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ISOLATION OF BRONCHIAL MUCINS FROM CYSTIC FIBROSIS SPUTUM BY USE OF CITRACONIC ANHYDRIDE

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ABSTRACT

Citraconylation was used to solubilize cystic fibrosis sputum and to dissociate its mucus glycoproteins from extraneous proteins. The mucin fraction was isolated by precipitation with Cetavlon, and characterized in terms of amino acid and carbohydrate composition. The data suggest that, in determining the physical properties of glycoproteins of cystic fibrosis mucus, aggregation by noncovalent forces may be as important as (or more important than) disulfide bonds.

INTRODUCTION

Cystic fibrosis (CF), the most common lethal genetic disease in the United States[1], is characterized by abundant and abnormally thick secretions of mucus. In the airways, chronic infection and pulmonary obstruction are major pathological findings[2].

Bronchial secretions from CF patients contain a decreased content of water, and, consequently, increased proportions of protein and other constituents which contribute to its thickness and tenacity[3]. The viscous nature of CF sputum, like that of all other bronchial secretions, is attributed to the presence of mucus glycoproteins which dictate much of the rheological and physicochemical properties of the sputum[4]. Detailed biochemical studies of the glycoproteins present in CF sputum have been made, but comparisons of these studies are difficult, as greatly differing isolation procedures have been employed[5–9]. Due to the excessively thick and sticky nature of these secretions, most of these procedures involve harsh methods which are often employed to disperse the gel in order to obtain the glycoproteins in a purified state.

We now report a new method for gently solubilizing the sputum and purifying the mucins in a fairly rapid and efficient procedure. This method is based on reports that mucins are often bound *in vivo* to various protein contaminants, some of which have been identified[10–12]. This binding has been found to be mainly of a noncovalent nature[13] between basic proteins, *via* ε-amino groups, and the negatively charged sialic acid and sulfate groups on the mucin[10,12]. Herein is described the use of citraconic anhydride to modify lysyl residues reversibly, thus freeing the sugar

residues and sulfate groups for precipitation by a quarternary ammonium salt. Modification of the ε-amino group of lysine residues by this procedure has been used for freeing nucleoprotein complexes[14]. The mucins that were precipitated were determined to be pure by analytical ultracentrifugation in solutions having a high concentration of salt and urea, in the presence and absence of 2-mercaptoethanol, and in SDS–poly(acrylamide) slab gel-electrophoresis, with and without reducing agents. The purified mucus glycoprotein was characterized in terms of amino acid and carbohydrate composition.

This method may be useful, not only for CF secretions, but also for the solubilization and purification of bronchial glycoproteins from normal sputum, and from sputum from other disease states.

EXPERIMENTAL

Source of CF secretions. — Sputum was freshly expectorated into sterile glass jars, immediately frozen, and kept at −20° until used. All collections were performed under the supervision of a medical doctor and a nurse.

Purification of mucus glycoprotein. — CF sputum (7.8 mL) was obtained from four children (2 boys and 2 girls). Ice-cold, sterile, saline solution (4 vol.) and one drop (40 μL) of 0.4% sodium azide were added, and the mixture was stirred for 3 h in the cold, centrifuged, the supernatant liquor collected, and the residue similarly re-extracted twice. The extracts were combined, 1mM EDTA was added, and the mixture was dialyzed against two changes of 0.01M NaCl and four changes of distilled water, and lyophilized. The dry residue was dissolved in 0.5M Tris · HCl buffer, pH 8.0 (20 mL) and, under constant stirring, 5 drops of citraconic anhydride were slowly added, the pH being continually kept at 8.0 by appropriate addition of NaOH. Following dialysis and lyophilization, the sample was dissolved in distilled water, the pH was adjusted to 4.8 with M acetic acid, Cetavlon (20% aqueous cetyltrimethylammonium bromide, 0.25 mL) was added dropwise, and the solution was kept overnight at 4°. The cloudy solution was centrifuged, the Cetavlon clot dissolved in a small volume of aqueous 0.5M NaCl, the mucin solution dialyzed and lyophilized, and the residue tested for purity as described next.

Analytical ultracentrifugation. — All sedimentation-velocity studies were conducted at a speed of 42,040 r.p.m., with a sample concentration of 5 mg/mL. Analyses were performed with Schlieren optics at a phase plate-angle of 60°, with solvents of either 0.5M NaCl or 6M urea, with and without added 2-mercaptoethanol (0.1%). Photographs were taken at 12-min intervals, and S values were calculated according to Svedberg and Peterson[15].

