Ultracentrifugation

Preparation of Intestinal Mucins Using the NVT 65 Near Vertical Tube Rotor

Umadevi Sajjan and J. Forstner

Division of Biochemistry
Hospital for Sick Children
Research Institute, Toronto

Introduction

Mucus secretions in the gastrointestinal, respiratory and reproductive tracts of mammals are complex mixtures of local secretions, sloughed cells, bacteria, water and various serum components. The viscoelastic properties of these secretions are due to the major gel-forming glycoprotein constituents called mucins, which are synthesized and secreted by specialized epithelial mucous cells. The capacity of mucins to protect epithelial surfaces depends upon their large size, and their rich and heterogeneous oligosaccharide composition, which determine the ability of mucins to polymerize and form gels. Abnormalities of mucins occur in many conditions including carcinomas, peptic ulcer, cystic fibrosis, chronic bronchitis, immune or infectious inflammatory diseases of the bowel and gallstone formation.

Research on the structure, function and pathology of mucins usually necessitates purification of these macromolecules, but purification procedures are time-consuming and labor-intensive. Sequential steps of cesium chloride density gradient ultracentrifugation, for example, are widely accepted as a means of purification, but the time required for each centrifugation is long, *i.e.*, often 48 to 72 h for each centrifugation. The Beckman NVT 65 rotor was investigated in the present study to determine if its use would permit a reduction in the centrifugation time required for mucin purification.

[1]Abbreviations used: PBS, phosphate buffered saline (0.1 M Na_2HPO_4, pH 7.2 in 0.15 M NaCl); PMSF, phenylmethylenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid disodium salt; NEM, *N*-ethylmaleimide; NaN_3, sodium azide; BSA, bovine serum albumin; BCA, assay for protein; PAS, periodic acid/ Schiff assay for sugar.

Methods

Human intestinal scrapings (124 g) were homogenized for 30 s in 3 L PBS containing 1 mM PMSF, 5 mM EDTA, 5 mM NEM and 0.02% NaN_3 and centrifuged at 27,000 x *g* for 30 min in a JA-14 rotor. The supernatant was concentrated by ultrafiltration using an Amicon Diaflow membrane (exclusion limit 100 kDa) to 300 mL. The density of the concentrate was adjusted to 1.4 g/mL with CsCl (total volume after density adjustment was 360 mL). A total of 210 mL (105 mL per run) were subjected to density gradient ultracentrifugation in an NVT 65 rotor at 4°C and 65,000 rpm for 16 h. See Figure 1 for flow diagram of the fractionation procedure.

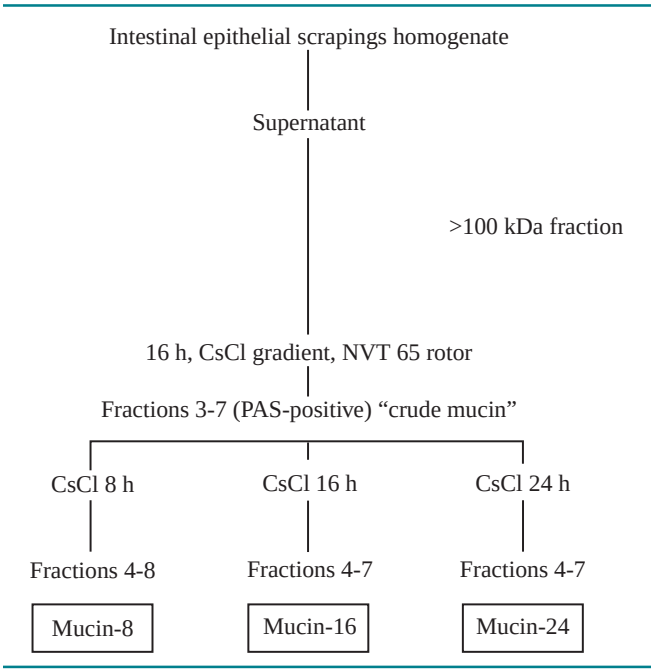

Figure 1. Protocol of mucin preparations using the NVT 65 rotor.

Each tube was fractionated into 12 fractions, and the density of each fraction was measured by refractometry with the use of a Milton Roy refractometer. A 0.5-mL aliquot of each fraction was dialyzed and analyzed for total protein and sugars by BCA (1) and PAS (2) assay, respectively. Figure 2 shows the density, PAS and BCA profiles of all 12 fractions. Fractions 3-7 (crude mucin) were pooled from both the spins (total volume 85 mL), density was adjusted again to 1.4 g/mL with CsCl (total volume after density adjustment was 103 mL) and centrifuged in the NVT 65 rotor at 4°C, 65,000 rpm for 8, 16 or 24 h. At each time interval two tubes were removed, fractionated into 12 fractions, and the density, PAS and BCA assays were carried out as described earlier. The profiles are given in Figure 3. Fractions 4-8 after an 8-h spin and fractions 4-7 after 16- or 24-h spins were pooled and dialyzed exhaustively against water. These fractions were designated as mucin-8, mucin-16 and mucin 24, respectively.

