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High-performance size-exclusion chromatography of porcine colonic mucins

Comparison of Bio-Gel® TSK 40XL and Sepharose® 4B columns

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ABSTRACT

A high-performance size-exclusion chromatography (HPSEC) method was developed for the separation of porcine colonic mucins using a Bio-Gel® TSK 40XL HPSEC column (300 mm × 75 mm). In addition, porcine gastric and bovine submaxillary mucin preparations were used to describe more fully the separation characteristics of the HPSEC column. For comparison, the same preparations were also separated using a Sepharose® 4B column (100 cm × 2.6 cm). The colonic and gastric mucins eluted in the void volume (V_0) of both columns. Bovine submaxillary mucin was in the elution volume (V_e) of both columns. Analytical HPSEC of fractions (V_0 and V_e) of the various preparations obtained by Sepharose 4B chromatography exhibited retention times identical to those for fractions obtained by HPSEC. After separation by both methods, purified mucins were obtained by $CsCl_2$ density gradient ultracentrifugation; analytical HPSEC profiles, protein contents, and monosaccharide compositions of both gastric and colonic mucins from either column were similar. The HPSEC method, however, is ideally suited to separate microgram to milligram quantities of colonic mucin preparations quickly: 2 to 4 h, compared with 24 to 30 h for the Sepharose 4B method.

INTRODUCTION

The mucus of the gastrointestinal tract is a complex mixture of glycoproteins, water, electrolytes, lipids, and proteins of serous or cellular origin. The visco-elastic character and lubricating nature of mucosal surfaces result mainly from the presence of high molecular weight, polydisperse glycoproteins, namely mucins, which are synthesized and secreted all along the gastrointestinal tract by goblet cells [1].

Gastrointestinal mucins generally have molecular weights greater than $2 \cdot 10^6$ dalton and carbohydrate compositions between 60% and 80%. Mucins also contain various amounts of sulfate and have various ratios of constituent monosaccharides (sialic acid, N-acetylglucosamine, N-acetylgalactosamine, galactose and fucose). The oligosaccharide moieties are generally linked to serine and threonine residues of the protein component through acetal bonds (O-glycosidic) [2,3].

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To study the developmental pattern of colonic mucins, we used miniature pigs as model organisms, because intestinal development and function in pigs resemble those in the human infant. Infant miniature pigs, however, can provide only limited amounts of tissue; therefore, a suitable micropreparative method was required. Porcine colonic mucins have a molecular weight of approximately $15 \cdot 10^6$ dalton and have been separated by Sepharose® 4B gel filtration [4]. The objective of this investigation was to determine whether high-performance size exclusion chromatography (HPSEC) could be used in lieu of gel filtration to separate colonic mucins. To describe the separation characteristics of the HPSEC column more completely and to compare its capabilities with those of the Sepharose 4B column, porcine gastric mucins ($2 \cdot 10^6$ dalton) and bovine submaxillary mucins (375 000 dalton) were also studied.

EXPERIMENTAL

Materials

Concentrated hydrochloric acid was purchased from Fisher (Pittsburgh, PA, U.S.A.). Durapore® hydrophilic filters (0.22 μm, 48 mm) were purchased from Waters (Milford, MA, U.S.A.). Molecular weight markers (MW-GF-1000 dit), enzyme inhibitors, deoxyribonuclease I (Type IV), ribonuclease (Type III-A), porcine gastric mucin Type II, bovine submaxillary mucin Type I, Trizma®, sodium azide, cesium chloride, Sepharose 4B, carbohydrate standards and all analytical procedure chemicals, with the exception of the BCA protein reagent (Pierce, Rockford, IL, U.S.A.) were purchased from Sigma (St. Louis, MO, U.S.A.). The Bio-Gel® TSK 40XL analytical column and guard columns were purchased from Bio-Rad (Richmond, CA, U.S.A).

Methods

Colonic specimen. A 6-month-old pig was killed by electrocution; the colon was rapidly removed and washed extensively with phosphate-buffered saline (PBS) that contained the following additives: sodium azide, 0.02%; phenylmethanesulfonyl fluoride (PMSF) (dissolved in 2.5% ethanol), 2 m*M*; N-ethylmaleimide, 10 m*M*; and sodium EDTA, 10 m*M*. The colon was opened along its longitudinal axis and the mucosa was scraped with a glass slide. The scrapings were suspended in 25 ml of 0.01 *M* Tris–HCl (pH 8.0, 0.02% sodium azide), homogenized for 30 s with a Tekmar Tissumizer Mark II (Cincinnati, OH, U.S.A.), then centrifuged at 34 000 rpm (105 000 *g*) for 1.0 h at 4°C. The supernatant was dialyzed against Milli-Q (Millipore, Houston, TX, U.S.A.) water for 2 days at 4°C and then lyophilized.

