Large-scale purification and characterization of the major phosphoproteins and mucins of human submandibular–sublingual saliva

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The major components of human submandibular–sublingual saliva (HSMSL) are mucins, amylases, cystatins, proline-rich proteins and statherin. Structure–function studies of these molecules have been hampered by the small amounts of purified materials that can be isolated from human secretions. The present study describes an integrated purification protocol for the large-scale preparation of many of these molecules. To dissociate partially heterotypic complexes among salivary molecules, HSMSL was initially fractionated into four pools by gel filtration with 6 M-guanidine hydrochloride. Subsequent fractionation of these four pools by gel-filtration and ion-exchange chromatography resulted in the purification of high- and low- M_r mucins, neutral and acidic cystatins, acidic and basic proline-rich proteins and statherin. Many variants or isoforms of these salivary molecules have been identified and biochemically characterized. Biochemical studies indicated that the low- M_r mucin exists as two isoforms which vary in their sialic acid to fucose ratios. Three isoforms of acidic cystatin S were characterized which differ in their phosphate content. Two isoforms of a basic proline-rich peptide were identified; the smaller peptide was a truncated form missing the first seven amino acids.

INTRODUCTION

It is well established that human submandibular–sublingual saliva (HSMSL), which can comprise at least 50 % of the total salivary secretions, contains several groups of families of proteins and glycoproteins which mediate various functions in the oral cavity. These include mucins, cystatins, amylases, proline-rich proteins (PRPs) and statherin. These families occur in saliva as multiple isoforms which originate from transcriptional, translational and/or post-translational modifications (Levine *et al.*, 1987; Minaguchi & Bennick, 1989). They participate in a wide array of biological functions including mineralization processes (Moreno *et al.*, 1982; Shomers *et al.*, 1982c; Bennick *et al.*, 1983), tissue coating (Kousvelari *et al.*, 1980; Bennick *et al.*, 1983; Fisher *et al.*, 1987; Al-Hashimi & Levine, 1989), lubrication (Hatton *et al.*, 1985; Levine *et al.*, 1987; Aguirre *et al.*, 1989a,b; Gans *et al.*, 1990), microbial attachment (Levine *et al.*, 1978; Stinson *et al.*, 1982; Murray *et al.*, 1982, 1986; Douglas, 1983; Gibbons *et al.*, 1983; Morris & McBride, 1984; Gibbons & Hay, 1988; Scannapieco *et al.*, 1989, 1990), anti-microbial activity (Mellersh *et al.*, 1979; Obenauf *et al.*, 1986) and digestion (Cohen & Levine, 1989). Understanding the functional relationships among individual variants or isoforms requires a knowledge of their biochemical structure which, in turn, is dependent on having sufficient amounts of purified materials for study. Investigators in salivary research have by and large focused their attention on purification of only those salivary proteins that are of particular interest to them. Consequently, there is no generalized protocol available for the efficient preparation of many of the different salivary molecules from intact or native secretions at the same time. In the present study,

an integrated protocol has been developed, based on separate procedures developed by several investigators, which permits the efficient purification of the major components of HSMSL saliva. These include high- and low- M_r mucins, representative acidic and basic PRPs, neutral and acidic cystatins and statherin. Data are presented that demonstrate the existence of multiple isoforms of these molecules and the biochemical differences of these variants.

EXPERIMENTAL

Materials

Acrylamide, *NNN'*-tetramethylethylenediamine (TEMED), ammonium persulphate, *NN'*-methylenebisacrylamide, Dowex 50-X4 (200–400 mesh), Dowex 1-X8 (200–400 mesh), horseradish peroxidase (HRP) colour-development reagent, HRP-conjugated goat anti-rabbit IgG (H + L) and HRP-conjugated rabbit anti-goat IgG (H + L) were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Sodium borohydride, *S*-ethylcysteine, Tris (TRIZMA), dithiothreitol, urea, SDS, guanidine hydrochloride, phosphoserine, 5'-AMP, 5'-UDP-Gal, ATP, *N*^α-benzoyl-L-arginine-7-amido-4-methylcoumarin, chicken egg white cystatin, alkaline phosphatase/agarose (bovine intestinal mucosa), papain (type III), Hepes and BSA (A7906) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). An electrophoresis calibration kit containing low- M_r protein standards, the f.p.l.c. system, Sephadex G-25, Sephadex G-200 (40–120 m), Sepharose CL-2B, Sephacryl S-300, DEAE-Sepharose CL-6B, Mono Q HR5/5 and Mono S HR5/5 columns were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). DEAE-cellulose DE-52 was from Whatman (Maid-

Abbreviations used: HSMSL, human submandibular–sublingual saliva; MG1, high- M_r salivary mucin; MG2, low- M_r salivary mucin; PRP, proline-rich proteins; TBS, Tris-buffered saline (0.01 M-Tris/HCl, pH 7.5, with 0.154 M-NaCl); DTT, dithiothreitol; PTH, *N*^α-phenylthiohydantoin; HRP, horseradish peroxidase; RM, reductive methylation; RA, reduction and alkylation; buffer 1, 6 M-guanidine/HCl in 100 mM-Tris/HCl, pH 7.5; buffer 2, 0.1 M-Hepes containing 6 M-urea, pH 7.5; buffer 3, 0.1 M-Tris/HCl containing 6 M-urea, pH 7.5; buffer 4, 5 mM-Tris/HCl, pH 7.6; buffer 5, 100 mM-Tris/HCl, pH 7.6; buffer 6, 1 M-NaCl in buffer 5; buffer 7, 0.2 M-NaCl in buffer 5; buffer 8, 0.1 M-NaCl in buffer 5; buffer 9, 0.3 M-NaCl in buffer 5.