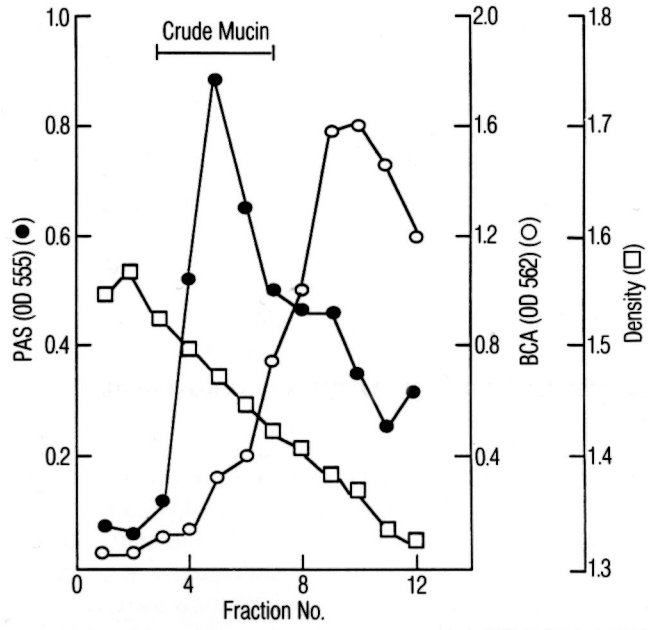

Figure 2. Density, PAS and BCA profile of fractions from first density gradient centrifugation in CsCl for 16 h.

Results

Total mucin recovered by immunoassay at each step, total protein as determined by BCA assay, and PAS/protein for each mucin preparation are given in Table 1. Table 2 represents the amino acid and amino sugar analyses of all three mucin

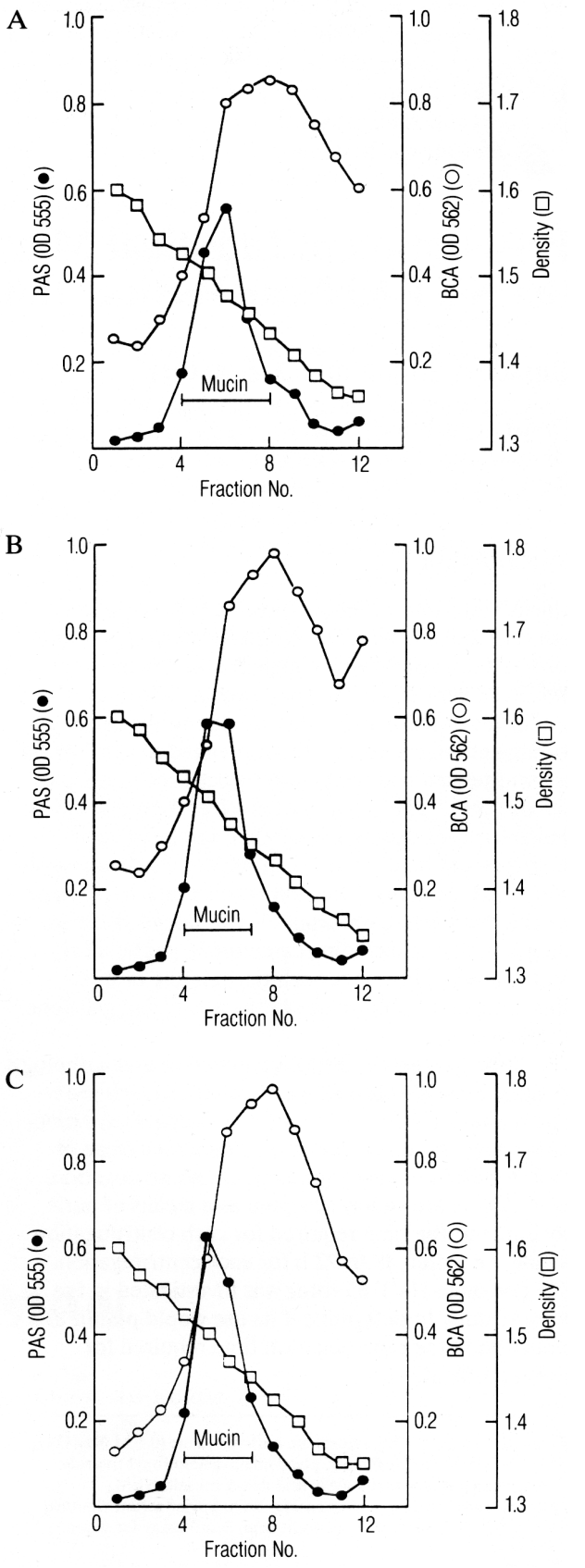

Figure 3. Density, PAS and BCA profiles of fractions obtained from CsCl density gradient centrifugation after 8 h (A), 16 h (B), or 24 h (C).

