Purification and Characterization of Rabbit Ocular Mucin

Scheffer C.G. Tseng,*† Andrew J.W. Huang,*† and David Sutler*

Ocular mucin, the major product of conjunctival goblet cells, constitutes the innermost layer of precocular tear film. Ocular mucin is known for its limited amount and inaccessibility. Using impression cytology, mucus strands collected from the inferior fornix of either rabbit or human eyes were found to contain inflammatory cellular debris. In order to circumvent these difficulties and to isolate native mucin molecule(s), we bathed rabbit eyes in fluid containing isotonic PBS and 5.5 × 10⁻⁴ M acetylcholine for 4 or 12 hr. Bathing fluids containing rabbit ocular mucin (ROM), 1 ml per eye, were pooled and combined with 1M guanidine HCl and protease inhibitors containing EDTA, PMSF, and sodium azide to avoid any possible enzymatic degradation, and then separated under the same conditions by Sepharose CL-4B. In parallel, commercial porcine stomach mucin (PSM) was purified and used to compare with ROM. We also developed nitrocellulose-based dot semi-quantitative assays for nucleic acid, protein, and glycoprotein. PAS-positive fractions monitored by such a dot assay were collected at CL-4B void volume and then separated from nucleic acid contaminants by CsCl-gradient ultracentrifugation. A protein fraction, 65K, poorly-glycosylated, with high contents of Asx, Glx, and Gly was found strongly associated with both ROM and PSM, and was only separable by ultracentrifugation in 4M guanidine HCl and CsCl. Purification of the ROM was verified by SDS-polyacrylamide gel electrophoresis, amino acid analysis, and carbohydrate analysis. These results will allow future exploration of the molecular mechanism by which tear film stability is achieved. Invest Ophthalmol Vis Sci 28:1473-1482, 1987

Mucin, a macromolecular glycoprotein, is the primary product of a special type of epithelial cell. Throughout the body, mucin is the major constituent of the mucous layer overlying all mucosal surfaces, and serves as a lubricative and protective barrier. In the eye, mucin is the product of conjunctival goblet cells and constitutes the innermost layer of the precocular tear film.¹ It plays an important role in maintaining the wettability and spreadability of the tear film² ³ and ocular surface integrity. In contrast to the studies of mucins of other organs in the body, detailed biochemical analysis has been limited and incomplete in ocular mucin due to its limited amount and relative inaccessibility. Several attempts have been made in the biochemical purification and characterization of human⁴ ⁵ and rabbit⁶ ⁷ ocular mucin. The majority of their starting materials were mucus strands harvested from inferior fornix or the inner caruncle.⁴ ⁶ As will be shown in this report, these materials contain polymorphonuclear and mononuclear inflammatory cells as well as desquamating epithelial cellular debris. It is conceivable that mucin molecules could have been degraded in these materials,⁵ and therefore may not represent their functional counterparts. For example, more than one components with molecular weights smaller than 1 X 10⁶ daltons,⁴ ⁶ some mucin components with a low carbohydrate/protein ratio,⁵ and a carbohydrate content without N-acetyl neuramic acid were reported in these earlier reports. In contrast, Kaura and Tiffany recently analyzed freshly-scraped rabbit ocular mucus, and preliminarily reported a single component, high molecular weight, carbohydrate-rich mucin.⁷ In order to explore the molecular interactions of mucin and ocular surface epithelium as well as the controlling mechanism of mucin biosynthesis and secretion, we sought as the first step to purify and characterize rabbit ocular mucin. Precautions were taken with specific modifications of the purification procedures so that the native, undegraded mucin macromolecules could be isolated and analyzed.

Materials and Methods

Chemical reagents such as cesium chloride (CsCl), ethylenediammine tetraacetic acid (EDTA) disodium salt, guanidine HCl, phenylmethylsulfonyl fluoride (PMSF), sodium azide, and Tris/HCl were obtained from Sigma Chemical Company (St. Louis, MO). Biological standards for nitrocellulose dot assays in...
cluded calf thymus DNA, bovine serum albumin, and porcine stomach mucin, as well as rabbit serum albumin, and were also from Sigma. Bio-dot apparatus and nitrocellulose acetate paper were products of Bio-Rad Laboratories (Richmond, VA). Electrophoretic reagents such as sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, TEMED, and dithiothreitol (DTT) were also from Bio-Rad. Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Acetylcholine chloride (Miochol®) was from Cooper Vision (Claremont, CA). Xylazine was obtained from Haver-Lockhart (Shawnee, KS), and ketamine HCl from Parke-Davis (Morris Plains, NJ).

