Characterization of High Molecular Weight Mucins of Rabbit Bladder

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Abstract: Epithelial mucin glycoproteins of bladder act as an effective barrier against invasion by pathogenic microorganisms and injury by toxic substances in urine. Although these glycoconjugates play important roles in the pathophysiology of bladder disorders such as intestinal cystisis, cancer, and urinary tract infections, they have not been characterized in detail either in humans or in animals. Rabbits could be useful for developing models for studying bladder disorders. In this study, we purified and partially characterized two major high molecular weight rabbit bladder mucin glycoproteins, designated RBM_1 and RBM_2, found in urine. Consistent with their mucin characteristics, amino acid compositions showed have high levels of serine, glutamic acid, proline, glycine and alanine, which together comprise 34% and 42% of the total amino acids in RBM_1 and RBM_2, respectively. Carbohydrate compositional analysis indicated that RBM_1 and RBM_2 consist of *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), galactose (Gal), *N*-acetylneuraminic acid (NeuAc) and fucose (Fuc) in the molar ratio of 1.0: 0.82: 0.12: 0.30: 0.02 and 1.0: 1.03: 0.46: 0.16: 0.05, respectively; mannose (Man) was not detected in either mucin. Both mucin fractions were strongly reactive to wheat germ agglutinin, but not to Ca2 antibody specific to a human tumor mucin antigen (asialylated carbohydrate linked to protein core), suggesting that most of the galactosyl residues of oligosaccharides are sialylated. Together, the data suggest that rabbit mucin glycoproteins characterized here are distinctively different from MUC1 mucin glycoprotein found in human urine.

Key Words: Rabbit bladder, Urothelium, Urine, Epithelial mucin glycoproteins, Biochemical characterization.

INTRODUCTION

Epithelial surfaces of the animals such as the tracheobronchial, gastrointestinal, and urogenital tracts that are in direct contact with the external environment are protected by a layer of mucus secretions [1, 2]. The bladder is lined by transitional epithelium (urothelium), which is a highly specialized tissue that has to accommodate changes in its luminal surface area when the bladder fills and contracts. More importantly, the transitional epithelium acts as a permeability barrier and provides protection from the hostile environment consisting of chemicals and microbes. There is considerable evidence that implicate the association of mucin glycoconjugates in the pathophysiology of bladder disorders such as urinary tract infections [3-5], interstitial cystitis [6, 7] and bladder carcinoma [8, 9]. The abundance of glycoproteins on the luminal surface of bladder is demonstrated by the very intense staining of rabbit and human bladders by anti-glycoprotein antibodies and lectins [10-12]. Previously, we have identified MUC1 glycoprotein (referred to as epitectin) as the major mucin glycoprotein of human [13] and rabbit urothelium [14]. These and other studies in our laboratory [15] demonstrate that mucin glycoproteins are the predominant glycoconjugates in mammalian bladder rather than glycosaminoglycans as suggested by Parsons and co-workers [16, 17]. However, in contrast to mucins on the tracheal, intestinal and cervical epithelium of animals, those associated with the bladder epithelium have not been well characterized. This could be due to the difficulty in obtaining adequate quantities of fresh bladder tissue.

The glycoconjugates of the bladder epithelium are continually shed to the urine. This enabled us to purify and structurally characterize a human bladder-associated mucin MUC1 from pooled human urine [18]. Sedimentation equilibrium analysis and gel filtration revealed that human urine MUC1 (epitectin) is a polydisperse preparation with molecular weights ranging from about 0.9 to 1.3 X 10^6. Epitectin was found to contain 50% carbohydrate by weight that is present as both sialylated and non-sialyated *O*-linked oligosaccharides having core 1 and 2 structures, Gal1→3GalNAc, GlcNAc1→6(Gal1→3)GalNAc and Gal1→4GlcNAc1→6(Gal1→3)GalNAc. One proposed function for MUC1 is the protection of the epithelium from extreme pH and osmolarity [13, 19]. Another bladder glycoprotein, GP51, resembling that reported to be decreased in the urine of interstitial cystitis patients compared to urine of healthy individuals, has been purified from rabbit bladder [20]. Previously, our laboratory has purified a major mucin glycoprotein with a molecular mass of about 245 kDa from radio labeled rabbit bladder explants and identified it as the rabbit homolog of human MUC1 [14]. Here, we report the purification of milligram quantities of two high molecular weight mucins from rabbit urine and biochemical characteristics of those mucins.