Table 1: Recovery of Mucin, Total Protein, and the Carbohydrate (PAS)/protein (BCA) Ratio of Mucins Purified Using the NVT 65 Rotor

Purification Steps	Total Mucin (immunoassay) (mg)	Total Protein (BCA Assay) (mg)	Recovery (%)	PAS/BCA (wt/wt)
Retentate (>IOO kDA)	7.24	NT	100	NT
lst CsCl density gradient				
16 h (fractions 3-7)	4.65	NT	64	NT
2nd CsCl density gradient				
8 h (fractions 4-8)	3.50	4.37	48	10.4
16 h (fractions 4-7)	4.20	4.32	58	13.5
24 h (fractions 4-7)	1.81	2.06	26	12.7

NT: not tested

Table 2: Composition of Mucins

Amino Acids (mol %)	Mucin-8	Mucin-16	Mucin-24	Standard Mucin
Asp	8.7	7.7	9.1	7.9
Glu	9.7	9.1	10.8	9.5
Ser	12.1	12.4	10.4	11.5
Gly	7.3	8.4	6.7	9.7
His	1.5	1.5	1.7	1.7
Arg	1.5	1.5	2.2	2.0
Thr	17.6	18.3	15.0	17.1
Ala	5.7	5.3	6.2	5.6
Pro	13.5	13.9	11.9	13.4
Tyr	1.2	1.1	1.6	1.5
Val	4.8	4.5	5.0	4.8
Met	0.4	0.9	0.7	0.7
Ile	4.4	4.9	4.1	3.8
Leu	5.0	4.7	6.4	5.1
Phe	2.4	2.2	2.8	2.1
Lys	3.6	2.9	4.8	2.8
Amino Sugars (nmol/µg of protein)				
GIcNAc	1.32	1.45	1.06	1.24
GaINAc	1.79	2.06	1.53	1.44

Standard mucin was prepared in the Type 50 Ti rotor run at 41,000 rpm for 48 h.

preparations, along with a "standard" mucin prepared by two centrifugations in CsCl using a Type 50.2 Ti fixed angle rotor at 41,000 rpm for 48 h (3). Figure 4 represents the immunoblots of mucins treated with an antibody to human small intestinal mucin (4), human serum proteins (Sigma Chem. Co., St. Louis) or human fibronectin (Boehringer GMBH, Mannheim, W. Germany).