Impression Cytology

To understand the contents of starting materials, we decided first to analyze the mucus strands collected from inferior fornix of both human and rabbit eyes using impression cytology according to the method we have previously described. The results are shown in Figure 1. Because of potential enzymatic degradation of mucin molecule(s) using such starting materials, we decided to take the following different approaches. We also chose to analyze a commercial porcine stomach mucin (PSM) in parallel for a comparison with rabbit ocular mucin (ROM).

Rabbit Ocular Bathing

In order to collect native ocular mucin, rabbit eyes were bathed with isotonic phosphate buffered saline (PBS), pH 7.4. In brief, the rabbit was anesthetized with an intramuscular injection of mixtures of 20 mg xylazine and 200 mg ketamine and subsequently maintained by hourly injections of 100 mg ketamine for a period of 4 hr. On selected occasions, bathing was extended to 10 to 12 hr. One eye at a time was used. Following initial anesthesia, the rabbit was put on one side and a total of four 4-0 black silk sutures, two on each lid, were placed subcutaneously along both the upper and lower lid margins and one 7-0 proline suture was used to elevate the nictitating membrane. All suture threads were then suspended and secured to four separate stands. By doing so, the ocular surface formed a basin into which was then added with 1 ml of PBS containing 5.5 × 10^{-4} M acetylcholine, which as shown will stimulate the secretion of native mucin by intestinal and conjunctival goblet cells. At the end of bathing, the fluid containing discharged mucin was then collected and added to a final concentration of 1 M guanidine HCl, and 50 mM Tris/HCl, pH 7.4, containing a mixture of protease inhibitors, 5 mM EDTA, 1 mM PMSF, and 0.02% (W/V) sodium azide to prevent enzymatic degradation. The samples were then stored at −20°C before use. Our study conformed to the ARVO Resolution on the Use of Animals in Research.

Nitrocellulose Dot Assays

In order to monitor the subsequent biochemical fractionations of mucin samples and to minimize the loss of mucin materials through various purification steps, nitrocellulose dot assays were developed. These assays allowed semi-quantitative measurements of nucleic acid, protein, and glycoprotein using respective, specific histochemical staining to each component. A Bio-Rad dot assay apparatus was used, in which a nitrocellulose acetate filter paper was sandwiched between an upper unit containing 96 wells and the lower collecting chamber, to which a powered vacuum was connected. The filter in each well was first wet by vacuum suction of 200 µl/well of PBS to remove any trapped bubbles. To each well was added 50 µl of sample containing either serially-diluted standards of nucleic acid (calf thymus DNA), protein (bovine serum albumin), and glycoprotein (PSM), or unknowns obtained from biochemical fractionations. The test materials were then trapped to the surface of the filter in each well by vacuum suction. The apparatus was untightened, and the samples were fixed by drying the filter paper in a pre-heated oven at 80°C for 5 min. The dried paper was then immersed in either 1% Gill’s hematoxylin, or 1% Fast Green in methanol/acetic acid/H₂O (20:7.5:72.5), or periodic acid-Schiff’s reagent (PAS) for 1 min for nucleic acid, protein, and glycoprotein assay respectively. The paper was destained with tap water for both Gill’s hematoxylin and PAS, and with methanol/acetic acid/H₂O (20:7.5:72.5) for Fast Green, and was dried again. The developed coloration was bluish-purple for nucleic acid, green for protein, and red for mucin, all of which could be well-preserved on this dried filter paper for more than a year. The sensitivity and specificity of such an assay is illustrated in Figure 2.

