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Structure of Canine Tracheobronchial Mucin Glycoprotein†

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ABSTRACT: Canine tracheal mucin glycoprotein was isolated from beagle dogs fitted with tracheal pouches. Following exclusion chromatography on Sepharose CL-4B, noncovalently associated proteins were further resolved by dissociative density gradient centrifugation in CsBr–guanidinium chloride, and the mucin was then extracted with chloroform–methanol. The delipidated high-density product obtained had a nominal molecular weight of about 10^6 and an overall composition characteristic for a mucin glycoprotein, viz., a high content of serine and threonine, about 80% carbohydrate by weight, the absence of mannose or uronic acid, measurable ester sulfate, and a Pronase-resistant domain of molecular weight $(1.75–3.0) \times 10^5$ which contains essentially all of the saccharide residues. Noncovalently bound lipid amounted to 6–10% by weight and was primarily cholesterol and cholesteryl esters. Cleavage of disulfide bonds by performic acid oxidation resulted in the release of a protein (M_r 65 000) not otherwise resolved by sodium dodecyl sulfate gel electrophoresis or the purification scheme.

The biological functions of upper respiratory tract secretions are critically dependent on their physical properties. For example, the effective functioning of the mucociliary transport system depends on the optimal viscosity and surface tension of the mucus secretion. The major component responsible for the desired properties of the mucus secretion is a single complex mucin glycoprotein. This macromolecule has binding domains for passive clearance of both protein and lipid and may also function as a ligand (via its saccharides) for cell surface receptors of microorganisms. Many previous studies have focused on the mucin isolated from the bronchial secretions of patients with cystic fibrosis or asthma (Chernick & Barbero, 1959; Feldhoff et al., 1979; Lamblin et al., 1977; Rose et al., 1979; Roussel et al., 1975) or normal individuals hospitalized for nonpulmonary problems (Sachdev et al., 1980; Lafitte et al., 1977; Boat et al., 1976; Woodward et al., 1982).

A model system used for collection of normal tracheobronchial secretions involves introduction of a subcutaneous tracheal pouch in dogs. This experimental procedure permits routine collection of mucus samples free of contaminants that might arise from salivary glycoproteins (Wardell et al., 1970). This method has been used for the isolation of canine tracheal mucus for investigating the rheological properties (Litt et al., 1974a,b; Khan et al., 1976) and for isolating and biochemically characterizing the glycoprotein components (Sachdev et al., 1978; Liao et al., 1979).

It has been indicated that mucus secretions from the canine tracheal pouch system show similarities to the secretions obtained from human sources (Liao et al., 1979). This report details chemical and physical studies on the mucin glycoprotein of canine tracheal secretions.

Materials and Methods

Crude Mucus Collection and Solubilization. Beagle dogs fitted with tracheal pouches following the general procedure

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described previously (Wardell et al., 1970) were donated to us by Dr. Mitchell Litt of the University of Pennsylvania, Philadelphia, PA. Mucus was collected every 1–2 weeks by aspiration with a hypodermic syringe fitted with an 18-gauge needle or by saline injection, physical mixing, and aspiration. In general, about 10 mL of aspirates was obtained; samples which were cloudy or discolored were not further studied.

The collected mucus was dialyzed at 4 °C vs. deionized water containing 0.1 mM α-toluenesulfonyl fluoride (TSF)[1] and lyophilized. Resolubilization of the lyophilized material was effected by stirring for 72 h in 50 mM Tris-HCl, pH 8.0, containing 0.02% NaN_3, 0.1 mM TSF, and 6 M urea. The volume of buffer employed was such as to give a concentration of 5 mg/mL lyophilized mucus. Insoluble material, less than 5% of the neutral sugar, was removed by centrifugation for 20 min at 12000*g*.

Analytical Methods. Analytical exclusion chromatography was generally carried out on 1.5 × 50 cm columns of Sepharose CL-4B equilibrated with the solubilization buffer. Fractions were screened for neutral sugar by the phenol–sulfuric acid method (Dubois et al., 1956) and for protein by the absorbance at 280 nm.

Amino acid analyses were carried out by automated ion-exchange chromatography on a Dionex MBF/SS amino acid analyzer following hydrolysis in vacuo at 110° C for 24, 48, or 72 h with 6 N HCl. Cysteine was quantitated as cysteic acid either by hydrolysis in 6 N HCl containing 0.25 M dimethyl sulfoxide (Spencer & Wold, 1969) or by treatment with performic acid (Hirs, 1967) prior to hydrolysis. Glucosamine and galactosamine were analyzed on the amino acid analyzer after hydrolysis in vacuo for 8 h at 100 °C in 4 N HCl.