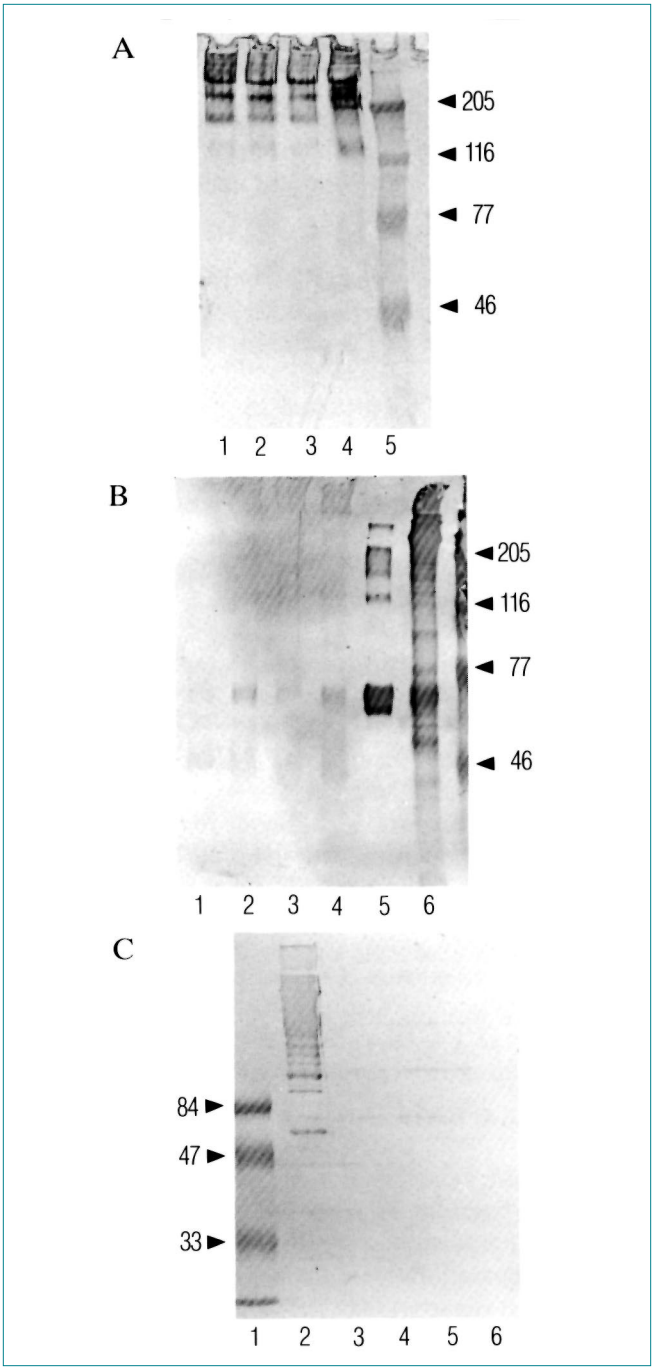

*Figure **4.** Western blots of mucins treated with antibody to (A) human small intestinal mucin, (B) human serum proteins or (C) fibronectin. Mucins (0.2 µg protein) were subjected to SDS-PAGE (7.5% polyacrylamide gels) under reducing conditions, transferred onto nitrocellulose and blocked with 3% gelatin. The blots were then treated overnight at 4°C with the various antibodies, and the antigenic bands were detected by a second antibody conjugated to alkaline phosphatase. (A) Lanes 1-4: mucin-24, mucin-16, mucin-8 and standard mucin, respectively; Lane 5: high molecular weight standards; (B) Lane 1: standard mucin; Lanes 2-4: mucin-8, mucin-16 and mucin-24, respectively; Lane 5: BSA (0.2 µg); Lane 6: fibronectin (0.2 µg); (C) Lane 1: low molecular weight standards; Lane 2: fibronectin (0.2 µg); Lanes 3-6: standard mucin, mucin-8, mucin-16 and mucin-24, respectively.*

Observations

1. The three mucins (designated as mucin-8, mucin-16 and mucin-24) prepared using an NVT 65 rotor showed similar amino acid and amino sugar analyses.
2. All three mucins prepared using the NVT 65 rotor showed similar SDS-PAGE patterns that were comparable to that of a standard mucin.
3. On an immunoblot with human small intestinal mucin antibody, all mucins, including the standard mucin, showed high molecular weight bands. In addition, a band at M_r = 118 kDa was observed, but the intensity of this band varied among the mucin preparations. (The variation is not considered significant.)
4. None of the mucins were immunoreactive with an antibody to human fibronectin.
5. Mucins prepared using an NVT 65 rotor gave a weakly positive band at 66 kDa when tested by western blotting with an antibody to human serum proteins. Comparison with a standard (0.2 μg) BSA preparation indicates that the contaminant is likely albumin, but is very minor (<0.002 μg). The least intense band was seen in mucin-16.
6. Mucin-16 gave the best yield of immunoreactive mucin.

Interpretation and Conclusions

Density gradient centrifugation in CsCl for 16 h gave a crude mucin (fractions 3-7) that was then subjected to a second CsCl gradient for 8 h, 16 h or 24 h. All three final preparations were similar in their amino acid and amino sugar profiles, SDS-PAGE patterns and carbohydrate (PAS)/protein ratios, suggesting that within limits (8 to 24 h), varying the centrifugation time during the second CsCl centrifugation does not significantly alter the composition of mucin. The yield of immunoreactive mucin after 24 h, however, was less than that after 16 h or 8 h of centrifugation. None of the mucin preparations reacted with an antibody to human fibronectin, but all of them gave a weakly positive band at 66 kDa (most likely albumin) on a western blot treated with antihuman serum proteins antibody. Thus, all the preparations appeared to have a very minor amount of albumin contamination. (By visual inspection we estimate this to be less than 0.1% of the mucin protein.)

These findings were compared with the standard mucin prepared using a Type 50.2 Ti rotor as described in Results. The three mucin preparations obtained using the NVT 65 rotor were almost identical with the standard mucin with respect to composition, SDS-PAGE and lack of contamination. Thus, an acceptably pure mucin preparation could be obtained after two sequential 16-h CsCl gradient runs in the NVT 65 rotor. This compares favorably with the two 48-h runs used to prepare standard mucin in a conventional fixed-angle rotor.

References

1. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C., *Anal. Biochem. 150*, 76-85 (1985)
2. Mantle, M., Allen, A., *Biochem. Soc. Trans. 6*, 607-609 (1978)
3. Mantle, M., Forstner, G. G., Forstner, J. F., *Biochem. J. 224*, 345-354 (1984)
4. McCool, D. J., Marcon, M. A., Forstner, J. F., Forstner, G. G., *Biochem. J. 267*, 491-500 (1990)

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