RNase and DNase digestion. Colonic, gastric, and submaxillary mucin preparations were dissolved in 20 to 40 ml of PBS, pH 7.0, that contained 0.02% sodium azide, 10 m*M* magnesium sulfate, 50 g/ml DNase, and 50 μg/ml RNase [5]. The mixtures were incubated at ambient temperature for 16.0 h. After incubation, each mixture was centrifuged for 30 min at 6000 *g* and 4°C. Each supernatant was then dialyzed extensively against Milli-Q water. The dialysates were frozen in liquid nitrogen and subsequently lyophilized.

Lipid extraction. Delipidation of the colonic mucin preparation was carried out by extraction with chloroform–methanol; two extractions were performed, first at 2:1, then 1:2 [6].

Sepharose 4B chromatography. Lyophilized colonic, gastric, and submaxillary mucin preparations (90 to 103 mg) were dissolved in 1.0 to 2.5 ml of 0.01 *M* Tris–HCl, pH 8.0, then loaded onto a 100 cm × 2.6 cm column containing Sepharose 4B that was previously equilibrated in 0.01 *M* Tris–HCl, pH 8.0, containing 0.02% sodium azide [7]. After elution at 18.0 ml/hour with equilibration buffer, 4.5-ml fractions were collected and peaks were located by determining the absorption of each fraction at 230 nm and 280 nm. Fractions that contained carbohydrates were detected at 492 nm using the phenol–sulfuric acid reaction [8].

High-performance size-exclusion chromatography. Size-exclusion chromatography was performed on a Waters 840 liquid chromatography system. A DEC 380 Professional computer (Digital Equipment Co., Maynard, MA, U.S.A.) was used for data acquisition and system control. Communication between the DEC 380 and the liquid chromatography components was established via a system interface module. A Model 510 pump was used for flow-rate control. Sample injections (10 to 150 μl) were performed by a Model 712 WISP auto-injector. Protein peaks were detected at 230 and 280 nm by using a Waters Model 490 Multiwavelength Detector.

A Bio-Gel TSK 40XL (7.5 mm × 300 mm, 10 μm) column was used to separate each mucin preparation. The column was equilibrated with 0.05 *m* Tris–HCl buffer (pH 8.0), and the proteins were eluted with the same buffer at a flow-rate of 1.0 ml/min. Before chromatography, the eluent was filtered through a Durapore hydrophilic filter (0.22 μm).

When the column was used preparatively, 4 to 12 mg of lyophilized material was dissolved in 100 to 150 μl of starting buffer and injected into the high-performance liquid chromatograph; 6 min after injection, 250-μl fractions were collected every 0.25 min. After separation of the sample, 20-μl aliquot was removed from each fraction and analyzed by HPSEC; the fractions that contained the void volume peak were pooled.

Molecular weight calibration. The HPSEC column was calibrated by injecting 20 μl of 1.0 mg/ml (in 0.05 M Tris–HCl, pH 8.0 buffer) solutions of carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase, sweet potato β-amylase, horse spleen apoferritin and bovine thyroglobulin. The peaks were detected by their absorbance at 230 nm.

To calibrate the Sepharose 4B column, the proteins were divided into two groups that were chromatographed separately. Carbonic anhydrase (3.0 mg), alcohol dehydrogenase (5.0 mg) and horse spleen apoferritin (10.0 mg) comprised the first group; the proteins were dissolved in 3.0 ml of Tris–HCl, pH 8.0, then loaded onto the column. The second group of proteins consisted of bovine serum albumin (10.0 mg), β-amylase (3.0 mg) and thyroglobulin (10.0 mg). After measuring the absorbance of each fraction at 230 nm, the elution volume of each peak was determined by measuring each fraction with a graduated cylinder.

