Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 61-67

ENZYMATIC SULFATION OF SALIVARY MUCINS: STRUCTURAL FEATURES OF 35S-LABELED OLIGOSACCHARIDES

B.L. Slomiany, J. Piotrowski, H. Nishikawa and A. Slomiany

Dental Research Center, New Jersey Dental School
University of Medicine & Dentistry of New Jersey
Newark, New Jersey 07103-2425

Received September 21, 1988

SUMMARY - Sulfotransferase activity catalyzing the transfer of sulfate ester group from 3'-phosphoadenosine-5'-phosphosulfate to salivary mucins was found associated with Golgi-rich membrane fraction of rat submandibular salivary glands. Alkaline borohydride reductive cleavage of the synthesized ^{35}S-labeled glycoprotein led to the liberation of the label into reduced acidic oligosaccharides. Most of the label was found incorporated in four oligosaccharides. These were identified as sulfated tri-, penta-, hepta- and nonasaccharides. The trisaccharide was characterized as SO_3H,6GlcNAcβ1,3Galβ1,3GalNAc-ol, the pentasaccharide as SO_3H,6GlcNAcβ1,3Galβ1,4GlcNAcβ1,3(NeuAcα2,6)GalNAc-ol and heptasaccharide as SO_3H,6Galβ1,4GlcNAcβ1,6(Fucα1)2Galβ1,4GlcNAcβ1,3Galβ1,3)GalNAc-ol.

INTRODUCTION - Among the constituents of saliva implicated in the maintenance of the health of oral cavity are mucins (1-5). The protective qualities of these highly glycosylated glycoproteins depend upon the features acquired during the synthesis and processing, one of which is decoration of certain sugar units with sulfate ester groups (6-8).

The carbohydrate chains bearing sulfated sugar units impart strong anionic character to mucus glycoproteins and hence influence the tenacity of their interaction with hard and soft oral tissue, and affect physicochemical and functional qualities of saliva such as viscosity, lubrication, waterproofing and bacterial clearance (3-5,9). Therefore, the process of salivary mucus glycoproteins sulfation appears to play a vital role in the maintenance of the health of oral cavity. Here, we report the enzyme system involved in the sulfation of submandibular salivary gland mucin, and the nature of carbohydrate chains bearing the sulfate ester group.

0006-291X/88 $1.50

MATERIALS AND METHODS - Submandibular salivary glands used for sulfotransferase enzyme preparation were obtained from 10 weeks old male Sprague-Dawley rats. The dissected glands were homogenized with 5 volumes of 3mM sodium phosphate buffer, pH 7.0, containing 0.25M sucrose and 1mM EDTA, and the homogenate was subjected to subcellular fractionation (10). For Golgi-rich membrane preparation, the microsomal pellet was subjected to sucrose gradient centrifugation and the Golgi-rich fraction was isolated from the interface between 0.32 and 1.20M sucrose layers. The pellet fractions were solubilized by stirring at 4°C for 30 min with 0.25M buffered sucrose, pH 7.0, containing 0.5% Triton X-100 (10).

For mucin preparation, the diced glands were homogenized with 50 volumes of 2M NaCl-50mM phosphate buffer, pH 7.0 and the mixture was centrifuged at 10,000 for 30 min. The supernatant was boiled for 3 min, recentrifuged, dialyzed, and lyophilized. The powder, dissolved in 6M urea, was chromatographed on a Bio-Gel A-50 column and the excluded mucus glycoprotein peak collected (11). Following rechromatography, the glycoprotein was subjected to equilibrium density gradient centrifugation in CsCl (10). Removal of the sulfate ester groups from mucins was accomplished by acid catalyzed solvolysis (12). The sulfate content of intact and desulfated preparations was determined turbidimetrically (13), while the composition and content of carbohydrates were determined by gas-liquid chromatography (11,12).