Quantitative analyses for carbohydrates other than amino sugars were by gas–liquid chromatography of the alditol acetates (Sawardekar et al., 1965) or trimethylsilyl derivatives (Clamp et al., 1971). Sialic acid was also separately estimated by the thiobarbituric acid method (Warren, 1959) or by HPLC (Silver & Karim, 1981) following acid hydrolysis or digestion with *Vibrio cholerae* neuraminidase. Sulfate was determined by the rhodizonate method following hydrolysis with 6 N HCl (Terho & Hartiala, 1971).

SDS–polyacrylamide gel electrophoresis was performed on a Hoefer Model 220 vertical slab gel unit. Gels were prepared according to the method of Haas and Kennett (1979) with several modifications. Separating gels, having an acrylamide concentration of 10% (w/v), and stacking gels, 5% (w/v) acrylamide, were prepared by dilution of a stock solution containing 30% acrylamide and 0.8% *N,N'*-methylenebis-(acrylamide). *N,N'*-Diallyltartardiamide was not used as a cross-linking agent due to gel instability during the subsequent periodic acid–Schiff staining procedure. Prior to electrophoresis, unless otherwise noted, the samples were incubated in buffer containing 2% (w/v) SDS for 3–5 min at 100 °C. After electrophoresis, gels were stained overnight with Coomassie blue, destained, and then thoroughly washed with 50% (v/v) methanol.

Silver staining (Wray et al., 1981) was subsequently performed on the gels which had been previously stained with Coomassie blue. Glycoproteins were then visualized on the same gel by staining with the PAS reagent (Fairbanks et al., 1971).

Amino-terminal group analysis was by the dansyl chloride procedure (Zanetta et al., 1976). Control experiments with proteins containing known NH_2 termini were done in parallel.

Alkaline borohydride elimination of O-linked saccharides was performed as previously described (Carlson, 1966).

Sedimentation equilibrium studies were performed in the indicated solvent in the Model E analytical ultracentrifuge using the meniscus depletion method (Yphantis, 1964). Samples were equilibrated with solvent by dialysis; solute concentrations were generally 2.0, 1.0, and 0.5 mg/mL. Molecular weights were calculated as described (Woodward et al., 1982); the partial specific volume employed was calculated from the amino acid and carbohydrate composition and was in agreement with the measured buoyant densities.

Radiolabeling of the glycoprotein was performed by (a) Chloramine-T-catalyzed iodination (Greenwood et al., 1963), (b) the Bolton–Hunter reagent (Bolton & Hunter, 1973), (c) reduction and alkylation using dithiothreitol and iodo[^{14}C]-acetamide (Sachdev et al., 1978), (d) carbodiimide-catalyzed amidation with [^{3}H]ethanolamine, and (e) reductive methylation with [^{3}H]HCHO (Jentoft & Dearborn, 1979).

Preparative Methods. A Sepharose CL-4B (5 × 95 cm) column equilibrated in solubilization buffer was used for preparative experiments. Routinely, 40 mL (200 mg) of crude material was fractionated at a flow rate of 100 mL/h; 21-mL fractions were collected and screened for neutral sugar by the phenol–sulfuric acid method and for protein by the absorbance at 280 nm.

Density gradient centrifugation was carried out for 72 h at 42000 rpm in a Beckman 60 Ti rotor at 14 °C at a nominal glycoprotein concentration of 1.5 mg/mL. The gradient solvent was 42% (w/w) cesium bromide and 16.7 mM sodium phosphate, pH 6.8, containing 4 M guanidinium chloride, 33 mM NaCl, and 0.02% (w/v) NaN_3 (Creeth et al., 1977). Samples were dissolved in this solvent by stirring at 4 °C overnight. Following centrifugation, 1-mL fractions were collected by aspiration from the bottom of the centrifugation tube. Neutral sugar and protein were analyzed by the phenol–sulfuric acid method and the absorbance at 280 nm, respectively. Density was measured for every fifth tube.

Isolated glycoprotein was delipidated by sequential extraction with chloroform–methanol (two extractions using a 2:1 mixture, followed by an extraction with a 1:2 mixture) as previously described (Woodward et al., 1982). Lipid components were resolved on a silicic acid column (Esselman et al., 1972) and identified by thin-layer chromatography.

Performic acid oxidation was carried out as described (Hirs, 1967). The performic acid oxidized mucin (PF_3) was reisolated by density gradient centrifugation. Reduction and alkylation were performed as previously described (Sachdev et al., 1978).

