Short Communications

Isolation and Characterization of Canine Submaxillary Mucin

By CHRISTIAN LOMBART and RICHARD J. WINZLER

*Laboratoire de Biochimie, E.R. Biomédicale, 45 Rue des Saints Péres, Paris 6, France, and
Department of Chemistry, Florida State University, Tallahassee, Fla. 32306, U.S.A.*

(Received 2 May 1972)

The isolation and properties of mucin from submaxillary glands of a number of species of animals have been reported (reviewed by Pigman & Gottschalk, 1966).

The present communication describes a method for preparation of purified canine submaxillary mucin involving gel filtration on Sephadex G-200 with an additional fractionation step using Bio-Gel CM-100. This method yields a native and highly purified material from submaxillary glands and from submaxillary secretions. The composition of the purified mucin indicates that it is a sulphated glycoprotein containing 35% protein, 61% carbohydrate and 3.8% sulphate. The findings extend previous work on canine submaxillary mucin reported by Hashimoto & Pigman (1962).

Canine submaxillary glands either were obtained from dogs under anaesthesia and immediately frozen in liquid N₂, or were obtained frozen from Pel-Freeze Inc. (Rogers, Ark., U.S.A.). The glands were decapsulated, freed from connective tissue and fat, cut into small pieces and extracted with 2 vol. of ice-cold distilled water containing NaN₃ (5mM) for 24h at 4°C (extract 1). Extracts 2 and 3 were obtained by repeating the extraction twice with 1 vol. of water for 48h. The extracts were combined and dialysed overnight in the cold against 0.1M-sodium acetate buffer, pH 3.9, and a small precipitate was removed by centrifugation.

Then 80ml of the combined dialysed supernatant, containing 610mg of protein, was applied to a Sephadex G-200 column (5cm×81cm) previously equilibrated with 0.1M-sodium acetate buffer, pH 3.9. Elution was carried out with the same buffer at a flow rate of 36ml/h. Fractions of volume 20ml were collected, and samples were analysed for protein by measuring the absorbance at 280nm, for hexoses by the orcinol method (Winzler, 1955) and for sialic acid by the direct Ehrlich method (Werner & Odin, 1952). Virtually all of the sialic acid, 93% of the hexose and 43% of the protein were eluted in the void volume. The remaining protein was eluted in two retarded peaks, one of which contained some hexose. Recoveries of sialic acid, hexose and protein were greater than 95%. For large-scale preparations 300ml (2.29g of protein) was applied to a column of larger size (9.7cm×105cm). The fractions constituting the

excluded peak were pooled and dialysed exhaustively against water. Freeze-drying gave 630mg of dry material, corresponding to 1.2% of the wet weight of the glands.

The mucin was then further purified by chromatography on Bio-Gel CM-100. Swelled Bio-Gel CM-100 was treated in succession with 0.1M-acetic acid, distilled water, 0.1M-NaOH and distilled water, and was then equilibrated overnight with 0.1M-sodium acetate buffer, pH 4.6, and poured to make a 5.6cm×16cm column. The freeze-dried material from the excluded peak on Sephadex G-200 (450mg), dissolved in 60ml of the acetate buffer, was applied to the column and eluted at a flow rate of 48ml/h, fractions of volume 13ml being collected. Samples were analysed for protein by measuring the absorbance at 280nm and for neutral sugars by the phenol-H₂SO₄ method (Dubois *et al.*, 1956). It was found that 85% of the hexose and 45% of the protein applied to the column were recovered in a non-retarded fraction. No other material was eluted from the column by increasing the ionic strength to 1 with NaCl. However, when treated batchwise with 0.1M-NaOH the gel released a tightly bound component that contained some carbohydrate. The non-retarded peak from the column was dialysed exhaustively against water and freeze-dried. It dissolved readily to give a very viscous solution. The yield was 0.7% of the wet weight of the glands. Larger samples (200-250ml) were also separated on larger columns (9.7cm×30cm).