Gel-Filtration Column Chromatography

Bathing fluid, 1 ml per eye, was then centrifuged at 1000 rpm, 4°C for 10 min to remove insoluble debris. The supernatant was added to a Sepharose CL-4B column (1.5 × 50 cm) which was pre-equilibrated with 50 mM Tris/HCl, pH 7.4, containing 1 M guanidine HCl with the same concentrations of protease inhibitors. The column was run at 4°C with a flow rate of 10 ml/hr. Fractions of each 1 ml were collected and monitored by a Gilford (Oberlin, OH) spectrophotometer at both 260 nm and 280 nm. Aliquots of 50 µl of each fraction were then subjected to
Fig. 1. Impression cytology appearance of mucus strands obtained from human (left) and rabbit (right) inferior fornix (original magnification ×160). Note the inflammatory cells, desquamating epithelial cells, and collapsed mucin.

nitrocellulose dot assay for nucleic acid, protein, and glycoprotein as described above.

CsCl-Density Gradient Ultracentrifugation

The PAS-positive fractions collected at the void volume of Sepharose CL-4B column were then pooled and subjected to isocratic CsCl-density gradient ultracentrifugation according to the procedure of Creeth and Horton. Void-volume materials were pooled from two to three Sepharose CL-4B runs to a total of 8 to 9 ml, to which guanidine HCl was added to a final concentration of 4 M and CsCl at a loading density of 1.39 gm/ml, and then centrifuged at 40,000 rpm, 4°C, for 64 hr using a Beckman SW 41 Ti rotor. After ultracentrifugation, tubes were punctured and a total of 16 fractions, each 0.8–0.9 ml, were collected, of which aliquots of 50 μl were removed for dot assays as described above. The specific gravity of each fraction was also monitored by weighing 200 μl of fluid in a microfuge tube.

SDS-Polyacrylamide Gel Electrophoresis

The pooled fractions from either Sepharose CL-4B or CsCl isocratic ultracentrifugation were then dialyzed and lyophilized. These samples were then subjected to SDS-polyacrylamide gel electrophoresis with 3% stacking gel and 5% or 10% separating gel according to the method of Laemmli. Samples, each of 10 μg, were reduced by adding 50 mM dithiothreitol (DTT) and heating at 100°C for 3 min prior to electrophoresis. After that, gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate by PAS method.

Amino Acid Analysis

The lyophilized samples collected during purification steps of both ROM and PSM were analyzed for their amino acid compositions. The samples were first hydrolyzed in 6 N HCl under nitrogen at 110°C for 24 hr. After removal of acid, the sample was analyzed by a Durrum (Sunnyvale, CA) D-500 automated amino acid analyzer. The analysis was conducted under a contract by William E. Brown, PhD, at Carnegie-Mellon University, Pittsburgh, Pennsylvania.

Carbohydrate Analysis

The lyophilized samples were also subjected to carbohydrate analysis using gas-liquid chromatography according to the method of Reinhold except that the methanalysis conditions followed those described by Chambers and Clamp. The analyses were conducted by the Laboratory for Carbohydrate Research, Massachusetts General Hospital, Boston, Massachusetts, through kind support provided by Roger W. Jeanloz, PhD.

Results

Using impression cytology, we studied the mucus strands collected from inferior fornix of both human and rabbit eyes. As shown in Figure 1, the mucus thus obtained actually contained inflammatory cellular debris. Both polymorphonuclear and mononuclear cells could be identified in addition to desquamated epithelial cells. The mucus materials appeared to be collapsed and tangled. Because of this finding, it became imperative to purify ocular mucin using a dif-
Fig. 2. Nitrocellulose dot assays for nucleic acid by Gill’s modified hematoxylin (upper panel), for protein by Fast Green (middle panel), and for glycoprotein by PAS (lower panels). Consult Methods for detailed staining procedures. Calf thymus DNA, bovine serum albumin (BSA), and porcine stomach mucin (PSM) were used as standards. Note PSM contained minor DNA contaminant.

