Carbohydrate Research, 115 (1983) C1–C5
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Preliminary communication

Murine submandibular mucin (MSM); a mucin carrying *N*- and *O*-glycosylically bound carbohydrate-chains

ARIE V. NIEUW AMERONGEN*, CLASINA H. ODERKERK, PIETER A. ROUKEMA,

Department of Oral Biochemistry, Vrije Universiteit, Van der Boechorststraat 7, Amsterdam (The Netherlands)

JOHANNES H. WOLF, JAN J. W. LISMAN, and BERNARD OVERDIJK

Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, Amsterdam (The Netherlands)

(Received December 10th, 1982; accepted for publication, February 18th, 1983)

The submandibular and sublingual salivary-glands of the mouse secrete mucins from their acinar cells[1]. Both mucins contained ~25% of sialic acid and >1% of fucose and sulphate[2]. The submandibular mucin also contains substantial quantities of mannose[2]. Moschera and Pigman[3] obtained similar results for the rat sublingual mucin and it was suggested that both *O*- and *N*-glycosylically bound oligosaccharide-chains might be present. We now report the isolation and composition of *N*- and *O*-glycosylically bound carbohydrate-chains from murine submandibular mucin (MSM).

MSM was isolated essentially as described previously[2], and its purity was established by electrophoresis[4] on 7.5% acrylamide gels at pH 8.9. The samples were solubilised either in distilled water, 0.1% SDS, 0.1% SDS plus 0.1% of 2-mercaptoethanol, or 0.1% SDS plus 0.05M *N*-acetylcysteine. The samples were also subjected to electrophoresis at pH 7.0 in SDS-acrylamide gel, using the conditions for the determination of molecular weight[5]. The gels were stained for protein with Amido Black and for carbohydrate with the periodic acid–Schiff reagent[6].

The effect of proteolytic enzymes was studied by incubating MSM, containing ~500 μg of sialic acid, with pronase or papain for up to 24 h followed by electrophoresis to detect degradation products. The incubation mixtures were chromatographed on columns (1.5 × 25 cm) of Biogel P-300, to separate the degraded material from the mucin.

β-Elimination of carbohydrate chains was effected by incubating[7] MSM (containing 1500 μg of sialic acid) with 0.1M KOH and M KBH_4 at 45° for 16 h. The reaction was stopped with Dowex 50W-X8 (H^+) resin (50–100 mesh), and boric acid was removed conventionally as the methyl ester. A solution of the dry product in 10mM formic acid was applied to a column of Dowex 50W-X2 and eluted with 10mM formic acid. Fraction I was

*To whom correspondence should be addressed.

0008-6215/83/$03.00

eluted in the void volume, and elution with M ammonium acetate gave fraction II. Fraction I was eluted from a column (1 × 120 cm) of Biogel P-4 with 0.5mM ammonium acetate (pH 5.0). Fractions of 0.5 mL were collected. Carbohydrate-containing fractions 67–70 (fraction Ia) and 113–119 (fraction Ib) were combined separately, and analysed by g.l.c.

Hydrazinolysis of MSM was effected by heating a suspension of MSM–sialic acid (455 μg) in anhydrous hydrazine (200 μL) at 100° for 10 h. After evaporation of excess of hydrazine with toluene, the oligosaccharides were precipitated with ethanol, reduced with borohydride, and re-*N*-acetylated.

Protein was determined according to the standard, Lowry method. Sialic acid was assayed as described by Warren[8].

Fig. 1. Electrophoresis of MSM before and after incubation with proteolytic enzymes. *a*–*e* were run in 7.5% gel at pH 8.9; *f* and *g* in 7.5% gel at pH 7.0 in 0.1% SDS. All gels were stained for carbohydrate with PAS-reagent, except gel *d*, which was stained for protein with Amido Black. Samples contained 10 μg of MSM–sialic acid, but gels *d* and *e* were loaded with 50 μg. Key: *a*, *d*, and *e*, native MSM; *b* and *c*, MSM after incubation with pronase for 7 h and with papain for 24 h, respectively; *f* and *g*, MSM after incubation with papain for 7 and 24 h, respectively.

The behaviour of the MSM preparations in electrophoresis is shown in Fig. 1. Overloading the gel showed no detectable carbohydrate (lane *e*) or protein (lane *d*) impurities. Incubation with pronase or papain gave a small amount of PAS-positive material, with a migration rate higher than that of the original MSM (lane *a*), both on gels of pH 8.9 (lanes *b* and *c*) and on SDS-acrylamide gels of pH 7.0 (lanes *f* and *g*). The migration rate of MSM did not change, which suggests that the major part of the molecule is not sensitive to proteolytic degradation, and that there are no significant amounts of contaminating (glyco)-proteins. No differences in migration rate were seen when alternative denaturing conditions were chosen. The finding that there are no subunits in MSM accords with our earlier findings[2] and those of others[9].

