Purification and Characterization of Monkey Salivary Mucin*

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Highly purified mucin was prepared from monkey (Macaca arctoides) extraparotid saliva by sequential chromatography on Sephadex G-200 (followed by reduction and alkylation of void volume materials), Sepharose CL-2B with 6 M urea, and CM52 cellulose with 6 M urea. Purity was critically ascertained by anion exchange chromatography, ultracentrifugal analysis, isoelectric focusing, sodium dodecyl sulfate-polyacrylamide electrophoresis, and crossed immunoelectrophoresis. Use of crossed immunoelectrophoresis to examine mucin preparations has not been previously reported. This technique was useful for assessing purity and displaying charge and size microheterogeneity in the purified N-carboxymethylated mucin. Threonine and serine comprised 37.8% of the total amino acids while the oligosaccharide moiety contained N-acetylgalactosamine, N-acetylgalactosamine, fucose, galactose, N-acetylneuraminic acid, and sulfate. Following alkaline borohydride treatment, the carbohydrate chains were found to be linked O-glycosidically between N-acetylgalactosamine and threonine (serine).

In the past, it has been difficult to isolate and assess the purity of human salivary mucins because of their large molecular weight, high viscosity, and poor solubility in aqueous solvents. Methods employed to increase mucin solubility include boiling (1, 2), proteolysis in acidic conditions (3), and precipitation with quaternary ammonium salts (4, 5). Mucin preparations so obtained were of questionable purity and may not represent the molecule as it exists in its native stage. Its close phylogenetic relationship makes the monkey a useful model for studying the oral ecology of man (6). The present study describes methods developed for the isolation of mucin from monkey (Macaca arctoides) extraparotid saliva. The mucin preparations obtained were highly purified when examined by several immunological and physical criteria. The methodologies described can be utilized for the purification of human salivary mucins in a form suitable for characterizing the role of these molecules in disease.

EXPERIMENTAL PROCEDURES

Materials

Sephadex G-200 and Sepharose CL-2B were obtained from Pharmacia Fine Chemicals. DE52 and CM52 were purchased from Whatman. Agarose (Industries A-45) was obtained from Fisher Scientific. The following were obtained from Sigma Chemical Co.: neuraminidase (Clostridium perfringens, type VI), Tris, sodium dodecyl sulfate (SDS), sodium borohydride, N-ethylmaleimide, phenylmethylsulfonfyl fluoride, bovine submaxillary mucin, phosphorylase a, catalase (bovine liver), ovalbumin, and myoglobin. Human IgG was obtained from Miles Laboratories. Bovine serum albumin was obtained from Schwarz/Mann. Chymotrypsinogen was obtained from Worthington Biochemicals. Iodoacetic acid was obtained from Eastman Organic Chemical and recrystallized with chloroform prior to use. Freund’s complete adjuvant was obtained from Difco Co. Unless otherwise noted, other chemicals were of highest reagent grade and purchased through commercial sources.

Methods

Analytical Procedures

Protein was determined by the method of Lowry et al. (7) and neutral sugars were determined by the anthrone reaction (8). Amino acid analyses were performed on a Beckman model 120C amino acid analyzer after hydrolysis with constant boiling HCl (1 to 2 mg in 4 ml) at 105°C for 28 h under a nitrogen atmosphere. Serine and threonine values were extrapolated to zero time following hydrolysis of samples for 24, 48, 72, and 96 h. Total protein was calculated from peptide residue weights. For the determination of neutral sugars and hexosamines, samples were hydrolyzed in 2 N HCl for 6 h at 100°C in sealed tubes. The hydrolysates were passed through coupled columns of Dowex 50-X4-H⁺ (200 to 400 mesh), and Dowex 1-X8 formate (200 to 400 mesh). Neutral sugars in the effluent wash were taken to dryness by lyophilization and then quantitated by means of automated borate-complex anion exchange chromatography as modified by Lee et al. (9). The amino sugars were eluted from the Dowex 50 columns with 2 N HCl and quantitated on the amino acid analyzer. Neutral sugar and hexosamine values were corrected for losses during hydrolysis. Galactosaminol was prepared according to the methods of Ciuffo (10) and quantitated on the amino acid analyzer with 0.35 M citrate buffer, pH 5.20, containing 0.3 M boric acid (11). Sialic acids released by hydrolysis in 0.1 N sulfuric acid at 80°C for 1 h or by neuraminidase treatment (12) were measured by the thioarbituric acid assay (13). For sulfate analyses, samples were hydrolyzed in 4 N HCl at 100°C for 22 h. Following hydrolysis, insoluble “humin” material was removed by low speed centrifugation and the sulfate separated from amino acids by passage of the hydrolysates through columns of Dowex 50-X4-H⁺ (200 to 400 mesh) with distilled water. Sulfate in the effluent wash was determined by the barium chloromolate procedure (14). Sulfate standards (K₂SO₄) analyzed under these experimental conditions gave values similar to the untreated standards.