A different approach with specific precautions to prevent any enzymatic degradation if we were to study the native functional molecule. Using a bathing technique, mucin was found to be collectable from the bathing fluid, isotonic PBS, in the presence of 5.5 × 10⁻⁴ M acetylcholine. The rationale for using acetylcholine can be found in previous works published by others.¹⁰¹¹ In a total of 1 ml fluid collected from one eye after 4 hr bathing, the amount of mucin was estimated by nitrocellulose dot assay to be in the range of 0.5–1.0 mg. Because of such a small quantity, we developed nitrocellulose dot assays for semi-quantitative measurements of nucleic acid, protein, and glycoprotein. These assays allowed us to monitor the biochemical fractionations using a very small aliquot without losing mucin materials. As shown in Figure 2, nucleic acid, protein, and glycoprotein standards could be differentiated by Gill’s hematoxylin, Fast Green, and PAS stains, respectively, of which each developed its own special color reaction. Serial dilutions of these standards demonstrated a gradual decrease of color intensity. The sensitivity of the nucleic acid assay by hematoxylin stain was noted to be 0.1–0.2 µg in 50 µl, 0.2–0.3 µg in 50 µl for protein, and 1–2 µg in 50 µl for mucin glycoprotein (Figure 2).

Immediately after bathing, mucin of the collected ocular surface fluid was solubilized by adding it to a final concentration of 1 M guanidine HCl in 50 mM Tris/HCl, pH 7.4, containing mixtures of protease inhibitors such as sodium azide, EDTA and PMSF. Since native mucin is one of the largest macromolecules, with a molecular weight exceeding 1 × 10⁶ daltons, one can crudely separate mucin from most other biological contaminants using gel-filtration Sepharose CL-4B column chromatography. A small peak detected at 280 nm was eluted at the void volume and separated from most other smaller-molecular-weight components (Figs. 3, 4). The void-volume fractions of ROM were noted to be PAS-positive (Fig. 4, lower). In parallel, commercial PSM was also analyzed by the same column and showed PAS-positive fractions at the void volume similar to those of ROM, and degraded mucin fragments in the included volume (Fig. 4, upper).

Since Sepharose CL-4B void-volume fractions also had a high 260 nm absorbance (Fig. 3), indicating possible nucleic acid contaminant, the pooled fractions were then subjected to CsCl-density gradient ultracentrifugation for further purification. As shown in Figure 5A, ROM collected after 4 hr bathing had two major peaks appearing at high and low specific gravity and one minor in between, according to 260 nm and 280 nm absorbances. The peak at a high CsCl gravity had a high 260 nm/280 nm absorbance ratio and was identified by dot assay as a nucleic acid contaminant (Fig. 6). The minor peak (labeled as I), noted as a shoulder prior to the last major peak (labeled as II), was identified by the dot assay as a glycoprotein(s) since it had positive staining with both PAS and Fast Green (Fig. 5A, 6). The last major peak (II) with a high 280 nm/260 nm absorbance ratio was identified as a pure protein fraction by the dot assay using Fast Green stain (Fig. 5A, 6). When 12 hr bathing fluid of ROM was analyzed, a similar pattern was noted (Fig. 5B), except that the protein peak II increased several-fold (cf Fig. 5A, B). The PSM was then analyzed and compared. It demonstrated two major peaks at mid (labeled as I) and low (labeled as II) specific gravity (Fig. 5C). There was no detectable nucleic acid contaminant either by 260 nm/280 nm absorbance ratio or by the dot assay using Hematoxylin (Fig. 6). Peak I was identified as PAS-positive glycoprotein(s) (Fig. 6), and peak II with a higher 280 nm/260 nm absorbance ratio was identified as a pure protein (Fig. 5C, 6). These results indicated that ultracentrifugation could effectively separate mucin glycoprotein from nucleic acid contaminant. Further
thermore, a pure protein fraction was also separated from mucin glycoprotein, suggesting the existence of a strong non-covalent binding between these two components. The binding appeared to be strong enough to withstand prior Sepharose CL-4B separation even in the presence 1 M guanidine HCl.

