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Isolation of different high-M_r mucin species from human whole saliva

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By using CsCl-density-gradient ultracentrifugation, two high-M_r mucin species were isolated from human whole saliva, having buoyant densities in 0.2 M-guanidinium chloride of approx. 1.56 g/ml (pool IA) and 1.48 g/ml (pool IIA). Analytical density-gradient centrifugation of submandibular, sublingual, labial and palatal saliva, followed by immunochemical analysis with anti-mucin monoclonal antibodies, indicated immunochemical and physicochemical similarities between the high-density mucins of pool IA and mucins from palatal salivary glands. Chemical analysis indicated that the putative palatal mucin was rich in sulphate, but poor in sialic acid. The lower-density mucins of pool IIA equated with the high-M_r mucins of submandibular–sublingual saliva, both immunochemically and physicochemically (buoyant density).

INTRODUCTION

Salivary mucins are secreted by the (sero)-mucous salivary glands, namely the submandibular and sublingual glands and the minor salivary glands of lip, cheek, tongue and palate. Salivary mucins are involved in the protection of oral surfaces against chemical, mechanical and microbial attacks (Tabak *et al.*, 1982; Levine *et al.*, 1987; Nieuw Amerongen *et al.*, 1987; Slomiany *et al.*, 1989). Relatively little is known about the structural and functional properties of the mucins secreted by the individual glands, particularly those originating from the minor glands. Using a panel of anti-mucin monoclonal antibodies (mAbs), we demonstrated that high-M_r palatal salivary mucins are clearly distinguishable immunochemically from those in sublingual, submandibular and labial saliva (Veerman *et al.*, 1991). In the present paper we describe the isolation and partial characterization of a high-M_r mucin fraction from human whole saliva, which immunochemically closely resembles the palatal salivary mucins.

MATERIALS AND METHODS

Materials

Phenylmethanesulphonyl fluoride, benzamidine hydrochloride, Tween-20, 6-aminohexanoic acid, *o*-phenylenediamine and guanidinium chloride (GuCl) (treated with activated charcoal and filtered before use) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephacryl HR-500 and Sepharose CL-2B were from Pharmacia (Uppsala, Sweden). CsCl was from Gibco (Paisley, U.K.). Horseradish-peroxidase-conjugated rabbit anti-(mouse immunoglobulin) antibody (RAMPO) and avidin conjugated to horseradish peroxidase were from Dako Patts (Glostrup, Denmark). BCA protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). All other chemicals were of the highest-purity grade available and were obtained from Merck (Darmstadt, Germany). High-avidity 96-well e.l.i.s.a. micro-titre plates were from Greiner (Frickenhausen, Germany).

Methods

Collection of human saliva. Human whole saliva of one individual (blood group A, non-secretor) was stimulated by chewing on Parafilm and collected in ice-chilled vessels, containing a set of proteinase inhibitors consisting of 5 mM-phenylmethanesulphonyl fluoride, 10 mM-benzamidine hydrochloride, 5 mM-EDTA and 10 mM-6-aminohexanoic acid. Palatal, labial, sublingual and submandibular salivas of the same individual were collected by aspirating fluid drops originating at the individual glandular orifices, using a micropipette [for a detailed description see Veerman *et al.* (1991)].

CsCl-density-gradient ultracentrifugation. CsCl-density-gradient centrifugation of whole saliva was performed essentially according to the method of Carlstedt *et al.* (1983). Approx. 25 ml of mechanically stimulated saliva, collected as described above, was adjusted to 4 M-GuCl by addition of an appropriate volume of a 7.8 M-GuCl stock solution. The resulting solution was gently stirred for 2 h at 4 °C. Any insoluble material present was removed by centrifugation (10000 *g* for 15 min). Then solid CsCl was added gradually with gentle stirring, until a final density of 1.45 g/ml was obtained. The solution was centrifuged at 100000 *g* for 100 h at 15 °C. Tubes were emptied from the bottom, fractions (approx. 1 ml) were collected and density was measured by weighing. Fraction density increased from approx. 1.30 g/ml (top) to 1.60 g/ml (bottom). The fractions were tested in e.l.i.s.a.s with anti-mucin mAbs F2 and E9 (see below). Fractions were tested 2-fold serially diluted, starting from 1:200. Immunoreactive peaks were pooled, dialysed against 0.2 M-GuCl and adjusted to a density of 1.5 g/ml with CsCl for a second density-gradient ultracentrifugation step (100000 *g* for 100 h). Fractions (1 ml) were collected and analysed for immunoreactivity with anti-mucin mAbs F2 and E9 as described above. Immunoreactive fractions were pooled, dialysed against distilled water and freeze-dried. The freeze-dried proteins were reconstituted to a concentration of approx. 1 mg dry wt/ml in 4 M-GuCl (pH 7.4). Samples (0.5 ml) were applied to a Sephacryl HR-500 column (30 cm × 1.5 cm) and eluted in 4 M-GuCl at a rate of 13 ml/h. Occasionally samples were applied to a Sepharose CL-2B column equilibrated in 4 M-GuCl, pH 7.4, and eluted in the same buffer at 2.5 ml/h. In both instances the fractions obtained (0.9 ml) were assayed 2-fold serially diluted in e.l.i.s.a.s with the various