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Fig. 1. (a) Gel filtration of freeze-dried HSMSL saliva (400–500 mg in 20–25 ml of buffer 1) on a column (2.6 cm $\times$ 95 cm) of Sephadex G-200 and (b) SDS/PAGE of resulting pools

Chromatography was carried out at room temperature using buffer 1 at a flow rate of 3–5 ml/h. Pools A–D were then screened by SDS/PAGE on 10% (w/v) gels. Immunoblotting identified the salivary components shown. M_r markers include: rabbit muscle phosphorylase *b* (94000), BSA (67000), ovalbumin (43000), bovine erythrocyte carbonic anhydrase (30000) and soya-bean trypsin inhibitor (20100).

stone, Kent, U.K.). [14 C]Formaldehyde (57.5 mCi/mmol) was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). Phenylthiohydantoin (PTH)-amino acid standards and polybrene were from Applied Biosystems (Foster City, CA, U.S.A.). 3% OV-225 on 80/100 Supelcoport was purchased from Supelco Inc. (Bellefonte, PA, U.S.A.). Immobilon-P transfer membrane was purchased from Millipore Corp. (Bedford, MA, U.S.A.). N.m.r. tubes were obtained from Wilmad Glass Co. Inc. (Buena, NJ, U.S.A.). All other reagents were of the highest commercial grade available.

Isolation of salivary components

HSMSL from a 30-year-old healthy female donor was collected and processed as previously described (Shomers *et al.*, 1982a). For all subsequent chromatography steps, pooled fractions were dialysed extensively against cold distilled water using Spectra/Por 3 tubing (Spectrum Medical Industries Inc., Los Angeles, CA, U.S.A.) and then freeze-dried. The proteins and glycoproteins of HSMSL were initially fractionated according to size by gel filtration on Sephadex G-200 using buffer with guanidine hydrochloride. Four pools (A–D) were obtained which comprised about 18, 6, 60 and 16% respectively of the total materials (dry weight) applied to the column (Fig. 1). Several columns were run in order to accumulate enough of these pools for the purification of the components described below. These pools were then screened by SDS/PAGE or by immunoblotting (Fig. 1). Pool A contained high- M_r salivary mucin (MG1), low- M_r salivary mucin (MG2), sIgA and amylase. Pool B contained unidentified 42000- and 36000- M_r components and small amounts of acidic PRPs. Pool C contained predominantly cystatins and acidic PRPs, and pool D contained mostly statherin and basic proline-rich peptides. Isoforms of several of these molecules were then purified and characterized.

Mucins. MG1-enriched pool A1 and MG2-enriched pool A2 were obtained by gel filtration of pool A on Sepharose CL-2B

(Fig. 2a). MG1 was then purified from pool A1 by the method of Loomis *et al.* (1987) using buffer 1.

For the purification of MG2, freeze-dried pool A2 (Fig. 2a) was subjected to reductive methylation as described by Jentoft & Dearborn (1979) and Prakobphol *et al.* (1982) with some modifications. Pool A2 was solubilized at a concentration of 20 mg/ml in buffer 2 which contained 6 M-urea. On occasion, the samples were not completely soluble in this buffer and a turbid solution resulted. This problem was overcome by the addition of small amounts of urea with gentle stirring. The clear solution was incubated at 37 °C with [14 C]formaldehyde (10-fold molar excess over the lysine content of the sample) in the presence of 100 mM-sodium cyanoborohydride. After 1–2 h, unlabelled formaldehyde (4 $\times$ the amount of [14 C]formaldehyde) was added and the mixture was further incubated for 24 h at 37 °C. The reaction mixture was fractionated (and desalted) on Sephacryl S-300 using buffer 3. MG2-containing fractions (RM MG2) were localized by SDS/PAGE/fluorography using [14 C]-labelled MG2 as a marker (Prakobphol *et al.*, 1982) and then pooled accordingly (Fig. 2b).

A second modification of the MG2 purification protocol was introduced which involved the reduction and alkylation of RM MG2 followed by chromatography on Sephacryl S-300 with 6 M-urea. For reduction and alkylation, 50 mg of RM MG2-containing materials were solubilized at a concentration of 10 mg/ml in 0.1 M-Tris/HCl with 2% disodium EDTA and 8 M-urea, pH 8.5, and reduced for 16 h with a 150-fold molar excess of dithiothreitol (DTT) (over half-cysteine content of the sample). The reduced sample was then alkylated for 1–2 h with a 2-fold molar excess (over the DTT content) of iodoacetic acid. Before addition, the pH of iodoacetic acid was adjusted to 8.0–8.5 with 1 M-NaOH. Both the reduction and alkylation procedures were carried out in the dark under N_2 . These materials were chromatographed (and desalted) on Sephacryl S-300 using buffer 3. Individual fractions were monitored by SDS/PAGE/

Fig. 2. Separation and purification of MG1 and MG2

(a) Gel filtration of pool A (150 mg in 5–6 ml of buffer 1) on a column (1.6 cm $\times$ 100 cm) of Sepharose CL-2B. Chromatography was carried out at room temperature using buffer 1 at 4–6 ml/h. (b) Gel filtration of ^{14}C -labelled reduced and methylated pool A2 (100 mg in 5 ml of buffer 2) on a column (1.5 cm $\times$ 160 cm) of Sephacryl S-300. Chromatography was carried out at room temperature using buffer 3 at a flow rate of 3–5 ml/h. Fractions were monitored by liquid-scintillation spectroscopy. SDS/PAGE/fluorography on 7.5% (w/v) gels was performed on alternate fractions (fraction no. 27 = lane 1; fraction no. 55 = lane 15). Fractions 31–37 (lanes 3–6) were pooled on the basis of the presence of a band with the same relative mobility as MG2. (c) Gel filtration of ^{14}C -labelled RM/RA MG2 on a column (1.5 cm $\times$ 160 cm) of Sephacryl S-300. Chromatography was carried out at room temperature using buffer 3 at a flow rate of 3–5 ml/h. Fractions were monitored by liquid-scintillation spectroscopy. ^{14}C -labelled RM/RA MG2 was localized by SDS/PAGE/fluorography as above.

fluorography, and MG2-containing fractions (RM/RA MG2) were pooled as shown in Fig. 2(c). The RM/RA MG2 was then subjected to ion-exchange chromatography on DEAE-Sepharose CL-6B as shown in Fig. 3.