Density gradient ultracentrifugation. Lyophilized material was dissolved in 15 ml of a 68% (68 g $CsCl_2$/100 ml PBS) solution; the final density of $CsCl_2$ was 1.42 g/ml. Density gradient ultracentrifugation was performed in a Beckman (Palo Alto, CA, U.S.A.) Model L2-70M ultracentrifuge using a vertical rotor VTi 50. The sample was spun at 36 000 *g* for 48 h at 4°C. After the run was completed, ten sequential 1.5-ml fractions were collected in a Beckman fraction recovery system. The density of each fraction was determined by weighing a 100-μl aliquot. Each fraction was exten-

sively dialyzed against Milli-Q water at 4°C. After dialysis to remove cesium chloride, the absorption of each fraction was measured at 230, 280 and 492 nm. In addition, 50-μl aliquots of each fraction were analyzed by HPSEC.

Protein assay. Protein concentrations were measured using the bicinchoninic acid (BCA) method [9].

Hydrolysis [10]. A 10-μg amount of separate mucin fractions (in 10 to 80 μl water) were placed into Waters Pico Tag® reaction vials; the vials were then placed into a hydrolysis chamber and dried under vacuum. For neutral monosaccharide determination, 200 μl of 2.0 *M* HCl were placed into the bottom of the hydrolysis chamber. The chamber was attached to a Waters Pico Tag hydrolysis station, and the interior of the chamber was evacuated, then flushed three times with nitrogen. The sealed chamber was placed into an oven and hydrolyzed for 4.0 h at 100°C. After gas-phase hydrolysis, the chamber was opened to release HCl. The residual HCl was removed under vacuum. The procedure was identical for the hydrolysis of amino sugars, except that 4.0 *M* HCl was used for a period of 6.0 h.

Neutral and amino sugar analysis [10].
After hydrolysis, 100 μl of Milli-Q water was added to each vial, and a 50-μl sample was analyzed by chromatography on a Dionex (Sunnyvale, CA, U.S.A.) CarboPak® anion-exchange column. An isocratic gradient using 16 m*M* NaOH at 1.0 ml/min was used to elute the monosaccharides. The monosaccharides were detected by a Dionex pulsed amperometric detector employing a gold electrode.

Sialic acid analysis. Sialic acid was determined by the method of Warren [11] after hydrolysis with 0.1 H_2SO_4 for 1 h at 80°C.

RNA, DNA and glucuronic acid determinations. RNA content was assessed by measuring the absorbance at 260 and 232 nm [12]. The DNA content was determined by using a fluorimetric method [13]. The glucuronic acid content was measured using the method of Bitter and Muir [14].

RESULTS AND DISCUSSION

As shown in Fig. 1, on both the Bio-Gel TSK 40XL and Sepharose columns, proteins with molecular weights between 150 000 and 669 000 dalton demonstrated a linear relationship between V_e/V_0 and the log of the molecular weight (where V_0 = void volume and V_e = elution volume). Neither carbonic anhydrase (29 000 dalton) nor bovine serum albumin (66 000 dalton) showed a linear relationship on either column. The deviation, however, was less for the HPSEC column than for the Sepharose 4B column. This result was expected because the approximate molecular weight fractionation range for globular proteins is 60 000 to $20 \cdot 10^6$ dalton [15] on the Sepharose 4B column and 10 000 to $4 \cdot 10^6$ dalton [16] on the Bio-Gel TSK 40XL (HPSEC) column.

The analytical profile (Fig. 2a) confirmed the presence of a high-molecular-weight component in the V_0 (6.74 min) of the HPSEC column after separation of the colonic mucin preparation. Non-mucin components that had a lower molecular weight eluted in a single peak at 10.0 min. The HPSEC micropreparative profile shown in Fig. 2b demonstrates column overloading (10.5 mg in 150 μl). Even so, Fig. 2d–f demonstrates the separation of the mucin fraction (V_0, 6.30 min) from the lower molecular weight components. The V_0 fraction (mucin) was obtained by pooling the