Collection and Handling of Monkey Extraparotid Saliva (EPS)

Three adult female stump-tail monkeys (Macaca arctoides) with blood and secretor group B activity were used as saliva donors. Animals were maintained and extraparotid saliva collected as we have recently described (12) whereby handling of the viscous pilocarpine-stimulated saliva was facilitated by a 1:10 dilution with cold saline. This enabled removal by centrifugation of the majority of glycosidase activity associated with bacterial and cellular debris.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; EPS, extraparotid saliva; PAS, periodic acid-Schiff; IgM, immunoglobulin M.

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Separate experiments were performed to determine the extent of protease activity during collection and handling of EPS. Extraparotid saliva was collected into chilled tubes containing 2 ml of 2% Na$_2$EDTA, 5 ml of 0.1 M Tris/acetate, pH 7.8, and 3 mg/ml each of N-ethylmaleimide and phenylmethylsulfonyl fluoride. These secretions were diluted and centrifuged as previously described (10). The clarified saliva containing protease inhibitors was desalted on columns (5 × 85 cm) of Sephadex G-25 (fine), equilibrated with 0.1 M pyridine/acetate buffer, pH 6.0, at 4°C. Fractions of 20 ml were collected at room temperature at a flow rate of 20 to 30 ml/h. Tubes were pooled as indicated in Fig. 1 and lyophilized.

Sephadex G-200 filtration with EPS collected without protease inhibitors. (See following section for details.)

**Purification of Monkey EPS**

**Gel Filtration on Sephadex G-200**—lyophilized materials, approximately 700 mg, were reconstituted at 30 mg/ml in 0.1 M pyridine/acetate buffer, pH 6.0, and stirred gently overnight at 4°C. The sample was clarified by centrifugation at 5000 g for 30 min at 4°C and was applied to a column (5 × 85 cm) of Sephadex G-200 equilibrated with 0.1 M pyridine/acetate, pH 6.0. Fractions of 20 ml were collected at room temperature at a flow rate of 20 to 30 ml/h. Tubes were pooled as indicated in Fig. 1 and lyophilized.

**Sephadex CL-2B Filtration of Peak A from Sephadex G-200**—The excluded materials (Peak A) from Sephadex G-200 (20 to 50 mg) were subjected to gel filtration chromatography on columns (1.5 × 85 cm) of Sephadex CL-2B utilizing three separate conditions: 1) elution with 0.1 M pyridine/acetate buffer, pH 6.0; 2) elution with dissociating conditions of 6.0 M urea in 0.1 M pyridine/acetate, pH 6.0; and 3) elution as in Condition 2 after reduction and alkylation (12) of Sephadex G-200 Peak A. Samples were dissolved at a concentration of 10 mg/ml in equilibrating buffer. Fractions of 2.3 ml were collected at room temperature at a flow rate of 2.5 to 3.0 ml/h. Fractions were pooled as indicated in Fig. 2 and either lyophilized directly (Condition 1) or dialyzed extensively against distilled water and then lyophilized.

**Ion Exchange Chromatography**—The high molecular weight Peak III a (Fig. 2, bottom) was further fractionated on columns (2.4 × 85 cm) of CM52 microgranular cellulose by a stepwise elution consisting of 0.1 M sodium phosphate, pH 7.0, with 6.0 M urea followed by 1.0 M sodium chloride in the initial buffer. Samples dissolved to 20 mg/ml in initial buffer were eluted into fractions of 4.6 ml at room temperature at a flow rate of 20 ml/h. Elution was monitored at 230 nm. Fractions were pooled as indicated in Fig. 5 (top), dialyzed extensively against distilled water, and lyophilized.