Pronase digestion of the mucin glycoprotein was performed for 72 h at 37 °C using 0.5 mg of enzyme per 10 mg of substrate (Feldhoff et al., 1979; Lamblin et al., 1977). The Pronase-resistant glycopeptides were isolated by chromatography on Sepharose CL-4B.

Results and Discussion

The initial purification step, exclusion chromatography, has been commonly employed for mucin glycoproteins (Feldhoff et al., 1979; Rose et al., 1979; Lafitte et al., 1977; Boat et al., 1976; Lamblin et al., 1979; LeTreut et al., 1981). Chromatography of the solubilized mucus on Sepharose CL-4B typically gave results as indicated in Figure 1. The major void

[1] Abbreviations: CTM, canine tracheal mucin; NeuNAc, *N*-acetylneuraminic acid; PAS, periodic acid–Schiff; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; TSF, α-toluenesulfonyl fluoride; HPLC, high-performance liquid chromatography; Gdn·HCl, guanidine hydrochloride; kDa, kilodalton(s); Me_2SO, dimethyl sulfoxide; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

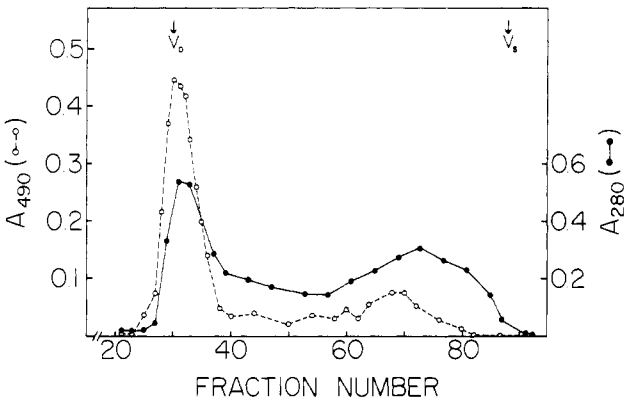

FIGURE 1: Chromatography of canine tracheal mucus on Sepharose CL-4B (5.0 × 95 cm). Crude mucus (200 mg) solubilized in 40 mL of 50 mM Tris, pH 8.0, containing 6 M urea, 0.02% NaN_3, and 0.1 mM TSF was loaded on the column and eluted with the same buffer at a flow rate of 100 mL/h. Fractions (21 mL) were collected and analyzed for neutral sugar (O) and protein (●).

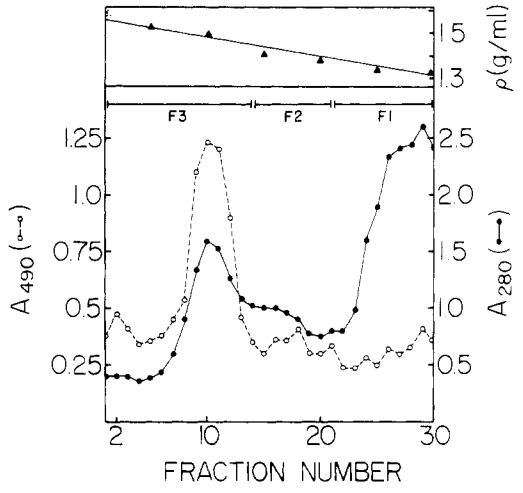

FIGURE 2: Density gradient centrifugation of the Sepharose CL-4B void volume material isolated as described for Figure 1. A solute loading concentration of 1–2 mg/mL was employed; solvent was 42% (w/w) CsBr in 16.7 mM sodium phosphate, pH 6.8, containing 4 M guanidinium chloride, 33 mM NaCl, and 0.02% NaN_3. Centrifugation was performed at 14 °C for 72 h at 42000 rpm in a 60 Ti rotor (Beckman Instruments). 1.0-mL fractions were collected by aspiration from the bottom of the tube; 50-μL aliquots were analyzed as in Figure 1; 100-μL aliquots of every fifth tube were weighed to define the densities. Fractions were pooled by density (in grams per milliliter) as indicated: F1 (1.30–1.40); F2 (1.40–1.45); F3 (1.45–1.60).

