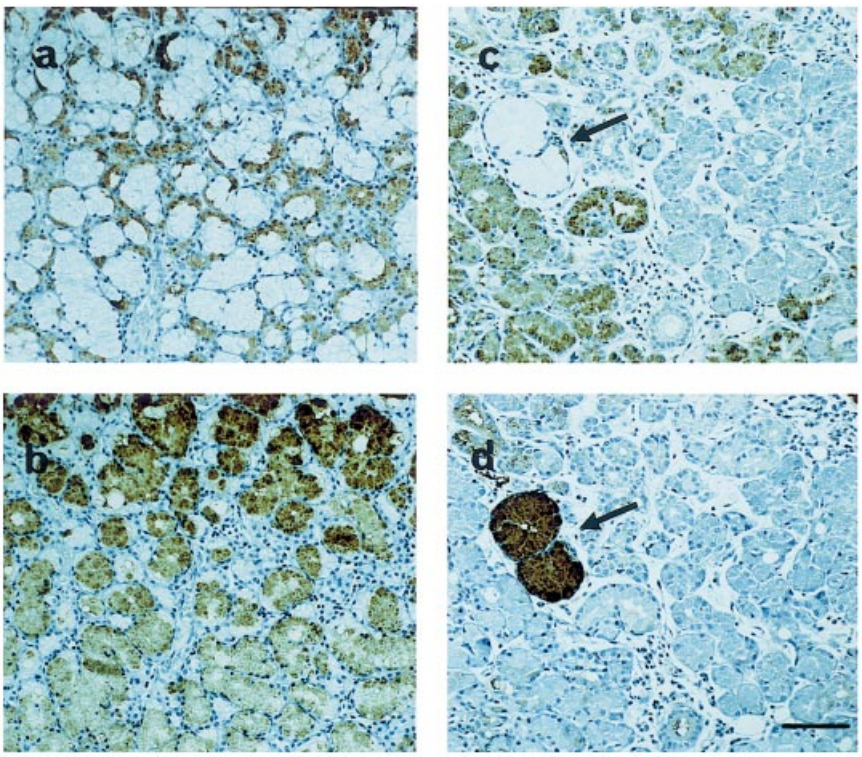

Figure 1 Sections of human sublingual gland stained with the LUM7-1 (a) and LUM5B-2 (b) antisera and of human submandibular gland stained with the LUM7-1 (c) and LUM5B-2 (d) antisera

Sections (4 μm) of paraffin-embedded tissue were stained with the antisera and counter-stained as described in the text. The arrows indicate MUC5B producing cells. The bar represents 100 μm.

the tops of the tubes and analysed for carbohydrate (glycan-detection method) and reactivity with the LUM5B-2 antiserum.

Gel chromatography

Gel chromatography was performed on a Superose-6HR column eluted at a flow rate of 0.25 ml/min with 4 M guanidinium chloride adjusted to pH 7. Fractions (0.25 ml) were analysed with antibodies against IgA, secretory component, lactoferrin and lysozyme.

ELISA

ELISA was carried out using the LUM5B-2, LUM7-1 and LUM7-2 antisera, as well as antisera against human IgA, secretory component, lysozyme, lactoferrin and the mAb 143 antibody. After dilution in 4 M guanidinium chloride/10 mM sodium phosphate buffer, pH 7, aliquots (100 μl) of fractions for analysis with the LUM5B-2 antiserum were reduced in dilution plates for 1 h at room temperature with 1 mM DTT (10 μl of a 10 mM solution in 6 M guanidinium chloride/1 M Tris/HCl buffer, pH 8, containing 5 mM sodium EDTA) and then alkylated for 1 h with iodoacetamide (10 μl of a 25 mM solution in 6 M guanidinium chloride/1 M Tris/HCl buffer, pH 8, containing 5 mM sodium EDTA). Fractions were coated on to multi-well assay plates (3912, Falcon) overnight at room temperature. After coating, plates were blocked for 1 h with PBS containing 0.05 % (v/v) Tween 20 and 0.5 % (w/v) BSA (blocking solution) and then incubated for 1 h with the LUM5B-2 (1:1000), LUM7-1 (1:500), LUM7-2 (1:2000), IgA (1:600), secretory component (1:300), lysozyme (1:200) or lactoferrin (1:100) antisera or the mAb 143 antibody (1:10000) diluted in blocking solution. Reactivity was detected with an AP-conjugated swine anti-rabbit antiserum, diluted 1:2000 in blocking solution (LUM5B-2, LUM7-1, LUM7-2, IgA, secretory component, lysozyme and lactoferrin) or with AP-conjugated anti-mouse IgG, diluted 1:40000 in blocking solution (mAb 143 antibody) using nitrophenyl phosphate (2 mg/ml in 1 M diethanolamine/HCl buffer, pH 9.8, containing 5 mM $MgCl_2$) as a substrate. Reactivity is expressed as absorbance at 405 nm after 1 h.

