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ROLE OF SIALIC ACID ON THE VISCOSITY OF CANINE TRACHEAL MUCIN GLYCOPROTEIN

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SUMMARY- The role of sialic acid on the viscosity of canine tracheal mucin (CTM) was investigated. The mucin glycoprotein, purified from canine tracheal mucus, was subjected to mild acid hydrolysis with aqueous acetic acid and autohydrolysis in water, in which ~50% drop in the relative viscosity (n_r) occurred. Carbohydrate compositional analysis before and after mild acid hydrolysis and autohydrolysis showed the complete removal of glycosidically bound sialic acid residues while all other sugar residues (i.e. galactose, N-acetyl galactosamine and N-acetyl glucosamine) remained unaltered, indicating that sialic acid residues are contributing towards the viscosity of CTM to a greater extent.

Tracheobrancial mucin glycoproteins are polyanionic macromolecules involved in the maintenance of mucociliary transport in the respiratory tract (1). These high molecular weight glycoproteins are composed of a protein core, covalently bound fatty acids and numerous O-glycosidically linked carbohydrate side chains (2). The protein core of mucin glycoproteins is rich in serine, threonine and proline (3). The oligosaccharide side chains range in size from one to over ten sugar units and carry various antigenic determinants, including those which endow the mucin with blood group ABH, Lewis and Forssman determinants (4). The mucin glycoprotein contained both neutral and acidic oligosaccharide chains

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in which the acidic oligosaccharides carry sialic acid and/or sulfate as their acidic groups (4).

The mucin glycoproteins forms highly viscous solutions with density ranges from 1.5-2.1 gm/mL (5). The constituent of mucus secretions of the alimentary, respiratory and reproductive tracts primarily responsible for the viscoelastic and permselective properties of the gel are the mucin glycoproteins (6). The gel forming properties of mucins apparently depend on their polymeric structure and the extent of intra- and intermolecular interactions between its carbohydrate side chains (6). As part of our goal to understand the role of individual glycosidically bound sugar residues in the polymeric structure of mucin glycoproteins, we purified canine tracheal mucin glycoprotein, which is similar to human tracheal mucin and studied its properties. Here we report the role of sialic acid residues in the viscosity of canine tracheal mucin glycoprotein.

MATERIALS AND METHODS

Canine mucus secretions were collected from beagle dogs having surgically constructed tracheal pouches (12). The collected mucus was dialyzed at 4^0C against deionized water containing 0.1 mM TSF and then lyophilized. The lyophilized mucus was solubilized in 0.1 mM Tris-HCl buffer, pH 8.0, containing 0.2% NaN_3, 0.1 mM TSF and 6 M urea, at a concentration of 5 mg/mL. The solution was chromatographed on a column of Sepharose CL-4B, equilibrated in and eluted with 50 mM Tris-HCl buffer, containing 0.02% NaN_3, 0.1 mM TSF, and 6 M urea. The eluted fractions were monitored for protein and carbohydrate, and the fractions containing mucin (void volume) were collected. The mucin was further purified by density gradient centrifugation followed by extraction with chloroform-methanol to remove associated lipids (12).

A 2% solution of purified mucin in water or 2 M acetic acid or 50 mM Tris-HCl buffer, pH 7.5, was heated with stirring at 80^0C for 1-2h (13). A portion of each hydrolyzate was dialyzed against distilled water, lyophilized, redissolved in water and then analyzed by HPAE-PAD for sialic acid.

Viscosity determinations were performed in a falling ball viscometer at constant temperature (25^0C). For the measurement of viscosity the intact and modified mucin preparations were dissolved at a concentration of 2% in water or 2 M acetic acid or 50 mM Tris-HCl buffer, pH 7.5 and the samples were brought to 25^0C. To calculate the relative viscosity (n_r), the measurements of solvent viscosity were also taken (6).

For carbohydrate analysis the glycoprotein samples (100 μg) were hydrolyzed in 2.5 M trifluoroacetic acid at 100^0C for 6h, the

hydrolyzate was lyophilized, redissolved in water and then analyzed at room temperature by high-performance anion-exchange chromatography with pulsed electrochemical detection (HPAE-PAD) (19). The system used for HPAE-PAD consisted of a Model PAD 2 detector (Dionex Corp., Sunnyvale, CA) and a Carbopac PA1 (4 X 250) pellicular anion-exchange column equipped with a Carbopac guard column. The Dionex eluant degas module was employed to sparge and pressurize the eluants with helium. In these experiments eluant 1 was 200 mM NaOH, eluant 2 was water, and eluant 3 was 100 mM NaOH containing 150 mM NaOAc. Sample injection was via Dionex micro injection valve equipped with a 25-μL sample loop operated by a controlled helium source of 100-120 psi.

