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Note

Analytical and semi-preparative high-performance liquid chromatography of oligosaccharides obtained by hydrazinolysis of hen ovomucoid

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Currently, monosaccharides and oligosaccharides are satisfactorily separated by paper, ion-exchange, thin-layer and gel filtration chromatography, however the methods are time-consuming High-performance liquid chromatography (HPLC) using a column of porous microparticles derivatized with amino groups allows a rapid separation of sugars[1–5] Since 1980, the HPLC on bonded primary amine packings of sugars derived from glycoprotein glycans have been described fractionation of oligosaccharides and glycopeptides excreted in the urine of patients with lysosomial diseases[6]; separations of oligosaccharides obtained by digestion with endoglycosidase H[7] or released by β-elimination of O-glycosylprotein[8 9], oligosaccharides derived from dolichol-linked oligosaccharide intermediates[10] Finally, clear separations of sialoglycopeptides or oligosaccharides have been obtained in less than 1 h by HPLC on a Micropak AX-10 ion-exchange column[11]

The present report shows that bonded primary amine packings are effective in the rapid separation of the mixture of neutral glycans liberated by hydrazinolysis of a hen ovomucoid neutral glycopeptide

MATERIALS AND METHODS

Glycoproteins, glycopeptides and oligosaccharides

Ovomucoid was prepared according to Fredericq and Deutsch[12] The asialoglycopeptide β was isolated after pronase hydrolysis of ovomucoid according to Monsigny *et al.*[13]. Oligosaccharides were released from asialoglycopeptide β by hydrazinolysis as previously described[14]. The resulting oligosaccharides were N-reacetylated according to Reading *et al* [15] and reduced with $NaBH_4$

Liquid chromatography on primary amine bonded silica

Analysis were carried out with a Spectra Physics Model 700 liquid chromatograph, equipped with an UV 8400 variable wavelength detector connected to a 4100 computing integrator.

HPLC was performed on a 5-μm Amino AS-5A column (0.4 × 25 cm, Chromatem 33; Touzart et Matignon). A 1-mg amount of oligosaccharides dissolved in 10

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μl of distilled water was injected into the column; for preparative chromatography, 3.5 mg of oligosaccharides dissolved in 20 μl of acetonitrile–water (50·50) were injected. The column was equilibrated with the initial solvent (acetonitrile–water, 65:35). After the injection, a linear gradient to acetonitrile–water (60:40) was applied for 30 min followed by isocratic conditions during 30 min and then a linear gradient to acetonitrile–water (50.50) for 30 min. The flow-rate was 1 ml/min. The oligosaccharides were detected at 200 nm under the following conditions: sensitivity of detec-