The reaction mixtures for mucus glycoprotein sulfotransferase assay, incubated at 37°C in a total volume of 100µl, consisted of 300µg desulfated glycoprotein, 7.8µM 3'-phosphoadenosine-5'-phospho[^{35}S]sulfate([^{35}S] PAPS), 0.5% Triton X-100, 4mM $MgCl_2$, 25mM NaF, 100mM imidazole-HCl buffer, pH 6.8, and enzyme fraction containing 20-80µg protein. The reaction was terminated after 60 min by addition of 0.4ml of ethanol and the precipitate was collected by centrifugation. The pellet was dissolved in 0.2ml of 0.1M citrate buffer, pH 3.6, and the ^{35}S-labeled glycoprotein product was separated from the components of the reaction mixture by chromatography on Bio-Gel P-30 column (14). The ^{35}S-labeled glycoprotein, eluted in the excluded volume, was then placed in vials containing scintillation solution and counted. For characterization of ^{35}S-products of enzymic reaction, a 60-fold scaled up assay system was used.

The isolated ^{35}S-glycoprotein was subjected to reductive cleavage of the carbohydrate chains using 0.05M KOH in 1.0M $NaBH_4$ (14), and the released oligosaccharide alditols were separated into neutral and acidic fractions on AG1x2(Cl^-) column (15). The oligosaccharides recovered in the acidic fraction were desalted on a Sephadex G-10 and chromatographed on thin-layer plates in 1-butanol-acetic acid-water (3:3:2). The plates were scanned for ^{35}S-label and the separated ^{35}S-labeled oligosaccharides were isolated. Desulfation of the isolated ^{35}S-oligosaccharides was performed by sonication with 0.05M HCl in dry methanol at 32°C for 4h (12).

Enzymatic hydrolysis of saccharide chains of the ^{35}S-labeled oligosaccharide alditols were performed by incubating the intact or desulfated substrates at 37°C for 24-36h with specific glycosidases. Reactions with α- and β-galactosidase, α-L-fucosidase and β-N-acetylhexosaminidase were carried out in 0.05M citrate buffer, pH 4.2, while the removal of sialic acid with neuraminidase was accomplished in 0.05M sodium acetate buffer pH 5.5 (15). Methylation of ^{35}S-labeled and desulfated oligosaccharide alditols was performed according to procedure

in (16) and the products were analyzed by gas-liquid chromatography (15,17).

RESULTS - The distribution of sulfotransferase activity for the synthesis of sulfated mucin in the subcellular fractions of rat submandibular salivary glands is presented in Table I. The enzyme activity was located in the Golgi-rich membrane fraction, the specific activity of which was about 10-fold higher than that of the total homogenate and, hence, this fraction was used as the enzyme source. The maximal sulfotransferase activity was obtained with 0.5% Triton X-100, 25mM NaF and 4mM $MgCl_2$. The apparent Km of the enzyme for sulfation of submandibular gland mucin was 11.1mg/ml.

Alkaline borohydride reductive cleavage of the ^{35}S-glycoprotein synthesized in vitro in the presence of submandibular gland enzyme resulted in the liberation of ^{35}S-label to the acidic oligosaccharide alditol fraction. On thin-layer chromatography, this fraction gave four ^{35}S-oligosaccharides (Fig.1). The composition and molar ratios of carbohydrates in these oligosaccharides are given in Table II. Based on the molar ratios, the oligosaccharides were identified as tri-, penta-, hepta- and nonasaccharides, each bearing one residue of sulfate.

Enzymatic degradation studies conducted with intact oligosaccharides II, III and IV (Table II), revealed their resistance to the action of α- and β-galactosidase, and β-N-acetylhexosaminidase. The oligosaccharide II was susceptible to α-L-fucosidase, while the oligosaccharide III was prone to the removal of sialic acid by neuraminidase. After desulfation oligosaccharides III and IV became susceptible to the action of

Table I. Distribution of mucus glycoprotein sulfotransferase activity in subcellular fractions of rat submandibular salivary glands

Fraction	Specific activity (pmol/mg protein 60 min)
Total homogenate	1.49 ± 0.16
Mitochondrial	0.47 ± 0.09
Microsomal	5.01 ± 0.57
Golgi-rich	16.00 ± 1.43
Cytosol	0.42 ± 0.08

Each value represents the means ± SD of four separate experiments performed in duplicate.