Cystatins. Neutral and acidic cystatins were purified from pool C (Fig. 1) by modifying our previously described procedures (Shomers *et al.*, 1982a; Al-Hashimi *et al.*, 1988). In particular, the use of guanidine hydrochloride in the fractionation of

Fig. 3. Ion-exchange chromatography of RM/RA MG2 on a column (1 cm $\times$ 15 cm) of DEAE-Sepharose CL-6B

The sample (40 mg) was solubilized at a concentration of 2–3 mg/ml in equilibrating buffer (0.005 M-sodium phosphate, pH 8.0). After elution with 2 bed vol. of equilibrating buffer, the column was eluted with 100 ml each of a linear gradient from 0.005 M- and 0.15 M-sodium phosphate, pH 8.0. Next, the column was eluted with 100 ml of 0.15 M-sodium phosphate, pH 8.0, followed by 100 ml of 0.15 M-sodium phosphate, pH 8.0, containing 1 M-NaCl. Chromatography was performed at 6 $^{\circ}$ C at a flow rate of 10–12 ml/h. Fractions were monitored by liquid-scintillation spectroscopy. SDS/PAGE/fluorography (lanes 1–3) and anionic-PAGE/fluorography (lanes 4–6) of MG2a (lanes 1 and 4), MG2b (lanes 2 and 5) and acidic peptides (lanes 3 and 6). All experiments used 7.5 % gels.

Fig. 4. Separation and purification of cystatins by DEAE-cellulose chromatography

(a) DEAE-cellulose DE-52 chromatography of pool C (about 600 mg in 50 ml of buffer 4). The column (2.5 cm $\times$ 50 cm) was eluted initially with 500 ml of buffer 4. Bound materials were eluted using a linear gradient consisting of 500 ml each of buffer 4 and buffer 5. After being washed with another 500 ml of buffer 5, additional materials were eluted with a linear salt gradient consisting of 500 ml of buffer 5 and 500 ml of buffer 6. Fractions were collected at 6 $^{\circ}$ C at a flow rate of 50–60 ml/h. (b) DEAE-cellulose DE-52 chromatography of pool C4 (200 mg in 8–10 ml of buffer 5). The column (1.5 cm $\times$ 120 cm) was eluted using a linear gradient consisting of 1 l each of buffer 5 and buffer 7. Fractions were collected at 6 $^{\circ}$ C at a flow rate of 15–20 ml/h.

HSMSL on Sephadex G-200 resulted in the localization of amylase in pool A instead of pool C. Therefore gel filtration of pool C in the presence of high salt was no longer needed to separate amylase from neutral cystatin (Al-Hashimi *et al.*, 1988). Pool C was subjected to anion-exchange chromatography on DEAE-cellulose DE-52. Fractions (C1–C4) were pooled as indicated in Fig. 4(a). Anionic-PAGE followed by immunoblotting with rabbit anti-(human neutral cystatin) localized neutral cystatin SA-I in pool C2, three acidic cystatins (designated C4a–4c) in pool C4 and a small amount of cystatin in pool C3.

Cystatins C4a–4c were then separated by chromatography on a longer column of DEAE-cellulose using a shallow linear gradient, and fractions were pooled as shown in Fig. 4(b). Final purification of acidic cystatins was attained by f.p.l.c. using an anion-exchange Mono Q HR5/5 column (Fig. 5a).

The thiol-blocking proteinase inhibitory activity of cystatins (Fig. 6) was evaluated by the procedure of Barrett & Kirschke (1981). Various amounts of cystatins (0–35 μ g in 250 μ l of 0.4 M-sodium/potassium phosphate buffer, pH 6.0, containing 8 mM-DDT and 4 mM-EDTA) were incubated for 10 min at 40 $^{\circ}$ C with

Fig. 5. Final purification of acidic cystatins by f.p.l.c.

(a) The composite profiles for acidic cystatins S (C4a), S1 (C4b), S2 (C4c) were run on a Mono Q HR5/5 column at a flow rate of 1 ml/min with 10 mM-Tris/HCl, pH 8.0. Initially, a chloride gradient (----) was introduced and increased to 0.2 M over 30 min and then continued at 0.2 M for 5 min. The gradient was increased to 0.5 M over 5 min, continued at 0.5 M for 5 min, and finally decreased to 0 M over 5 min. Typically, samples were dissolved at 10 mg/ml and approximately 0.05–0.10 ml was applied to the column. (b) Anionic-PAGE on 7.5% gels of salivary cystatins. The gels were stained with Coomassie Blue. Lane 1, neutral cystatin SN; lane 2, acidic cystatin S1 (C4b); lane 3, acidic cystatin S (C4a); lane 4, acidic cystatin S2 (C4c); lane 5, alkaline phosphatase-treated acidic cystatin S1.

Fig. 6. Thiol-blocking proteinase inhibitory activity of cystatins

Cystatin SN and cystatin S1 were reduced and alkylated by the procedures used for pool MG2. ■, Cystatin SN; ◆, RA, cystatin SN; ▲, cystatin S1; ◇, RA cystatin S1; □, dephosphorylated cystatin S1; ●, cystatin S; ○, cystatin S2; △, egg-white cystatin.

200 munits of papain (in 500 μ l of 0.1% Brij 35 in water). To this was added 250 μ l of 20 μ M-*N* $^{\alpha}$ -benzoyl-L-arginine-7-amido-4-methylcoumarin (in deionized water) and samples were incubated for an additional 10 min at 40 $^{\circ}$ C. The enzyme reaction

was terminated by the addition of 1.0 ml of 0.1 M-sodium monochloroacetate (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) in 0.1 M-sodium acetate, pH 4.3. The release of the 7-amido-4-methylcoumarin was measured on a Perkin-Elmer model 650-40 fluorescence spectrophotometer using an excitation wavelength of 345 nm and an emission wavelength of 438 nm.

Acidic PRPs. These proteins were localized in pool C3 by their characteristic metachromatic staining with Coomassie Blue after SDS/PAGE or anionic-PAGE and by immunoblotting with goat anti-(human PRP). Subsequent purification of acidic PRP was achieved by DEAE-cellulose DE-52 chromatography of pool C3 (Fig. 7a). Examination of these pools by anionic-PAGE revealed an incomplete separation of components (Fig. 7b). Nevertheless, pool C3i (lane 11) was more homogeneous than the others. Minor contaminants in pool C3i were removed by f.p.l.c. using a Mono Q HR5/5 column (Fig. 7c).