**Materials which were not absorbed onto CM-cellulose (peak IIIa, Fig. 5, top) were chromatographed on columns (1.5 × 12.5 cm) of DE55 microgranular cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.0. The sample (9 mg/ml) was eluted using a gradient consisting of 150 ml of equilibrating buffer and 150 ml of 3.0 M sodium chloride in 0.01 M sodium phosphate, pH 7.0, with 1.0 M urea. Fractions of 1.67 ml were collected at room temperature at a flow rate of 26 ml/h. Elution was monitored at 230 and 280 nm. Fractions were pooled as indicated in Fig. 6 (bottom), dialyzed extensively against distilled water and lyophilized.

**SDS-Polyacrylamide Gel Electrophoresis**

Electrophoresis was carried out in gels of 3.0, 5.0, and 7.5% acrylamide following the procedures of Hudson and Spiro (15). Samples were prepared by incubation at 37°C for 2 h at a concentration of 2 mg/ml, lyophilized weight/ml in 0.1 M sodium phosphate buffer, pH 7.0, with 1% SDS and 1% (v/v) 2-mercaptoethanol. Glycoproteins were fixed and stained by Coomassie blue and periodic acid-Schiff (PAS) reagents (16). Molecular weights were calculated for salivary glycoproteins from plots of the log molecular weight versus relative mobility of standard proteins (17). The molecular weights employed for the standards were: unreduced IgG, 147,000; phosphorylase a, 94,000; bovine serum albumin, 66,000; catalase, 60,000; ovalbumin, 46,000; chymotrypsinogen, 25,700; myoglobin, 17,200.

**Preparation of Antisera**

Antisera to monkey EPS and monkey serum were prepared in goats by previously described immunization techniques using Freund’s complete adjuvant (18). Antisera to mucin Fraction IIIa (Fig. 2, bottom) were prepared in Hartley-Albino guinea pigs as follows. One milligram of mucin material was dissolved in 0.5 ml of 0.184 M NaCl and emulsified with an equal volume of Freund’s complete adjuvant. The mixture was equally distributed to four subcutaneous sites on the back and into each hind footpad. Two booster immunizations (1 mg each) at several new sites on the back were performed at 10 day intervals. Swollen footpads were not reinjected. Preimmune and test bleedings were taken by cardiac puncture. Antisera were tested by immunoelectrophoresis against the mucin and intact EPS.

**Immunological Procedures**

Immunoelectrophoresis and immunodiffusion were performed as previously described using slides containing 0.8% agarose (Indubiose A-45) in barbital/acetate buffer, pH 8.2 (18). Electrophoresis was conducted at 100 V (approximately 3 mA/slide) for 2 h. After incubation and washing, slides were stained with a solution of 0.025% Coomassie blue, 10% isopropanol alcohol, and 10% glacial acetic acid and destained with methanol/acetic acid/water (5:1:5).

Cropped and tandem crossed immunoelectrophoresis (19) and fused rocket immunoelectrophoresis (19) were performed on an LKB Multiphor apparatus equipped with a water cooling plate (2-4°C). The first dimension of crossed immunoelectrophoresis was carried out in 1% agarose gel with 0.02 M barbital/Tris/glycine, pH 8.6, at 10 V/cm gel for approximately 30 min. Electrophoresis in the second dimension was carried out at 4 V/cm gel for 12 h in 1% agarose containing either 1:40 or 1:100 dilution of antiserum. For fused rocket immunoelectrophoresis, 5-150 ng aliquots from isoelectric focusing fractions were placed in wells cut from 1% agarose prepared in barbital/Tris/glycine buffer. Samples were allowed to diffuse at 4°C for 60 min after which 1% agarose containing a 1:10 dilution of antiserum was poured and permitted to gel. Electrophoresis was carried out for 12 h at 4 V/cm gel. After each procedure, plates were air-dried, washed with 0.154 M NaCl, then distilled water, stained, and destained as described above.