MATERIALS AND METHODS

Materials

Healthy female rabbits were maintained at the Animal facility of the Milton Hershey Medical Center. Sepharose CL-4B and cesium trifluoroacetate (CsTFA) were purchased from Pharmacia (Piscataway, NJ). Carbo Pac PA1 column

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and sugar reference standards were from Dionex (Sunnyvale, CA). Amino acid standards were from Pierce Biotechnology (Rockford, IL). Biotinylated wheat germ agglutinin and HRP conjugated streptavidin were from Vector Laboratories (Burlingham, CA). Enhanced chemiluminescent reagent was from GE Health Care (Pittsburg, PA). Ca2 monoclonal antibody specific to human mucin glycoprotein antigen was a gift from Professor Henry Harris, University of Oxford. All other chemicals were of analytical grade.

Collection and Processing of Rabbit Urine

Urine samples collected from the healthy rabbits were centrifuged to remove cell debris, and stored in -20°C until used. After accumulating about 4 liters, the urine was thawed, concentrated by ultrafiltration at 4°C using Amicon YM-30 membrane with a molecular weight cutoff of 30,000. The concentrated samples were then dialyzed against distilled water at 4°C, freeze dried, and stored at -20°C until used.

Perchloric Acid Precipitation

The freeze-dried rabbit urine was dissolved in double distilled water at 4°C. While maintaining the solution at 4°C (ice bath), an equal volume of 1.3 N perchloric acid was added with stirring [21]. The cloudy solution was incubated for 30 min with occasional mixing and then centrifuged at 7,000 rpm for 15 min at 4°C to remove the precipitated protein. The precipitate was discarded and the supernatant, kept in an ice bath, was neutralized by addition of ice-cold 1.2 M potassium hydroxide solution. The precipitated potassium perchlorate was removed by centrifugation at 7,000 rpm for 15 min at 4°C. The supernatant was dialyzed against double distilled water at 4°C for 48 h and lyophilized to recover the glycoconjugates (mucins and glycosaminoglycans) containing high carbohydrate content.

Gel Filtration Chromatography

The perchloric acid-soluble material was reconstituted and fractionated on a Sepharose CL-4B column (7 x 83 cm) pre-equilibrated with phosphate buffered saline (PBS). Fractions (16.5 ml each) were collected and aliquots analyzed for protein and sialic acid. The sialic acid containing fractions were pooled, dialyzed, and lyophilized.

CsTFA Density Gradient Centrifugation

The material obtained from gel filtration chromatography was re-dissolved in 50 mM Tris-HCl buffer, pH 8.0 containing 4 M guanidine-HCl by stirring overnight at 4°C [22]. The solution was centrifuged at 3000 rpm in a Sorvall centrifuge for 5 min to remove a small amount of insoluble material. The density of the supernatant was adjusted to 1.35 g/ml with CsTFA, and the solution centrifuged at 14°C in a Beckman 70 Ti rotor for 72 h at 42,000 rpm. Fractions (1 ml) were collected by gentle aspiration from the bottom of tube and densities of every fifth fraction determined by weighing 100 µl in a calibrated micropipette. Aliquots of each fraction were analyzed for sialic acid. The fractions positive for sialic acids were pooled and the material recovered by exhaustive dialysis and lyophilization.

Cellulose Acetate Electrophoresis

Cellulose acetate electrophoresis was performed in a Beckman R-101 microzone electrophoresis cell with pyridine-formic acid buffer, pH 5.0 at 4 mA for 20 min [23]. The mucins and glycoprotein standards on the cellulose acetate strips were detected by staining with 0.1% Alcian blue in 0.5% aqueous acetic acid [24] or the periodate acid-Schiff reagent [25].