Analytical methods

Density measurements were performed on fractions from the density gradients using a Carlsberg pipette as a pycnometer. Sialic acid was determined with an automated version [31] of the method described in [32]. Carbohydrate content was determined either by slot-blotting samples on nitrocellulose membranes (pore size 0.2 μm) and staining with PAS as described in [33] or by using the glycan-detection method which was performed in multi-well assay plates (3912, Falcon), coated with salivary samples overnight at room temperature. Plates were washed with PBS containing 0.05 % (v/v) Tween 20, treated with 25 mM sodium periodate in 0.1 M sodium acetate buffer, pH 5.5, for 20 min, washed with PBS/Tween and incubated for 1 h with digoxigenin-succinyl-ϵ-amido-caproic acid hydrazide. After an additional wash, the plates were incubated with AP-conjugated anti-digoxygenin antibodies for 1 h and developed using nitrophenyl phosphate as the substrate, as described for ELISA above.

RESULTS

Immunohistochemistry

The LUM7-1 and LUM7-2 antisera were studied using immunohistochemistry on tissue sections of salivary glands. In the sublingual salivary gland, the LUM7-1 antiserum stained the seromucous demilunes (Figure 1a), and the same pattern was seen for the LUM7-2 antiserum (results not shown). The LUM5B-2 antiserum reacted with almost all seromucous end pieces (Figure 1b). In the submandibular salivary gland, reactivity