**Isoelectric Point Determination**

pl was determined using an LKB model 8101 110-ml electrofocusing column. Mucin (3.8 mg of Peak IIIa-1, Fig. 5, top) was applied to a glycerol gradient from 87% to 0% containing 4 M urea and focusing was performed between 2-4°C with 2% Bio-Lyte carrier solution (Bio-Rad), pH 3 to 10. Voltage was adjusted to maintain a wattage between 3.6 and 4.8 for 96 h after which the sample was eluted into 2-ml fractions and the pH measured. Each fraction was then dialyzed extensively against distilled water and protein localized by absorbance at 230 nm and fused rocket immunoelectrophoresis.

**Ultracentrifugal Analysis**

Sedimentation velocity measurements were made in a Beckman model E analytical ultracentrifuge, equipped with an analytical H rotor, 12 mm 2° sector standard cells, and schlieren optics. At 50.8 × 10$^6$ rpm, photographs were taken at 4, 14, 34, and 46 min at angles of 60°, 50°, 50°, and 40°, respectively. Purified mucin (Fig. 5 Peak IIIa-1), 10 mg/ml in 0.01 M sodium phosphate buffer, pH 6.0, with 2.0 M sodium chloride, and several dilutions were analyzed for homogeneity.

**Characterization of Glycopeptide Linkage**

Mucin Fraction IIIa-1 (Fig. 5, 19.8 mg) was made 4 mg/ml with 1.0 M sodium borohydride in 0.1 M sodium hydroxide. After incubation at 37°C for 60 h, the solution was titrated slowly on ice with glacial acetic acid to pH 4.0. Excess BH$_4^-$ was converted to borric acid by a 10 fold addition of 0.1 N formic acid. Neutral and acidic oligosaccharides were then separated from peptides by batchwise elution through columns (2.4 × 0.1 cm) of Dowex 50-X4 H$^+$ (200 to 400 mesh) with 5 to 6 column volumes of cold 0.01 N formic acid. Following lyophilization, boracic acid in the oligosaccharide fraction was volatilized as methyl borate on a rotary evaporator by repeated additions of methanol. Peptides were eluted from Dowex 50 with 1.0 M pyridine/acetate, pH 5.1, and lyophilized. Samples were then analyzed to determine the extent of alkaline cleavage.

**RESULTS**

**Collection of Monkey Extraparotid Saliva**

Extraparotid saliva collected with and without protease inhibitors gave similar elution profiles on Sephadex G-200. No appreciable difference in the relative amino acid and carbohydrate compositions of the void volume fractions from these Sephadex G-200 columns was detected.
Purification of Mucin

Gel Filtration on Sephadex G-200 and Sepharose CL-2B—Fig. 1 shows the elution profile following chromatography of extraparotid saliva on Sephadex G-200. The recoveries of neutral sugar and protein were 102% and 97.2%, respectively. Peak A contained 172, 110, 194, 190, 15, 207, 274, 82, and 85 residues per 1000 amino acids of threonine, serine, N-acetylglucosamine, N-acetylgalactosamine, mannose, fucose, galactose, sialic acids, and sulfate, respectively. This composition identified its mucin-like character. Examination of Peak A by SDS-7.5% polyacrylamide gel electrophoresis revealed intense PAS staining at the origin and additional glycoproteins with estimated molecular weights of 107,000, 100,000, and 82,000.

Cross-linked Sepharose 2B was utilized to further resolve the glycoprotein components in Sephadex G-200 Peak A. Initial chromatography without dissociating conditions resulted in retarded elution and inadequate resolution of high molecular weight mucins (Ia) (Fig. 2, top) from smaller glycoproteins. Elution with 6 M urea (Fig. 2, middle) showed separation of a mucin fraction, IIa, from lower molecular weight proteins Ic. Fraction IIa eluted 20.7 ml before Ia, reflecting a diminished interaction with the gel matrix in the presence of 6.0 M urea. When Sephadex G-200 Peak A was reduced and alkylated and chromatographed as per Condition 2, additional disulfide-cleaved glycoproteins (IIIb) were separated from the high molecular weight mucin (IIa) (Fig. 2, bottom). A quantitative recovery of protein (82.1%) and neutral sugars (96.9%) was obtained using chromatography Condition 3 while recoveries from Conditions 1 and 2 were considerably less (Table I).

Fractions Ia, IIa, and IIIa were compared by immunodiffusion with goat anti-EPS. A single antigen was detected in Fraction IIIa which was immunologically identical with one of two antigens found in Fractions Ia and IIa.

