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STRUCTURAL STUDIES OF THE CARBOHYDRATE COMPONENT OF A HUMAN, PAROTID-SALIVA, PROLINE-RICH GLYCOPROTEIN

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ABSTRACT

The carbohydrate chains of the human, parotid-saliva, proline-rich glycoprotein were released as oligosaccharides by hydrazinolysis and were fractionated by high-pressure-liquid chromatography. Four oligosaccharides were characterized. On the basis of compositional analysis, sequential enzymic degradation, and methylation data, the carbohydrate moiety of the glycoprotein was found to be a complex-type oligosaccharide containing a tri-D-mannosyl-di-*N*-acetylchitobiose core with two and three lactosamine branches, and an L-fucosyl group linked to O-6 of the asparagine-linked 2-acetamido-2-deoxy-D-glucose residue. In addition, several L-fucosyl groups, linked at O-6 of the penultimate D-galactose residues, are present in variable proportions. The biological significance of these results is discussed.

INTRODUCTION

We reported earlier[1] the presence of a tri-D-mannosyl-di-*N*-acetylchitobiose core in the carbohydrate chains of human-parotid, proline-rich glycoprotein (PRGP). The principal difficulty in structural studies of the carbohydrate chains of PRGP has been in obtaining pure oligosaccharides representative of the glycoprotein. Recently, we reported[2] a convenient method for the separation of neutral oligosaccharides by high-pressure-liquid chromatography (h.p.l.c.). In this paper, we describe the isolation and structural studies of the carbohydrate chains from human, parotid-saliva PRGP which, in addition to the structure described earlier[1], contains L-fucosyl groups linked to penultimate D-galactose residues. The identification of terminal L-fucosyl groups, and their location only on certain sugar chains suggest a significant role.

EXPERIMENTAL

Materials. — Human-parotid saliva from a single donor and the proline-rich

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glycoprotein were obtained as previously described[3]. The glycopeptides were prepared by enzymic proteolysis with papain and Pronase[4].

Preparation of oligosaccharides. — Carbohydrate chains of PRGP were obtained by hydrazinolysis. Glycopeptides (20 mg) were treated with anhydrous hydrazine[5] (1 mL) in screw-capped vials for 24 h at 100 °. The hydrazine was evaporated, and the dry residue was dissolved in 15% acetic acid (1 mL), previously cooled to 4 °. The *N*-deacetylated oligosaccharides were separated from amino acid hydrazides in a column (50 × 2 cm) of Bio-Gel P-6 (200–400 mesh). The column was washed with distilled water. The purified *N*-deacetylated oligosaccharides were *N*-reacetylated in 0.1M sodium hydrogencarbonate (0.5 mL) with acetic anhydride (10 μL). After a 12-h incubation, the mixture was saturated with solid sodium hydrogencarbonate, and acetic anhydride (5 μL) was added. The mixture was kept for 15 h at room temperature and then desalted by chromatography on Bio-Gel P-2 (50–100 mesh). The oligosaccharides fractions were reduced with sodium borohydride (10 mg) in 0.05M sodium hydroxide (0.5 mL) as described previously[4].

High-pressure-liquid chromatography separation. — Separations were performed with a Waters Associates instrument equipped with a two-pump Model 6000A solvent-delivery system, a Model U6K manual injector, and a Model 660 solvent programmer. Absorbance detection at 206 nm was obtained with a Uvicord detector, LKB model S2138, coupled to an LKB model 2250 recorder. The chromatograph was equipped with a column (25 × 0.32 cm, i.d., Alltech Associates) packed with silica-NH_2 (5 μm). The elution of the oligosaccharides was performed with a linear gradient of 7:3 to 3:2 (v/v) acetonitrile–water for 1 h at room temperature, at a flow rate of 1.5 mL/min.

Carbohydrate composition — Neutral sugars and hexosamines were determined as per-*O*-(trimethylsilyl) derivatives by g.l.c. with a Perkin–Elmer instrument (flame-ionization detector) by the slightly modified procedure[6] of Reinhold[7]. The hexosamines were obtained by hydrolysis for 2 h at 100 ° with 2M trifluoroacetic acid, followed by *N*-reacetylation.