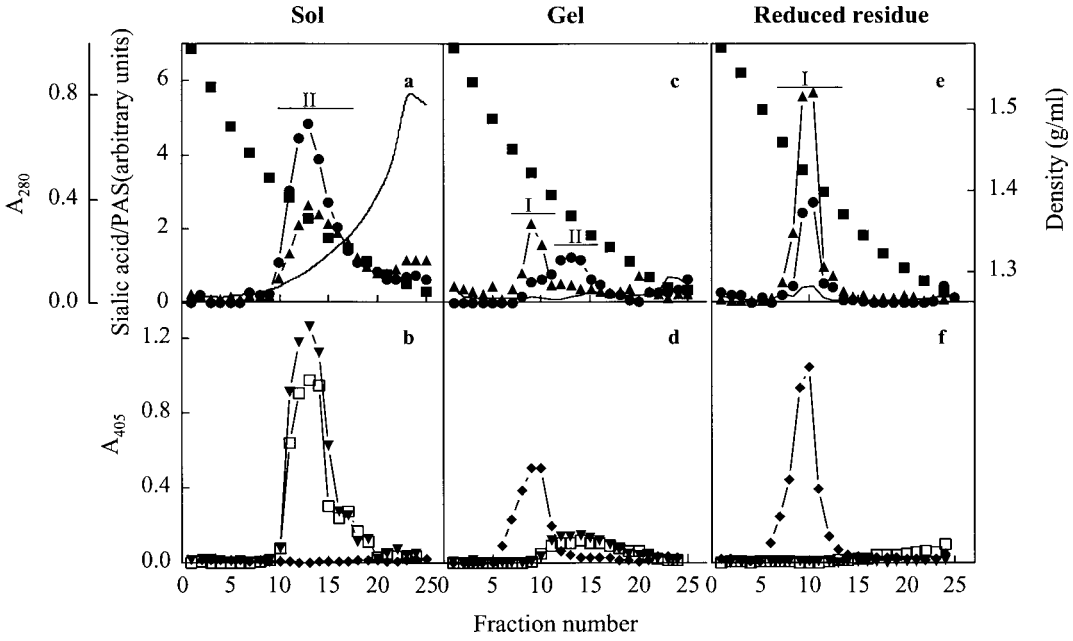

Figure 2 Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride of mucins from the sol phase (a, b), the gel phase (c, d) and reduced residue (e, f) of HSMSL saliva

Samples were subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (257 000 ***g***, 15 °C, 75 h Beckman 70.1 Ti rotor, Beckman Optima L-70 centrifuge). Fractions were analysed for density (■), sialic acid (●), PAS reactivity (▲), absorbance at 280 nm (—), and reactivity with the LUM5B-2 (◆), LUM7-1 (▼) and LUM7-2 (□) antisera.

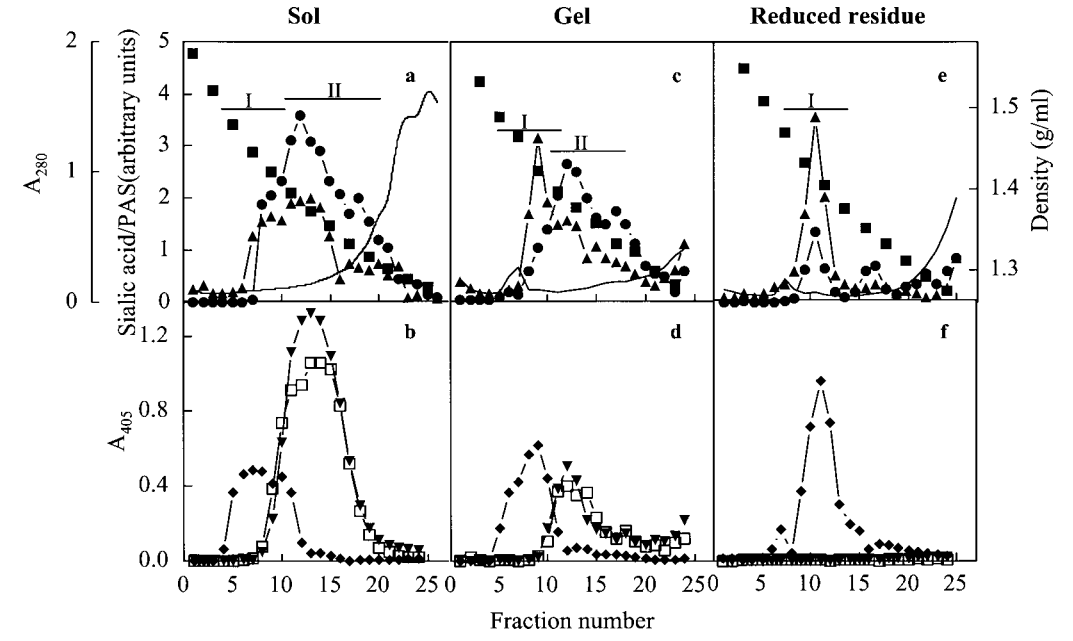

Figure 3 Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride of mucins from the sol phase (a, b), the gel phase (c, d) and reduced residue (e, f) of whole saliva

For details see Figure 2.

Table 1 Relative amounts of MUC5B in fractions of HSMSL and whole saliva

The PAS reactivity corresponding to the LUM5B-2-positive populations was integrated and expressed as a percentage of the total PAS/MUC5B material. nd, not detected.

