Carbohydrate Research, 95 (1981) 227–236
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

EVIDENCE FOR THE PRESENCE OF THE TRIMANNOSYL-DI-*N*-ACETYL-CHITOBIOSE CORE IN THE CARBOHYDRATE CHAINS OF HUMAN-PAROTID, PROLINE-RICH GLYCOPROTEIN

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(Received December 17th, 1980; accepted for publication, February 20th, 1981)

ABSTRACT

The carbohydrate chains of the human-parotid, proline-rich glycoprotein are linked through a single type of carbohydrate–peptide linkage (asparaginyl–*N*-acetylglucosamine). The structure of the internal part of the carbohydrate chains, determined by chemical, enzymic, and g.l.c.–m.s. methods, includes the trimannosyl-di-*N*-acetylchitobiose core involved in the carbohydrate–peptide linkage. Furthermore, an L-fucose residue is linked to the 2-acetamido-2-deoxy-D-glucosyl residue linked to the L-asparaginyl residue. The sequence of the peripheral part of the chains has also been determined as α-L-Fuc*p*→β-D-Gal*p*→β-D-Glc*p*NAc→α-D-Man*p*, suggesting a double-branched, basic carbohydrate structure.

INTRODUCTION

Previous investigations of the composition of human-parotid saliva have shown that two main components are present; an α-amylase and a proline-rich glycoprotein. We have reported ealier[1] the fractionation of individual samples of parotid saliva, and described the isolation and chemical composition of a parotid glycoprotein. This molecule has a molecular weight of ~36 000. Its approximate composition is 60% of protein and 40% of carbohydrate (D-mannose, L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose). A preliminary report[2] described the presence of a single type of carbohydrate–peptide linkage, 2-acetamido-1-*N*-(4-L-aspartyl)-2 deoxy-β-D-glucopyranosylamine (asparaginyl–*N*-acetylglucosamine). This paper offers information on the structure of the oligosaccharide chains.

EXPERIMENTAL

Materials. — Human-parotid saliva from a single donor and the proline-rich glycoprotein were obtained as previously described[1]. The glycopeptides were prepared by enzymic proteolysis with papain and Pronase[2]. Jack-bean β-D-galactosidase

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(EC 3.2.1.23), α-D-mannosidase (EC 3.2.1.24), and *N*-acetyl-β-D-hexosaminidase (EC 3.2.1.52) were prepared by a slight modification[3] of the procedure of Li and Li[+]. β-D-Mannosidase (EC 3.2.1.25) and *N*-acetyl-β-D-hexosaminidase from rat epididymis were prepared according to a published procedure[5]. β-Aspartylacetylglucosaminidase (EC 3.5.1.37), which cleaves the asparaginyl-*N*-acetylglucosamine linkages of glycopeptides, was prepared from almond emulsin (Sigma) according to the procedure described by Takahashi[6]. The enzyme preparation also contained the following exoglycosidases: α-L-fucosidase (EC 3.2.1.51), β-D-galactosidase, and *N*-acetyl-β-D-hexosaminidase. β-D-Galactosidase and *N*-acetyl-β-D-hexosaminidase activities were determined by use of the corresponding *p*-nitrophenyl glycoside. α-L-Fucosidases from almond emulsin did not act on *p*-nitrophenyl α-L-fucopyranoside[7], and their activities were determined with L-fucose-rich glycopeptides[2] as substrate. One unit (U) of activity is defined as that which causes the cleavage of 1 μmol of *p*-nitrophenol per min from the corresponding *p*-nitrophenyl glycoside at 37°, under the appropriate assay conditions.

Amino acid and amino sugar composition. — The amino acid composition was determined with a Multichrom B Beckman analyzer. Separation of the amino acids, and 2-amino-2-deoxy-hexose and -hexitol was performed on a single column. The preparation to be analyzed was hydrolyzed with 5.6M hydrochloric acid in a sealed vacuum tube, for 24 h at 100°. Samples analyzed for 2-acetamido-2-deoxy-D-glucitol were hydrolyzed for 4 h at 100° with 4M hydrochloric acid, and determined as 2-amino-2-deoxy-D-glucitol with the amino acid analyzer.

Sugar composition. — Neutral sugars and 2-amino-2-deoxyhexoses were analyzed as the per-*O*-(trimethylsilyl) derivatives by g.l.c. with a Perkin–Elmer instrument (flame-ionization detector) by the slightly modified procedure[8] of Reinhold[9]. The samples for 2-amino-2-deoxyhexoses were hydrolyzed with 2M trifluoroacetic acid for 2 h at 100°, followed by *N*-reacetylation.