Examination of Fractions Ia, IIa, and IIIa by SDS-5% polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol is shown in Fig. 3 A to C. Each displayed PAS-positive materials that neither penetrated the gel nor stained appreciably with Coomassie blue. Additional glycoprotein components were detected in Ia and IIa. The smaller disulfide-linked components of IIa possessed mobility (180,000 to 250,000 estimated molecular weights) similar to the components found in Fraction IIIb. When IIa was examined in the absence of 2-mercaptoethanol, no PAS-positive components penetrated the gel. As Ia and IIa are immunologically related, Ia likely represents a mucin whose larger and smaller disulfide-linked subunits could be separated into IIa and IIIb following reduction and alkylation.

Table I compares the percentage compositions of mucin-containing peaks Ia, IIa, and IIIa. The carbohydrate (neutral sugars plus hexosamines) to protein ratios for Ia, IIa, and IIIa were 2.2, 2.4, and 3.4, respectively. The peptide moiety of these glycoproteins was increasingly enriched in hydroxyamino acids and depleted of aromatic amino acids as the chromatographic conditions were changed from 1 to 3. Residues of threonine plus serine per 1000 total amino acids were 283, 333, and 382 while tyrosine plus phenylalanine were 37, 21, and 9, respectively in Ia, IIa, and IIIa. These properties identified the increasingly mucin-like character of the fractions obtained by Conditions 1, 2, and 3, respectively.

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**Table I**

| Condition | Protein% | Neutral sugar% |
|-----------|----------|----------------|
| 0.1 M pyridine/acetate buffer, pH 6.0 | 38.5 | 65.0 |
| 6.0 M urea in 0.1 M pyridine/acetate buffer, pH 6.0, following reduction and alkylation of Peak A | 34.0 | 49.5 |

a Determined by the Lowry method (7).

b Determined by the anthrone reaction.
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Fig. 3. SDS-polyacrylamide gel electrophoresis of mucin fractions. Samples were prepared as described in the text. Total carbohydrate, 40 to 60 μg, was applied to each gel. Gels A to C represent Ia, IIa, and IIIa, respectively (5% acrylamide), while Gels D and E represent IIIa and IIIa-1 (3% acrylamide). Electrophoresis was carried out for 6 to 7 h at 8 mA/gel. Gels were stained with PAS.

Table II
Percentage composition of mucins obtained by Sepharose CL-2B gel filtration

| Component       | Ia  | IIa | IIIa |
|-----------------|-----|-----|------|
| Protein*        | 22.8| 18.7| 18.7 |
| N-Acetylglucosamine | 13.1| 13.1| 15.3 |
| N-Acetylgalactosamine | 10.8| 10.8| 12.5 |
| Mannose         | 0.6 | 0.2 | 0.2  |
| Fucose          | 10.2| 9.5 | 13.9 |
| Galactose       | 14.6| 11.7| 18.6 |
| Sialic acids    | 7.1 |     |      |
| Sulfate         |     |     | 6.6  |

* Calculated from peptide residue weights.

The purity of mucin Peak IIIa was assessed by attempting to prepare a monospecific antiserum in guinea pigs. Fig. 4 shows the reaction of guinea pig anti-IIIa and goat anti-EPS with intact extraparotid saliva and the mucin. With each antiserum, the mucin displayed a characteristic acidic immunoprecipitate. However, the guinea pig antiserum now revealed an additional unrelated cationic antigen in intact extraparotid saliva and mucin IIIa (see arrows, Fig. 4).

CM-Cellulose Chromatography of Mucin Peak IIIa—Mucin was separated from the cationic antigen by fractionating Peak IIIa on CM52 cellulose (Fig. 5, top). A single acidic peak, IIIa-1, was obtained upon elution with the equilibrating buffer. Subsequent stepwise elution with 1.0 M sodium chloride in equilibrating buffer gave a cationic peak, IIIa-2. IIIa-1 and IIIa-2 comprised 95 and 5%, respectively, of the peptide residue weight protein of Fraction IIIa. Immunoelectrophoretic examination of IIIa-1 and IIIa-2 with the guinea pig antiserum revealed that the acidic mucin and cationic antigen had been separated.