		MUC5B (%)		
Fraction	Individual	Sol phase	Gel phase	Reduced residue
HSMSL	1	nd	35	65
	2	nd	24	76
	3	nd	4	96
	4	nd	51	49
	5	nd	47	53
Whole	1	30	47	23
	2	16	41	43
	6	11	41	48

with the LUM7-1 antiserum was seen in many secretory acini (Figure 1c), whereas in an adjacent section, the LUM5B-2 antiserum stained scattered focal points (Figure 1d, an example is marked with an arrow) that did not coincide with those showing LUM7-1 reactivity.

Distribution of mucins between sol, gel and reduced residue

HSMSL saliva (five individuals) and whole saliva (three individuals) were collected and, after an initial separation into gel and sol phases, the gel phase was solubilized with 6 M guanidinium hydrochloride and the residue brought into solution by reduction with DTT. To eliminate the possibility that sol material was entrapped in the gel phase, the gel was carefully washed twice with PBS prior to extraction. No MUC7 was detected in the second wash, suggesting that the gel phase was free from contamination by sol material (results not shown). Three samples (sol, gel and reduced residue) were thus obtained and mucins were isolated from each of them with isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride. In the sol phase of HSMSL saliva, a population banding between 1.31 and 1.40 g/ml was identified by carbohydrate analyses (Figure 2a) and this component reacted with the LUM7-1 and LUM7-2 antisera (Figure 2b). No MUC5B mucin was detected in this sample. In the gel phase, carbohydrate analysis showed two partially resolved populations banding between 1.40 and 1.45, and between 1.34 and 1.38 g/ml (Figure 2c). The 'high-density' one reacted more strongly with PAS relative to the sialic acid assay whereas the 'low-density' population reacted mainly with the assay for sialic acid. Reactivity with the LUM5B-2 antiserum followed the 'high-density', PAS-reactive peak (Figure 2d), whereas the LUM7-1 and LUM7-2 reactivity showed a broad distribution coinciding with the 'low-density' sialic acid-rich population (Figure 2d). In the reduced residue, carbohydrate analysis revealed a unimodal mucin population between 1.40 and 1.46 g/ml (Figure 2e). Reactivity with the LUM5B-2 antiserum followed the distribution obtained with the chemical analyses. No MUC7 could be detected (Figure 2f).

In whole saliva, carbohydrate analysis of the sol phase showed a complex pattern with a broad distribution of glycoproteins between 1.30 and 1.45 g/ml (Figure 3a). The LUM5B-2 antiserum recognized mucins on the 'high-density' side of the distribution whereas the LUM7-1 and LUM7-2 antisera reacted with a broad peak of mucins towards the 'low-density' side of the distribution (Figure 3b). In the gel phase, three partially resolved populations were detected with the carbohydrate analyses. As in the gel phase from HSMSL saliva, a 'high-density'