In order to evaluate the purity of the mucin component during purification steps, we analyzed their dialyzed and lyophilized samples by 5% or 10% SDS-polyacrylamide gel electrophoresis. As shown in Figure 7, under reduction by 50 mM DTT, crude PSM showed a protein doublet with molecular weights of 64K and 65K daltons, respectively (lane 1). They became more intensified in the void-volume fractions obtained from Sepharose CL-4B, as compared to their included-volume fractions which consisted of presumed partially-degraded mucin (cf lanes 2 and 3, see also Fig. 4, upper). It is interesting to point out that after subsequent ultracentrifugation, the 64K protein, the lower band of this doublet, was primarily noted after reduction in the PSM mucin Peak I (lane 4). This result indicates that this protein component might be associated with mucin core protein by disulfide bond(s). Nevertheless, the 65K protein, the upper band of the doublet, was noted in the Peak II (lane 5). Both of them were proteins with low carbohydrate contents, as evidenced by dot assay (Fig. 6), PAS staining of 10% gel (not shown), and subsequent amino acid and carbohydrate analysis (Table 1). Since this 65K protein was separated from mucin molecules only by ultracentrifugation in the presence of 4 M guanidine HCl and CsCl, it suggests that this protein had a strong and probably specific binding
with mucin molecules. When ROM was analyzed, Sepharose CL-4B indeed effectively removed the majority of small-molecular-weight components (cf lanes 6, 7, and 8). After ultracentrifugation, a faint protein band was noted after reduction of the mucin fraction 1 which had a similar mobility to the 64K protein of the PSM doublet (lane 9). Similarly, the protein fraction obtained from peak II at a low specific gravity was found to be a single protein band with mobility comparable to the 65K protein of PSM doublet (cf lanes 10 and 5). Due to the limited loading amount, both samples on lanes 7 and 9 showed

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**Fig. 5A, B.** CsCl-gradient ultracentrifugation of rabbit ocular mucin after 4 hr (A) and 12 hr (B) bathing. Peaks I and II indicate mucin and its associated protein fraction respectively.

**Fig. 5C.** CsCl-gradient ultracentrifugation of porcine stomach mucin. Peaks I and II indicate mucin and its associated protein fraction respectively.

**Fig. 6.** Nitrocellulose dot assays of fractions in Figure 5 (1 to 16) for nucleic acid (upper), pure protein (middle), and glycoprotein (lower). Consult Methods for details.
only faint bands. Throughout all these gel analyses, both PSM and ROM were found in the well of upper 3% stacking gel and not in the 5% lower gel by PAS stain, due to their high molecular weights (not shown). Because of the absence of other small-molecular-weight components in the 10% lower gel, their purity was verified (lanes 4 and 9).

For further analyses of their purity, the above lyophilized samples were then subjected to analyses of amino acid composition and carbohydrate content. For PSM, amino acid analysis of the purified mucin showed high contents of threonine (28.4%), serine (13.4%), and proline (20.7%), as well as high contents of N-acetyl glucosamine (42.7%) and N-acetyl galactosamine (30.5%) in a total of 1000 amino acids (Table 1). This result was generated after the separation of a tightly-associated protein fraction by ultracentrifugation in the presence of 4 M guanidine HCl and CsCl (Figs. 5, 6, 7). This 65K protein was found to have a totally different amino acid composition with a relatively high content of aspartic acid/asparagine and glycine (cf CL-4B Vo fraction, Peak I purified mucin, and Peak II protein fraction), and a low carbohydrate content of N-acetyl glucosamine (2%) and N-acetyl galactosamine (<0.6%). For ROM, three consecutive fractions (labeled as 1, 2, and 3, Fig. 5B) from the mucin fraction I were analyzed. It is noteworthy that the amino acid pattern changed from fractions 1 to 3 in a gradual manner. Fraction 3 had the highest content of carbohydrate (18.6% N-acetyl glucosamine and 26.8% N-acetyl galactosamine), and amino acids with high contents of serine (22.8%), glutamic acid/glutamine (16.9%), and glycine (23.8%). These results indicate that this fraction might represent the most purified ROM. From fractions 3 to 1, one begins to see the decreasing trend of the above amino acids, and the increasing trend of other amino acids such as aspartic acid/asparagine and proline, which were of a high content in the protein obtained from peak II (Table 1). This notion was also supported by the results of the contents of N-acetyl glucosamine and N-acetyl galactosamine. These results strongly indicate that a strong non-covalent binding existed between mucin and this 65K protein. The bound form would result in a higher molecular weight, a low carbohydrate content, and a higher CsCl specific gravity (Fraction 1). In contrast, the unbound purified mucin would be of a lower molecular weight, a high carbohydrate content, and a lower CsCl specific gravity (Fraction 3). Some similarities were also noted between the 65K protein of PSM and ROM when their amino acids and carbohydrates were compared (Columns 3 and 7, Table 1). It should also be noted that this protein was not a serum albumin when amino acids composition and carbohydrate content were compared with those of bovine serum albumin (BSA) (Table 1). The electrophoretic mobility was also dif-
Table 1. Amino acid composition of mucins (per 1000 A.A.)