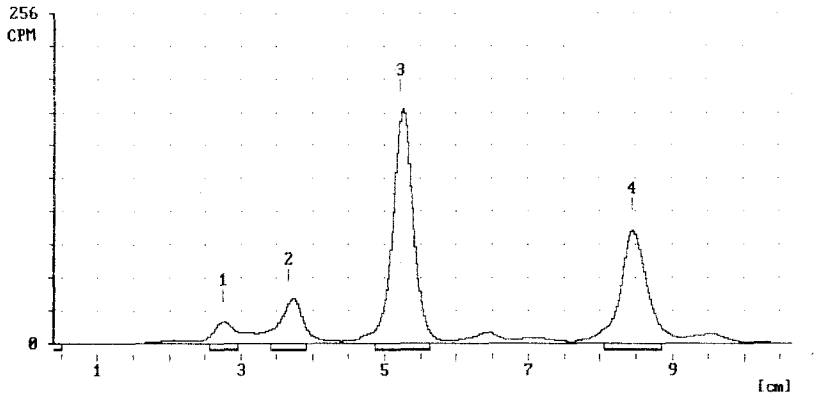

Fig. 1. Radioactivity scan of thin-layer chromatogram of the acidic oligosaccharide alditols obtained from submandibular salivary gland ^{35}S-mucin synthesized in vitro in the presence of submandibular gland mucus glycoprotein sulfotransferase. The positions of the labeled oligosaccharides are indicated by numbers. 1, oligosaccharide I; 2, oligosaccharide II; 3, oligosaccharide III; 4, oligosaccharide IV.

β-N-acetylhexosaminidase and when followed by β-galactosidase, oligosaccharide IV was degraded to galactose, N-acetylglucosamine and N-acetylgalactosaminitol, while oligosaccharide III yielded galactose, N-acetylglucosamine and a trisaccharide composed of N-acetylglucosamine, sialic acid and N-acetylgalactosaminitol. This was degraded to N-acetylglucosamine, sialic acid and N-acetylgalactosaminitol by β-N-acetylhexosaminidase followed by neuraminidase. Stepwise treatment of oligosaccharide II with α-L-fucosidase, β-galactosidase, β-N-acetylhexosaminidase and β-galactosidase resulted in its conversion to sulfated trisaccharide. This trisaccharide, following desulfation was degraded to galactose, N-acetylglucosamine and N-acetylgalactosaminitol when subjected to β-galactosidase and

Table II. The composition and molar ratios of carbohydrates in the ^{35}S-oligosaccharides obtained from ^{35}S-glycoprotein synthesized in vitro in the presence of submandibular salivary gland mucus glycoprotein sulfotransferase

Oligosaccharide	Molar ratios					
	Fuc	Gal	GlcNAc	GalNAc-ol	NeuAc	SO_4^{2-}
I	0.88	3.75	1.92	1.00	0.90	0.94
II	0.95	2.81	1.89	1.00	---	0.92
III	---	1.06	1.97	1.00	0.93	0.95
IV	---	1.12	1.03	1.00	---	0.91