Methylation analysis. — Methylation of oligosaccharides was accomplished according to the method of Hakomori[10]. The alditol acetates and 2-deoxy-2-(*N*-methylacetamido)alditol acetates were prepared according to the method of Björndal *et al.*[11]. The partially *O*-methylated alditol acetates and 2-deoxy-2-(*N*-methylacetamido)alditol acetates were identified by g.l.c.–m.s. analyses with a Hewlett–Packard 5993 B 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 heated at a rate of 6°/min from 130 to 260° the injection port was kept at 270°, the separator temperature was set 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 matched against a library of known standards for positive identification.

Periodate oxidation. — Samples (10 mg) of glycopeptides were oxidized at 4° in the dark with 10mM sodium metaperiodate (10 mL). Periodate consumption was

followed by monitoring absorbance at 310 nm and, after 9 h of oxidation, no more variation of the absorbance was observed. The excess of sodium metaperiodate was decomposed by the addition of 1,2-ethanediol (50 μL), and then the sample was treated overnight at 4° with sodium borohydride (0.21M final concentration). The remaining sodium borohydride was decomposed by the addition of 4M acetic acid, and salts were eliminated by gel filtration on Sephadex G-25. After lyophilization, the glycopeptides were hydrolyzed with 0.1M sulfuric acid, for 2 h at 80°, and the residual glycopeptides were desalted on a column of Bio-Gel P-2 (50–100 mesh).

Removal of L-*fucose.* — L-Fucose-free glycopeptides were obtained by treating the glycopeptides (10 mg) with M hydrochloric acid for 4 min at 100°, in a sealed vacuum-tube, and then chromatography on a column of Bio-Gel P-2 (50–100 mesh).

Enzymic degradation of glycopeptides. — Treatment with jack-bean β-D-galactosidase, α-D-mannosidase, and *N*-acetyl-β-D-hexosaminidase was performed in 50mM sodium citrate buffer at 37° for 48 h, at pH 4.5, 5.0, and 5.5, respectively. One unit of enzyme was added for 3 mg of glycopeptides. When sequential treatment was performed, the remaining glycopeptides were separated from the released sugars by chromatography on a column of Bio-Gel P-2 (50–100 mesh).

Treatment of the periodate-oxidized glycopeptides (4 mg) with β-D-mannosidase (1 U) and *N*-acetyl–β-D-hexosaminidase (6 U) was performed in 50mM phosphate–citrate buffer (pH 7.0) for 48 h at 37°. The products were chromatographed on a column of Bio-Gel P-2 (100–200 mesh). The effluent was monitored for hexose by the phenol–sulfuric acid reaction[12], and for free 2-acetamido-2-deoxy-D-glucose by the method of Good and Bessman[13].

A β-aspartylacetylglucosaminidase preparation from almond emulsin was used to cleave the β-asparaginyl-*N*-acetylglucosamine linkages of the glycopeptides. The hydrolysis was performed in 0.2M sodium acetate buffer (pH 6.0) for 48 h at 37°. The enzyme supplied for 40 mg of glycopeptides corresponds to the product obtained by fractionation of 250 mg of almond emulsin. The oligosaccharides released were fractionated on a column of Bio-Gel P-6 (—400 mesh) in distilled water. Determination of a 2-acetamido-2-deoxy-D-glucose residue at the reducing terminus was performed by sodium borohydride reduction (1 mg) of the oligosaccharide fractions (2 mg). After 24 h at 20°, the solution was treated with 4M acetic acid, and boric acid was removed by addition of methanol and subsequent evaporation. 2-Acetamido-2-deoxy-D-glucitol was determined with a Multichrom B Beckman analyzer.

RESULTS

Fractionation and characterization of the glycopeptides. — The glycopeptides obtained by papain–Pronase proteolysis of the human-parotid, proline-rich glycoprotein were fractionated by a two-step, gel-chromatography procedure on Bio-Gel P-4 (—400 mesh) and Bio-Gel P-6 (—400 mesh) (Fig. 1). After fractionation on a column of Bio-Gel P-4 (Fig. 1A), one major peak was detected with the phenol–sulfuric acid reaction (M-P-4). The carbohydrate-containing fractions were combined,