Fucose, glucosamine, galactosamine, galactose and mannose were eluted isocratically with 16 mM NaOH. N-Acetyl and N-glycolyl neuraminic acid were eluted isocratically with 100 mM NaOH containing 150 mM NaOAc. Unless otherwise mentioned, the flow rate was 0.9 mL/min. The column was regularly checked by measurement of the retention times of fucose, galactosamine, glucosamine, galactose, glucose and mannose which were eluted with 16 mM NaOH at 0.9 mL/min.

The following pulse potentials and durations were used: E_1 = 0.05 V (t_1 = 300 ms); E_2 = 0.65 V (t_2 = 180 ms); E_3 = -0.65 V (t_3 = 60 ms). Detection was with 1000 nm full scale. No post column base addition was used as no baseline drift was observed with these detector setting; a Dionex 4270 integrator was used to record the data.

For sialic acid analysis, the glycoprotein samples were hydrolyzed with 2 M acetic acid at 100^0C for 3-4 h, freeze dried and then analyzed by HPAE-PAD. Sodium dodecyl sulfate (SDS) -polyacrylamide gel-electrophoresis was performed on slab gels using a Bio-Rad mini-Protean cell by the procedure of Laemmli (20) with subsequent Coomassie blue and/or periodate-Schiff or silver staining (21).

RESULTS AND DISCUSSION

The primary structure of CTM polypeptide is rich in serine and threonine residues, and has a carbohydrate content of ~70% by weight (Table 1). The purified CTM runs as a polydisperse band on SDS-PAGE indicative of molecular heterogeneity, a typical nature of mucin glycoproteins and the Mr. is ~(1.75-3.0)X10^5 (12). CTM contained ~7% N-acetyl neuraminic acid (sialic acid), by weight (by weight) and forms a highly viscous solution in water and in aqueous buffers. Because of the gel forming nature, sialidases are ineffective to remove the glycosidically bound sialic acid residues from CTM. Hence, mild acid hydrolysis was chosen to selectively remove the

Table 1: Carbohydrate compositional analysis of Canine tracheal mucin

Sugars	A	B	C	D
Fuc	11.9	9.9	9.9	11.6
GalN#	10.0	10.0	10.0	10.0
GlcN	21.3	21.2	21.4	21.3
Gal	14.7	14.6	14.7	14.7
Man	0.8	0.8	0.8	0.8
NeuAc	6.5	0.1	0.2	6.1
%Carb-ohydrate	69.5	61.9	61.5	69.2

A= Native CTM.
B= CTM after autohydrolysis in water.
C=CTM after hydrolysis in 2M acetic acid.
D=CTM after solvolysis in Tris-HCl buffer pH7.0.
# Normalized with respect to D-GalN residues. Values are not corrected for destruction or incomplete hydrolysis.

glycosidically bound sialic acid residues from CTM to study the viscoelastic properties of this mucin glycoprotein. CTM was hydrolyzed in aqueous acetic acid to remove the glycosidically bound sialic acid residues (13). The carbohydrate compositional analysis (Table 1) of CTM by HPAE-PAD, after hydrolysis with aqueous acetic acid and dialysis to remove the released sialic acid, showed the absence of sialic acid residues indicating that the mild acid treatment quantitatively removed glycosidically bound sialic acid residues. Further the analysis also indicated that all other sugar residues remained intact during the hydrolysis except a very little loss of fucose residues. Furthermore, the amino acid analysis (Table 2) of CTM, before and after solvolysis in aqueous acetic acid showed no significant change indicating that the primary structure of CTM remained unaltered during solvolysis. Measurement of viscosity of

Table 2: Amino acid composition of Canine tracheal mucin#

Amino acid	A	B	C	D
Thr	230	231	230	230
Ser	208	206	208	208
Glu	97	96	97	97
Gly	93	94	95	93
Pro	132	134	133	132
Ala	40	40	39	40
Val	50	50	51	50
Met	3	3	3	3
Ile	25	24	24	25
Leu	30	31	30	30
Tyr	10	11	11	10
Phe	13	12	13	13
His	13	13	12	13
Lys	27	28	27	28
Arg	28	28	28	27

A= Purified CTM.
B= CTM after autohydrolysis in water.
C=CTM after hydrolysis in 2M acetic acid.
D=CTM after solvolysis in Tris-HCl buffer, pH7.0.
# Data are in residues per 1000 residues. Values are not corrected for destruction or incomplete hydrolysis.