The preparation from Bio-Gel CM-100 dissolved in 0.1M-sodium acetate buffer, pH 3.9, gave a single hypersharp peak in a Spinco model E analytical ultracentrifuge run at 59000rev./min. However, at 29500rev./min a normal symmetrical peak was obtained. Sedimentation was carried out at concentrations from 0.5 to 10mg/ml. Extrapolation to zero concentration gave $s_{20,w}^{0}$ 10S. The sedimentation rate was markedly concentration-dependent, but the variation of $s_{20,w}$ with mucin concentration was linear at concentrations lower than 0.35%.

The mucin molecules do not penetrate even dilute polyacrylamide gels (Tettamanti & Pigman, 1968; De Salegui & Plonska, 1969). However, by using a mixed gel of 2% polyacrylamide and 0.5% agarose (Peacock & Dingman, 1968; Holden *et al.*, 1971a,b)

the mucin migrated into the gel, and showed a single rather diffuse band when stained with Coomassie Blue for protein or by the Schiff-periodate stain for carbohydrates.

When tested by immunoelectrophoresis with anti-serum to crude gland extract, the purified mucin gave one long precipitin arc that stained with Amido Black.

The amino acid composition of the purified canine submaxillary mucin, determined with a Beckman amino acid analyser after hydrolysis of a 0.2% solution of the mucin in 6M-HCl at 100°C for 22 and 70h under N₂, is given in Table 1. The values

for serine and threonine were corrected by extrapolation of the hydrolysis to zero time. The protein content of the mucin, both by amino acid composition and by the colorimetric method of Lowry *et al.* (1951), was about 33%. As has been reported for other submaxillary mucins, threonine, serine, proline, glycine and alanine are the major amino acids, accounting for 71% of the amino acid residues.

The carbohydrate components of the purified canine submaxillary mucin are also tabulated in Table 1. Individual neutral sugars were determined by g.l.c. of the alditol acetates (Lehnhardt & Winzler, 1968). Hexosamines were assayed by using the amino

Table 1. *Chemical composition of canine submaxillary mucin*

Experimental details are indicated in the text. Results are expressed as g of amino acid or sugar released by hydrolysis of 100g of canine submaxillary mucin dried to constant weight. No correction is made for ash.

Component	Composition	
	(g/100g of mucin)	(mol/100mol of amino acids)
Lysine	0.5	1.0
Histidine	0.6	1.2
Arginine	3.1	5.0
Aspartic acid	1.1	2.4
Threonine	5.6	14.0
Serine	3.8	11.0
Glutamic acid	2.8	5.4
Proline	3.7	9.5
Glycine	5.8	25.5
Alanine	3.2	11.3
Cystine (half)	0.5	1.2
Valine	1.9	4.9
Methionine	0.1	0.2
Isoleucine	0.4	0.9
Leucine	1.7	3.8
Tyrosine	0.4	0.6
Phenylalanine	1.3	2.2
Protein*	36.5	100.0
Protein†	34.5	
		(mol/100mol of monosaccharides)
N-Acetylneuraminic acid	8.6	9.0
Galactose	14.6	27.6
Fucose	10.4	21.7
N-Acetylgalactosamine‡	15.2	22.8
N-Acetylglucosamine‡	12.5	18.8
Uronic acid	0.0	0.0
Total carbohydrate	61.3	99.9
Sulphate	3.8	12.8

* Sum of the residue weights of the amino acids.

† Protein determination by the method of Lowry *et al.* (1951).