MSM was treated with pronase, and the products were chromatographed on Biogel P-300 (Fig. 2). A major protein-containing peak that appeared in the void volume (fractions 9–15) had the same electrophoretic mobility as MSM, and a protein–sialic acid ratio of 1.3, contained D-mannose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose in molar ratios comparable to those of MSM, and had an amino

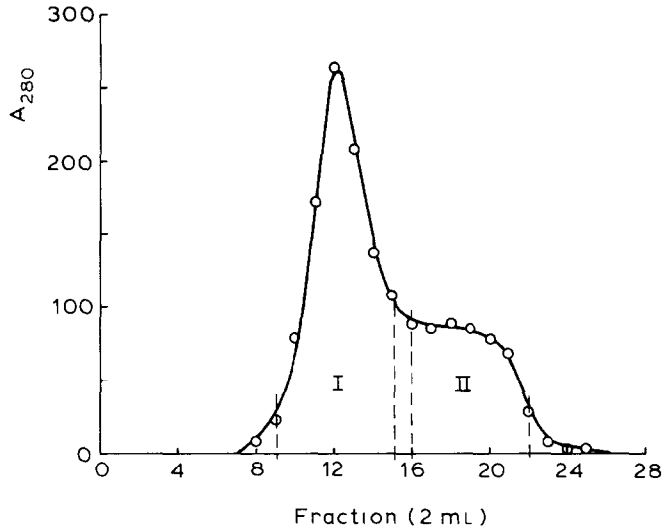

Fig. 2. Chromatography of MSM (after incubation with pronase for 24 h) on a column (1.5 × 25 cm) of Biogel P-300; elution with 0.154M sodium chloride. Fractions of 2 mL were collected. The protein-containing fractions were determined by measuring the absorption at 280 nm. Fractions 9–15 and 16–22 were combined and analysed.

acid composition comparable to that of MSM. Fractions 16–22 contained material having a protein–sialic acid ratio of 9.6. The protein content of this fraction was too low to permit further analysis. Thus, incubation of MSM with pronase does not remove D-mannose-containing material, suggesting that D-mannose is an integral component of the carbohydrate moiety of MSM.

TABLE I

MOLAR CARBOHYDRATE RATIO OF MSM FRACTIONS OBTAINED AFTER β-ELIMINATION AND HYDRAZINOLYSIS, AS DETERMINED BY G.L.C.[a]

	β-Elimination			*Hydrazinolysis*
	Fraction Ia	*Fraction Ib*	*Fraction II*	
Man	1.8	N.d.[b]	1.7	0.8
Gal	1.4	1.2	0.8	0.6
Glc	0.3	0.4	N.d.	0.2
GalNAc	0.4	0.1	0.2	N.d.
GlcNAc	1.2	0.5	0.9	0.6
Sialic acid	1.0	1.0	1.0	1.0
GalNAc-OH	N.d.	0.4	N.d.	N.d.

[a] Analysis was performed as described by Reinhold[14].
[b] Not detectable.

MSM was subjected to β-elimination with KBH_4. The product mixture was eluted from Dowex 50W-X2 with 10mM formic acid, to give a carbohydrate-containing fraction I. The material remaining on the column, which could be eluted with M ammonium acetate (fraction II), seemed to be largely undegraded material (Table I). Fraction I was further fractionated on Biogel P-4, to give two carbohydrate-containing fractions (Ia and Ib) that were analysed for carbohydrate (Table I). Fraction Ia was eluted in the void volume, suggesting a molecular weight higher than 800. Fraction Ib was eluted in the trisaccharide zone. On a weight basis, the ratio of fractions Ia and Ib was 1 : 1. Fraction Ia contained D-mannose and 2-acetamido-2-deoxy-D-glucose, but no 2-acetamido-2-deoxy-D-galactitol, and fraction Ib contained 2-acetamido-2-deoxy-D-galactitol, but no D-mannose, indicating the presence in MSM of *N*- and *O*-glycosylically bound carbohydrate-chains, respectively.

Hydrazinolysis of MSM gave oligosaccharides that consisted of D-mannose, D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and sialic acid. No 2-acetamido-2-deoxy-D-galactose was detected. The presence of D-glucose might be ascribed to incompletely processed carbohydrate-chains.

The fact that MSM contains substantial proportions of D-mannose and 2-acetamido-2-deoxy-D-glucose, in addition to 2-acetamido-2-deoxy-D-galactose, D-galactose, and sialic acid, led Roukema *et al.*[2] to suggest the presence of two types of oligosaccharide chains. This is now substantiated. The occurrence of both *O*- and *N*-glycosylically bound carbohydrate-chains on one polypeptide chain has been described only for glycoproteins of the serum-type, *e.g.*, fetuin[10], thyroglobulin[11], and erythrocyte-membrane glycoproteins[12]. However, the presence of D-mannose in mucin-type glycoproteins has also been reported[3] for rat sublingual mucin, but not for rat submandibular mucin. The latter substance does contain 2-acetamido-2-deoxy-D-glucose[13]. Our results on MSM indicate that the simultaneous occurrence of *O*- and *N*-glycosylically bound carbohydrate-chains can also be found on mucins.

ACKNOWLEDGMENTS

We thank Dr. B. Fournet (Lille, France) for valuable suggestions, and Mr. L. A. W. Trippelvitz and Mr. C. J. van der Wal for performing carbohydrate and amino acid analyses.

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