Fig. 4. Immunoelectrophoretic analyses of extraparotid saliva and mucin Fraction IIIa with goat anti-EPS and guinea pig anti-IIIa. Anode is to the right.

Fig. 5. Top, chromatography on CM52 cellulose of mucin Fraction IIIa (Fig. 2 bottom). The sample, containing 32 mg, was applied to a column (2.4 × 25 cm) in 0.01 M sodium phosphate buffer with 6.0 M urea at pH 7.0. After elution with this buffer, a stepwise gradient (Fraction 100) was applied as described in the text. Lettered areas designate fractions which were pooled, dialyzed, and lyophilized. Bottom, chromatography on DE52 cellulose of mucin Fraction IIIa-1 (Fig. 5, top). The sample, containing 9 mg, was applied to a column (1.5 × 12.5 cm) in equilibrating buffer of 0.01 M sodium phosphate with 6 M urea, pH 7.0. Elution was carried out using a linear gradient as described in the text.
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Evaluation of Purity of Mucin Preparation

Mucin preparation IIIa-1, which contained a single antigen as identified by the guinea pig antiserum, was further evaluated for purity by DEAE-cellulose chromatography, SDS-polyacrylamide electrophoresis in 3% gels, ultracentrifugal analysis, isoelectric focusing, and crossed immunoelectrophoresis.

Chromatography on DE52 using a continuous gradient in 6 M urea is shown in Fig. 5, bottom. A single sharp peak eluted between 0.2 and 0.3 M NaCl, suggesting minimal charge heterogeneity under these experimental conditions.

Comparison was made between mucins IIIa and IIIa-1 by SDS-3% polyacrylamide gel electrophoresis (Fig. 3, D and E). Both materials penetrated the 3% gels. Mucin IIIa-1 penetrated the gels farther than mucin IIIa, suggesting that IIIa-1 and IIIa-2 remain associated in this electrophoretic system. Both IIIa and IIIa-1 appeared to contain two mucin species. The higher molecular weight component in Fraction IIIa-1 had an $R_F$ identical with bovine submaxillary mucin examined under similar conditions.

Sedimentation velocity studies were performed in 0.01 M sodium phosphate buffer, pH 6.0, with 2.0 M NaCl. Serial dilutions of a 10 mg/ml solution were examined, with 1.25 mg/ml being the smallest concentration for which a defined peak was seen. At these concentrations, schlieren patterns revealed a single sharp symmetrical peak (Fig. 6).

Isoelectric focusing revealed a peak between pH 1.82 and 1.88 (Fig. 7). A reaction of identity among every fraction in this peak was demonstrated by fused rocket immunoelectrophoresis using goat anti-EPS. No additional components were detected in any fraction having a pH greater than 1.88. The low isoelectric point may in large part be attributed to the presence of sialic acid and sulfate.

Crossed and tandem crossed immunoelectrophoresis were utilized to evaluate the purity of the mucin preparations and assess the effects of S-carboxymethylation and neuraminidase treatment on the mucin's interaction with antisera. The reaction of mucin IIIa-1 with goat anti-EPS and guinea pig antiserum is shown in Fig. 8, A and D. The goat antiserum revealed a single component while the guinea pig antiserum detected two immunologically related components (designated X and Y). Contamination of IIIa-1 with the lower molecular weight fraction, IIIb, (Fig. 2, bottom) could also be detected by this technique (Fig. 8G) where an additional component Z was immunologically cross-reactive with X. These data were con-
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The chemical composition and physical properties of mucin preparation IIIa-1 are given in Table III. Of the total weight, 62.0% was accounted for. This relatively low recovery may reflect losses occurring through interactions between amino acids and sugars during acid hydrolysis conditions (21). Threonine, serine, proline, glycine, and alanine made up 68.9% of the total amino acid residues. Approximately 4 residues of N-acetylglucosamine (267), N-acetylgalactosamine (227), fucose (216), galactose (283), and N-acetylneuraminic acid (153 residues/1000 amino acid residues) were present.

The carbohydrate moiety contained N-acetylglucosamine (267), N-acetylgalactosamine (227), fucose (216), galactose (283), and N-acetylneuraminic acid (153 residues/1000 amino acid residues). Approximately 183 residues of sulfate were present. The percentage of total EPS protein, carbohydrate, and sulfate found in mucin preparation IIIa-1 is given in Table IV.