Basic proline-rich peptides and statherin. Amino acid analysis of the low- M_r materials in pool D revealed that proline and tyrosine comprised approx. 26% and 10% of the total amino acids, suggesting the presence of proline-rich peptides and the tyrosine-rich peptide or statherin. Consequently, pool D was fractionated on DEAE-cellulose into two pools, D1 and D2 (Fig. 8a). Amino acid analysis indicated that pool D1, which did not bind to DEAE-cellulose, contained a high content of proline and glycine and therefore represented basic proline-rich peptides. Further fractionation of this material into two basic fractions,

Fig. 7. Purification of acidic PRPs

(a) DEAE-cellulose DE-52 chromatography of pool C3 (350–400 mg in 15–20 ml of buffer 5). The column (1.5 cm $\times$ 120 cm) was eluted initially with buffer 5 and then with a linear gradient consisting of 1 l each of buffer 5 and buffer 8. Fractions were collected at 6 $^{\circ}$ C at a flow rate of 15–20 ml/h. (b) Anionic-PAGE on 7.5% gels of acidic proline-rich fractions. The gels were stained with Coomassie Blue. Lane 1, HSM SL; lane 2, pool C3; lanes 3–13, pools C3a–k respectively; lane 14, f.p.l.c.-purified PRP1. (c) The elution profile of pool C3i on a Mono Q HR5/5 column run at a flow rate of 1 ml/min over 30 min in 50 mM-Tris/HCl, pH 8.0. After 5 min, a chloride gradient was introduced and increased to 0.22 M (over 10 min). Next the chloride gradient (---) was increased to 0.25 M-NaCl (over 30 min). Typically, samples were dissolved at 2 mg/ml in 50 mM-Tris/HCl, pH 8.0, and approximately 0.1 ml was applied to the column.

D1a and D1b, was achieved by f.p.l.c. with a Mono S HR5/5 column (Fig. 8b).

Pool D2 consisted almost exclusively of statherin as determined

by amino acid analysis. After dialysis and freeze-drying, statherin was further desalted using Sephadex G-25 with 0.02 M-acetic acid. Further purification of statherin-rich pool D2 was accomplished by f.p.l.c. on a Mono Q HR5/5 column as shown in Fig. 8(c).

General chemical and analytical methods

For amino acid composition, samples were hydrolysed with constant boiling HCl for 24 h at 110 $^{\circ}$ C in evacuated sealed tubes. Hydrolysates were analysed by a Beckman model 6300 amino acid analyser using α -amino- β -guanidinopropionic acid (Pierce Chemical Co., Rockford, IL, U.S.A.) as an internal standard. For determination of neutral sugars and hexosamines (Loomis *et al.*, 1987), samples were hydrolysed in 2 M-HCl at 100 $^{\circ}$ C for 6 h. The hydrolysate was passed through coupled columns of Dowex 50-X4, H⁺ (200–400 mesh) and Dowex 1-X8, HCOO⁻ (200–400 mesh). Neutral sugars in the effluent were quantified as alditol acetates by g.l.c. on a Varian model 3700 gas chromatograph with an OV-225 column (180 $^{\circ}$ C isothermal for 5 min followed by an increase in temperature at 4 $^{\circ}$ /min to 200 $^{\circ}$ C and then held at that temperature for 40 min) using arabinose as internal standard. Peaks were quantified by a Gilson model 620 Data Master integrated to an Apple IIe computer. Hexosamines were eluted from Dowex 50 columns with 2 M-HCl and quantified by the amino acid analyser using α -amino- β -guanidinopropionic acid as internal standard. Sialic acid was determined by the thiobarbituric acid procedure after hydrolysis with 0.025 M-H₂SO₄ for 1 h at 80 $^{\circ}$ C (Warren, 1959).

The tryptophan content of salivary proteins was determined after hydrolysis with 200 μ l of 4.0 M-methanesulphonic acid containing 0.2% tryptamine, for 24 h at 115 $^{\circ}$ C. The hydrolysate was partially neutralized with 3.5 M-NaOH before amino acid analysis (Simpson *et al.*, 1976).

Automated protein sequencing was carried out on an Applied Biosystems model 471A protein sequencer controlled by a microprocessor. Samples (1 nmol) were dissolved in water and dried on to a glass-fibre filter precycled with polybrene. The PTH derivatives were analysed using a 5 μ m PTH-C₁₈ reversed-phase cartridge-style column (0.46 cm $\times$ 22 cm) with an on-line isocratic PTH analyser. The column was eluted with a mobile phase consisting of 10% tetrahydrofuran, acetonitrile and sodium acetate buffers (pH 4.6 and 3.8) according to the manufacturer's instructions. The PTH-amino acids were monitored at 270 nm using a variable-wavelength detector interfaced to a Hewlett-Packard HP3394A integrator. The average overall yield was 50–60% of the total protein loaded, with a repetitive cycle yield of greater than 90%.

SDS/PAGE was carried out according to the procedure of Laemmli (1970) while the procedure of Ornstein (1964) was adopted for anionic-PAGE. Electrophoresis was performed using a Hoefer SE 280 Tall Mighty Small electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Proteins were visualized by staining with a 0.1% solution of Coomassie Brilliant Blue R250 in methanol, water and acetic acid (5:5:1, by vol.) ¹⁴C-labelled components were visualized by fluorography whereby the gels were fixed in trichloroacetic acid (10%, w/v) for 1–2 h at room temperature and then soaked in EN³HANCE (New England Nuclear, Boston, MA, U.S.A.) for 1 h at room temperature (Loomis *et al.*, 1987).

For immunoblotting (Towbin *et al.*, 1979; Burnette, 1981), samples were subjected to SDS/PAGE and transferred to Immobilon-P transfer membrane (room temperature; 100 mA; 0.1 M-Tris containing 0.192 M-glycine and 20% methanol as transfer buffer) with Hoefer Semi-phor model TE-70 transfer system. The membrane was treated with 5% BSA in TBS for 30 min at 37 $^{\circ}$ C, washed three times with 0.1% BSA in TBS,

Fig. 8. Purification of basic PRPs

(a) DEAE-cellulose DE-52 chromatography of pool D (110 mg in 10 ml of buffer 4). The column (2.5 cm $\times$ 50 cm) was subjected to the same gradients that were used for pool C (Fig. 4a). Fractions were collected at 6 $^{\circ}$ C at a flow rate of 15–20 ml/h. (b) The elution profile of pool D1 on a Mono S HR5/5 column run at a flow rate of 1 ml/min in 10 mM-Tris/HCl, pH 8.0. After 10 min, a chloride gradient (----) was introduced and increased to 0.05 M over the next 30 min. Next, the chloride gradient was increased to 0.5 M (over

incubated with an appropriate dilution of antiserum at room temperature for 1 h with gentle agitation, and then washed three times for 10 min each with 0.1% BSA in TBS. Next, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG for 1 h followed by washing for 10 min three times with 0.1% BSA. Antigens were revealed with HRP colour-development reagent (0.5 mg of 4-chloronaphthol/ml in 50 mM-TBS and 0.03% hydrogen peroxide).