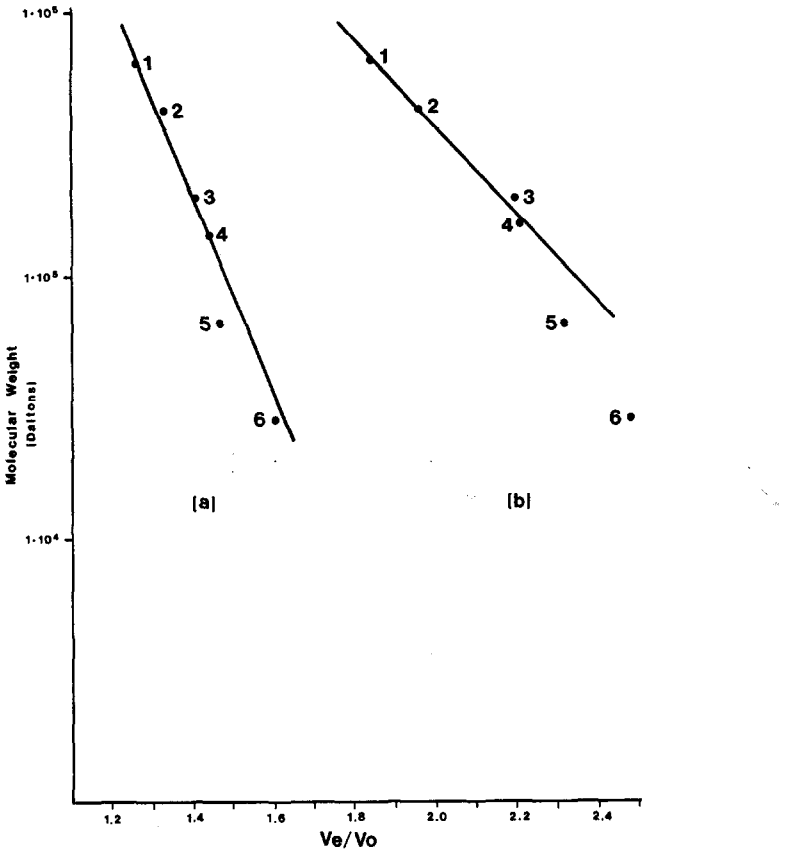

Fig. 1. Molecular weight calibration of the HPSEC (a) and Sepharose 4B (b) columns. The proteins were separated and the elution volumes were determined as described in the *Methods* section. Proteins: 1 = thyroglobulin (669 000); 2 = apoferritin (443 000); 3 = β-amylase (200 000); 4 = alcohol dehydrogenase (150 000); 5 = bovine serum albumin (66 000); 6 = carbonic anhydrase (29 000).

tubes collected from 6.00 to 7.50 min. Tubes collected from 7.75 to 12.0 min (Fig. 2e and f) demonstrated the presence of lower-molecular-weight components. The results obtained by Sepharose 4B chromatography (Fig. 2c) reflected those obtained by analytical HPSEC; the V_0 was easily separated from the lower-molecular-weight components. Both the analytical HPSEC and the Sepharose 4B chromatograms reflected, on the basis of absorbance at 230 and 280 nm respectively, the low amounts of V_0 material present in the colonic mucin preparation. After pooling, dialysis, lyophilization and weighing of the V_0 material, 2.7 and 4.7% (w/w) of the material applied to the HPSEC and Sepharose 4B columns, respectively, were recovered.

The results obtained in the separation of the gastric mucin preparation (Fig. 3a–f) were similar to those observed with colonic mucin. Although the molecular weight of porcine gastric mucin has been reported to be approximately $2 \cdot 10^6$ dalton [17], the mucin fraction eluted in the V_0 of both the HPSEC and Sepharose 4B columns. On the basis of the absorbance at 230 nm, analytical HPSEC reflected a