While the mucin only contained 1% of the total protein in intact EPS, it contains a considerable amount of the total carbohydrate and sulfate.

Nature of Glycopeptide Linkages

Following alkaline borohydride treatment, oligosaccharides could be separated from peptides by passage through Dowex 50 columns. The recoveries of protein and component sugars are given in Table V. The majority of sugars and only a trace of protein were eluted from Dowex 50 with 0.01 N formic acid. When the Dowex columns were next eluted with 1 M pyridine/acetate, pH 5.0, 56.5% of the starting protein was recovered, as calculated from peptide residue weights. Control experiments where protein determinations were made on alkali borohydride-treated mucin prior to Dowex chromatography gave comparable results. Correcting for this recovery, the percentage conversion of threonine and serine to γ-aminobutyric acid and alanine was 41.7%. Approximately 86% of the recoverable galactosaminol was converted to galactosaminol.

Preliminary gel filtration studies on Sephadex G 25 of the Dowex 50 eluate yielded a major oligosaccharide fraction (Fig. 9, Peak 2) with a molar ratio of sugars to galactosaminol of 1.1, 1.4, 2.1, and 0.5 for N-acetylglucosamine, fucose, galactose, and N-acetylneuraminic acid, respectively. Sulfate was not determined. Based upon a single residue of galactosaminol, this oligosaccharide fraction had an average calculated minimum molecular weight of 1080.

| Component | Intact mucin | Alkaline borohydride-treated mucin |
|-----------|-------------|----------------------------------|
| Total protein | 40.2 | 0.7 |
| Threonine | 8.4 | 0.06 |
| Serine | 5.5 | 0.08 |
| α-aminobutyric acid | | 1.5 |
| Alanine | 3.6 | 0.1 |
| N-acetylgalactosaminol | 8.1 | 0.6 |
| N-acetylglucosaminol | 10.0 | 5.4 |
| Fucose | 8.7 | 7.4 |
| Galactose | 11.8 | 10.8 |
| N-acetylneuraminic acid | 2.2 | 2.2 |

* Actual recovery was 101%.

Chemical Composition and Physical Properties of Purified Monkey Mucin

TABLE III

| Component | Mucin residues/1000 amino acid residues |
|-----------|---------------------------------------|
| Aspartic acid | 25 |
| Threonine | 222 |
| Serine | 156 |
| Glutamic acid | 69 |
| Proline | 59 |
| Glycine | 167 |
| Alanine | 85 |
| Half-cystine | 3.5 |
| Valine | 70 |
| Methionine | 1.2 |
| Isoleucine | 30 |
| Leucine | 37 |
| Tyrosine | 3.5 |
| Phenylalanine | 9.4 |
| Lysine | 19 |
| Histidine | 16 |
| Arginine | 27 |
| N-Acetylglucosamine | 267 |
| N-Acetylglucosaminol | 227 |
| Mannose | ND |
| Fucose | 216 |
| Galactose | 283 |
| N-Acetylneuraminic acid | 153 |
| Sulfate | 183 |

* Extraplolar to zero hydrolysis.

* As N-carboxymethylcysteine.

* ND, not detected.

* Identified by descending paper chromatography using n-butyl alcohol: n-propyl alcohol: 0.1 N HCl (1:2:1, v/v) (22).

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Our previous studies (23) have shown a marked similarity in the biochemical and immunological characteristics of monkey and human salivas. These findings, coupled with a greater yield of lyophilized materials from monkey saliva, made this secretion an excellent model to study mucin purification. We report here the isolation of highly purified monkey salivary mucins by procedures which minimized endogenous protease and glycosidase degradation during handling and exogenous degradation during purification. Such precautions are necessary in order to obtain molecules suitable for biochemical and functional characterization.