Antisera

Rabbit anti-(human neutral cystatin) was prepared in New Zealand White rabbits by the immunization protocol of Shomers *et al.* (1982a). Rabbit antibody against partially deglycosylated MG2 was prepared as described by Cohen *et al.* (1991). Goat anti-PRP was kindly provided by Dr. A. Bennick (Department of Biochemistry, University of Toronto, Toronto, Ont., Canada). Rabbit anti-sIgA was purchased from DAKO Corp. (Carpenteria, CA, U.S.A.). Rabbit anti-(human salivary amylase) was obtained from Sigma.

Phosphate analyses

The phosphate content of salivary proteins was determined colorimetrically by the procedure of Chen *et al.* (1956) and/or by n.m.r. spectroscopy as described by Vogel & Bridger (1982). For n.m.r., standard curves were generated from solutions (0.1–5.0 mM) of phosphoserine prepared in 50 mM-Tris/HCl, pH 7.6, with 1 mM-EDTA and 33% 2H_2O (ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.). The standard curve was validated with 1.2 mM solutions containing one residue (5'-AMP), two residues (5'-UDP-Gal) and three residues (ATP) of phosphate. Cystatins and statherin were dissolved at 0.35 mM-protein in 2H_2O . For all experiments, 0.6 ml portions of standard or sample solutions were placed in 5 mm XR-55 n.m.r. tubes fitted with a Teflon chuck having a capillary tube containing 85% phosphoric acid as an external standard. N.m.r. experiments were performed at 25 $^{\circ}$ C on a Varian VXR-500 spectrometer at 202 MHz at a 90 $^{\circ}$ flip angle and a 2.5 s recycle time. Proton decoupling during acquisition (gated) was used unless otherwise indicated. ^{31}P n.m.r. spectra were obtained keeping all acquisition parameters constant. The linewidth was measured at half-height and corrected for an additional line broadening introduced by the computer digital filtering. Integrations were carried out in absolute mode to determine peak areas and a standard curve was generated.

The location of phosphoserine residues in the *N*-terminal region of acidic cystatins was assessed after the conversion of phosphoserine into *S*-ethylcysteine (Meyer *et al.*, 1987). This was accomplished by the addition of 100 μ l of a mixture consisting of 80 μ l of ethanol, 65 μ l of 5 M-NaOH, 60 μ l of ethanethiol (J. T. Baker, Phillipburg, NJ, U.S.A.) and 400 μ l of water to dried samples (2 nmol) in a 1.0 ml reaction vial. The mixture was incubated for 1 h at 50 $^{\circ}$ C under nitrogen, then acidified with 10 μ l of acetic acid. The location of *S*-ethylcysteine was then determined by *N*-terminal sequencing of the derivatized cystatins.

For alkaline phosphatase digestion, salivary statherin and

5 min) and then down to 0 M (over 5 min). Typically, samples were dissolved at 2 mg/ml in 10 mM-Tris/HCl, pH 8.0, and approximately 0.1 ml was applied to the column. (c) The elution profile of pool D2 (—) and alkaline phosphatase-treated D2 (....) on a Mono Q HR5/5 column run at a flow rate of 1 ml/min with 10 mM-Tris/HCl, pH 8.0. A chloride gradient (----) was introduced and increased to 0.15 M over 5 min and then to 0.3 M (over 30 min). Typically, samples were dissolved at 2 mg/ml in 10 mM-Tris/HCl, pH 8.0, and approximately 0.05–0.10 ml were applied to the column. Inset: anionic-PAGE of D2a (left lane) and D2b (right lane).

cystatins (100–140 nmol in 100 μ l of 0.5 M-Tris/HCl, pH 9.0) were incubated for 24–36 h at 37 °C with 2 units of alkaline phosphatase/agarose (5 μ l of toluene was added as a preservative). After removal of the immobilized enzyme by centrifugation at 3000 g for 1 min, the dephosphorylated materials were analysed by anionic-PAGE or f.p.l.c. on a Mono Q HR 5/5 column.

RESULTS AND DISCUSSION

Mucins

Our previous studies on salivary mucin purification have shown that both MG1 and MG2 can undergo non-covalent complexing with several constituents in saliva including sIgA (Prakobphol *et al.*, 1982; Loomis *et al.*, 1987 respectively). We employed several approaches to dissociate these complexes including urea and reductive methylation. However, these procedures usually required several time-consuming recycling steps through various gel matrices to ensure mucin purification. This resulted in lower yields and did not completely resolve the poor solubility properties of these molecules. Consequently, we have modified our mucin-purification protocols. The first modification involves the use of guanidine hydrochloride (buffer 1) instead of urea in the initial gel-filtration steps. The guanidine hydrochloride greatly enhanced the solubility of freeze-dried HSMSL (see above) and crude mucin-containing fractions. As shown in Fig. 2(a), gel filtration of pool A on Sepharose CL-2B separated MG1 (pool A1) from MG2 (pool A2). MG1 which was then purified from pool A1 using guanidine-containing buffers (results not shown) was identical in composition with that obtained using urea-containing buffers (Loomis *et al.*, 1987). Previously for MG2 purification, reductive methylation alone was used to dissociate the smaller peptides whereas repeated recycling through Sephadex G-200 with 6 M-urea or immunoaffinity columns of anti-sIgA was used to dissociate the sIgA (Prakobphol *et al.*, 1982). These recycling steps greatly diminished the yields of MG2. To maximize the dissociation of sIgA from MG2 and increase the yields of this mucin, reductive methylation was followed by a reduction and alkylation step and each was accompanied by gel filtration under dissociating conditions. Finally, MG2-enriched materials (RM/RA MG2, Fig. 2c) were subjected to ion-exchange chromatography on DEAE-Sepharose CL-6B (Fig. 3). A first pool was obtained during gradient elution at approx. 0.025 M-sodium phosphate, pH 8.0, a second pool was eluted with 0.15 M-sodium phosphate, and a third pool was eluted with 1 M-NaCl. As shown by SDS/PAGE/fluorography, the first two pools contained predominantly materials with an R_f comparable with MG2 (Prakobphol *et al.*, 1982) and represent two isoforms designated