Methylation analysis. — The methylation of oligosaccharides was carried out according to the method of Hakomori[8]. The alditol acetates and 2-deoxy-2-(*N*-methylacetamido)alditol acetates were prepared according to the method of Björndal *et al.*[9]. The partially *O*-methylated alditol and 2-deoxy-2-(*N*-methylacetamido)alditol acetates were identified by g.l.c.–m.s. with a Hewlett-Packard 5993B g.l.c.–m.s. instrument operated with 5985 software. G.l.c. was performed with a glass column (0.3 mm i.d. × 1.5 m) packed with 3% of OV 17 on 100–200 mesh Gas Chrom Q (Supelco, Inc., Bellefonte, PA 16823). The column was programmed to be heated at a rate of 6 °/min from 130 to 260 °. The injector part was kept at 270 °, the separator at 280°, and the ionizing voltage at 70 eV. Two minutes after sample injection, repetitive scanning was started from *m/z* 40 to 500 in a 2.1-s cycle. The methylated alditol acetates were identified by a combination of g.l.c. retention times, selected ion recording, and mass spectra. The spectra were compared to known standards for positive identification.

TABLE I

SUGAR COMPOSITION[a] OF OLIGOSACCHARIDES AND METHYL ETHERS[b] OBTAINED FROM METHYLATED OLIGOSACCHARIDES

Sugar	*Methyl ether*	*Oligosaccharide fraction*							
		3		*4*		*6*		*7*	
L-Fucose			0.7		0.6		2.2		2.9
	2,3,4-[c]	0.8		0.7		0.8		1.2	
D-Galactose			2		2.5		2.3		2.9
	2,3,4,6-[c]	1.6		2.2		1.2		0.7	
	2,3,4-[c]					0.8		0.8	
D-Mannose			3		3		3		3
	3,4,6-[c]	1.8		1.3		1.6		0.8	
	3,6-[c]			0.7				1	
	2,4-[c]	1		1		1		1	
2-Acetamido-2-deoxy-D-glucose		2.7		3.3		2.4		3	
	3,6[d]		4.2		5.3		4		4.6
2-Acetamido-2-deoxy-D-glucitol									
	1,3,5-[d]	1		1		1		1	

[a]Molar ratio relative to D-mannose taken as 3. [b]The molar ratios obtained for the methylated sugars by g.l.c.–m.s. may not correspond to those obtained for carbohydrate analysis, as no correction factors for the methylated sugars were used. The molar ratio for a specific series of methylated hexoses were calculated according to Sweet *et al.*[10]. [c]Molar ratio of methyl ethers of L-fucose, D-galactose and D-mannose relative to 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannose. [d]Molar ratio of 2-acetamido 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-D-glucose relative to 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-1,3,5-tri-*O*-methyl-D-glucitol taken as 1.

RESULTS

Separation and characterization of the carbohydrate chains. — The carbohydrate chains, linked to asparagine residues of the glycopeptides, were liberated with anhydrous hydrazine. The released oligosaccharides were reduced with sodium borohydride and then separated by h.p.l.c. The oligosaccharides were recovered quantitatively, from the column eluate, to give 7 fractions (Fig. 1). The fractions obtained from several runs were pooled, evaporated, and analyzed for carbohydrate components (see Table I). Oligosaccharide Fractions 3, 4, 6, and 7, obtained by h.p.l.c., were analyzed by methylation (see Table I).

The results of Fraction 3 indicate that D-galactose and L-fucose are present as terminal residues, and that the 2-acetamido-2-deoxy-D-glucose residues are linked at O-4, and the 2-acetamido-2-deoxyglucitol residues at O-4 and -6. As L-fucose had been shown previously[1] to be linked at O-6 of the asparagine-linked 2-acetamido-2-deoxy-D-glucose residue, these results indicate two residues of 2-substituted D-mannose and one residue of 3,6-disubstituted D-mannose. In combination with the results of the sequential exo-glycosidase digestion described[1] previously, they suggest for Fraction 3 oligosaccharide structure **1**.