β-N-acetylhexosaminidase. The above results, besides establishing the sequential arrangement of sugar units, also suggest that the sulfate ester group in oligosaccharides III and IV is situated at the terminal N-acetylglucosamine residue, while in oligosaccharide II the sulfate group is attached to the terminal galactose. The results of permethylation analyses performed on the intact oligosaccharide IV showed the presence of C-3 substituted galactose, C-6 substituted N-acetylglucosamine and C-3 substituted N-acetylgalactosaminitol wherease C-3 substituted galactose, C-3 substituted N-acetylgalactosaminitol and unsubstituted residue of N-acetylglucosamine were obtained from the desulfated oligosaccharide IV. The oligosaccharide III gave on permethylation partially methylated alditol acetates of 2,4,6,-tri-O-methylgalactitol, 1,4,5-tri-O-methyl-N-methylacetamidogalacitol, 3,4-di-O-methyl-N-methylacetamidoglucitol and 3,6-di-O-methyl-N-methylacetamidoglucitol. Following desulfation, the permethylated oligosaccharide III gave rise to one terminal and one C-4 substituted residue of N-acetylglucosamine, C-3 substituted galactose, and C-3,6 substituted N-acetylgalactosaminitol. The data on the sulfated oligosaccharide II indicated the presence of C-2, C-3 and C-6 substituted resides of galactose, C-4 substituted residues of N-acetylglucosamine, C-3,6 substituted N-acetylgalactosaminitol and unsubstituted residue of fucose, while one terminal residue of galactose, one unsubstituted fucose and C-2 and C-3 residues of galactose, C-4 substituted N-acetylglucosamine and C-3,6 substituted N-acetylgalactosaminitol were present in the desulfated oligosaccharide II.

DISCUSSION - The results of our study demonstrate the presence in rat submandibular salivary glands a sulfotransferase enzyme that catalyzes the transfer of sulfate ester group from PAPS to salivary mucins. This sulfotransferase is associated with Golgi membrane, displays optimum activity at pH 6.8, and requires detergent, Mg^{2+} and F^{-}. The data on the synthesized ^{35}S-glycoprotein established that the sulfate ester group in the presence of the sulfotransferase enzyme incorporated into the carbohydrate chains of mucus glycoprotein. Thin-layer chromatography of the oligosaccharide alditols obtained through alkaline borohydride reductive cleavage of the ^{35}S-glycoprotein revealed that most of the incorporation occurred in four oligo-

saccharides. These oligosaccharides ranged in size from three to nine sugar units and contained one sulfate ester group. Based on the susceptibility to exoglycosidases and permethylation data of the intact and desulfated compounds, the sulfated sugars identified as galactose-6-sulfate and N-acetylglucosamine-6-sulfate. From structural analysis data, the ^{35}S-oligosaccharides were identified as SO_3H,6GlcNAcβ1,3Galβ1,3GalNAc-ol, SO_3H,6GlcNAcβ1,3Galβ1,4GlcNAcβ1,3(NeuAcα2,6)GalNAc-ol and SO_3H,6Galβ1,4GlcNAcβ1,6(Fucα1,2Galβ1,4GlcNAcβ1,3Galβ1,3)GalNAc-ol. Limited availability of sulfated nonasaccharide precluded structural evaluation of this compound.

Although data on the structure of sulfated carbohydrate chains in other salivary mucins are not available, the occurrence of N-acetylglucosamine-6-sulfate has been suggested in the mucins from rat saliva and rabbit submandibular gland (2, 18), whereas N-acetylglucosamine-4-sulfate was identified in salivary mucins of monkey and dog (19,20). On the other hand, sulfated galactose was found to be present along with sulfated N-acetylglucosamine in carbohydrate chains of respiratory tract and gastric mucins (21,22). In gastric mucins N-acetylglucosamiie-6-sulfate appears to be located internally (22), while in the oligosaccharides bearing galactose-6-sulfate recently characterized from tracheobronchial mucus glycoprotein, this sugar occupies the terminal positions (23). Our data show that in rat salivary mucins, the sulfate ester group can occur at the C-6 of galactose and C-6 of N-acetylglucosamine and that these sugars occupy the terminal positions in the carbohydrate chains. Furthermore, from the results obtained, it is apparent that the presence of sialic acid in the carbohydrate chains does not preclude the sulfaltion, since two of the ^{35}S-labeled oligosaccharides contained sialic acid. This would indicate that in processing salivary mucins through cellular assembly line some of the carbohydrate chains undergo sialyation and sulfation while the others are either sulfated or sialylated. What controls the preference of one over the other is not clear.