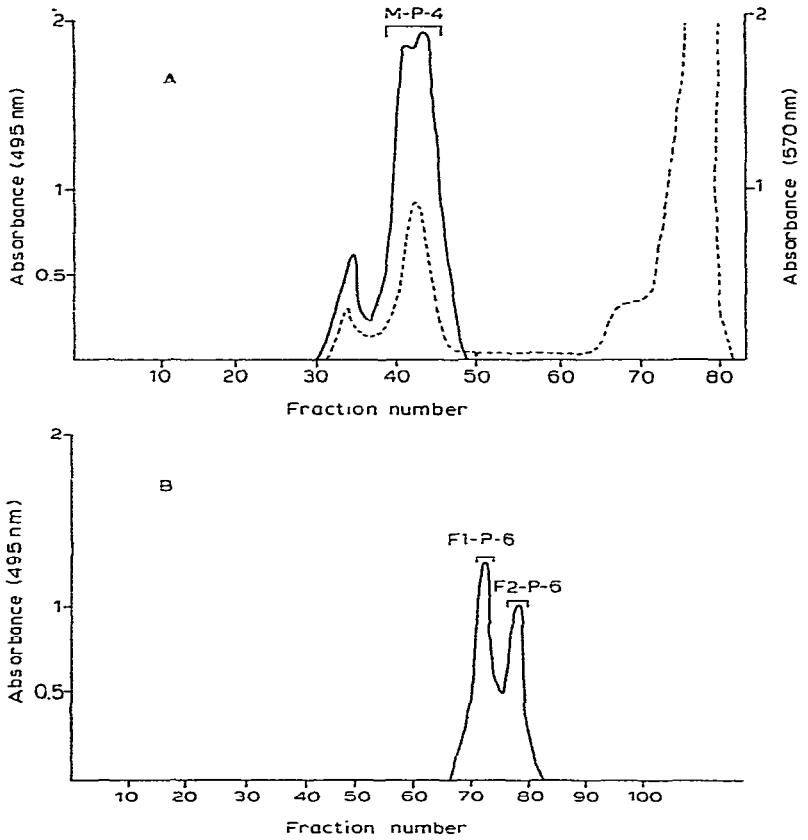

Fig. 1. (A) Elution profile of papain–Pronase hydrolyzate of proline-rich glycoprotein from a column (2.3 × 100 cm) of Bio-Gel P-4 (—400 mesh). The column was eluted with distilled water and fractions (4 mL) were collected. Each fraction was examined for the presence of carbohydrate by the phenol–sulfuric acid test[12] (———), and for amino acid by the ninhydrin reaction (– – – –). The major phenol-positive peak was collected (M-P-4) and lyophilized. (B) Elution pattern of fraction M-P-4 from a column (150 × 1.8 cm) of Bio-Gel P6 (—400 mesh). The column was eluted with distilled water, and fractions (2.5 mL) were collected. Each fraction was examined for hexoses by the phenol–sulfuric acid test[12]. Fractions indicated by bars were combined to give Fraction F_1-P-6 and Fraction F_2-P-6.

lyophilized, and rechromatographed on a column of Bio-Gel P-6 (Fig. 1B). Two carbohydrate-containing fractions were partially separated. Each one was combined and lyophilized to give Fractions F_1-P-6 and F_2-P-6. Amino acid and sugar composition are given in Table I. We have previously reported[2] that the papain–Pronase glycopeptides of the proline-rich glycoprotein contain one asparagine-linked carbohydrate chain and only one type of amino acid sequence immediately around the carbohydrate peptide linkage, Gly-Asn-Gln-Ser. The results given in Table I show that the molar ratio of D-mannose to aspartic acid is 3:1. This result suggests that, although no evidence for the purity of the F_1-P-6 and F_2-P-6 glycopeptides is available, the carbohydrate chains of the proline-rich glycoprotein contain the trimannose core encountered in many glycoproteins having the β-asparaginyl–*N*-acetylglucos-

TABLE I

AMINO ACID AND CARBOHYDRATE COMPOSITION OF F_1-P-6 AND F_2-P-6 GLYCOPEPTIDES

Amino acid residue	*F_1-P-6 (μmol/g)*	*F_2-P-6 (μmol/g)*
Aspartic acid	290	330
Serine	160	210
Glutamic acid	220	260
Glycine	270	340

Carbohydrate residue	*F_1-P-6*			*F_2-P-6*		
	%	*(μmol/g)*	*Molar ratio[a]*	%	*(μmol/g)*	*Molar ratio[a]*
N-Acetylneuraminic acid[b]	traces			0		
L-Fucose	19.4	1180	4.6	18.8	1150	3.5
D-Mannose	13.9	770	3	17.9	990	3
D-Galactose	16.3	900	3.5	14.6	810	2.4
2-Acetamido-2-deoxy-D-glucose	25.6	1160	4.5	26.4	1200	3.6
Ratio of D-mannose to aspartic acid[c]			2.7			3