MG2a and MG2b respectively (Fig. 3). The third pool contained mostly low- M_r components and only trace amounts of MG2 (Fig. 3). Anionic-PAGE/fluorography of MG2a and MG2b also indicated that these preparations were free of acidic peptides (Fig. 3). MG2b had a higher sialic acid content than MG2a (Table 1) and migrated faster than MG2a during both SDS/PAGE and anionic-PAGE. The existence of two isoforms of MG2 which differ in their charge properties explains the charge heterogeneity previously reported for this mucin (Prakobphol *et al.*, 1982).

The amino acid compositions of MG2-containing fractions obtained during various steps of purification are compared with that of MG2a and MG2b (Table 2). The content of acidic amino acids (Asx and Glx) decreased as purity of the MG2 preparations increased. These two residues comprised the major amino acids present in the acidic peptide fractions. In general, the amino acid compositions of MG2a and MG2b were similar to each other and comparable with that reported previously for MG2 (Prakobphol *et al.*, 1982), suggesting that the two species may have identical peptide moieties. Characteristically, purified MG2a and MG2b preparations have a high content of proline and alanine and very low amounts of glycine. Major differences between MG2a and MG2b were found in their carbohydrate content where MG2a had a lower content of sialic acid and a higher content of fucose than MG2b (Table 1). The chemical composition of MG2a and MG2b does not appear to vary among different individuals since the MG2 isoforms prepared from five different subjects had identical elution profiles on DEAE-Sepharose CL-6B and comparable amino acid profiles.

The effects of reductive methylation and/or reduction and alkylation on the purity of MG2a and MG2b were determined by eliminating each of these steps during the purification protocol. The amino acid compositions of mucins prepared by eliminating the reductive methylation step were comparable with those prepared as outlined above. However, for ease of monitoring the column eluates, radiolabelling by reductive methylation may be desirable. In contrast, mucins that had not undergone reduction and alkylation had a lower content of Ser + Thr and Pro and a higher content of Gly, indicating that this step is necessary to dissociate and/or prevent co-elution of acidic peptides with mucins from DEAE-Sepharose CL-6B.

Cystatins

Earlier studies have shown that human saliva contains at least three genetically distinguishable forms of cystatins, namely cystatin S (HPS-12), cystatin SA and cystatin SN (SA-I) (Isemura *et al.*, 1984a,b, 1987; Hawke *et al.*, 1987; Al-Hashimi *et al.*, 1988). HSMSL contains at least seven cystatins (one neutral and six acidic isoforms) which have not undergone post-translational proteolytic processing (Shomers *et al.*, 1982a; Al-Hashimi *et al.*, 1988). However, the acidic cystatins differ in their overall charge and in their degree of phosphorylation (Shomers *et al.*, 1982b).

The protocol for the isolation of neutral salivary cystatin, previously designated SA-I, has been modified slightly from that previously described by Al-Hashimi *et al.* (1988). As before (Al-Hashimi *et al.*, 1988), final purification of the neutral cystatin from pool C2 was achieved by Mono Q anion-exchange chromatography (Fig. 5a). N-terminal sequencing revealed a single tryptophan residue. Since cystatin SA-I (Al-Hashimi *et al.*, 1988) has an identical amino acid composition and sequence with cystatin SN described by Saitoh *et al.* (1987, 1988) and to eliminate confusion among nomenclatures from different laboratories, this neutral cystatin will be referred to as cystatin SN.

The three most acidic cystatins localized in pool C4 were separated by sequential anion-exchange chromatography on

Table 1. Chemical composition of MG2a and MG2b

Results are the percentage of recovered weight. Values in parentheses were normalized to 100% recovery.			
Component	MG2a	MG2b	MG2*
Protein	16.3 (25.2)	15.6 (20.3)	22.1 (31.4)
N-Acetylgalactosamine	10.1 (15.6)	13.1 (17.2)	12.8 (18.2)
N-Acetylglucosamine	7.8 (12.0)	11.8 (15.4)	7.3 (10.4)
N-Acetylneuraminic acid	9.3 (14.4)	20.1 (26.2)	6.9 (9.8)
Fucose	10.5 (16.2)	5.3 (6.9)	7.1 (10.1)
Galactose	9.7 (15.1)	9.8 (12.8)	12.0 (17.1)
Mannose	0.9 (1.4)	0.9 (1.2)	1.0 (1.4)

* Results taken from Prakobphol *et al.* (1982).

Table 2. Amino acid composition of low- M_r mucin fractions

Values are not corrected for destruction due to hydrolysis.

Amino acid	Residues/1000 residues						Acidic peptides (Fig. 4)
	Pool A2 (Fig. 2a)	RM MG2 (Fig. 2b)	RM/RA MG2 (Fig. 2c)	MG2a	MG2b	MG2*	
Aspartic acid	127	107	92	61	67	49	139
Threonine	71	120	141	186	171	209	56
Serine	65	81	87	105	113	162	68
Glutamic acid	87	79	76	71	75	69	111
Proline	87	148	179	230	223	237	57
Glycine	88	53	38	10	11	14	85
Alanine	68	100	114	133	129	144	61
Half-cysteine	11	4	7	14	14	1	29
Valine	64	58	50	34	36	31	71
Methionine	14	9	6	1	1	1	11
Isoleucine	44	36	32	21	23	15	46
Leucine	64	59	56	46	51	28	91
Tyrosine	35	21	14	5	6	1.4	28
Phenylalanine	45	34	28	17	18	9	42
Histidine	26	23	22	21	24	9	27
Lysine	49	31	28†	23†	24†	14†	36†
Arginine	56	37	29	22	14	7	40

* From Prakobphol *et al.* (1982).