Gel Electrophoresis

SDS-polyacrylamide electrophoresis was performed by using 4-15% mini gradient gels (Bio-Rad) and 2% polyacrylamide gels containing 0.5% agarose [26]. After electrophoresis, the gels were stained with Coomassie brilliant blue followed by silver reagent [27] for detection of proteins or with the periodate acid-Schiff reagent for detection of glycosylated proteins.

Lectin Blot and Western Blot Analyses

After SDS-PAGE, the proteins from the gels were transferred to nitrocellulose membrane using Tris-glycine buffer containing 0.037% SDS and 20% methanol at 80 mA overnight at 4°C. The membrane was incubated with biotinlylated wheat germ agglutinin (50 µg/ml) for 1 h at room temperature, washed and probed with HRP-conjugated streptavidin (50 ng/ml) for 1 h at room temperature. The bound streptavidin-HRP was detected using the enhanced chemiluminescence system.

For Western blot analysis, membranes containing rabbit bladder mucin fractions were probed with Ca2 antibody [28] followed by HRP-conjugated anti-mouse IgG secondary antibody. The bound antibody was detected using the enhanced chemiluminescence system as above.

Colorimetric Analysis for Proteins and Carbohydrates

Chromatographic column effluents and CsTFA density gradient fractions were screened for protein by absorbance at 280 nm. Sialic acid in the fractions was measured by thiobarbituric acid assay after hydrolysis with 0.1 N H_2SO_4 for 1 h at 80°C [29].

Amino Acid Analysis

Amino acid composition was determined by Pico-Tag analysis using reverse phase HPLC [26]. The mucin samples (200 µg) were acid hydrolyzed with 6 N HCl (Pierce Biotechnology, Rockford, IL) *in vacuo* at 110°C for 18 h and the hydrolysate dried. The material was dissolved in a solution consisting of ethanol, water, and triethylamine in the ratios of 2:2:1 (v/v/v) and redried. These samples were dissolved in derivatizing solution consisting of ethanol, water, triethylamine, and phenylisothiocyanate in the ratios of 7:1:1:1 (v/v/v/v). The mixture was incubated at room temperature for 5 min and dried under vacuum in a Speed-Vac. The derivatized samples were analyzed by HPLC on a 3.9 x 300-mm C18 Pico-Tag column (Waters, Millipore) using aqueous sodium acetate and acetonitrile gradient as described [30] and detected at 245 nm. The amino acid peaks were quantitated by correcting for response factors of standard amino acids derivatized and analyzed as above.

Carbohydrate Compositional Analysis

Hexose and hexosamine content of the samples were analyzed by high-performance anion-exchange chromatography using a Dionex HPLC equipped with a pulse amperometric detector [31]. For neutral sugars and amino sug-

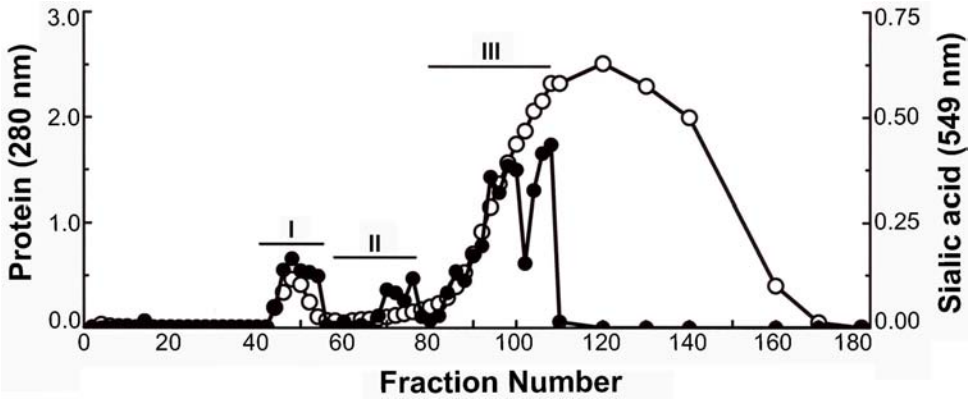

Fig. (1). Gel filtration chromatography of glycoproteins isolated from rabbit urine by perchloric acid precipitation on Sepharose CL-4B columns (7 x 83 cm). The column was eluted with PBS and fractions (16.5 ml) collected and aliquots analyzed for protein at 280 nm (o) and *N*-acetylneuraminic acid at 549 nm (•) by thiobarbituric acid assay. Fractions in Peaks I, II and III were pooled as indicated by horizontal bars.