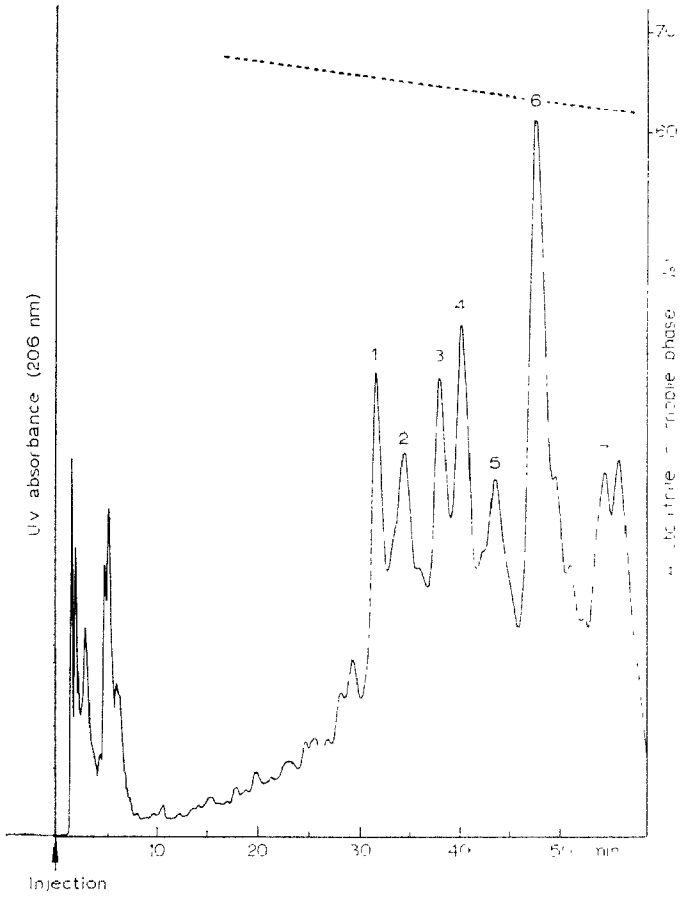
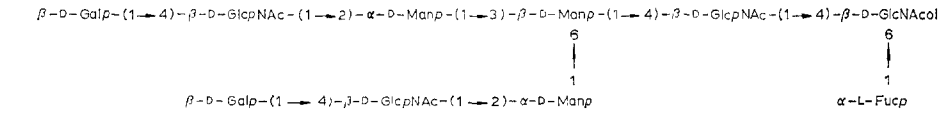


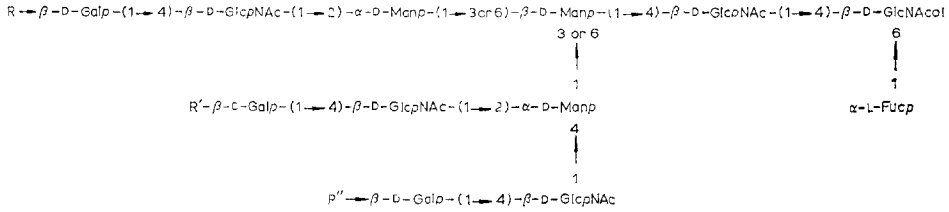
Fig. 1. High-pressure liquid chromatography of PRGP-derived oligosaccharides. The chromatogram was developed with a linear gradient decreasing the acetonitrile concentration (---) in the mobile phase. The flow rate was 1.5 mL/min. Fractions 3, 4, 6, and 7 were characterized. Fractions 1 and 2 contained, in addition to a small proportion of carbohydrate, contaminating material, which limited the carbohydrate analysis. Fraction 5 was obtained in very small amount and characterization of this oligosaccharide was not possible.

The carbohydrate analysis of Fraction 4 (Table I) showed a composition similar to that of Fraction 3, except that Fraction 4 contained an additional residue of D-galactose and of 2-acetamido-2-deoxy-D-glucose suggesting the presence of an additional outer-chain. The results of the methylation analysis (Table I) indicate the presence of a 2,4-disubstituted D-mannose residue in addition to the methylated derivatives obtained for Fraction 3. Combination of compositional analysis and of the results of methylation strongly suggest a tribranched structure (**2**), which is an extension of **1**.

Carbohydrate composition of oligosaccharide Fraction 6 (Table I) showed the same components as those of oligosaccharide 3 and an additional L-fucose residue. The results of the methylation (Table I) indicate a 6-substituted D-galactose

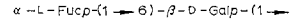


1



2 R = R′ = R″ = No residue

3 R,R′,R″ = 2 α-L-Fuc*p*-(1→6)- residues



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residue, and, combined with the results of enzymic degradation earlier reported[1], suggest the presence of a D-galactose residue linked at O-6 by an L-fucosyl group (**4**).