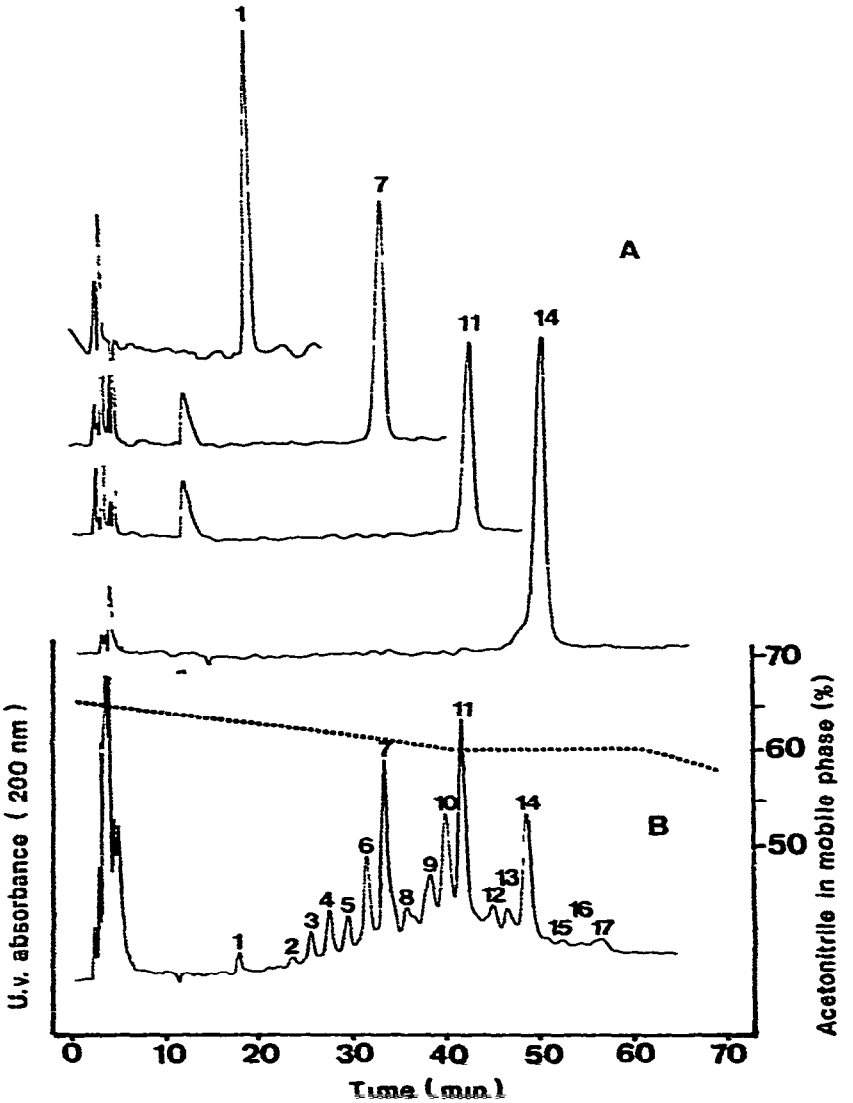

Fig. 1. Analysis (A) of oligosaccharides 1, 7, 11 and 14 obtained by semi-preparative chromatography (B) of oligosaccharides from the hydrazinolysis of hen ovomucoid neutral glycopeptide *β* on 5-μm Amino AS-5A (Chromatem 33, Touzart et Matignon) For chromatographic conditions, see Material and Methods.

tor, 0.35; integrator attenuation, 4, for analytical chromatography; sensitivity of detector, 0.32, integrator attenuation, 50, for semi-preparative chromatography.

Molar composition of oligosaccharides

The molar composition of oligosaccharides was determined by gas–liquid chromatography (GLC) of trifluoroacetylated methylglycosides according to Zanetta *et al.*[16].

Thin-layer chromatography (TLC) of oligosaccharides

TLC of oligosaccharides was performed on silica gel plates (pre-coated silica gel 60, Merck) using ethanol–*n*-butanol–pyridine–acetic acid–water (100.10 10.3 30 v/v/v/v/v)[17] during 7 h. Oligosaccharides were revealed with an orcinol–sulphuric acid reagent[18].

RESULTS AND DISCUSSION

The separation of a mixture of reduced oligosaccharides obtained by hydrazinolysis from hen ovomucoid is shown in Fig. 1. The effective separation of seventeen fractions was obtained within 90 min on an Amino AS-5A column (Fig. 1B) and semi-preparative chromatography on the same column allows one to obtain pure fractions 1, 7, 11 and 14 (Fig. 1A). The results of the semi-preparative chromatography of 4.2 mg of ovomucoid-derived oligosaccharides are given in Table I.

TABLE I

WEIGHTS OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF 4.2 mg OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS FROM OVOMUCOID NEUTRAL GLYCOPEPTIDE β

Fraction number	*Weight (μg)*
F-0	312.4
F-1	44
F-2	36.3
F-3	95.7
F-4	169.4
F-5	73.7
F-6	265
F-7	510.4
F-8	146.3
F-9	283.8
F-10	400.4
F-11	644.6
F-12	186
F-13	242
F-14	284
F-15	48.4
F-16	36.3
F-17	83.7
Recovered	3862

The use of a 0.4 × 25 cm column filled with silica gel modified by organic amines provides quantitative recovery (92%) of the oligosaccharides without loss of resolution. Each fraction was analysed by TLC (Fig. 2) and the carbohydrate composition was determined by GLC (Table II). Four fractions (1, 7, 11 and 14) are homogeneous on the basis of HPLC and TLC and of the monosaccharide molar composition. The latter is characterized by the absence of the presence in low amounts of galactose and by the relative high content of N-acetylglucosamine. Of interest is the ratio (N-acetylglucosamine + N-acetylglucosaminitol)/mannose, *i.e.*, (GlcNAc + GlcNAc-ol)/Man, the value of which is related to the number of N-acetylglucosamine branches on the mannose residues. The limiting values are 1.35 and 2.7 in the case of classical biantennary structures such as human serum transferrin[19] and of pentaantennary structures such as turtle-dove ovomucoid[20], respectively.