CTM in aqueous acetic acid, before and after hydrolysis, showed ~50% decrease (Table 3) in relative viscosity (n_r) indicating that the solvolysis caused a significant effect on the viscoelastic properties of CTM.

The mucin glycoprotein was subjected to autohydrolysis (13) in which an aqueous solution of CTM was heated and then measured the viscosity of the resulting solution. Surprisingly, the results (Table 3) showed ~50% decrease in relative viscosity (n_r) indicating that the

Table 3: Comparison of relative viscosity (n_r) of Canine tracheal mucin

Medium	A	B	C
Water	3.05	1.42	54
2M HOAc	3.15	1.37	56
50 mM Tris buffer, pH 7	3.02	2.85	15

A= Relative viscosity (n_r) before heating the solution.

B= Relative viscosity (n_r) after heating the solution.

C= % of decrease in relative viscosity (n_r) after solvolysis.

autohydrolysis of CTM also caused a significant change in viscoelastic properties of the mucin glycoprotein. The carbohydrate compositional analysis of autohydrolyzed CTM by HPAE-PAD showed the complete absence of glycosidically bound sialic acid residues (Table 1). Further, the amino acid analysis of autohydrolyzed CTM showed no significant change in composition (Table 2) indicating that the protein core remained unaltered during autohydrolysis. Furthermore, the sugar composition showed that the proportions of Fuc, Gal, GlcNAc and GalNAc was similar to the native CTM indicating that the sugar chains didn't undergo considerable change during autohydrolysis except the loss of glycosidically bound sialic acid residues.

In order to confirm the decrease in the n_r of CTM, after solvolysis in aqueous acetic acid hydrolysis and autohydrolysis is due to the removal of glycosidically bound sialic acid residues, a solution of CTM in buffered solution at pH 7.0 was heated so that the glycosidically bound sialic acid residues would not undergo hydrolysis. The n_r of the resulting solution was measured and the results showed only a 15% decrease (Table 3). Further, the carbohydrate and amino acid analysis of the resulting CTM (Tables 1&2) showed no changes indicating that the slight decrease in n_r is not due to any primary structural change in CTM and may be due to the change in secondary structure of the mucin glycoprotein.

These results demonstrates that the removal of glycosidically bound sialic acid residues from CTM by mild acid hydrolysis and

autohydrolysis causes a significant change in n_r. However the data didn't showed the significance of unbound sialic acid residues in the mucin solution. In order to obtain such data the mucin solution, after mild acid hydrolysis and autohydrolysis, was dialyzed to remove the released sialic acid and then the n_r was measured. The results showed no change in the n_r indicating that unbound sialic acid seems to have no effect on the viscosity of CTM at that concentration.

The foregoing results demonstrates that the glycosidically bound sialic acid residues plays a significant role in the viscosity of CTM. Glycosidically bound sialic acid residues has been shown to play several important biological functions (14). To our knowledge this is the first report to show the significance of sialic acid in the viscosity and hence gel forming nature of CTM. Although several studies have been reported, in which the role of sialic acid and the carbohydrate side chains, on the viscosity of several mucin glycoprotein is described (6, 15-18). In such studies the sialidases and other exoglycosidases were employed to remove the glycosidically bound sugar residues. Because of the gel forming nature of mucin glycoproteins, the exoglycosidases are not very effective and hence there is a possibility that such reactions would be incomplete. In case of CTM, the commercially available sialidases were not effective to remove sialic acid residues, quantitatively. The data presented in this study indicates that the mild acid hydrolysis and also autohydrolysis removed the glycosidically bound sialic acid residues from CTM, selectively and completely without significant change in other sugar residues and in the amino acid composition. Although a small amount of fucose residues (~3%) were removed during mild acid hydrolysis, the significance of such side reaction is very limited because during autohydrolysis, release of fucose residues was not observed. Further, the free sialic acid seems to have no significant effect on the viscosity of CTM indicating that the glycosidically bound sialic acid residues help the mucin glycoprotein to be in the proper folding and contributes towards the viscosity to a greater extent.

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