The composition of oligosaccharide Fraction 7, as compared with that of oligosaccharide Fraction 4, showed an increase of ~2 residues of L-fucose. The methylation data (Table I) showed, in addition to other derivatives, the presence of a 6-substituted D-galactose residue. These data, combined with the results of enzymic degradation reported earlier[1], suggest a terminal L-fucosyl group linked to O-6 of a D-galactose residue. The oligosaccharide of Fraction 7 possesses a tribranched structure similar to that of Fraction 4, the difference being in the terminal L-fucosyl group. In Fraction 4 oligosaccharide, only one chain has a terminal L-fucosyl group, whereas Fraction 7 oligosaccharide has two chains terminating in L-fucosyl groups. It is, yet, not clear which of the three chains terminate in L-fucosyl groups (**3**).

DISCUSSION

The proline-rich glycoprotein (PRGP) of human parotid saliva[3,11] has a molecular weight of ~36 000, and consists of approximately six or seven repeating glycopeptides[12]. According to the carbohydrate content (~40%), the present results of the chemical and structural analysis of the carbohydrate moiety are in fair agreement with the presence of 6 or 7 carbohydrate chains. The carbohydrate chains were released from the protein moiety by hydrazinolysis and separated by h.p.l.c. On the basis of compositional analysis and methylation data we propose two basic

structures corresponding to two- and three-branched complex-type, asparagine-linked, sugar chains[13]. One of the main features of the carbohydrate moiety of PRGP is the high proportion of L-fucose residues. L-Fucose residues, in this glycoprotein like in many others, are present as terminal groups and are linked to D-galactose residues, a feature unique to *N*-glycosylated glycoproteins. In addition, to the best of our knowledge, no other glycoprotein is known to have L-fucose groups linked to O-6 of D-galactose residues (3). Furthermore, no blood-group H activity[14] was detected in PRGP, which supports the location and linkage of the L-fucosyl groups. The study of different donors has demonstrated[14,15] the extent of individual carbohydrate variations of PRGP. Per chain, the number of L-fucosyl groups usually varies from 1 to 4, that of D-galactose residues from 2 to 3, and that of 2-acetamido-2-deoxy-D-glucose residues from 4 to 5. According to the present results, it would be expected that heterogeneity in the proportion of D-galactose and 2-acetamido-2-deoxy-D-glucose residues correspond to variation in the relative number of two- and three-branched chains. An important observation made during this investigation was the absence (by g.l.c. analysis) of neuraminic acid residues. It is conceivable that the glycoproteins, in which two- or three-branched sugar chains terminate with L-fucosyl groups, are devoid of sialic acid.

The results described in this paper are quite different from the results reported by Misaki *et al.*[16], who also reported a carbohydrate structure for PRGP. Their results show, for the carbohydrate chains, only one structure having (a) one terminal L-fucosyl group linked at O-2 of a D-galactose residue, (b) one L-fucosyl group linked at O-3 of the 2-acetamido-2-deoxy-D-glucitol residue, and one of the peripheral 2-acetamido-2-deoxy-D-glucose residues substituted at O-3 and -4 by D-galactose residues.

The structural characterization of the salivary components should lead to a better understanding of their biological roles. The carbohydrate component of soluble glycoproteins appear to act in a variety of ways[17]. In particular, it may modify the physicochemical properties of proteins, such as changing their hydrophobicity (high proportion of L-fucose). The multiple structures of the sugar-chain moieties of many glycoproteins may not be a simple reflection of microheterogeneity caused by incomplete biosynthesis, but may be the results of strictly controlled mechanisms[18]. Salivary macromolecules[19] are involved in diverse functions, all providing a protective environment for the teeth[20]. Genetic polymorphism of closely related compounds as the proline-rich protein (PRP) has been recognized and described[21]. PRP and PRGP constitute about two thirds of the parotid salivary proteins[20]. The extent of individual carbohydrate-variations of PRGP may lead to a further understanding of the post-translational modifications (glycosylation) of such salivary proteins as PRGP, many of which may have important biological consequences, for example in the caries process.

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