ars, the samples were hydrolyzed in 2 M trifluoroacetic acid at 100°C for 8 h. The dried hydrolysates were dissolved in HPLC grade water and analyzed on a Carbo Pac PA-1 column by isocratic elution with 14 mM sodium hydroxide. Sialic acids (NeuNAc and NeuNGly) were determined after hydrolysis with 0.1 N H_2SO_4 at 80°C for 1 h. The hydrolysates were neutralized with NaOH and an aliquot analyzed on the above column. Elution was with 10 mM NaOAc/102 mM NaOH for 10 min followed by a gradient of 10 mM NaOAc/102 mM NaOH to 40 mM NaOAc/60 mM NaOH over the next 20 min and finally 200 mM NaOAc/60 mM NaOH for 10 min. These analyses were performed at ambient temperature at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Rabbit bladder mucin was purified from pooled urine because urine from an individual rabbit was insufficient for biochemical characterization. Pooled urine samples were subjected to ultrafiltration using YM-30 membrane to desalt and remove low molecular weight material and the concentrate freeze-dried. In a typical experiment, about 4 liters of urine yielded 633 mg of dry powder. This material was then treated with perchloric acid to precipitate proteins and glycoproteins of low carbohydrate content. Mucins that have high carbohydrate content are soluble in perchloric acid and therefore, remain in the supernatant. After perchloric acid precipitation, the supernatant gave 30 mg of glycoconjugates. Sepharose CL-4B column chromatography of the perchloric acid-soluble material yielded three fractions; the high molecular size CL-4B excluded fraction (I), the intermediate size material just included in CL-4B (II) and protein-rich low molecular weight material (III) (Fig. **1**).

Material corresponding to Peak I in Fig. (**1**) that contained high molecular weight mucins was subjected to density gradient centrifugation under dissociative conditions using CsTFA [22]. This procedure resolved Peak I material into two distinct fractions; a typical sedimentation profile for the CsTFA density gradient centrifugation of Peak I material is illustrated in Fig. (**2**). Thus, a high-density (~1.47) fraction (designated RBM_1) and a well-resolved lower density (~1.42) fraction (RBM_2) were obtained (Fig. **2**). The purified RBM_1 and RBM_2 fractions were used for biochemical characterization.

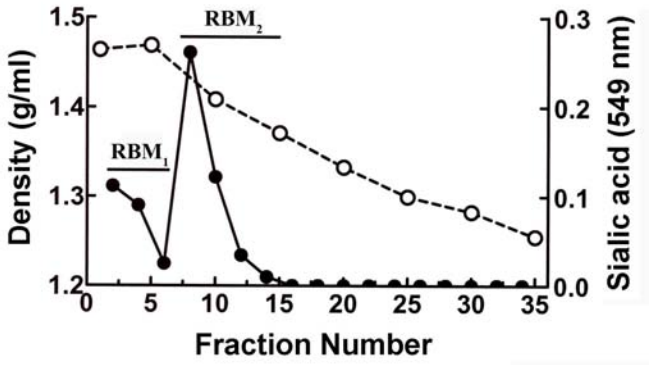

Fig. (2). CsTFA density gradient centrifugation of the high molecular weight mucins from rabbit urine. The material eluting in the void volume (Peak I) of Sepharose CL-4B column was subjected to CsTFA density gradient centrifugation. The gradients were fractionated and fractions analyzed for density (○) and *N*-acetylneuraminic acid (●). The mucin glycoproteins in the Peaks RBM_1 and RBM_2 were pooled as indicated by the horizontal bars, dialyzed, and lyophilized.