Abbreviations used: mAb, monoclonal antibody; GuCl, guanidinium chloride; PBST, 150 mM-NaCl/50 mM-potassium phosphate buffer, pH 7.4, containing 0.1% (v/v) Tween-20.

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anti-mucin mAbs. Starting dilution was 1:100. Carbohydrate analysis was performed as described previously (Nieuw Amerongen *et al.*, 1987). Protein was determined with the BCA protein assay (Smith *et al.*, 1985), with BSA as a reference. Sulphate content was determined according to the method of Terho & Hartiala (1971), after hydrolysis of mucins in 0.5 M-HCl at 100 °C for 4 days.

Buoyant densities of mucins of individual glandular salivas were determined as follows: submandibular saliva (100 μl), sublingual saliva (10 μl), palatal saliva (2 μl) and labial saliva (2 μl) were dissolved in 4 M-GuCl, pH 7.4, and CsCl was added to a starting density of 1.45 g/ml (final volumes approx. 20 ml). Solutions were ultracentrifuged at 100000 *g* for 100 h. Fractions were collected and analysed for density and immunoreactivity as described above.

Monoclonal antibodies. A detailed description of the production and characterization of anti-mucin mAbs used in the present study has been given elsewhere (Rathman *et al.*, 1989; Veerman *et al.*, 1991). In short, mAb F2 was directed to a neuraminidase-insensitive periodate-insensitive as well as papain-insensitive epitope present on the glycopeptide moiety of high-M_r salivary mucins (MG1). mAb E9 (Veerman *et al.*, 1991) and mAb 19-9 (Brockhaus *et al.*, 1985) were directed to (different) sialic acid-containing epitopes, present on both high-M_r (MG1) and low-M_r (MG2) salivary mucins. mAb B11 was directed towards a neuraminidase-insensitive periodate-sensitive epitope, present on both MG1 and MG2.

E.l.i.s.a. of salivary mucins. E.l.i.s.a. of salivary mucins was carried out as previously described (Veerman *et al.*, 1991): a dose–response curve of salivary samples was made by diluting the sample (in duplicate) directly in the wells of a 96-well microtitre plate. Samples were 2-fold serially diluted in 0.1 M-$NaHCO_3$, pH 9.6, starting at a concentration of approx. 2.5 μg dry wt./ml (for purified mucins) or a starting dilution of 1:200 (for submandibular saliva) or 1:1000 (for sublingual, labial and palatal saliva). Plates were incubated overnight at 4 °C. After incubation, wells were rinsed twice with phosphate-buffered saline (150 mM-NaCl/50 mM-potassium phosphate buffer, pH 7.4) containing 0.1 % (v/v) Tween-20 (PBST), followed by three rinses with demineralized water. This washing procedure was performed between each incubation step. Then mAbs to salivary mucins were added, appropriately diluted in PBST containing 1 % gelatin (PBST/gelatin). After incubation (for 1 h at 37 °C) and subsequent washing, RAMPO (diluted 1:2000 in PBST/gelatin) was added. After incubation (for 45 min at 37 °C), wells were rinsed, and substrate-containing solution was added (1,1′-*o*-phenylenediamine/0.03 % H_2O_2 in 0.1 M-citrate/phosphate buffer, pH 5.0). Colour was allowed to develop for 45 min or until sufficient intensity was reached. Reaction was stopped by addition of H_2SO_4 (final concentration 0.2 M), and absorbance was measured at 492 nm, using a micro-titre plate reader. Quantification of apparent mucin concentration was performed with a purified MG1 preparation as a reference (Veerman *et al.*, 1989). Relative (normalized) mucin concentration as determined with a particular mAb was calculated as the apparent mucin concentration determined with that particular mAb divided by the sum of apparent mucin concentrations (determined with all four mAbs) × 100 % (Veerman *et al.*, 1991).