ACKNOWLEDGEMENTS - This work was supported by Grant DE-05666-09 from the National Institute of Dental Research and Grant AA-05858-06 from the National Institute of Alcoholism and Alcohol Abuse, National Institutes of Health, U.S. Public Health Service.

REFERENCES

1. Mandel, I.D. (1977) in: The Glycoconjugates (Horowitz, M. I. and Pigman, W., eds.) pp. 153-179, Academic Press, New York.
2. Tabak, L.A., Levine, M.J., Mandel, I.D., and Ellison, S.A. (1982) J. Oral Pathol. 11, 1-17.
3. Slomiany, B.L., Murty, V.L.N., Slomiany, A., Zielenski, J., and Mandel, I.D. (1986) Biochim. Biophys. Acta 882, 18-28.
4. McGhee, J.R., and Michalek, S.M. (1981) Annu. Rev. Microbiol. 35, 595-638.
5. Sarosiek,J., Bilski,J., Murty, V.L.N., Slomiany, A., and Slomiany, B.L. (1988) Biochem. Biophys. Res. Commun. 152, 1421-1427.
6. Schachter, H., and Williams, D. (1982) in: Mucus in Health and Disease II (Chantler, E.N., Elder, J.B., and Elstein, M., eds.) pp. 3-28, Plenum Press, New York.
7. Slomiany, B.L., Tsukada, H., and Slomiany, A. (1986) Biochem. Biophys. Res. Commun. 141, 387-393.
8. Neutra, M.R., and Forstner, J.F. (1987) in: Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.) Vol. II, pp. 975-1009, Raven Press, New York.
9. Roukema, P.A., and Nieuw-Amerongen, A. (1979) in: Saliva and Dental Caries (Kleinberg, I., Ellison, S.A., and Mandel, I.D., eds.) pp. 67-80, IRL, New York.
10. Liau, Y.H., Murty, V.L.N., Gwozdzinski,K., Slomiany, A., and Slomiany, B.L. (1986) Biochim. Biophys. Acta 880, 108-116.
11. Slomiany, B.L., Liau, Y.H., Carter, S.R., Zielenski, J., and Slomiany, A. (1986) Arch. Oral Biol. 31, 463-468.
12. Slomiany, B.L., and Slomiany, A. (1978) J. Biol. Chem. 253, 3517-3520.
13. Clark, A. E., and Denborough, M.A. (1971) Biochem. J. 121, 811-816.
14. Slomiany, B.L., Liau, Y.H., Carter, S.R., Sarosiek, J., Tsukada, H., and Slomiany, A. (1987) Digestion 38, 178-186.
15. Slomiany, A., and Slomiany, B.L. (1978) J. Biol. Chem. 7301-7306.
16. Stellner, K., Saito, M., and Hakomori,S.I. (1973) Arch. Biochem. Biophys. 155, 464-472.
17. Slomiany, B.L., Zdebska, E., and Slomiany, A. (1984) J. Biol. Chem. 259, 2863-2869.
18. Embery, G., and Ward, C. (1976) Arch. Oral Biol. 21,627-629.
19. Lombart, C.G., and Winzler, R.J. (1974) Eur. J. Biochem. 49, 77-86.
20. Tabak, L.A., and Levine, M.J. (1981) Arch. Oral Biol. 36, 315-317.
21. Roussel, P., Lamblin, G., Degand, P., Walker-Nasir, E., and Jeanloz, R. (1975) J. Biol. Chem. 250, 2114-2122.
22. Slomiany, B.L., and Meyer, K. (1973) J.Biol. Chem. 248, 2290-2295.
23. Mawhinney, T.P., Adelstein, E., Morrison, D.A., Mawhinney, A.M., and Barbero, G.J. (1987) J. Biol. Chem. 262, 2994, 3001.