volume peak contained the bulk of the neutral sugar (62–84%) applied on the column. A broad, protein-rich, included fraction was always present as well. Although use of an exclusion matrix with higher porosity (e.g., Sepharose 2B, glyceryl-CPG 3000, Bio-Gel A 5m) allowed some penetration by the mucin glycoprotein, the bulk of the material was still excluded. Thus, additional component resolution by this approach appeared not to be feasible. The anticipated composition of the glycoprotein under study, approximately 80% carbohydrate by weight, allows selective fractionation by density gradient methods. Therefore, the material excluded from Sepharose CL-4B was further resolved by density gradient centrifugation under dissociative conditions (Woodward et al., 1982). Typical data for such an experiment are illustrated in Figure 2, and compositional analyses are presented in Table I. The high-density fraction (ρ = 1.5 g/mL; F3 in Figure 2) containing essentially all of the carbohydrate corresponds to the mucin glycoprotein while the lower density material (ρ = 1.3–1.4 g/mL; F1 in Figure 2) appeared to be a very heterogeneous

Table I: Compositional Analyses of Canine Tracheal Mucin Fractions[a]

amino acid	Sepharose CL-4B V_0	CsBr gradient F1	CsBr gradient F3
cysteic acid	63	51	41
aspartic/asparagine	84	95	70
threonine	94	69	147
serine	115	89	145
glutamic/glutamine	111	118	104
proline	74	59	89
glycine	101	79	85
alanine	70	79	61
valine	53	65	53
methionine	9	9	9
isoleucine	21	24	23
leucine	67	102	56
tyrosine	20	27	14
phenylalanine	26	34	20
histidine	22	22	22
lysine	36	45	31
arginine	34	33	30
saccharide			
fucose	4.1		15.2
galactose	5.1		20.5
GlcNAc	5.6		9.4
GalNAc	2.3		6.4
NeuNAc	0.4		1.8
sulfate			2.6

[a] Amino acid data are in residues per 1000 residues; saccharide and sulfate data are weight percent.

mixture of proteins and glycoproteins. Examination of the low-density components by gel electrophoresis showed a variety of proteins, all of which were included when rechromatographed on Sepharose CL-4B (data not shown). Thus, these components either are mechanically entrapped in the matrix created by the mucin glycoprotein or have specific associative interactions with it that are disrupted by the conditions employed for the gradient. The reactivity of the F1 components with anti-canine serum antibodies (Miles Scientific, lot R714) by Ouchterlony immunodiffusion (Williams & Chase, 1971) (data not shown) suggests that the majority of these are circulating proteins as in the case of the comparable human material (Woodward et al., 1982). It is not presently known if any specific products of the tracheal epithelial cells are present in this mixture, nor is there any evidence for specific associative behavior between an F1 component and the mucin glycoprotein (F3).

Previous results obtained from studies on human tracheobronchial material have shown the presence of noncovalently associated lipid in the high buoyant density fraction. Thus, the high-density canine fraction was examined for the presence of associated lipid by extraction with chloroform–methanol. The amount of lipid varied somewhat in different preparations but was generally about 10% by weight (6–11% in several isolates). The lipid material recovered from the organic phase was examined by thin-layer chromatography. The data summarized in Table II are representative of a single sample, but all isolates showed similar patterns. The major lipids are cholesterol and cholesteryl esters with less amounts of triglycerides, phospholipids, and glycolipids. Attempts to demonstrate specific reassociation by exposure of radiolabeled lipid to delipidated F3 (extraction with $CHCl_3$–CH_3OH) were not successful for comparable human material. Since the bound lipid could have affected aggregation, delipidated high-density fractions were reexamined by density gradient centrifugation and exclusion chromatography on Sepharose CL-4B. The results are shown in Figure 3 and Figure 4, respectively.

Table II: Lipid Analysis of Canine Tracheal Mucin[a]

class of lipid	wt %
cholesterol	50–65
cholesteryl esters	20–30
triglyceride	less than 1
phospholipid	3–6
lysophospholipid	2–5
glycolipids/gangliosides	less than 1
sulfatides	less than 1

[a] Data are ranges from eight separate analyses and are expressed as weight percent of the total lipid fraction isolated from CTM by extraction with chloroform–methanol (twice with 2:1 mixtures followed by once with a 1:2 mixture), concentrated by rotary evaporation, and resolubilized in either 2:1 chloroform–methanol or 1:1:0.15 chloroform–methanol–water.

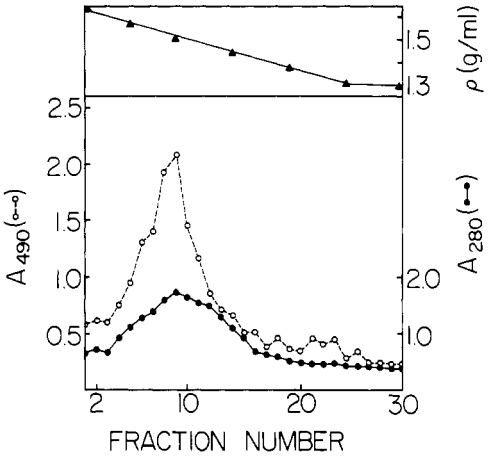

FIGURE 3: Density gradient centrifugation of high-density material (F3, Figure 2) following delipidation by extraction with chloroform–methanol. Details are as described in Figure 2.