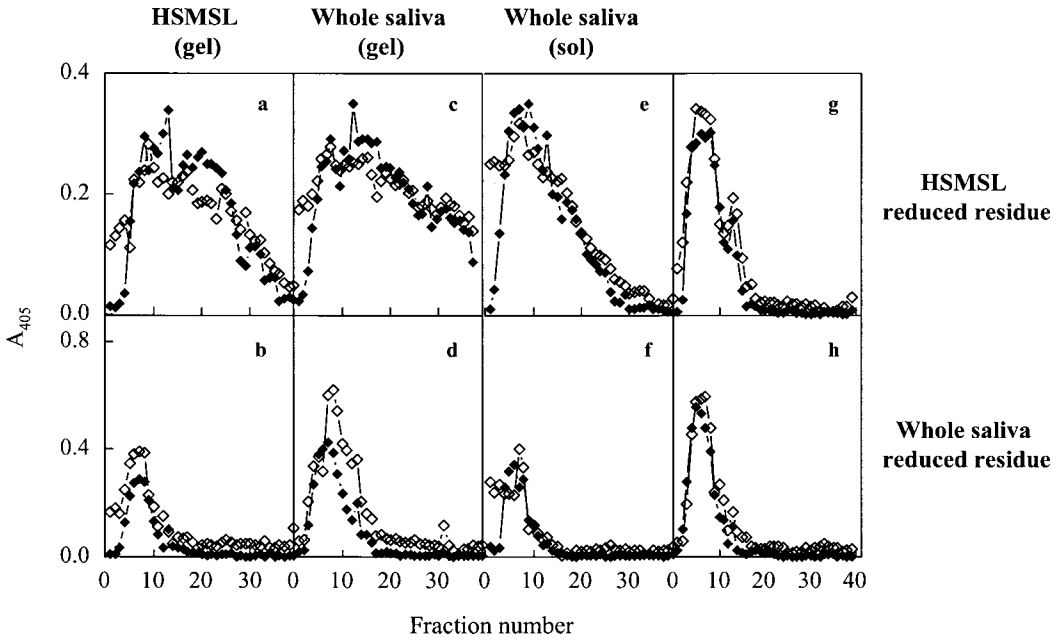

Figure 4 Rate-zonal centrifugation of whole mucins and subunits from HSMSL and whole saliva

Whole mucins from the gel phases of HSMSL (**a**) and whole saliva (**c**) as well as from the sol phase of whole saliva (**e**) pooled as shown in Figures 2(c), 3(a) and 3(c) (pool I) respectively as well as the cognate subunits (**b**, **d**, **f**), and subunits from the reduced residues of HSMSL and whole saliva [Figures 2e and 3e (pool I)] (**g**, **h**), were subjected to rate-zonal centrifugation in a Beckman L-70 Optima centrifuge (287 000 ***g***, 20 °C, 2 h 45 min, Beckman SW41 Ti rotor). Fractions (300 μl) were collected from the tops of the tubes and analysed for carbohydrate using the glycan-detection method (◇) and reactivity with the LUM5B-2 antibody (◆).

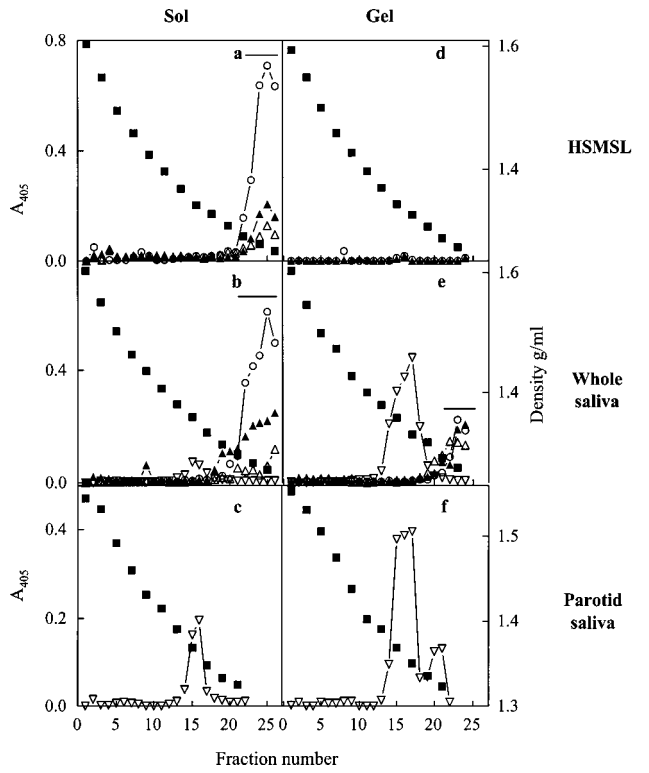

Figure 5 Isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride of HSMSL saliva (a, d), whole saliva (b, e) and parotid saliva (c, f) from the sol (a–c) and gel (d–f) phases

Samples were subjected to isopycnic density-gradient centrifugation in CsCl/4 M guanidinium chloride (287 000 ***g***, 15 °C, 70 h Beckman 70.1 Ti rotor, Beckman Optima L-70 centrifuge). Fractions were analysed for density (■), reactivity with antisera for IgA (○), lysozyme (△), lactoferrin (▲) and for reactivity with the mAb 143 (▽) antibody.