RESULTS

Density-gradient equilibrium centrifugation of glandular salivas

Fig. 1 shows the results of analytical density-gradient equilibrium runs, performed on palatal (Fig. 1*a*), sublingual (Fig. 1*b*), submandibular (Fig. 1*c*) and labial (Fig. 1*d*) saliva in 4 M-GuCl,

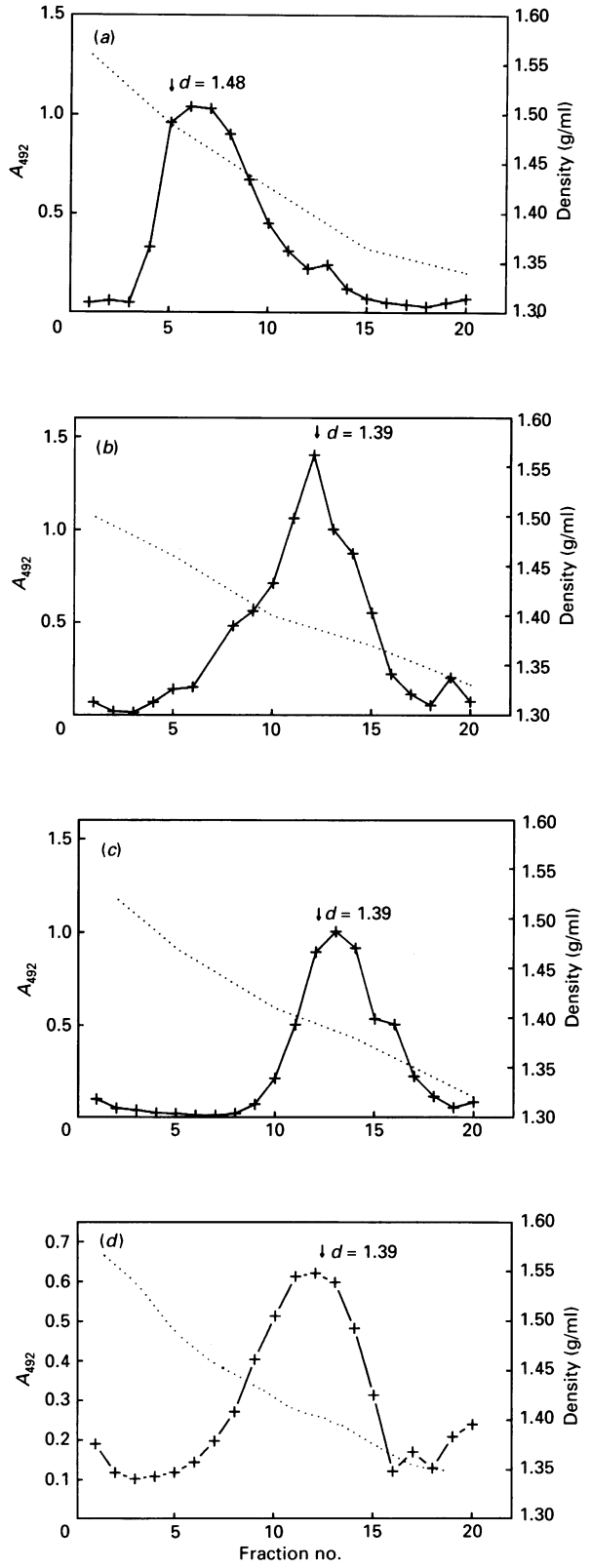

Fig. 1. CsCl-density-gradient ultracentrifugation of human glandular salivas in the presence of 4 M-GuCl, pH 7.4

· · · · ·, Fraction density; +, response in e.l.i.s.a. with mAb F2 at 492 nm. (*a*) Palatal saliva; (*b*) sublingual saliva; (*c*) submandibular saliva; (*d*) labial saliva. Tubes were emptied from the bottom and all fractions (approx. 1 ml) were analysed serially diluted in e.l.i.s.a. with mAb F2. Palatal mucins float at a higher buoyant density than those in the other salivas.