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<th>ROM</th>
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<tr>
<td>N-acetyl galactosamine</td>
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<td>305</td>
<td>&lt;6</td>
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* Abbreviations: PSM, porcine stomach mucin; ROM, rabbit ocular mucin; 4B + Vo, void-volume fractions after Sepharose CL-4B; 4B + Ucf, fraction collected after CL-4B and CsCl-gradient ultracentrifugation; I, II, (1) (2) (3), consult Figure 5 A, B, C.

The detailed carbohydrate content was measured and is shown in Table 2. The mucin fraction purified by both Sepharose CL-4B and ultracentrifugation exhibited high carbohydrate/protein ratio, (87.3% vs 12.7%) for PSM and (84.2% vs 15.8%) for ROM. In addition, both mucins had five major sugar components: N-acetyl galactosamine, N-acetyl glucosamine, galactose, fucose, and N-acetyl neuramic acid (NANA), and were devoid of mannose and xylose.

Discussion

The major difficulty in the biochemical studies of ocular mucin is its limited amount and inaccessibility. In addition, we also demonstrated by impression cytology that mucus strands collected from inferior fornix contained inflammatory cells, epithelial cellular debris, and collapsed mucus. We therefore suspected that such starting materials may not contain the native functional mucin molecule due to potential proteolytic degradation. To circumvent the above problems, we made the following modifications in the biochemical purification of rabbit ocular mucin (ROM).

We first developed the nitrocellulose dot assay for semi-quantitative measurements of nucleic acid, protein, and glycoprotein (mucin). This allowed us to monitor fractionations obtained from Sepharose CL-4B as well as CsCl-gradient ultracentrifugation. Only a small quantity of sample was required so that there was a minimal loss of mucin during these purification steps. Herein, we demonstrated the feasibility and efficiency of these assays. Second, we collected ROM by bathing rabbit ocular surface with isotonic PBS containing acetylcholine. As shown in this study, mucin was recoverable from this bathing fluid. The use of acetylcholine has been helpful in obtaining sufficient amounts of native mucins from intestine and conjunctiva. The bathing of isotonic fluid for 4 hr is not physiological and may have included plasma membrane glycoproteins in the crude extracts. Nevertheless, the purified mucin has a molecular weight exceeding $1 \times 10^6$ daltons and is devoid of any mannose, a common monosaccharide present in membrane glycoproteins, indicating that the subsequent purification steps have effectively eliminated all contaminants. Third, it was important to add protease inhibitors throughout the biochemical purification steps so that any possible enzymatic degradations could be minimized. Finally, we confirmed the effectiveness of purifying mucin by the use of a combina-

different from that of rabbit serum albumin on a 10% SDS-polyacrylamide gel (not shown).

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Table 2. Carbohydrate composition of mucins (Wt. %)

<table>
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<th>ROM</th>
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<tr>
<td>Carbohydrate content</td>
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<tr>
<td>Protein content</td>
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<td>N-acetyl galactosamine</td>
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<td>N-acetyl glucosamine</td>
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<tr>
<td>N-acetyl neuramic acid</td>
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tion of Sepharose CL-4B and CsCl-gradient ultracentrifugation. This point will be further discussed below.