PAS-reactive population was found at 1.43 g/ml while two partially separated sialic acid-rich populations were found at 1.38 and 1.33 g/ml (Figure 3c). The LUM5B-2 antiserum again reacted with the 'high-density' PAS-rich component whereas the LUM7-1 and LUM7-2 antisera reacted with both sialic acid-rich populations (Figure 3d). In the reduced residue, a unimodal mucin distribution between 1.39 and 1.45 g/ml was detected with the carbohydrate analyses (Figure 3e) and reactivity with the LUM5B-2 antiserum followed this distribution (Figure 3f). No MUC7 was found in this fraction. The relative amounts of MUC5B in the different fractions in HSMSL and whole saliva were estimated from the areas under the curves of the PAS-rich peaks from the density gradients corresponding to the populations showing reactivity with the LUM5B-2 antibodies (Table 1). In HSMSL saliva, no MUC5B was seen in the sol phase, between 4 and 51 % was found in the gel phase and most of the MUC5B was 'insoluble' and thus present in the reduced residue. In whole saliva, significant amounts of MUC5B mucins were identified in all three phases. It should be pointed out that differences in glycosylation between mucins in the various fractions, and/or between individuals, could, if present, influence the PAS reactivity of the molecules and thus the estimation of the relative proportions. The presence of significant amounts of so-far unidentified salivary glycoprotein could also introduce a bias; however, this possibility appears less likely since MUC5B appears to be the major high-density glycoprotein in human saliva [10].

Rate-zonal centrifugation of MUC5B mucins

Fractions from the gel phase of HSMSL and whole saliva that contained MUC5B were pooled and subjected to rate-zonal centrifugation. 'Whole' mucins from the gel phase of HSMSL saliva (pool I, Figure 2c) displayed a broad range of molecular sizes by carbohydrate analysis and LUM5B-2 reactivity was seen over the entire distribution (Figure 4a). Those from the gel phase of whole saliva (pool I, Figure 3c) had a similar size distribution (Figure 4c). MUC5B mucins from the sol phase of whole saliva (pool I, Figure 3a) showed a narrower distribution closer to the top of the gradient, suggesting that they were smaller than those in the gel phase (Figure 4e). After reduction, the gel-phase mucins from both sources, as well as those from the sol phase of whole saliva, were present at the top of the gradient, indicating a reduction in molecular size (Figures 4b, 4d and 4f). MUC5B from the reduced residues of both HSMSL and whole saliva (Figures 4g and 4h) showed a similar distribution to those of the subunits from the respective gel phases (Figures 4b and 4d). However, in some samples of the reduced residue of HSMSL (pool I, Figure 2e) a partial separation was observed, suggesting the presence of two subunit populations.

Distribution of non-MUC5B components in saliva

In HSMSL saliva, IgA, lysozyme and lactoferrin were found at the top of the gradient in the sol phase (Figure 5a), while the gel phase was devoid of reactivity (Figure 5d). The secretory component showed the same pattern (results not shown). In whole saliva, the pattern was the same as for HSMSL saliva in the sol phase (Figure 5b). In the gel phase, all four proteins were detected at the top of the gradient and the major part of lysozyme was found there (result for secretory component not shown; Figure 5e). The reduced residue in both HSMSL and whole saliva was completely devoid of reactivity for all proteins studied (results not shown). An antibody (mAb 143) against a parotid salivary agglutinin reacted with a component at a density of 1.34 g/ml in the gel phase of whole saliva (Figure 5e), whereas in the corresponding sol phase very little agglutinin reactivity was present (Figure 5b). In the sol phase of saliva from the parotid gland, the mAb 143 antibody reacted with a peak at 1.34 g/ml (Figure 5c). The major part of the agglutinin was found in the gel phase and two populations at 1.36 and 1.33 g/ml were identified with the mAb 143 antibody (Figure 5f). No reactivity was seen in the reduced residue (results not shown).