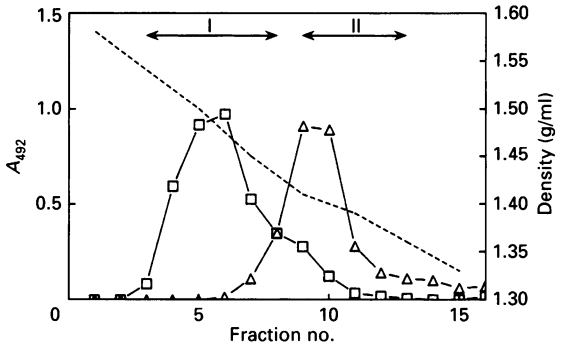

Fig. 2. CsCl-density-gradient ultracentrifugation of human whole saliva in the presence of 4 M-GuCl, pH 7.4

Fractions were assayed 2-fold serially diluted with mAbs F2 and E9 as probing antibodies. -----, Fraction density; ——, mAb reactivity as measured in e.l.i.s.a. at 492 nm with mAb F2 (□) and mAb E9 (△). Pool I, fractions 3–8; pool II, fractions 9–13.

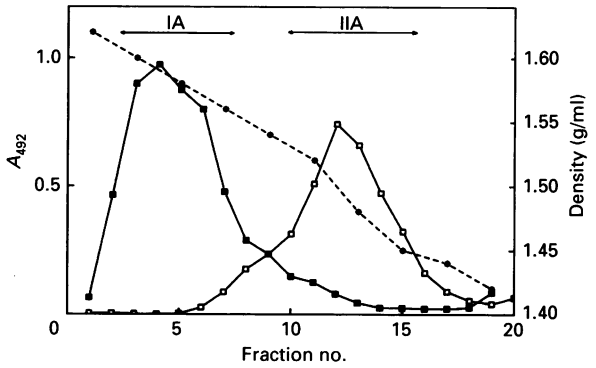

Fig. 3. CsCl-density-gradient ultracentrifugation of pool I (■) and pool II (□) (see Fig. 2) in 0.2 M-GuCl, pH 7.4

Starting density: 1.5 g/ml. Fractions were assayed 2-fold serially diluted with mAb F2 as probing antibody. -----, Fraction density; ——, mAb reactivity as measured in e.l.i.s.a. at 492 nm with mAb F2 as probing antibody. Pool IA, fractions 2–8. Pool IIA, fractions 10–16. The results of the two separate runs are presented in one Figure.

pH 7.4. The results indicate a distinctly higher buoyant density for only the palatal mucins, which floated at approx. 1.48 g/ml, as compared with submandibular, sublingual and labial mucins, having under these conditions a buoyant density of approx. 1.39 g/ml.

Isolation of mucins from human whole saliva

Fig. 2 shows the immunoreactivity of the fractions obtained after the first CsCl-gradient-density equilibrium centrifugation of human whole saliva in 4 M-GuCl (see the Materials and methods section). Fractions were tested for immunoreaction with mAbs F2 and E9. The immunoreactive peaks floating at densities of approx. 1.48 g/ml (pool I) and 1.40 g/ml (pool II) were collected for further purification. The high-density peak (pool I) was predominantly recognized by mAb F2, whereas pool II reacted with both mAbs F2 and E9. In addition, material immunoreactive only with mAb E9 was present at a lower density. Gel-filtration analysis (results not shown) of the latter material indicated that it largely comprised MG2 (Prakobphol *et al.*, 1982; Loomis *et al.*, 1987), and it was not further analysed. Fractions were pooled as indicated in Fig. 2. Then a second