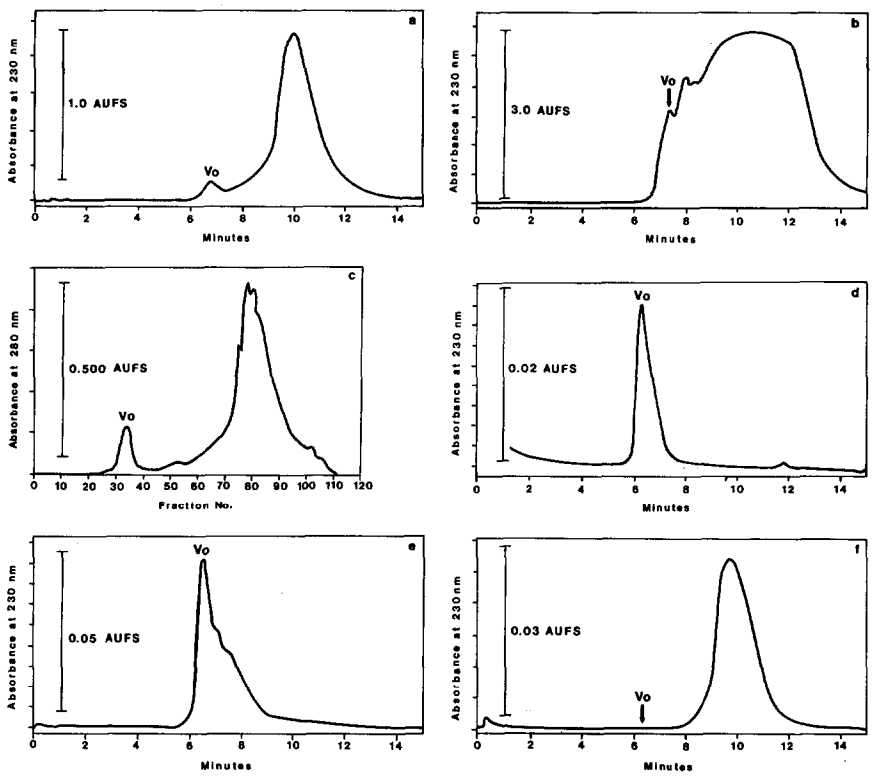

Fig. 2. Representation of the HPSEC and Sepharose 4B separations of adult porcine colonic mucin preparation after DNase and RNase treatment. (a) An analytical HPSEC injection (700 μg, 10 μl) was separated as described in the *Methods* section. The retention time of the mucin-containing fraction (V_0) was 6.74 min. (b) HPSEC micropreparative separation of 10.5 mg (150 μl). (c) Sepharose 4B separation of 101 mg (1.95 ml). Tubes 30–40 (V_0, fraction A), 48–54 (fraction B), and 70–95 (fraction C) were pooled. (d–f) 50-μl injections of HPSEC fractions collected between 6.75 and 7.0 min, 7.75 and 8.0 min, and 9.5 and 12.0 min, respectively. Tubes collected from 6.50 to 7.75 min (V_0, fraction A) and 9.75 to 12.0 min (fraction B) were pooled.

much larger V_0 population (relative to the included peak) than was present in the colonic preparation. After pooling, dialysis, lyophilization, and weighing, 16.8 and 19.4 (w/w) of the material applied to HPSEC and Sepharose 4B columns, respectively, was recovered in the V_0 fractions.

The molecular weight of bovine submaxillary mucins is approximately 375 000 dalton [18]; they would therefore be included in the elution volume of both columns. The analytical HPSEC profile (Fig. 4a) demonstrated the presence of one broad peak which eluted at 8.96 min. In contrast, the Sepharose 4B profile (280 nm monitoring) revealed a V_0 peak as well as an included peak. The Sepharose 4B V_0 fraction was collected, dialyzed, and lyophilized. The phenol sulfuric acid determination demonstrated the absence of carbohydrate, and because the material did not absorb at 230 nm (Fig. 4a), it was concluded that the V_0 fraction did not contain mucins. No further determinations were therefore made on the material.