CONCLUSIONS

HPLC on bonded primary amine packings gives excellent fractionation of a

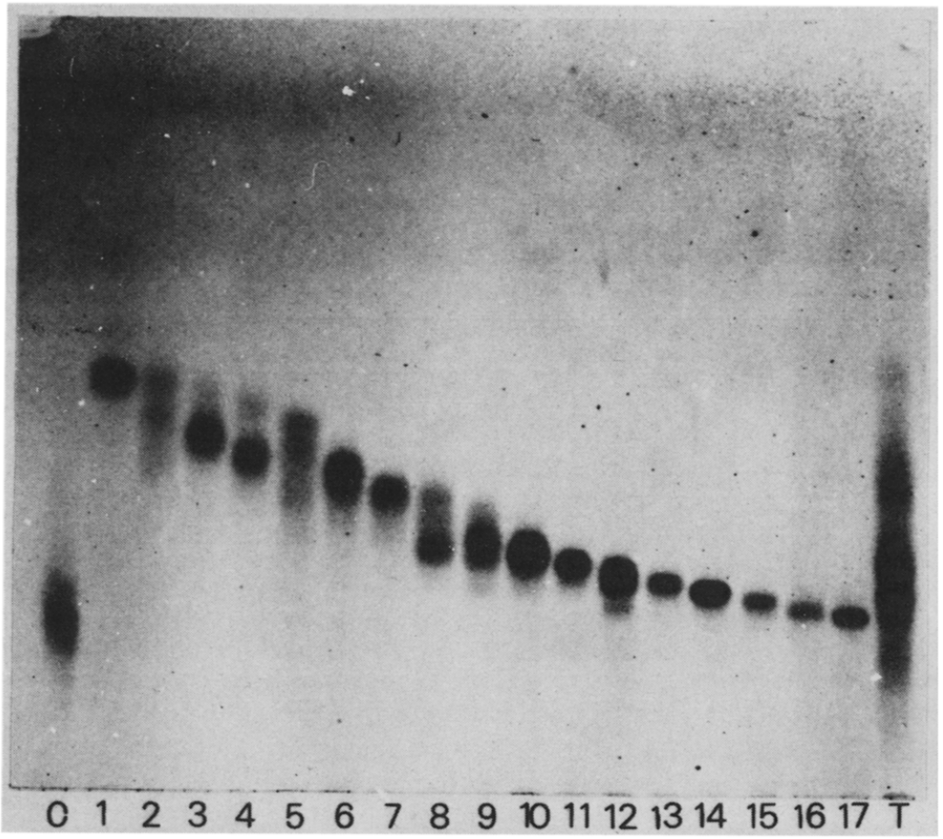

Fig. 2. TLC on silica gel plates (Merck) of seventeen fractions obtained by semi-preparative chromatography of oligosaccharides from the hydrazinolysis of hen ovomucoid neutral glycopeptide. Solvent: ethanol–*n*-butanol–pyridine–acetic acid–water (100:10:10:3:30 v/v/v/v/v). Development time: 7 h. Oligosaccharides revealed with orcinol–sulphuric acid reagent.

TABLE II

CARBOHYDRATE COMPOSITION OF FRACTIONS OBTAINED BY SEMI-PREPARATIVE CHROMATOGRAPHY OF OLIGOSACCHARIDES LIBERATED BY HYDRAZINOLYSIS OF HEN OVOMUCOID NEUTRAL GLYCOPEPTIDES β

Fractions	*Molar ratio**				*GlcNAc + GlcNAc-ol* / *Man*
	Gal	*Man*	*GlcNAc*	*GlcNAc-ol*	
1	0	3	1 3	0 94	0 74
2	0	3	2 94	0 81	1 25
3	0	3	3 4	0 50	1 3
4	0	3	3 63	0 66	1 43
5	0	3	3 4	0 2	1 2
6	0	3	3 9	0 60	1 5
7	0	3	4 64	0 94	1 86
8	0 44	3	5 02	0 61	1 87
9	0 44	3	5 38	0 65	2 01
10	0 59	3	4 63	0 64	1 75
11	0 19	3	6 8	1 01	2.6
12	0 72	3	5 70	0 57	2.09
13	1 03	3	5 99	0 30	2 09
14	1 07	3	6 8	1 07	2 62
15	1 52	3	6 9	0 71	2 53
16	1 5	3	6 21	0 57	2 26
17	1 85	3	6 8	0 84	2 54
Native glyco-peptide β	0 6	3		6	2

* The ratio of mannose (Man) was taken as 3 Gal = Galactose, GlcNAc = N-acetylglucosamine, GlcNAc-ol = N-acetylglucosaminitol

new class of glycans constituting a complex mixture of about twenty reduced neutral oligosaccharides liberated by hydrazinolysis from hen ovomucoid. These glycans are in general N-acetylglucosamine-rich and highly branched Our results illustrate again the usefulness of HPLC as a powerful tool to investigate the various compounds encountered in the field of glycoproteins. glycans of the "N-acetyl-lactosaminic type"[7 11], of the "oligomannosidic type"[7], of the "mucin type"[8 9] and lipid intermediate-derived oligosaccharides[10], dolichyl pyrophosphoryl oligosaccharides[21] and peracetylated mono- and oligosaccharides[22]

The results obtained demonstrate the high microheterogeneity of hen ovomucoid, which was not revealed by our first investigations[23] The primary structure of the numerous glycans isolated is now under investigation in order to understand the significance of their amazing microheterogeneity better and define the characteristics of the new class of glycans

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