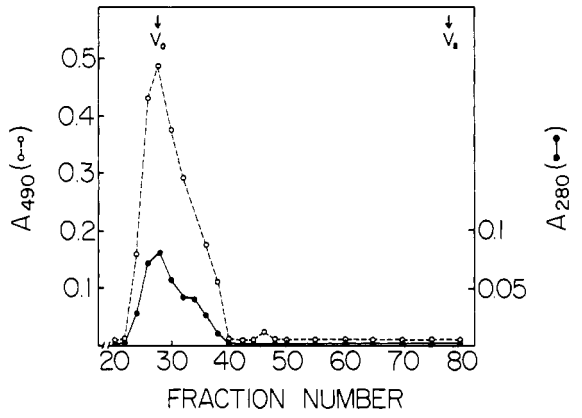

FIGURE 4: Sepharose CL-4B (1.5 × 50 cm) chromatography of delipidated high-density mucin (F3, Figure 2). Details are as described in Figure 1.

Except for a small shift in buoyant density, expected as a result of lipid removal, patterns were the same as those of the nondelipidated F3 material. It can be reasoned that a subsidiary property of the tracheal mucin glycoprotein is to provide nonspecific binding sites for lipid, thus facilitating the clearance of this class of molecules from the upper airway.

Examination of the density gradient fractions by gel electrophoresis gave the results shown in Figure 5. The high buoyant density component, identified as the mucin glycoprotein, apparently contains no material of lower molecular weight detectable by Coomassie blue or PAS staining. As expected for a component with a nominal molecular weight in the range of 10^6, penetration into the gel matrix is not detectable for any species reactive with Coomassie blue, silver nitrate, or PAS reagents.

Table III: Cysteine–Cystine Concentration of Canine Tracheal Mucin Glycoprotein[a]

cysteine–cystine	residues per 1000 residues		
total half-cystine	48–56[b]	56–60[c]	42[d]
free –SH	4.5		
reduced/alkylated	54		

[a] Data are presented for delipidated F3, the high-density mucin glycoprotein isolated after cesium bromide density gradient centrifugation. Total half-cystine was determined by amino acid analysis as cysteic acid; free –SH was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid), and reduced/alkylated was determined by amino acid analysis as (carboxymethyl)cysteine. [b] Analysis in the presence of Me_2SO. Range for eight separate samples. [c] Analysis after performic acid oxidation. [d] Analysis of high-density material isolated after a second density gradient containing 20 mM β-mercaptoethanol, fraction βF_3.

Table IV: Molecular Weights of Canine Tracheal Mucin Samples[a]

sample	A	B
Sepharose CL-4B void	$(1.0–2.4) \times 10^6$	$(1.2–1.3) \times 10^6$
delipidated F3	$(0.60–1.0) \times 10^6$	$(0.75–1.2) \times 10^6$
PF_3[b]		$(3.2–6.0) \times 10^5$
Pronase core[c]	$(1.8–3.0) \times 10^5$	

[a] Values from sedimentation equilibrium experiments performed in phosphate-buffered saline, pH 7.2 (column A), or 6 M urea in the same solvent (column B). From the shape of the curves, log c vs. r^2 (data not shown), it is evident that there is considerable size heterogeneity. Data are presented as a range of molecular weights. [b] Performic acid oxidized, delipidated F3, after reisolation by density gradient centrifugation. [c] Material isolated following Pronase digestion of delipidated F3 and chromatography on Sepharose CL-4B.

Equilibrium sedimentation experiments in both nondissociating and dissociating (6 M urea) buffer systems (Table IV) substantiate the heterogeneity and aggregation tendency of the Sepharose CL-4B void fraction, as well as the high molecular weight suggested by gel filtration methods. Delipidated F3, after disruption of both protein and lipid interactions (by CsBr–Gdn·HCl treatment and $CHCl_3$–MeOH extraction, respectively), has a lower average molecular weight (Table IV) than the Sepharose CL-4B void fraction while still maintaining considerable size heterogeneity. It is possible that the high molecular size arises from aggregation of an unknown nature. Interactions of mucin glycoproteins with albumin have been reported, and it is clear from the exclusion chromatography data that substantial amounts of noncovalently associated protein are present in the mucin fraction.