† As mono- and di-methyl lysine.

Table 3. Amino acid composition of cystatins

Amino acid	Residues/molecule				
	Cystatin SN	Cystatin S (C4a)	Cystatin S1 (C4b)	Cystatin S2 (C4c)	Cystatin S*
Aspartic acid	13	13	13	13	13
Threonine	5	5	5	5	5
Serine	7	8	8	8	8
Glutamic acid	19	23	23	23	22
Proline	6	5	5	5	5
Glycine	5	5	5	5	5
Alanine	6	7	7	7	7
Half-cysteine	4	4	4	4	4
Valine	8	7	7	7	7
Methionine	0	1	1	1	1
Isoleucine	6	5	5	5	6
Leucine	8	8	8	8	8
Tyrosine	6	6	6	6	6
Phenylalanine	5	6	6	6	6
Histidine	2	2	2	2	2
Lysine	7	5	5	5	5
Arginine	11	9	9	9	9
Tryptophan	3	2	2	2	2
M_r ...	14056				14190
Phosphate...	0	0	1	2	0

* Deduced from the nucleotide sequence (Saitoh *et al.*, 1987).

DEAE-cellulose (Fig. 4b) and Mono Q (Fig. 5a). The acidic cystatins C4a, C4b and C4c were eluted from Mono Q at 0.18 M-, 0.20 M- and 0.45 M-NaCl respectively. On the basis of relative yields, it was found that C4b was the most abundant of the acidic cystatins. Their amino acid compositions and *N*-terminal sequences were identical with each other and with that of cystatin S (Table 3 and Fig. 9). The neutral cystatin and acidic cystatin C4b contained less than 0.1 residue of hexosamines per molecule, indicating that they are not glycosylated.

Our previous studies suggested that differences in anionic mobility among the three acidic cystatins was probably due to varying levels of phosphorylation (Shomers *et al.*, 1982b). Alkaline phosphatase treatment of pool C4b and C4c followed by anionic-PAGE revealed that their relative mobilities were now equivalent to that of C4a suggesting that C4a is not phosphorylated (Fig. 5b). Analysis of the phosphate content of the acidic cystatins by a colorimetric assay and by ^{31}P n.m.r. spectroscopy indicated that C4a, C4b and C4c contain zero, one

	1	5	10	15	20	25
SN:	Trp-Ser-Pro-Lys-Glu-Glu-Asp-Arg-Ile-Ile-Pro-Gly-Gly-Ile-Tyr-Asn-Ala-Asp-Leu-Asn-Asp-Glu-Trp-Val-Gln-					
S:	Ser-Ser-Ser-Lys-Glu-Glu-Asn-Arg-Ile-Ile-Pro-Gly-Gly-Ile-Tyr-Asp-Ala-Asp-Leu-Asn-Asp-Glu-Trp-Val-Gln-					
	26	30	35			
SN:	Arg-Ala-Leu-His-Phe-Ala-Ile-Ser-Glu-Tyr-					
S:	Arg-Ala-Leu-His-Phe-Ala-Ile-Ser-Glu-Tyr-					

Fig. 9. *N*-Terminal sequence comparison of cystatin SN and acidic isoforms of cystatin S (pools C4a, C4b or C4c)

Table 4. Phosphate content of cystatins and statherin

Values for the colorimetric assay are the average of duplicate analyses.

	Residues per molecule	
	Colorimetric assay	N.m.r.
Cystatin SN (C2)	ND	0
Cystatin S (C4a)	0.41	0
Cystatin S1 (C4b)	0.86	0.97
Cystatin S2 (C4c)	1.44	1.57
Statherin (D2)	ND	1.87

ND, Not determined.

Table 5. Amino acid composition of acidic PRP1, basic PRP and statherin*

Amino acid	Residues/100 residues				
	Acidic PRP1 (C3i)	Basic PRP D1a	Basic PRP D1b*	Statherin D2a	Statherin D2b
Aspartic acid	7.2	0.5	—	2.8	2.7
Threonine	0.6	—	—	2.4	2.3
Serine	3.3	1.1	—	3.8	3.1
Glutamic acid	25.4	6.3	5.6	24.7	24.3
Proline	24.6	54.2	52.4	17.2	16.2
Glycine	22.3	14.5	16.1	9.8	10.3
Alanine	1.7	4.1	3.6	—	—
Half-cysteine	0.1	0.5	—	—	—
Valine	2.4	2.4	1.9	3.0	2.7
Methionine	—	—	—	—	—
Isoleucine	1.3	3.8	3.3	2.5	2.2
Leucine	2.6	2.0	1.8	5.3	4.8
Tyrosine	0.2	4.2	4.5	13.9	15.7
Phenylalanine	0.9	6.4	5.6	6.0	6.5
Lysine	1.7	—	—	2.3	2.3
Histidine	1.8	—	—	—	0.1
Arginine	4.1	—	5.1	6.4	6.8

* The analysis for D1b is similar to peptide P-B described by Isemura *et al.* (1979).

and two phosphate residues per molecule (Table 4). In addition, ³¹P n.m.r. spectroscopy indicated that no phosphate was present in cystatin SN whereas two residues of phosphate were found in purified statherin. Several studies have indicated that the consensus sequence for phosphorylation is Ser-X-Y where Y is an acidic residue, most notably glutamine (Henderson *et al.*, 1981;

Nisbet *et al.*, 1981; Hamazume *et al.*, 1985; Laber *et al.*, 1989). With cystatin S, Ser-X-Glu occurs at positions 3–5 and 99–101 (Saitoh *et al.*, 1987). Derivatization of C4b with ethanethiol and subsequent *N*-terminal sequencing indicated that the serine at position 3 was phosphorylated. If position 3 is phosphorylated, then the consensus sequence of Ser-X-Y could also be fulfilled for position 1. In fact, derivatized C4c could not be sequenced. In this context, Meyer *et al.* (1987) have indicated that an *N*-terminal phosphoserine is not transformed into *S*-ethylcysteine and instead pyruvate is formed resulting in a blocked *N*-terminus. Although these studies suggest that serine at position 1 in C4c might be phosphorylated, the location of the second phosphoserine remains to be definitely determined. Interestingly, Laber *et al.* (1989) have shown that the structural difference between two isoforms of chicken cystatin was due to the presence of phosphoserine at position 80. In contrast with other salivary phosphoproteins (e.g. PRP and statherin), where phosphoserine occurs in *N*-terminal domains, it is possible that certain isoforms of acidic salivary cystatins contain phosphoserine in *C*-terminal regions. Nevertheless, the three acidic cystatins represent variants or isoforms of cystatin S which differ in their degree of phosphorylation and can thus be designated cystatin S, S1 and S2.