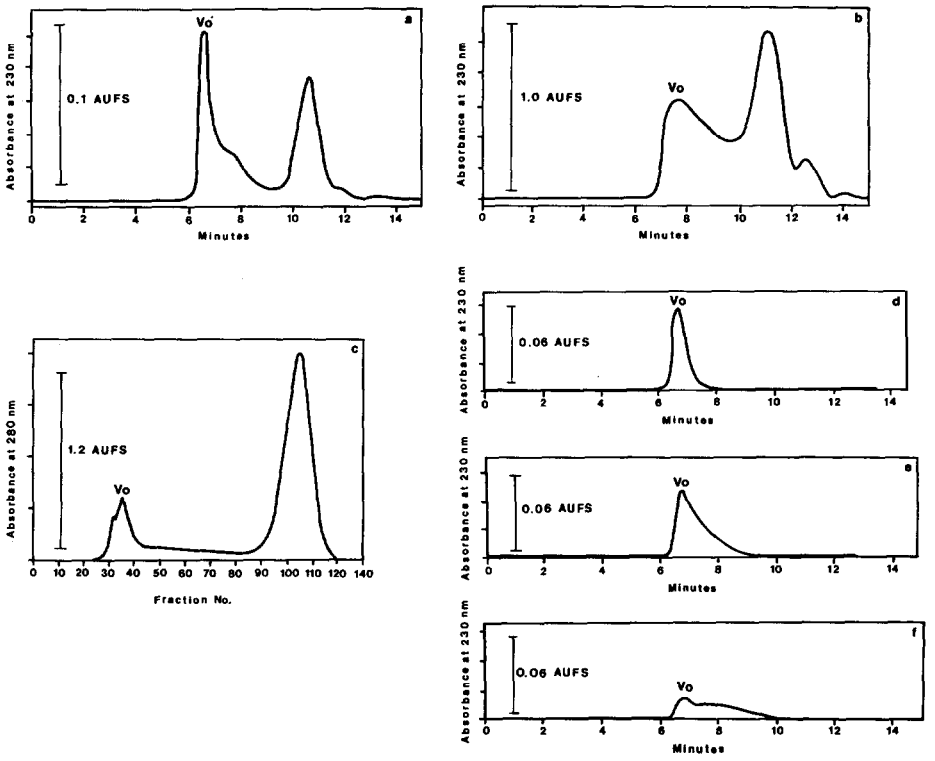

Fig. 3. Representation of the HPSEC and Sepharose 4B separations of porcine gastric mucin preparation after DNase and RNase treatment. (a) An analytical HPSEC injection (1.0 mg, 25 μl) was separated as described in the *Methods* section. The retention time of the mucin-containing fraction (V_0) was 6.61 min. (b) HPSEC micropreparative separation of 6.0 mg (150 μl). (c) Sepharose 4B separation of 209 mg (4.9 ml). Tubes 32–42 (fraction A) and 90–120 (fraction B) were pooled. (d–f) 50-μl injections of HPSEC fractions collected between 7.25 and 7.50 min, 8.00 and 8.25 min, and 9.25 and 9.50 min, respectively. Tubes collected from 6.75 to 7.75 min (V_0, fraction A) and 9.75 to 12.0 min (fraction B) were pooled.

The fractions obtained by HPSEC and Sepharose 4B separation were identical in retention times (Table I). Once the preparations were separated by HPSEC and Sepharose 4B chromatography, the pooled mucin-containing fractions were purified by $CsCl_2$ density gradient ultracentrifugation. After separation with either gel filtration or HPSEC followed by ultracentrifugation, the purified mucins were shown to be free of DNA, RNA and glucuronic acid. The monosaccharide and protein compositions of the HPSEC- and Sepharose 4B-separated colonic and gastric mucins (after ultracentrifugation) were similar to one another. The literature value for galactose in procine colonic mucin was 7% higher than the value we obtained, and the N-acetylglucosamine content was approximately 7% lower than either the HPSEC- or Sepharose 4B-separated mucins [4]. The composition of the HPSEC- and Sepharose 4B-separated porcine gastric mucins also agreed well with each other. However, the literature values were approximately 5% lower for N-acetylgalactosamine and 6% lower for sialic acid [17]. In contrast, the protein content reported in the literature was