On the basis of results obtained with human tracheal mucin glycoprotein (Woodward et al., 1982), the role of disulfide bonds in the macrostructure was examined. Quantitation of free SH groups as well as disulfide bridges was carried out by reduction–alkylation, alkylation prior to reduction, and titration with 5,5′-dithiobis(2-nitrobenzoic acid). Results are summarized in Table III. The effect of cleavage of disulfide bonds on molecular weight was also examined by analytical ultracentrifugation and SDS–PAGE of performic acid oxidized glycoprotein. Data are shown in Figures 6 and 7 and Table IV. The role of disulfide bonds in mucin aggregate formation seems to vary with the source. Relatively simple mucins such as those of the salivary system appear not to have significant quantities of sulfur-containing amino acids, and their aggregation behavior thus involves other associative mechanisms (Hill et al., 1977; Bhavanandan & Hegarty, 1987). In contrast, the macrostructure of human tracheobronchial mucin appears to involve disulfide bonds, either between homologous molecules or between the mucin glycoprotein and subsidiary proteins, which form the basis for an extended network (Lamblin et al., 1977; Rose et al., 1979; Roussel et al., 1975;

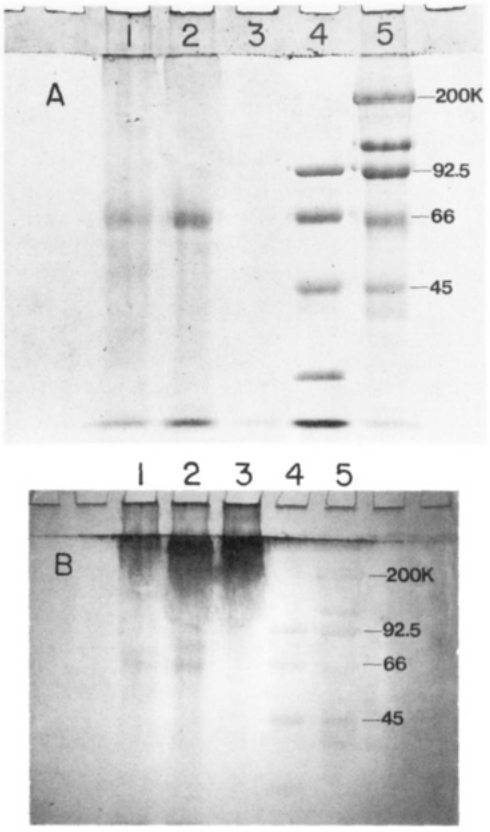

FIGURE 5: SDS–polyacrylamide slab (using 10% running and 5% stacking gels) electrophoresis of density gradient fractions. Lane 1, F1 (Figure 2); lane 2, F2 (Figure 2); lane 3, delipidated F3 (Figure 3); lane 4 and 5, low (96 000–21 000) and high (200 000–43 000) molecular weight standards, respectively. 500 μg of each fraction was applied. Panel A, Coomassie blue staining; panel B, PAS staining.

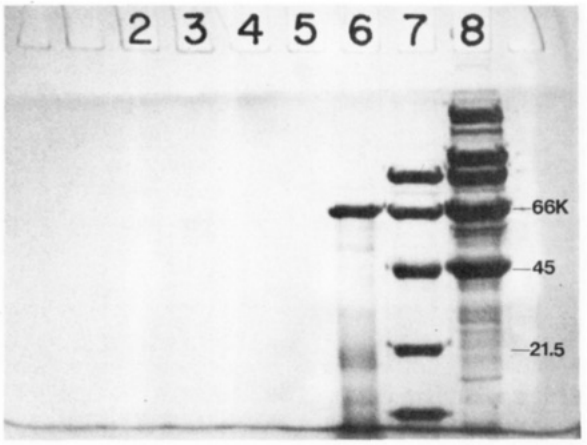

FIGURE 6: SDS–polyacrylamide slab gel electrophoresis (Coomassie blue staining) of fractions derived from Sepharose CL-4B chromatography of PF_3 (high-density mucin isolated by density gradient centrifugation after delipidation and performic acid oxidation of CTM). Lane 2 contains 250 μg of the fully included fraction, lane 3 contains 300 μg of the partially included fraction, and lane 4 contains 400 μg of the void volume fractions. Lanes 5 and 6 contain unfractionated PF_3. Lanes 2–5 were treated prior to electrophoresis by heating for 3 min in sample buffer containing 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS. Lane 6 was pretreated by heating for 10–15 min in the same sample buffer. Lanes 7 and 8 contain standards (as in Figure 5).