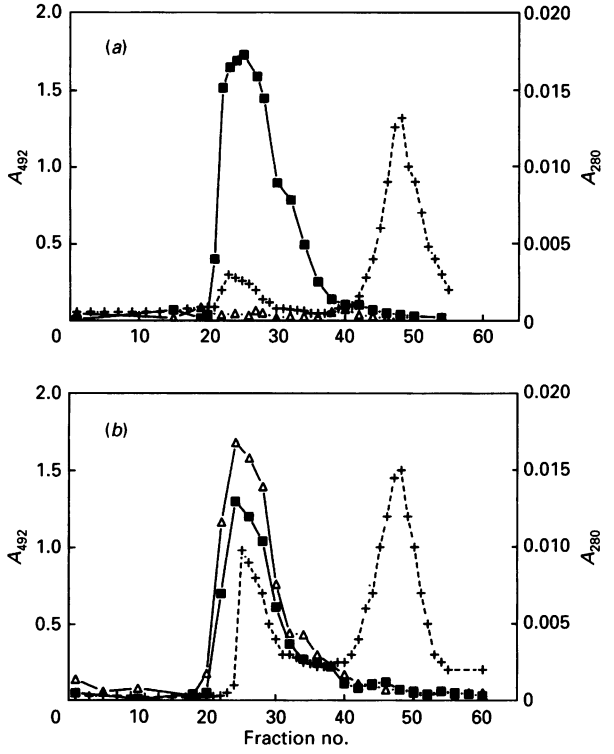

Fig. 4. Sephacryl HR-500 chromatography of pool IA (*a*) and pool IIA (*b*) in 4 M-GuCl, pH 7.4

Pools IA and IIA were obtained after repetitive CsCl-equilibrium-density-gradient centrifugation of human saliva (see Figs. 2 and 3). -----, A_{280}; ——, A_{492}. Fractions were tested 2-fold serially diluted, starting from 1:100, in e.l.i.s.a.s with mAb F2 (■) and mAb E9 (△). Fractions 20–30 were pooled, dialysed against distilled water and (immuno)chemically analysed. Amount applied: 1 mg (dry wt.) in 0.5 ml. Immunoreactive material was eluted only in the void volume.

density-gradient ultracentrifugation step was performed on the two separate pools in 0.2 M-GuCl, at a starting density of 1.50 g/ml (Fig. 3). Under these conditions, the immunoreactive peaks floated at higher densities of approx. 1.56 g/ml (pool IA) and 1.48 g/ml (pool IIA). A similar dependence of buoyant density of mucins on the guanidine concentration has been reported by Carlstedt *et al.* (1983). Immunoreactive fractions were pooled as indicated in Fig. 3 legend, dialysed against distilled water and freeze-dried. After reconstitution in 4 M-GuCl, mucins (approx. 1 mg dry wt./ml) were gel-filtered over Sephacryl HR-500 in 4 M-GuCl. Fig. 4 shows the elution profiles monitored at 280 nm as well as in e.l.i.s.a.s with mAbs F2 and E9. For both species, immunoreaction was only observed in the void-volume fractions, indicating that only high-M_r mucins (MG1) were present. Essentially identical results were obtained after gel filtration over Sepharose CL-2B under dissociative conditions (results not shown). Typically, from 25 ml of Parafilm-stimulated human whole saliva, approx. 1 mg (determined as dry weight) of each species could be recovered. In this respect it should be noted that Parafilm-stimulated saliva contained more of the high-density mucin species (relative to the low-density species) then did unstimulated saliva. The immunochemical characterization of these species, as probed with the various mAbs, indicated again that the high-density species (pool IA) was recognized only by mAb F2. In contrast, the mucin species floating at densities 1.46–1.48 g/ml (pool IIA) reacted with mAbs 19-9, E9 and B11 as well as with mAb F2 (Fig. 5). For comparison, in Fig. 5 are also shown the immunoreactivity

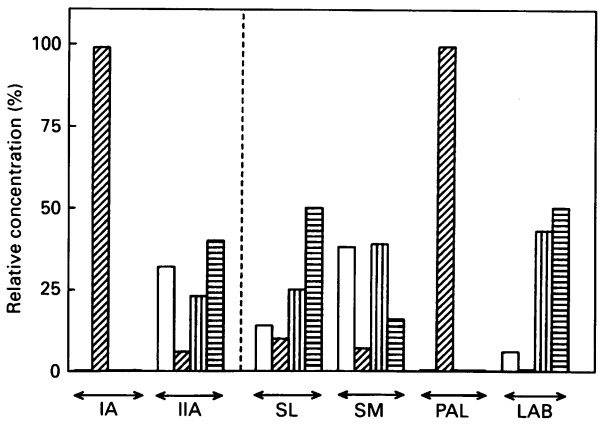

Fig. 5. Relative immunoreactivities of (salivary) mucin species towards anti-mucin mAbs

IA and IIA, pooled void-volume fractions of Sephacryl HR-500 elution of high-density and low-density mucins (see Figs. 4*a* and 4*b*); SL, sublingual; SM, submandibular, PAL, palatal; LAB, labial. □, mAb B11; ▨, mAb F2; ▥, mAb E9; ▤, mAb 19-9. Relative mucin concentrations in each sample were calculated as described in the Materials and methods section.