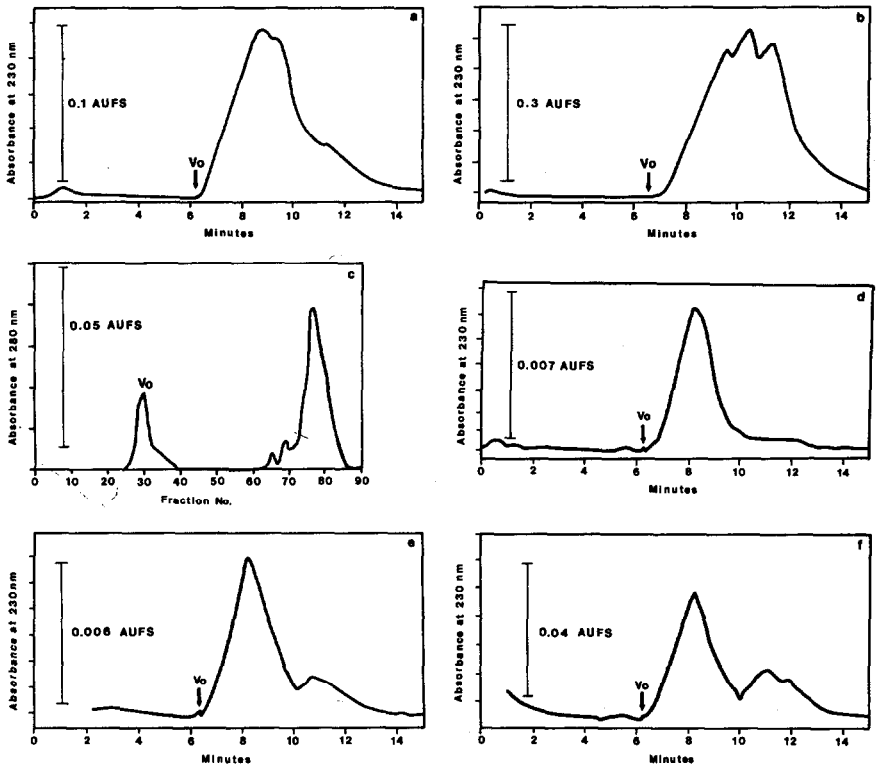

Fig. 4. Representation of the HPSEC and Sepharose 4B separations of bovine submaxillary preparation after DNase and RNase treatment. (a) An analytical HPSEC injection (2.12 mg, 20 μl) was separated as described in the *Methods* section. The single broad peak had a retention time of 8.96 min. (b) HPSEC micropreparative separation of 15.9 mg (150 μl). (c) Sepharose 4B separation of 103 mg (0.98 ml). Tubes 27–36 (V_0, fraction A) and 70–82 (fraction B) were pooled. (d-f) 50-μl injections of HPSEC fractions collected between 9.50 and 9.75 min, 10.75 and 11.0 min, and 11.75 and 12.0 min, respectively. Tubes collected from 9.0 to 10.75 min (fraction A) and 10.75 to 15.0 min (fraction B) were pooled.

TABLE I

RETENTION TIMES (t_R) OF POOLED FRACTIONATED MUCINS SEPARATED ON HPSEC AND SEPHAROSE 4B COLUMNS AND ANALYZED ON AN HPSEC COLUMN

Mucin	HPSEC		Sepharose 4B	
	Fraction	t_R (min)	Fraction	t_R (min)
Colonic	A (V_0)	6.43	A(V_0)	6.43
Gastric	A (V_0)	6.51	A (V_0)	6.49
Colonic	B	9.56	B	9.67
Gastric	B	10.6	B	10.6
Submaxillary	B	8.33	B	8.32

approximately 10% higher than the value we found. The starting material for this study was a commercial preparation, and the method by which the preparation was obtained was not available. Consequently, the differences between the values determined in this study and those in the literature may be result of different methods for isolation of the mucin fraction before chromatography and ultracentrifugation.

The compositions of the HPSEC- and Sepharose-separated submaxillary mucins were in fair agreement with each other. The relatively poorer agreement between the values of the two methods probably resulted from the inclusion of the mucins by both columns, and from the fact that the separations were not equivalent. The separation of submaxillary mucins was included in this investigation only for the purpose of comparing the HPSEC and Sepharose 4B methods; the values for submaxillary mucins reported in the literature (Table II) were obtained by using non-chromatographic methods of separation [18].

TABLE II

PROTEIN AND MONOSACCHARIDE COMPOSITION OF PURIFIED PORCINE COLONIC (PC), PORCINE GASTRIC (PG) AND BOVINE SUBMAXILLARY (BS) MUCINS (%, w/w)

Literature values are given without the sulfate content.

	Composition (%, w/w)								
	PC			PG			BS		
	HPSEC	Sepharose	Lit. [12]	HPSEC	Sepharose	Lit. [17]	HPSEC	Sepharose	Lit. [18]
Fucose	14.6	12.0	12.0	12.3	12.3	14.3	1.88	2.35	0.91
Galactose	17.3	17.4	24.0	29.0	30.6	32.9	6.51	5.31	5.15
N-Acetyl galactosamine	10.9	11.8	9.58	12.9	13.0	10.5	15.6	11.3	–
N-Acetyl glucosamine	32.4	34.8	27.6	32.6	29.2	24.7	–	–	18.8[a]
Sialic acid	13.0	11.4	11.4	6.51	8.72	0.25	25.5	28.1	21.2
Protein	11.8	12.8	15.3	6.74	7.10	17.2	50.6	52.8	53.9

[a] Total hexosamine content.

The objective of this study was to determine whether the HPSEC method could replace Sepharose 4B in the purification of colonic mucins. Preliminary experiments (data not shown) showed that very small amounts of mucins were present in colonic mucosal scrapings of infant miniature pigs. To harvest more mucins, we decided to see whether we could collect more of the lower molecular weight fractions and remove the contaminant components with the density gradient ultracentrifugation step; separation would be contingent on the lower-molecular-weight protein/glycoprotein having a lower density. Secondly, the density gradient ultracentrifugation step was originally implemented to separate a low-density, high-molecular-weight, non-mucin glycoprotein (present in the HPSEC or Sepharose 4B V_0 fractions) from the high-density, high-molecular-weight mucins [19]. To date, only high-molecular-weight, high-density colonic mucins have been described [11]. In Fig. 5a, one can observe the