The purity of the high-molecular weight rabbit bladder mucin fractions (RBM_1 and RBM_2) were analyzed by gel electrophoresis in the presence of SDS using 4-15% gradient polyacrylamide mini gels, and 2% polyacrylamide and 0.5% agarose composite vertical slab gels. The polyacrylamide gels did not reveal any Coomassie blue or PAS stained low molecular weight material confirming that the preparations were free of low molecular protein/glycoprotein contaminants. As expected for high molecular weight and highly glycosylated mucins, both RBM_1 and RBM_2 neither entered the gel nor stained appreciably with Coomassie blue (not shown). On polyacrylamide-agarose composite gels, RBM_1 and RBM_2, each gave a single broad band that stained strongly with the PAS reagent and had mobility similar to ovine submaxillary mucin of molecular weight of about 2 million; no other bands were detectable on these gels by any of the staining reagents used (Fig. **3**, lanes 1 and 2). Because of the lack of high-molecular-weight glycoprotein standards and the abnormal behavior of highly glycosylated proteins in SDS-PAGE, it was not possible to estimate the molecular weight of rabbit bladder mucin fractions based on their electrophoretic mobility. However, the bladder mucins eluted in

the void area of Sepharose CL-4B and had mobility similar to that of ovine submaxillary mucin in polyacrylamide-agarose gels. Therefore, it can be estimated that RBM_1 and RBM_2 have 'apparent' molecular masses of 2.0 X 10^6 daltons or greater. On cellulose acetate electrophoresis, the mucin fractions, RBM_1 and RBM_2, each gave a single band of mobility intermediate between that of the porcine gastric mucin and hyaluronic acid; no glycosaminoglycan contaminants were detected in the mucin fractions (not shown). On the lectin blot analysis, the rabbit bladder mucin fraction RBM_1 showed high reactivity with wheat germ agglutinin (Fig. **3**, lane 7) suggesting the presence of a high density of terminal NeuAc and/or GlcNAc residues [32, 33]. Rabbit bladder mucin fraction RBM_2 also strongly reacted with wheat germ agglutinin (not shown). Western blot analysis of the rabbit bladder mucin fractions using Ca2 antibody did not show any cross reactivity. These results taken together with carbohydrate composition indicate that the oligosaccharide moieties of RBM_1 and RBM_2 are different from those in the high molecular weight MUC1 (epitectin) purified from human urine and the MUCI homolog of rabbit bladder explant [13, 18].

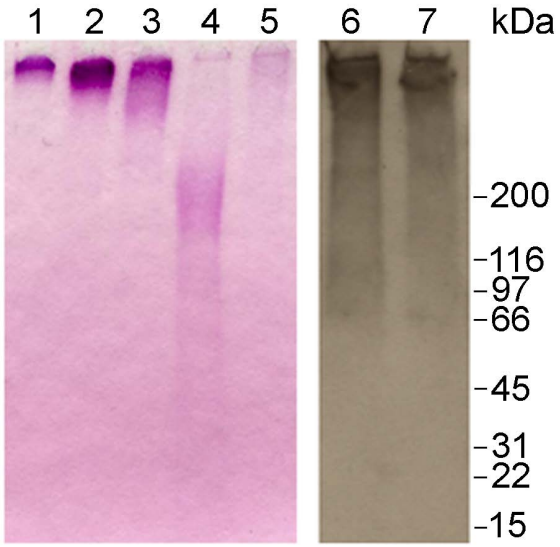

Fig. 3.

Fig. (3). SDS-polyacrylamide gel electrophoresis and Western blot analysis of rabbit bladder mucins. The purified mucin fractions RBM_1 and RBM_2 (see Fig. **2**) and lower molecular weight glycoproteins fractions (Peak II and III in Fig. **1**) were analyzed on 4-15% gradient polyacarylamide gels and the gels stained with the PAS reagent. Lanes 1 and 2, RBM_1 and RBM_2. Lanes 3 and 4, glycoprotein fractions eluted in Peaks II and III (Fig. **1**), respectively. Lane 5, ovine submaxillary gland mucin. Lanes 6 and 7, ovine submaxillary gland mucin and RBM_1, respectively were electrophoresed as above, transferred to nitrocellulose membranes, treated with biotinylated wheat germ agglutinin followed by streptavidin-HRP conjugate, and detected with the chemiluminescent reagent. Similar result was observed with rabbit bladder mucin fraction RBM_2 (not shown).