Information derived from sequence data (Saitoh *et al.*, 1987; Al-Hashimi *et al.*, 1988) indicates that the ratios of Asp+Glu and Asn+Gln in neutral cystatin SN and acidic cystatin S are 18/14 and 22/13 respectively. In addition, there are 18 residues of Lys+Arg in cystatin SN as compared with 14 residues of Lys+Arg in cystatin S. Collectively, these differences contribute to the acidic nature of cystatin S.

The purified cystatins described in this study (SN, S, S1 and S2) were all found to inhibit the proteolytic activity of papain with cystatin SN being the most active inhibitor (Fig. 6). This biological activity was not affected by previous exposure to 6 M-guanidine hydrochloride or 8 M-urea or by alkaline phosphatase treatment. In contrast, disruption of the disulphide bonds in cystatin SN and cystatin S1 by reduction and alkylation abolished thiol-blocking proteinase inhibitory activity.

Acidic PRPs

Previous studies have identified acidic PRPs in human submandibular-sublingual secretions, but these have not been completely characterized (Bennick, 1977). In the purification protocols described in this study, the acidic PRPs were localized in pool C3 by SDS/PAGE, anionic-PAGE and immunoblotting. This material was subsequently separated into 11 pools by anion-exchange chromatography (Fig. 7a). The high content of Pro, Gly and Glx in these pools indicated the presence of acidic PRPs. The PRP in pool C3i was purified to homogeneity by Mono Q anion-exchange chromatography (Fig. 7c). This PRP displayed a single band on anionic-PAGE (Fig. 7b, lane 14) and had an amino acid composition comparable with PRP1 described by Hay *et al.* (1988) (Table 5).

Several PRP-containing pools (primarily C3d and C3g) also

Fig. 10. *N*-Terminal sequence of basic PRPs

PCA, pyrrolidone 5-carboxylic acid. D1b is peptide P-B described by Isemura *et al.* (1979).

contained acidic cystatins when examined by anionic-PAGE/Western transfer. These cystatins exhibited anionic mobility between cystatins SN and S and may be comparable with those cystatin isoforms originally described by Shomers *et al.* (1982a) as the C2 group of cysteine-containing phosphoproteins. Their relationship to one of the three genetically distinguishable forms of cystatins, cystatin SA (Isemura *et al.*, 1987), remains to be determined.

Basic PRPs and statherin

The basic PRPs in pool D1 were separated into two components, D1a and D1b, by Mono S cation-exchange chromatography (Fig. 8b). Amino acid analysis revealed that D1b was similar in composition to a basic peptide, P-B, isolated from human whole saliva (Isemura *et al.*, 1979) (Table 5). *N*-Terminal sequencing indicated that the *N*-terminus of D1b was blocked, which is consistent with the findings of Isemura *et al.* (1979). *N*-Terminal sequence analysis of D1a revealed that this PRP was a truncated form of peptide P-B which is missing the first seven amino acids (Fig. 10). These peptides represent a second group of basic PRPs described in HSMSL saliva. Robinson *et al.* (1989) have described a 44-residue basic PRP from HSMSL saliva which is presumably derived by the proteolytic processing of acidic PRPs. The sequence of this protein is identical with the *C*-terminal segment (residues 107–150) of human parotid PRP1, PRP2 and PIF-S (Wong & Bennick, 1980; Schlesinger & Hay, 1986; Hay *et al.*, 1988) and identical with a basic PRP isolated from human parotid saliva by Isemura *et al.* (1980). Thus it appears that some of the basic PRPs of HSMSL saliva may be derived by proteolytic processing, presumably before their exit from the salivary ducts.

The amino acid composition and the first 30 residues of the *N*-terminal sequence of pool D2 were identical with that reported for statherin (Schlesinger & Hay, 1977). When this statherin pool was subjected to Mono Q anion-exchange chromatography, it was found to be comprised of two components, designated D2a and D2b, which were eluted at 0.15 and 0.18 M-NaCl respectively [Fig. 8(c); see inset for anionic-PAGE of these materials]. The amino acid profiles of D2a and D2b were comparable (Table 5) and the sequences of their *N*-terminal 35 residues were identical. Analysis of alkaline phosphatase-treated D2 by f.p.l.c. indicated that the dephosphorylated statherins now had anionic mobilities that were less than the intact components (Fig. 8c). These data indicated that the charge variation, as deduced from closely migrating but distinct peaks on f.p.l.c. (Fig. 8c), was not due to differences in phosphate content. It is likely that the variation between these two statherin isoforms may be due to differences in their *C*-terminal sequences. Indeed, a study by Jensen *et al.* (1991) has identified an isoform of statherin which lacks the *C*-terminal phenylalanine residue.

In summary, we have described an integrated protocol for the efficient purification of the mucins and major phosphoproteins from HSMSL saliva. In general, this protocol was derived from procedures used by several different laboratories for the purification of individual salivary components. It uses an initial

step of gel filtration under dissociating conditions which minimizes heterotypic complexing among molecules. Final purification was achieved by conventional ion-exchange chromatography and/or f.p.l.c. As part of this protocol, we report that the smaller human salivary mucin, MG2, exhibits microheterogeneity in its oligosaccharide chains. Two isoforms have been identified which differ primarily in their content of the terminal sugars, sialic acid and fucose. We also report that acidic salivary cystatins can show various degrees of phosphorylation. The first phosphorylation site appears to be serine at position 3. Further work is required to locate definitely the other sites of phosphorylation. Two isoforms of a basic PRP have been purified; one of these represents a truncated form of a previously described basic PRP which is probably derived by proteolytic processing. In general, the multiple isoforms described in this study appear to be the result of post-translational modifications in glycosylation, phosphorylation and proteolytic processing.

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