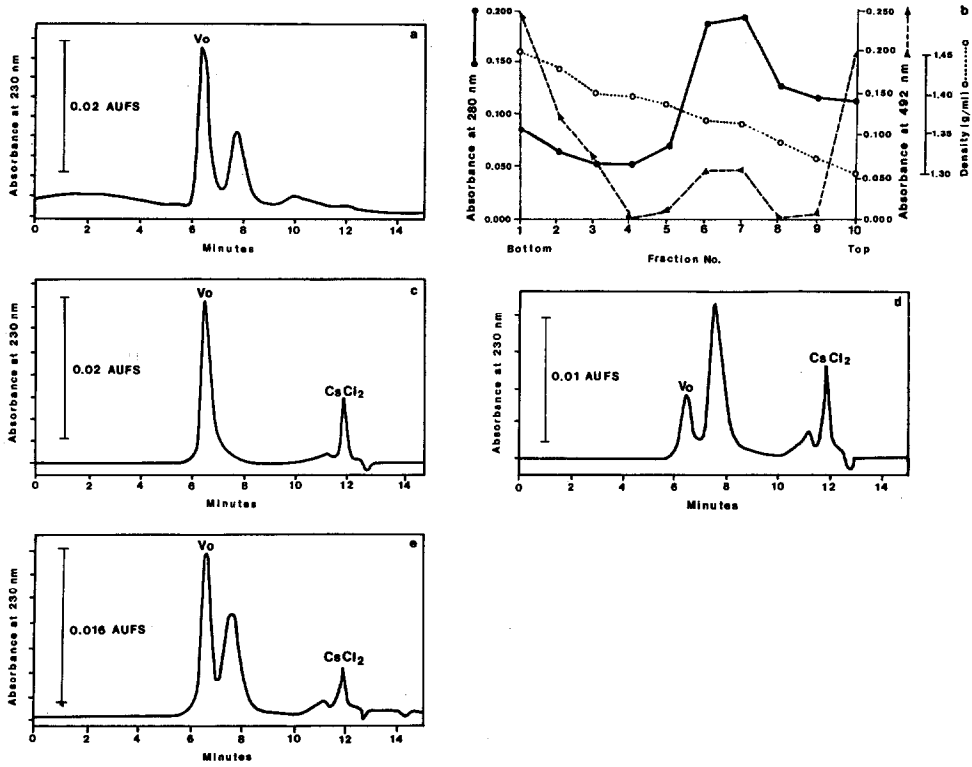

Fig. 5. HPSEC demonstration of the removal of two non-mucin components by density gradient ultracentrifugation. (a) Depiction of an HPSEC analytical profile of an HPSEC-purified preparation obtained by pooling fractions from 6.50 min to 8.35 min (instead of 6.5 min to 7.75 min). The V_0 peak eluted at 6.42 min and the second peak eluted at 7.77 min. (b) Density gradient ultracentrifugation separation of the sample depicted in (a). (c–e) Representation of HPSEC analytical profiles of ultracentrifuge fractions: density = 1.452 (highest density), 1.338 and 1.323 g/ml (lowest density), respectively. The retention time of the V_0 peak found in the density = 1.452 g/ml fraction was 6.49 min while the retention times of the two peaks found in the density = 1.338 g/ml and density = 1.32 g/ml fractions were 6.48, 7.62 min, and 6.58, 7.62 min, respectively.

lower-molecular-weight peak at 7.77 min that was purposely included into the pooled mucin fraction. Fig. 5b represents the density gradient separation of the mixture depicted in Fig. 5a. The high-density fraction (Fig. 5c) contained only the V_0 (mucin) peak. The middle density fractions (data not shown) contained trace amounts of V_0 material. The two low-density fractions (Fig. 5e and f) contained the high-molecular-weight V_0, non-mucin, glycoprotein and the lower-molecular-weight contaminant which eluted at 7.77 min.

Our results indicate that the HPSEC method is ideally suited for the separation of colonic mucins, and although the composition determined for gastric mucins differed from literature values, the monosaccharide and protein compositions of the HPSEC and Sepharose 4B mucins were similar. In contrast to the Sepharose 4B separation, which requires larger samples and longer elution times (24 to 30 h), the

HPSEC method can separate microgram to milligram quantities in 15.0 min. Even if 5 to 10 preparative injections must be made for the same sample, total separation time will require only 2 to 3 h. In addition, the HPSEC method can be used analytically to locate mucins in various chromatographic or ultracentrifugation fractions, which enable conservation of small quantities of mucins.

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