To further study the proteins present in the various fractions, the low-density material at the tops of the gel- and sol-phase gradients of whole saliva as well as the sol phase of HSMSL saliva were pooled as shown in Figure 5 and subjected to gel chromatography on a Superose-6HR column. In the sol phases of both HSMSL and whole saliva (Figures 6a and 6b), sIgA (i.e. a molecule reacting with the antibodies against both IgA and the secretory component) eluted close to the void volume. A second peak of the free secretory component was more included. Lactoferrin reactivity followed that of the secretory component while lysozyme eluted as a single peak that was significantly more retarded than the second lactoferrin one. In the gel phase of whole saliva (Figure 6c), the pattern was broadly similar, although only one lactoferrin population was present. An IgA population additional to that eluting in the void volume was partially included. When comparing the data from density-gradient centrifugation (Figure 5) with those obtained by using gel chromatography (Figure 6), a difference is evident in the relative amounts of the various proteins studied. The most likely explanation for this discrepancy is 'competitive coating' in the ELISA assay of the density gradients where the proteins occur in

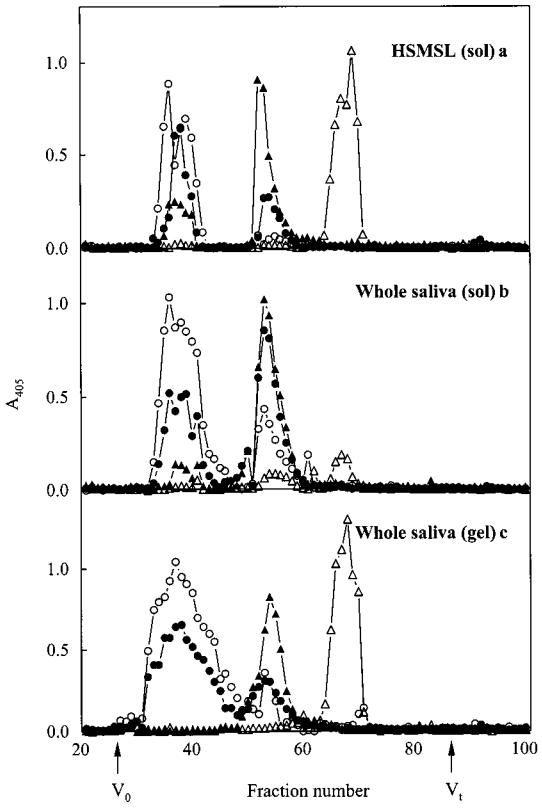

Figure 6 Gel chromatography on Superose-6HR of low-density fractions from the sol phase of HSMSL saliva (a) and the sol (b) and gel (c) phases of whole saliva

Fractions pooled as shown in Figures 5(a), 5(b) and 5(e) were subjected to gel chromatography on a Superose-6HR column eluted with 4 M guanidinium chloride at a flow rate of 0.25 ml/min. Fractions (0.25 ml) were analysed for IgA (○), secretory component (●), lysozyme (△) and lactoferrin (▲) antibodies. V_0, void volume; V_T, total volume.

the same fractions. During gel chromatography, the separation of the components would minimize this problem and thus ensure a more representative estimation.

DISCUSSION

Antisera raised against sequences within the MUC7 and MUC5B apoproteins were used to study the distribution of these mucins in HSMSL and whole saliva. Both LUM7-1 and LUM7-2 were shown to stain the seromucous demilunes in the sublingual gland, whereas the LUM5B-2 antisera stained the seromucous end pieces. In the submandibular gland, LUM7-1 and LUM7-2 stained many acini, whereas LUM5B-2 only reacted with a few focal points. The data are in accordance with other studies showing that MUC7 and MUC5B are produced by different cells within the submandibular and sublingual glands [19].

To allow identification of the components forming the salivary gel matrix, saliva was separated into a sol and a gel. Electron-microscopy studies have indicated the presence of a network structure in saliva [34] and it is highly likely that this network largely comprises this matrix. In both HSMSL and whole saliva, MUC7 was present mainly in the sol phase, although some MUC7 appeared consistently in the gel. Since the gel phase had been washed carefully, it appears unlikely that MUC7 was present as a sol-phase contaminant and the finding thus suggests either that an interaction between MUC7 and the gel-forming matrix may occur or that this mucin may itself form large structures. The latter possibility is supported by reports that MUC7 is capable of self-association [4]. In the density gradients of the gel-phase material from whole saliva, reactivity with the LUM7-1 and LUM7-2 antisera showed a partial separation into two populations, possibly corresponding to the two putative glycoforms identified previously [5].