Amino acid composition of rabbit bladder mucin fractions RBM_1 and RBM_2 is shown in Table **1**. RBM_1 and RBM_2 have composition characteristics of mucins, that is, a high content of serine, glutamic acid, proline, and glycine, which together comprise about 34% and 42% of the total, respectively. The monosaccharide compositions of RBM_1 and RBM_2 are shown in Table **2**. The molar ratios of GalNAc, GlcNAc, Gal, NeuAc, and Fuc in fractions RBM_1 and RBM_2 were 1.0: 0.82: 0.12: 0.30: 0.02 and 1.0: 1.03: 0.46: 0.16: 0.05, respectively. These values are typical of mucins in that they had high content of GalNAc and no detectable quantity of Man. Analysis for uronic acids by carbazole assay [34] was negative. These results confirm that the mucin fractions RBM_1 and RBM_2 were free of nucleic acid and glycosaminoglycans contaminants.

Table 1. Amino Acid Analysis of Rabbit Bladder Mucins

	Amino Acid Residues[a]/1000 Residues	
	RBM_1	**RBM_2**
Aspartic acid	ND	ND
Glutamic acid	16	92
Serine	39	65
Glycine	213	115
Histidine	172	277
Arginine	ND	45
Threonine	ND	ND
Alanine	ND	42
Proline	72	147
Tyrosine	32	ND
Valine	ND	ND
Methionine	32	ND
Cystine	177	ND
Isoleucine	144	181
Leucine	20	36
Phenylalanine	82	ND
Lysine	ND	ND

[a]Estimated by Pico-Tag analysis using reverse phase HPLC.
ND: Not detected.

The glycoproteins in the urine have been analyzed extensively in order to evaluate the disease process affecting the urinary bladder, kidneys and urogenital tract [35]. Several low molecular weight glycoproteins such as Tamm-Horsfall glycoprotein (THGP)/Uromodulin, GP51, Fetuin-A, beta 2-glycoprotein-1, C-reactive protein, Amyloid beta A4 protein, Alpha-1-inhibitor 3, Vitamin D-binding protein, Kallikrein 3, uronic-acid rich protein (UAP) are found abundantly in mammalian urine [14, 20, 35-39]. However, the results of our studies reveal that the high molecular weight RBM_1 and RBM_2 mucin fractions we have characterized are distinct from these glycoproteins including THGP as well as the MUC1 mucins of human and rabbit bladder that we have previously reported [13, 18].

Table 2. Carbohydrate Compositions of Rabbit Bladder Mucins

Monosaccharides[a]	Monosaccharide Composition			
	RBM_1		RBM_2	
	% weight	molar ratio	% weight	molar ratio
Fucose	1	0.02	2	0.05
Galactose	6	0.12	17	0.46
Galactosamine	44	1.00	37	1.00
Glucosamine	36	0.82	38	1.03
N-Acetylneuraminic acid	13	0.30	6	0.16

[a]Analyzed by the high-pH anion-exchange HPLC.

The nature of glycoconjugates eluting in Peaks II and III of Sepharose CL-4B column (Fig. **1**) were also examined. SDS-PAGE (not shown) and agarose gel electrophoresis (Fig. **3**, lanes 3 and 4) showed the presence of several low molecular weight proteins and glycoproteins. Monosaccharide analysis indicated that Peak II and Peak III materials contain significant amount of *N*-linked glycoproteins as revealed by the presence of substantial amount of mannose. Since our focus in these studies was mucins, further purification and characterization of peaks II and III material was not undertaken.

In summary, we have purified and characterized two high molecular weight mucins designated as RBM_1 and RBM_2 from rabbit urine. The results show that it is possible to purify milligram quantities of bladder epithelial mucin glycoproteins from rabbit urine for detailed biochemical characterization. Based on analogy to previous work on MUC1 glycoprotein purified from human urine [18] and evidence that high molecular weight mucin synthesized by rabbit bladder in explants cultures were shed into the media [12], it can be inferred that these mucins originate from the bladder epithelium. The oligosaccharide of rabbit mucins appears to be relatively small in size terminating with either sialic acid or GlcNAc. Further detailed biochemical studies should aid in understanding the roles of epithelial mucins in the pathophysiology of bladder.

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