Mucins have been purified and characterized from various other organ systems including cervix, bronchus, saliva, stomach, intestine, and colon. These studies indicate that mucins or mucus glycoproteins are macromolecular complex structure with molecular weights exceeding $1 \times 10^6$ daltons, except that of saliva, and consist of a core protein rich in serine, threonine, and proline residues, oligosaccharide chains O-glycosyl-linked through N-acetyl galactosamine residue to either serine or threonine, and with the possible presence of a carbohydrate-free portion that may be linked to the glycosylated peptide by covalent disulfide bonds.

In this report, we noted that both ROM and commercial porcine stomach mucin (PSM) had a molecular weight exceeding $1 \times 10^6$ daltons as judged by their void-volume elution from Sepharose CL-4B. Using CsCl-gradient ultracentrifugation, ROM was found to have a specific gravity of 1.36 gm/ml, which was of slightly smaller molecular weight than that of PSM, which had a specific gravity of 1.39 gm/ml. The mucin purified by Sepharose CL-4B and CsCl-gradient ultracentrifugation had a high ratio of carbohydrate content vs protein content. For example, the purified ROM and PSM had a carbohydrate content of 84.2% and 87.3% respectively (Table 2). These values were comparable to the reported values of mucins in other organ systems, which were in the range of 66% to 81%, except for rat colon mucin. These values were certainly higher than those of human ocular mucus reported by Chao et al, which had a fluctuating range of carbohydrate contents from 57% depending on the sample preparation and solubility. Other previous studies on ocular mucins did not report data on carbohydrate content. The discrepancy between our data and those of Chao et al may be due to the fact that a 65K protein was still tightly associated with mucin after Sepharose CL-4B. This protein component could only be separated from mucin after ultracentrifugation, a procedure not used by Chao et al. This explanation was supported by our data that carbohydrate content, as viewed by SDS-polyacrylamide gel electrophoresis under a reduced state, we also observed a faint band constituting part of the doublet together with the 65K protein band in PSM (Figure 7), suggesting a disulfide-bond-rich domain may be present in PSM, in contrast to all other reported mucins which form such a linkage through both threonine and serine, the former more frequently than the latter. When amino acid composition was compared among PSM samples before and after ultracentrifugation, it also indicated that further purification was achieved by dissociating the 65K protein (Table 1). Similarly, when sequential ultracentrifugation fractions 1 to 3 of ROM are compared, one can also find the gradual change in the proportional amount from mucin-related amino acids such as serine, glutamine/glutamic acid, and glycine to the 65K protein-related ones such as aspartic acid/asparagine and proline (Table 1). Using SDS-polyacrylamide gel electrophoresis under a reduced state, we also observed a faint band constituting part of the doublet together with the 65K protein band in PSM (Figure 7), suggesting a disulfide-bond-rich domain may be present in PSM. Whether this finding is also true in ROM is not clear at this time. In summary, we have verified the identity and purity of the isolated ROM and PSM.

It is worthwhile to further discuss this tightly-associated 65K protein. The tight association of this protein with mucin explains why the results of mucin composition reported in the literature are variable,
depending on whether this protein has been totally removed. We have also investigated the nature of this protein. The results of amino acid composition, carbohydrate contents, and electrophoretic mobility indicate that this 65K protein is not albumin. The fact that both PSM and ROM had such a similar protein, ie, a molecular weight of 65K daltons, amino acids rich in aspartic acid/asparagin, glutamic acid/glutamine, and glycine, and a low carbohydrate content suggests this protein may be commonly associated with mucin molecules. The quantity of this protein also increased when ocular bathing was extended from 4 to 12 hr. For these reasons, we speculate that this protein may be a putative "mucin binding protein," a protein present on the apical membrane of epithelial cells with an active role in maintaining mucin on the mucosal surface. Currently, we are testing this hypothesis using a monoclonal antibody approach.

In summary, we have taken the first step in purifying and characterizing rabbit ocular mucin. With this information, we hope that we can then explore the molecular mechanisms by which the interactions between mucin molecule and apical membrane proteins or glycoproteins are operated, and by which the precellular tear film stability is ensured.

**Key words:** conjunctiva, goblet cells, mucin, mucus, rabbit eyes

**References**