‡ Amino sugars were presumed to occur as the N-acetyl derivatives.

acid analyser (Weber & Winzler, 1969), with occasional checks to demonstrate that the hexosamine peaks disappeared after periodate oxidation of the hydrolysates. Sialic acid was determined by the direct Ehrlich reaction (Werner & Odin, 1952) or by the thiobarbituric acid method of Aminoff (1961) after hydrolysis of samples with 10M-HCl at 100°C for 10 min. Hyaluronate and chondroitin sulphates are absent, since no uronic acid was detectable by the carbazole reaction (Bitter & Muir, 1962). The carbohydrate content of the mucin was 61% and consisted of *N*-acetylneuraminic acid, galactose, fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine in the molar proportions 0.4:1.2:0.95:0.83:1.00. The mucin contained 3.8% sulphate, as determined by the method of Antonopoulos (1962).

Gel filtration of mucins on Sephadex G-200 in 0.25M-NaCl has been carried out by Bhargava & Gottschalk (1966). In the present method gel filtration was carried out at pH 3.9. At this pH the viscosity of the mucin was qualitatively less than at pH 7. Chromatography on Bio-Gel CM-100 resulted in removal of protein of low carbohydrate content from the Sephadex G-200 void-volume eluate. The final product appeared to be homogeneous by sedimentation in the ultracentrifuge. However, by electrophoresis on polyacrylamide-agarose gels and by immunoelectrophoresis the preparation spread to give a single somewhat diffuse band or arc. It is likely that this results from micro-heterogeneity due to variations in carbohydrate content. The chemical composition of the purified mucin was very similar to that isolated from canine submaxillary glands by Hashimoto & Pigman (1962) by a procedure involving precipitation of the mucin with cetyltrimethylammonium bromide. The content of amino sugars observed in the present work is slightly higher than that reported by Weber & Winzler (1969), and the ratio of aspartate to serine+threonine is also somewhat different. This no doubt is a result of the greater purity of the present preparation. Canine submaxillary mucin is a sulphated glycoprotein, as was suggested by the work of Bignardi *et al.* (1964). The

sulphate content corresponds to 12.8 mol/100 mol of monosaccharide, an amount that is only slightly less than the molar content of *N*-acetylglucosamine.

The preparation method described above has also been successfully applied to bovine submaxillary mucin and may be proposed as a general method for mucin isolation.

This work was supported by National Institutes of Health Grant 2R01CA11803-02, American Cancer Society Grant BC-25E and Armed Forces Epidemiological Board Grant MD2803.

- Aminoff, D. (1961) *Biochem. J.* **81**, 382
Antonopoulos, C. A. (1962) *Acta Chem. Scand.* **16**, 1521
Bhargava, A. S. & Gottschalk, A. (1966) *Biochim. Biophys. Acta* **127**, 223
Bignardi, C., Aureli, G., Balduini, C. & Castellani, A. A. (1964) *Biochem. Biophys. Res. Commun.* **17**, 310
Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330
De Salegui, M. & Plonska, H. (1969) *Arch. Biochem. Biophys.* **129**, 49
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350
Hashimoto, Y. & Pigman, W. (1962) *Ann. N.Y. Acad. Sci.* **93**, 541
Holden, K. G., Yim, N. C. F., Griggs, L. J. & Weisbach, J. A. (1971a) *Biochemistry* **10**, 3105
Holden, K. G., Yim, N. C. F., Griggs, L. J. & Weisbach, J. A. (1971b) *Biochemistry* **10**, 3110
Lehnhardt, W. F. & Winzler, R. J. (1968) *J. Chromatogr.* **34**, 471
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Peacock, A. C. & Dingman, C. W. (1968) *Biochemistry* **7**, 668
Pigman, W. & Gottschalk, A. (1966) in *Glycoproteins, their Composition, Structure and Function* (Gottschalk, A., ed.), p. 434, Elsevier, Amsterdam, London and New York
Tettamanti, G. & Pigman, W. (1968) *Arch. Biochem. Biophys.* **124**, 41
Weber, P. & Winzler, R. J. (1969) *Arch. Biochem. Biophys.* **129**, 534
Werner, I. & Odin, L. (1952) *Acta Soc. Med. Upsal.* **57**, 230
Winzler, R. J. (1955) *Methods Biochem. Anal.* **2**, 279