During the progress of these studies, several reports have appeared which utilized gel filtration techniques and disulfide bond cleavage as a means of preparing mucin from human sputum (24) and aspirated tracheobronchial secretions (25). Purity of these preparations was not rigorously ascertained. In addition, the presence of leukocytic proteases and hydrolases in human sputum (26) hampers the isolation of undegraded products from this fluid. In our studies, mucin was isolated by sequential gel filtration on both Sephadex G-200 and cross-linked Sepharose 2B, with the latter performed using dissociating conditions. Quantitative recoveries were obtained by chromatography on Sephacryl CL-2B in 6 M urea after reduction and alkylation of the mucin-like materials from Sephadex G-200. This was in contrast to yields obtained without prior cleavage of disulfide bonds where mucin materials either clogged or interacted with the gel matrix. Reduction and alkylation also simplified removal of immunoglobulin contaminants since this procedure resulted in the dissociation and subsequent separation of light and heavy chain subunits (cf. IgG marker, Fig. 2 bottom) by gel filtration. Tests of purified mucin with goat anti-mucin serum substantiated these findings.

Interestingly, disulfide bond cleavage produced a lower molecular weight component (IIIb) with mucin-like composition which was immunologically identical with the high molecular weight mucin. This material comprised 40.2% of the protein and 34.9% of the neutral sugar recovered from the Sepharose column. Our finding of negligible protease and glycosidase activity during the handling of saliva suggests that these fractions represent authentic biosynthetic subunits and are not the products of enzymatic degradation.

In the present studies, purity of the mucin preparation was evaluated by several criteria. Homogeneity was displayed by ultracentrifugation in high ionic strength buffer, anion exchange chromatography on DEAE-cellulose in urea, and isoelectric focusing in urea. Thus, charge heterogeneity was not apparent utilizing conditions which promote dissociation of noncovalent interactions. In addition, enzymatic removal of sialic acid and subsequent immunoelectrophoretic analysis revealed no change in the mobility of the mucin immunoprecipitate. However, charge microheterogeneity was evidenced when intact and desialized mucin were compared by crossed immunoelectrophoresis (Fig. 5, II and I) where removal of this sugar resulted in a more symmetrical precipitin pattern.

Although finding two glycoprotein components by SDS-gel electrophoresis suggested size heterogeneity of the mucin preparation, the anomalous behavior of highly glycosylated molecules in SDS gel systems makes this interpretation uncertain (27). Recently, Silverberg and Marchesi (28) have shown that the electrophoretic behavior in SDS gels of the majorialoglycoprotein from human erythrocytes depended on protein concentration, SDS concentration, and buffer ionic strength composition. Our data do not exclude the possibility that the electrophoretic patterns observed with the mucin resulted from the electrophoresis conditions. Stronger evidence for size heterogeneity was obtained by crossed immunoelectrophoresis with the guinea pig antiserum (Fig. 8D). As described previously, the guinea pig antiserum possessed at least two specificities directed against the S-carboxymethylated mucin. In the second dimension of crossed immunoelectrophoresis, larger mucin molecules possessing more antibody combining sites precipitated earlier during electrophoresis resulting in a smaller peak (Y) while smaller molecules precipitated later into a larger peak (X). Similar findings have been obtained by crossed immunoelectrophoresis when comparing polymeric 19 S IgM with its 7 S subunits (29). Further, the two mucin components (X, Y) so visualized did not result from antiserum protease activity since mucin that was not S-carboxymethylated revealed only a single component.

Our results re-emphasize the value of examining purified salivary material using antisera prepared in more than one species (18, 30). The guinea pig antiserum detected a cationic contaminant and revealed size heterogeneity in the S-carboxymethylated mucin preparation, properties which were not displayed in reactions with the goat antiserum. In contrast, the goat antiserum was useful during initial purification steps and helped identify charge heterogeneity. Guinea pigs were chosen for the preparation of mucin antiserum since our previous experience has shown that rabbits respond poorly when immunized with monkey and human salivary mucin. The monkey salivary mucin reported here has an amino acid composition not unlike mucins of ovine (31), bovine (32), and canine (33) submandibular glands; human sputum (24, 25) and saliva (6, 12); and monkey cervical mucus (34). In general, these mucins are enriched in threonine and serine and contain only small amounts of basic and aromatic amino acids. The amounts of aspartic acid, glutamic acid, proline, glycine, alanine, and leucine are variable. The monkey salivary mucin which possessed blood group B activity as determined by hemagglutination inhibition assays, had a carbohydrate...
composition similar to monkey cervical mucin (34) and contained oligosaccharides linked $\alpha$-glycosidically through $N$-acetylgalactosamine and threonine (serine).

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