LeTreut et al., 1981; Bhaskar & Reid, 1981). The canine tracheal mucin appears to be similar in this respect to the human tracheal mucin. The molecular weights of the intact canine mucin glycoprotein and the overall compositional data suggest that the nonglycosylated polypeptide core should be

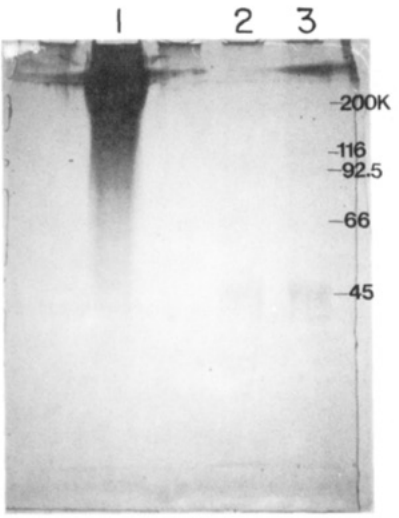

FIGURE 7: SDS–polyacrylamide slab gel electrophoresis (PAS staining). Lane 1 contains PF_3 (high-density mucin isolated by density gradient centrifugation after delipidation and performic acid oxidation). Lanes 2 and 3 contain standards (as in Figure 5).

Table V: Amino Acid Composition of the CTM Pronase Core and the 65-kDa Protein

amino acid[a]	Pronase core[b]	65-kDa protein[c]	canine serum albumin[d]
$CySO_3H$	23	0	64
Asp	55	46	84
Thr	191	33	43
Ser	209	121	49
Glu	126	129	140
Gly	138	188	55
Ala	75	73	126
Val	37	49	52
Met	1	4	8
Ile	15	37	9
Leu	33	68	112
Tyr	0	89	51
Phe	15	41	60
His	23	40	24
Lys	23	59	79
Arg	36	23	42
$GlcNH_2$[e]	7.38		
$GalNH_2$	4.46		

[a] Data are expressed as residues per 1000 residues. Cysteine is determined as cysteic acid after hydrolysis in 6 N HCl containing 0.25 M Me_2SO. [b] Analysis of the protease-resistant glycopeptides isolated by Pronase digestion of performic acid oxidized CTM followed by gel filtration. [c] Analysis of the 65 000-dalton protein isolated after treatment of CTM with β-mercaptoethanol. [d] The composition of canine serum albumin is listed for comparison. [e] Hexosamine data are expressed as weight percent.

about 100 000 daltons. In fact, chemical deglycosylation of the purified canine mucin glycoprotein yielded a product of about 110 000 daltons (Woodward et al., 1987). Accordingly, such a structure will contain about 20 disulfide linkages.

The appearance of a low molecular weight (65 000) protein subsequent to disulfide bond cleavage is notable in Figure 6. This protein was isolated from the PF_3 fraction by preparative SDS–PAGE essentially as described by Hager and Burgess (1980). A yield of about 500 μg was obtained starting with 10 mg of PF_3. The amino acid composition of the 65 000 molecular weight protein is presented in Table V. Since this component is not resolved by exclusion chromatography, the density gradient step, or SDS gel electrophoresis prior to treatment with reducing agents, we believe the association with the mucin glycoprotein is either directly via one or more disulfide linkages or indirectly through a domain whose integrity is dependent on intact S–S bridges. Preliminary data indicate

Table VI: Amino Acid Analysis of Canine Tracheal Mucin Glycoprotein before and after Treatment with Alkaline Sodium Borohydride[a]

amino acid	βF_3	$NaBH_4$–βF_3
Ser	144	61 (−83)
Thr	164	90 (−74)
Ala	57	121 (+64)
α-aminobutyric acid		58 (+58)

[a] βF_3 is high-density material isolated after delipidation and recentrifugation in cesium bromide containing 20 mM β-mercaptoethanol. Data are in residues per 1000 amino acid residues. Values in parentheses indicate changes in composition.

that this 65 000-dalton component is not recognized by antibodies to canine serum. This negative experiment, however, does not serve to establish the origin of this material but does rule out canine serum albumin. The amino acid composition (Table V) also indicates that this protein is not canine serum albumin. An explicit stoichiometric relationship between the 65K protein and the mucin glycoprotein remains undefined. An analogous protein component has been isolated from human lung mucin (N. J. Ringler et al., unpublished results).