Amino acid analyses. — Samples were each combined with an equal volume of 12M HCl in a hydrolysis tube, and this was evacuated. After 22 h at 110°, the hydrolyzates were evaporated to dryness, and the residues dissolved in small volumes of citrate buffer, pH 2.2. Aliquots (100 μg) were analyzed by the two-column procedure of Spackman *et al.*[16], using Dionex DC-1A resin and a Beckman Model 120C Amino

Acid Analyzer. Threonine and serine were corrected (for decomposition during hydrolysis) by factors of 1.06 and 1.10, respectively[17].

Chemical analyses. — Protein was determined by the method of Lowry *et al.*[18], using crystalline bovine serum albumin as the standard. Neutral hexoses were assayed by the phenol–sulfuric acid method of Dubois *et al.*[19], and sialic acid by the resorcinol procedure according to Svennerholm[20]. Hexosamines were determined, after hydrolysis in 6M HCl for 4 h at 104°, by gas–liquid chromatography (g.l.c.), and also by means of the Amino Acid Analyzer. G.l.c. was performed by using the alditol acetate derivatives of the sugars in a column (91.44 cm) of Supelco SP-2340, with nitrogen as the carrier gas[21]. Sulfate was assayed by the rhodizonate method[22]. SDS–poly-(acrylamide) gel-electrophoresis was performed in 6.25% acrylamide gels in Tris–glycine buffer, pH 8.6, as described by Weber and Osborn[23], and stained for proteins with Coomassie Brilliant Blue R-250, and for carbohydrates with periodic acid–Schiff stain.

RESULTS

Purification of mucin by using citraconic anhydride and Cetavlon. — After dissolution of the sputum in saline, the extracts were found to contain over 85% of the total protein, sialic acid, and neutral hexose. Based on previous observations, the contamination by proteins was found to be much greater in CF sputum than in bronchial secretions from normal and chronic bronchitis patients[5]. In order to remove these extraneous proteins from the saline extract, we adapted a procedure described by Shetty and Kinsella[14], who used citraconic anhydride to separate nucleoprotein complexes from their basic histones, and to precipitate the nucleic acids selectively. Citraconic anhydride minimizes noncovalent interactions by reversibly modifying the lysyl residues present in the proteins, the action being primarily restricted to the extraneous proteins, as mucins contain only very small proportions of lysine[24]. Chemical analysis revealed that the mucins dissociated from the positively charged proteins, and became precipitable with Cetavlon, whereas the extraneous proteins remained in the supernatant liquor. Deacylation was achieved by adjusting the pH to 4.8, and was complete within 2 h. The supernatant (CF-S) and the mucin (CF-C) fractions were separately dialyzed, lyophilized, and subjected to chemical characterization.

Chemical analyses. — The gross chemical composition of each of the two fractions (CF-S and CF-C) is shown in Table I. The mucin fraction contained ~70% (by weight) of sugars and ~30% of protein, which corresponds well to the characteristic chemical composition of mucus glycoproteins[24,25]. The Cetavlon supernatant liquor contained only 22% of sugar constituents, the rest being protein.

The molar sugar ratios of each fraction are given in Table II. Whereas the mucin fraction contains a large proportion of fucose, but no mannose, the supernatant fraction shows mannose as the preponderant sugar, with very little fucose, and only a trace of 2-acetamido-2-deoxygalactose; this suggests that the CF-S fraction con-

TABLE I

GROSS, CHEMICAL COMPOSITIONS OF GLYCOPROTEIN FRACTIONS FROM CF SPUTUM

Component	*CF-C*	*CF-S*
Protein	4.14	13.50
Neutral hexose	5.15	2.30
Sialic acid	1.80	0.70
2-Acetamido-2-deoxygalactose	0.78	0.06
2-Acetamido-2-deoxyglucose	1.78	0.73
Protein/sialic ratio[a]	2.3	19.3

[a]Weight/weight. Values expressed in mg.

TABLE II

SUGAR COMPOSITION OF GLYCOPROTEIN FRACTIONS FROM CF SPUTUM

Sugar	*Molar ratios*	
	CF-C	*CF-S*
Fucose	5.7	0.3
Galactose	3.5	0.9
Mannose	0	1.2
2-Acetamido-2-deoxyglucose	2.3	1.0
2-Acetamido-2-deoxy-galactose	1.0	0.1
Sialic acid	1.3	0.6

tains contaminating serum glycoproteins that are asparagine-linked, and the CF-C fraction consists of mucin uncontaminated with mannose-containing oligosaccharides.

The amino acid analysis of each fraction is given in Table III. Threonine, serine, and proline account for almost 50% of the total amino acids of the mucin fraction. The supernatant protein fraction contained 16% of serine and threonine, and $<10\%$ of proline.