In HSMSL saliva, MUC5B was found only in the gel phase with the major part (65–75 %) of the mucins being 'insoluble' in guanidinium chloride and thus present in the extraction residue. However, in whole saliva MUC5B was present also in the sol and only about 30–45 % was 'insoluble' in guanidinium chloride, suggesting that MUC5B from HSMSL saliva constitutes the major part of the 'insoluble' fraction of whole saliva and/or that MUC5B from the minor glands is more 'soluble'. Alternatively, proteolytic 'processing' in the oral cavity may cause MUC5B from HSMSL saliva to become more 'soluble'. The major part of intestinal MUC2 also occurs as an 'insoluble' glycoprotein complex that is resistant to extraction in guanidinium chloride [35–37], showing that this feature is not unique for salivary MUC5B. The macromolecular organization of the 'insoluble' MUC2/MUC5B complexes is currently not known.

Rate-zonal centrifugation showed the 'soluble' MUC5B mucins to be large and oligomeric in both HSMSL and whole saliva, as expected from previous investigations [15]. However, the MUC5B mucins from the sol phase of whole saliva appear to be smaller than those from the gel phase although the cognate subunits from both populations were of similar sizes, suggesting that the size differences are due to either different degrees of oligomerization or proteolytic degradation. In the reduced residue of HSMSL saliva, the subunits were seen to separate into different populations. However it is not known whether these populations reflect glycosylation differences or correspond to oligomers joined by reduction-insensitive bonds as has been shown to occur in MUC2 [37].

Separation of saliva into a sol and a gel phase also allows identification of non-mucin components that are associated with the salivary gel matrix. Secretory component, sIgA, lysozyme and lactoferrin were found in the sol phases of both HSMSL and whole saliva but were also present in the gel of whole saliva, suggesting these proteins may bind non-covalently to the salivary gel matrix after secretion. Secretory IgA was found mainly in the sol phase, lactoferrin seemed to distribute equally between the sol and gel phase of whole saliva while lysozyme was enriched in the gel. Lysozyme has been shown previously to bind to respiratory mucins via ionic bonds [23,24]. The parotid salivary agglutinin was also identified in the gel phase of whole saliva, suggesting that it might interact with the gel matrix. However, when parotid saliva (containing very little or no mucin) was separated into gel and sol phases, the agglutinin was also present in the gel, suggesting that it may itself form very large structures consistent with the previously described oligomeric structure of the agglutinin [1].

The data presented here suggest the presence of a salivary mucin matrix 'decorated' with protective factors such as lactoferrin, lysozyme and sIgA. MUC7, as well as the parotid salivary agglutinin, appear either to be associated with this matrix or to form separate supramolecular structures. The protection of the oral cavity may thus be enhanced through the enrichment of protective factors close to the mucosal membrane. Oral bacteria have been demonstrated to interact with MG2 and since these mucins carry the sialyl-Le^x determinant they may thus also interact with neutrophils [3,6,8]. A second role envisaged for the salivary gel matrix is thus to mediate specific interactions between elements of the host defence system and micro-organisms. Since

other investigators have shown that MUC5B is the major 'large' mucin in saliva [10], it seems highly likely that this glycoprotein provides the backbone of this matrix.

In conclusion, we have identified two forms of gel-forming salivary MUC5B, one of which is 'insoluble' in guanidinium chloride. sIgA, lactoferrin and lysozyme, and possibly also MUC7 and the agglutinin originating from the parotid gland, are to varying extents associated with the salivary gel matrix. The presence of such a 'decorated' matrix provides a mechanism with which to enrich protective agents close to the mucosal surface and possibly also to enhance interactions between bacteria and neutrophils.

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