The nature of the covalent linkage between the peptide and saccharide moieties was determined by treatment of the mucin glycoprotein with alkaline borohydride (Carlson, 1966). Typical data are summarized in Table VI. As anticipated, the saccharide units of the tracheal mucin glycoproteins are linked via *N*-acetylgalactosaminyl residues to both serine and threonine hydroxyls, and about half of the hydroxyamino acids are glycosylated. Given the limitations of current technology, it is not possible to explicitly identify those residues nor is it possible to state that a particular locus (Ser or Thr) is or is not always glycosylated. Sulfate analyses gave values of 2.5–3.0% by weight, presumably ester sulfate on the sugar residues. The location of these groups is not known.

As in the case with the analogous human material (Feldhoff et al., 1979), the mucin glycoprotein obtained from canine sources does not have a detectable free amino terminus. Attempts were made to radiolabel both the peptide and saccharide parts of the isolated glycoprotein. Reaction with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (Bolton et al., 1976) gave negligible incorporation. In addition, neither Chloramine-T-catalyzed iodination, alkylation with iodo[^{14}C]acetamide, nor reductive methylation with [^{3}H]HCHO proved to be effective labeling procedures. The poor yield in the various radiolabeling studies is consistent with the lack of a free amino terminus and low tyrosine and lysine content. Tracer was incorporated from [^{3}H]ethanolamine utilizing a carbodiimide coupling procedure, but it was established that this label was primarily restricted to the sialyl residues (unpublished results). The isolated 65-kilodalton protein could, however, be radioiodinated without difficulty. Preliminary results suggest that incorporation of label into the protein moiety can be achieved by the use of radioactive amino acids in canine tracheal organ culture, an approach that may resolve the current ambiguity with respect to the proteins remaining associated with the mucin following the CsBr–guanidinium gradient.

The Pronase-digested glycoprotein eluted as a heterogeneous included peak on Sepharose CL-4B similar to the comparable human material (Feldhoff et al., 1979). The carbohydrate-containing material (90% of the neutral sugar) was recovered by lyophilization following dialysis. The amino acid composition of the Pronase core is presented in Table V. As is the case with the human material, proteolytic digestion and analysis of the protease-resistant, saccharide-rich core suggest that many of the disulfide bonds are present in a sugar-free domain of the glycoprotein (Table V).

The molecular weights of the various mucin fractions were measured by equilibrium sedimentation. Results for the Sepharose CL-4B void, delipidated F3, PF_3 (high-density material isolated after delipidation, performic acid oxidation, and recentrifugation in cesium bromide), and the Pronase core are summarized in Table IV.

In previous studies, isolation of canine tracheal mucin glycoproteins involved treatment of the pouch secretions with reducing agents followed by gel filtration and ion-exchange chromatography (Sachdev et al., 1978; Liao et al., 1979). The reduction releases many low molecular weight components, the origins of which are difficult to ascertain. In addition, the reduction may disrupt the macromolecular architecture of the mucin molecule. We have attempted to isolate the major glycoprotein by gel filtration using buffer containing 6 M urea followed by density gradient centrifugation in CsBr–4 M guanidinium chloride. Both these steps resulted in the release of a considerable quantity of low molecular weight protein and glycoprotein components, the majority of which appear to be of circulating (serum) origin. Treatment of the apparently homogeneous delipidated high molecular weight mucin fraction with disulfide-reducing or oxidizing agents resulted in an almost 50% decrease in the size of glycoprotein and the release of a 65 000-dalton protein which is not recognized by antibodies to canine serum components. The specific association of this 65 000-dalton component and the noncovalent association of lipids with the canine mucin glycoprotein are findings not previously reported. The carbohydrate and amino acid composition of the purified high molecular weight glycoprotein is typical of mucins and resembles (in an overall manner) those reported for the major fractions obtained by Sachdev et al. (1978) and Liao et al. (1979). In contrast, our preparation had no detectable free α-amino group whereas arginine was reported to be the amino-terminal amino acid in the preparation obtained by Sachdev et al. (1978).

The properties of the CTM glycoprotein reported in this study resemble in many respects those of the human tracheobronchial mucin glycoproteins investigated by us (Feldhoff et al., 1979; Woodward et al., 1982). Mucin glycoproteins from both sources had relatively nonspecific lipid binding domains, blocked amino termini, specifically associating 65 000-dalton protein components, involvement of disulfide bonds in the macrostructure, no available sites for in vitro labelling of the protein core, comparable molecular size and carbohydrate composition, clustered O-glycosidically linked saccharides giving rise to pronase-resistant saccharide-rich cores, and protein cores of about 100 000 daltons (Woodward et al., 1987). These remarkable similarities confirm that canine tracheal pouch secretions provide an ideal system for obtaining uncontaminated respiratory mucin glycoproteins for detailed investigations.

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