Analytical ultracentrifugation. — The mucin fraction (CF-C) was subjected to high-speed analytical ultracentrifugation. The glycoprotein, at a concentration of 5 mg/mL of 0.5M NaCl, was centrifuged at 42,040 r.p.m., and the Schlieren pattern obtained had one peak, which broadened with time—a typical feature of mucins in this system. However, when the sample was dialyzed, and the product dissolved in 6M urea, a hyperfine peak was obtained that exhibited considerably less diffusion. When a reducing agent (0.1% 2-mercaptoethanol) was present, in 6M urea, no appreciable change in Schlieren pattern or S value was observed (see Table IV). A comparable S value of 6.08 was obtained by Boat and Cheng[26] using bronchial washings from a CF patient. Because the S value obtained in 0.5M NaCl is approximately twice that of the sample dissolved in 6M urea, it is suspected that a considerable

TABLE III

AMINO ACID ANALYSES OF GLYCOPROTEIN FRACTIONS FROM CF SPUTUM

Amino acid	*Mol/100 mol*	
	CF-C	*CF-S*
Lys	2.4	5.2
His	0.3	0.7
Arg	0.5	2.8
Asp	8.9	10.4
Thr	19.8	6.2
Ser	15.2	9.4
Glu	8.1	13.0
Pro	12.4	9.7
Gly	9.7	9.7
Ala	8.1	6.9
Cys	1.2	2.1
Val	4.0	4.3
Met	0.4	0.5
Ile	2.4	2.4
Leu	6.5	7.3
Tyr	trace	6.9
Phe	trace	2.4

TABLE IV

S VALUES OF FRACTION CF-C IN DIFFERENT SOLVENTS

Solvent	$S_{20,w}$
0.5M NaCl	12.6
6M urea	6.9
6M urea + 0.1% 2-mercaptoethanol	6.4

degree of aggregation occurs in the salt solution. These data indicate that disulfide bonding probably does not play a major role in determining the size and physical nature of the CF mucin described here; this finding has been reported by others working with bronchial mucins[7,8,25,27].

Poly(acrylamide) gel-electrophoresis. — Slab gel electrophoresis of the initial crude extract, the mucin, and the non-mucin fractions was performed in the presence of sodium dodecyl sulfate, with and without either 1,4-dithiothreitol or 2-mercaptoethanol. The non-mucin fraction (CF-S) showed one major protein band, corresponding to a molecular weight of 48,000, and several minor bands when 100 μg of protein was applied to the gel. The initial crude extract showed numerous protein-stainable bands. However, the CF-C mucin (200 μg) did not enter the gel matrix, as would be expected (due to its high molecular weight and high carbohydrate content),

but no contaminating protein or carbohydrate-stainable bands were seen, even in the presence of agents that reduce disulfide bonds. This supports the ultracentrifugal data, which suggest that disulfides are not linking subunits of this glycoprotein.

DISCUSSION

Separation of mucin glycoproteins from extraneous proteins has been attempted by several methods[5,9,25]. Gel filtration in denaturing solvents is often used, resulting in a void-volume mucin peak. However, some insolubility may result during the concentration procedure, and the high viscosity of this material often necessitates loading of the column several times with low concentrations. Various precipitants, such as ethanol and quaternary ammonium salts, have also been employed, but ethanol usually precipitates other proteins present[6], and quaternary ammonium salts precipitate only half of the mucins present[28].

We report here a new method for isolating bronchial mucus glycoproteins by using citraconic anhydride and Cetavlon. Although Cetavlon has been used in the past for isolation of mammalian mucins, its use with human mucus has been limited, because the yield was poor[28]. We have greatly improved the yield of pure mucus glycoprotein, using Cetavlon, by treatment of the crude extract with citraconic anhydride prior to precipitation. A variety of criteria was employed in order to determine the nature and homogeneity of the glycoprotein obtained. Chemical analysis of both the carbohydrate and amino acid constituents revealed a typical mucin profile, with no mannose and only traces of aromatic amino acids. Although the purity of large macromolecules is difficult to ascertain, our preparation appeared pure by sedimentation-velocity, analytical ultracentrifugation using different solvents, as well as by SDS–poly(acrylamide) gel-electrophoresis. These are criteria that are generally used as a guide in establishing the degree of polydispersity of such high-molecular-weight compounds[29].

In addition to being simple, this procedure allowed us to obtain the glycoprotein components in high yield, as determined by the recovery of hexosamines and sialic acid. After the initial solubilization, $\sim 90\%$ of the hexosamines present were isolated in the mucin fraction.