Table 1. Chemical composition of isolated human salivary mucins

IA and IIA were pooled void-volume fractions obtained during Sephacryl HR-500 gel chromatography (Fig. 4) of salivary mucins with buoyant densities of 1.56 and 1.48 g/ml (in 0.2 M-GuCl) respectively.

	Chemical composition (%)	
	Pool IA	Pool IIA
Protein	13.8	14.4
Carbohydrate	76.6	84
Fucose	12.7	6.3
Mannose	0.7	1.3
Galactose	24.1	21.3
N-Acetylgalactosamine	10.9	18.4
N-Acetylglucosamine	25.7	28.2
Sialic acid	2.5	8.5
Sulphate	9.5	1.2

profiles of individual palatal, submandibular, sublingual and labial saliva, illustrating the great similarity between palatal mucins and the high-density mucin species.

Analysis of the carbohydrate composition of the isolated mucin species (Table 1) indicated that the sialic acid content of the high-density species (pool IA) was at least 3-fold lower than the other species. In contrast, the sulphate content of pool IA was 8-fold higher than that of the other species.

DISCUSSION

A substantial part of the mucin population in human whole saliva is derived from the numerous minor mucous salivary glands of palate, lip, cheek and tongue (Tabak *et al.*, 1982). However, little is known on the specific properties of these minor glandular mucins, because of difficulties in obtaining sufficient material from individual minor glands. In a previous paper we have shown that distinct immunochemical differences are present between notably palatal mucins and mucins from labial, submandibular and sublingual glands (Veerman *et al.*, 1991). Now we present evidence that palatal mucins also differ from labial, submandibular and sublingual mucins in buoyant density (Fig. 1), thus enabling their isolation from whole saliva by using density-gradient equilibrium centrifugation. The similarities in both buoyant density (Figs. 1 and 2) and immunochemical profiles (Fig. 5) between palatal mucins and the high-density mucin fraction of pool IA lead us to the conclusion that this mucin fraction mainly comprises palatal mucins. In view of their immunochemical profile (see Fig. 5), the pool IIA mucins seem to represent a mixture of the high-M_r mucins of submandibular, sublingual and labial saliva. In this context we want to point out that the relative (normalized) concentrations of the epitopes in the pool II mucins presented in Fig. 5 were calculated on the basis of apparent concentrations, and therefore do not reflect the real ratio in which the various epitopes are present on these molecules (see also Veerman *et al.*, 1991).

The possibility exists that pool IA contains other mucin species that are not detectable with the mAbs used in this study. Direct demonstration is therefore needed to verify whether the putative palatal mucin of pool IA is comprised of palatal material. In this respect it is noteworthy that Green & Embery (1987) found in rat that mucins from palatal tissue contain hardly any sialic acid, but are relatively rich in sulphate. These data concur with the present findings that the putative palatal mucins were not recognized by sialic acid-dependent mAbs (E9 and 19-9), as well as with their observed high buoyant density, which for gastric mucins has been correlated with the degree of sulphation (van Beurden-Lamers *et al.*, 1989). This is further corroborated by the chemical analysis of the putative palatal mucin (Table 1), which indicated that they contained low amounts of sialic acid, but were rich in sulphate. As a consequence, the high-density salivary mucin may easily be overlooked when sialic acid is used as a tracer to monitor isolation steps. For example, if mAb E9 or 19-9 had been used to monitor the density-gradient equilibrium centrifugation of salivary mucins, we would have completely missed this mAb F2-positive high-density mucin species (see Fig. 2). The possibility that the sialic acid-poor species has originated from a sialic acid-rich species, e.g. by bacterial neuraminidase activity, is unlikely, since it was found that the buoyant density of purified salivary mucins shifted towards a lower value on desialylization (E. C. I. Veerman, unpublished work).

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