Based on the variety of conditions used in the purification of the CF-C mucin, we suggest that, although intermolecular disulfide bonding may play a role in determining the physical properties of CF mucins, noncovalent forces may be as, or even more, significant in the aggregation of bronchial mucins and other proteins in sputum. Although these interactions are weak in comparison to the disulfide bond, these electrostatic forces would, because of their large number, be major contributing factors in maintaining the stability of the aggregate. We consider that a crucial purification step involves the dissociation of mucin–protein complexes with citraconic anhydride, a reversible modifier of lysyl ε-amino groups. Although citraconic anhydride has been implicated in some irreversible modifications of active sulfhydryl groups[30], its effect upon the mucin is presumed to be minimal, as there are only

traces of cysteine in mucus glycoproteins from bronchial secretions. It is suspected that whatever potential sulfhydryl groups are present are shielded from the direct effect of the citraconic anhydride by the numerous oligosaccharide side-chains. Probably more important is the effect of citraconic anhydride upon the active sulfhydryl groups of the extraneous proteins.

We have utilized this procedure to purify mucin from sputum solubilized in saline. However, it may also be suitable for bronchial secretions that are subjected to a preliminary solubilization in other solvents, such as urea and guanidine, with prior dialysis.

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REFERENCES

1 P. A. Di Saint'Agnese and P. B. Davis, *N. Engl. J. Med.*, 295 (1976) 481–485.
2 P. B. Davis and P. A. Di Saint'Agnese, *Pediatr. Res.*, 14 (1980) 83–87.
3 M. T. Lopez-Vidriero and L. Reid, *Am. Rev. Respir. Dis.*, 117 (1978) 465–477.
4 J. A. Alhadeff, *Clin. Genet.*, 14 (1978) 189–201.
5 T. F. Boat, P. W. Cheng, R. N. Iyer, D. M. Carlson, and I. Polony, *Arch. Biochem. Biophys.*, 177 (1976) 95–104.
6 G. P. Roberts, *Eur. J. Biochem.*, 50 (1974) 265–268.
7 G. Lamblin, M. Lhermitte, P. Degand, Y. H. Sergeant, and P. Roussel, *Biochim. Biophys. Acta*, 332 (1973) 372–382.
8 P. A. Feldhoff, V. P. Bhavanandan, and E. A. Davidson, *Biochemistry*, 18 (1979) 2430–2436.
9 M. C. Rose, W. S. Lynn, and B. Kaufman, *Biochemistry*, 18 (1979) 4030–4037.
10 J. M. Creeth, J. L. Bridge, and J. R. Horton, *Biochem. J.*, 181 (1979) 717–724.
11 R. Picot, I. Das, and L. Reid, *Thorax*, 33 (1978) 235–242.
12 A. O. Jenssen, O. Smidsrød, and O. Harbitz, *Scand. J. Clin. Lab. Invest.*, 40 (1980) 727–731.
13 C. E. Snyder, C. E. Nadziejko, and A. Herp, *Int. J. Biochem.*, in press.
14 J. K. Shetty and J. E. Kinsella, *Biochem. J.*, 191 (1980) 269–272.
15 T. Svedberg and K. O. Peterson, *The Ultracentrifuge*, Clarendon Press, Oxford, England, 1940.
16 D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30 (1958) 1190–1206.
17 A. M. Wu and W. W. Pigman, *Biochem. J.*, 161 (1977) 37–47.
18 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
19 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
20 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1956) 604–611.
21 W. Niedermeier, *Anal. Biochem.*, 40 (1971) 465–475.
22 T. T. Terho and K. Hartala, *Anal. Biochem.*, 41 (1971) 471–476.
23 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.
24 A. Herp, A. M. Wu, and J. Moschera, *Mol. Cell. Biochem.*, 23 (1979) 27–43.
25 G. P. Sachdev, F. J. Meyers, F. O. Horton, D. F. Fox, G. Wen, R. M. Rogers, and R. Carubelli, *Biochem. Med.*, 24 (1980) 82–94.
26 T. F. Boat and P. W. Cheng, *Fed. Proc.*, 35 (1976) 1444.

27 G. LAMBLIN, M. LHERMITTE, A. BOERSMA, P. ROUSSEL, AND V. REINHOLD, *J. Biol. Chem.*, 255 (1980) 4595–4598.
28 I. OEMRAWSINGH, *Studies on High-molecular Secretory Sialoglycoproteins from Human Submandibular Salivary Glands*, Bronder-Offset, Rotterdam, 1972.
29 V. B. HATCHER, G. O. H. SCHWARZMANN, R. W. JEANLOZ, AND J. W. MCARTHUR, *Biochemistry*, 16 (1977) 1518–1524.
30 A. C. BRINEGAR AND J. E. KINSELLA, *Int. J. Pept. Protein Res.*, 18 (1981) 18–25.