[a]Molar ratio relative to D-mannose taken as 3. [b]Determined by the Aminoff method. [c]Molar ratio relative to aspartic acid (value has been corrected for ~20% destruction of aspartic acid during acid hydrolysis[2]).

amine-linkage type. Differences between the two fractions F_1-P-6 and F_2-P-6 consist essentially of the presence of approximatively one additional residue of L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose in the carbohydrate moiety of the F_1-P-6 glycopeptides.

Periodate oxidation. — After periodate oxidation and mild acid-hydrolysis of the glycopeptides, the carbohydrate content of the remaining core glycopeptide showed only 2-acetamido-2-deoxy-D-glucose and D-mannose in a 2:1 molar ratio. The sequence of the carbohydrate residues of the remaining core-glycopeptides obtained after periodate oxidation and mild acid-hydrolysis was also determined. Jack bean α-D-mannosidase and *N*-acetyl-β-D-hexosaminidase failed to release D-mannose and 2-acetamido-2-deoxy-D-glucose. β-D-Mannosidase and *N*-acetyl-β-D-hexosaminidase from rat epididymis were able to release 98% of the D-mannose and 47% of the 2-acetamido-2-deoxy-D-glucose. Bio-Gel P-2 (100–200 mesh) chromatography of the hydrolyzate resulted in the isolation of a ninhydrin-positive peak containing approximatively equal proportions of glycine, aspartic acid, glutamic acid, serine, and 2-acetamido-2-deoxy-D-glucose. Another piece of evidence for the presence of a di-*N*-acetylchitobiose unit was the susceptibility of the glycopeptides to a human liver endo-β-*N*-acetylglucosaminidase characterized in our laboratory[14]. On the basis of

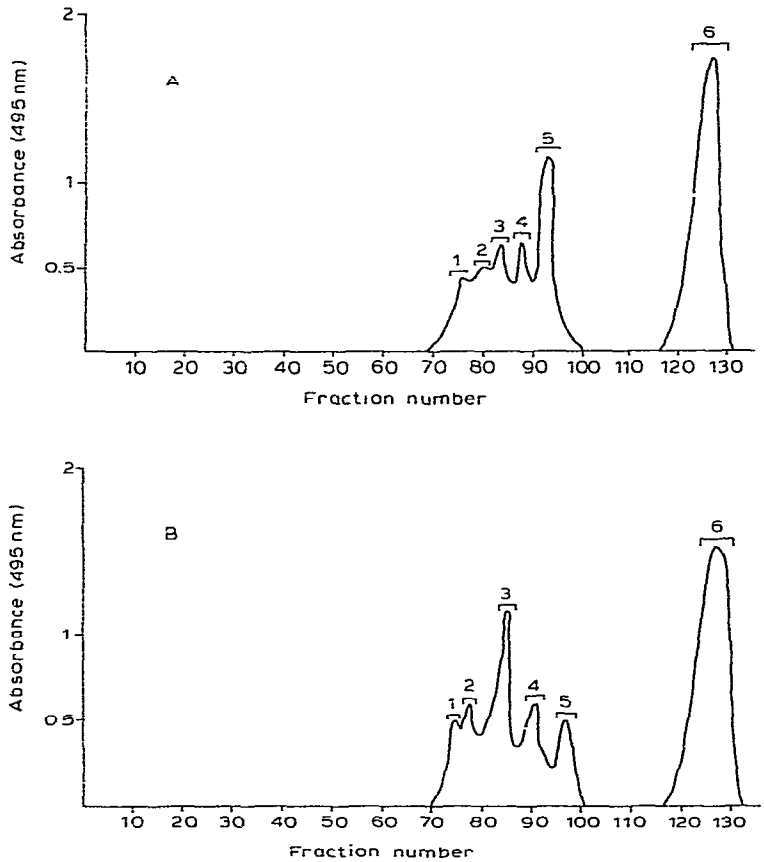

Fig. 2. (A) Elution profile of oligosaccharides obtained after treatment of F_1-P-6 glycopeptides with the almond-emulsin preparation. The column was eluted with distilled water, and fractions (2.5 mL) were collected. Each fraction was examined for hexose by the phenol–sulfuric acid test[12]. Fractions indicated by bars were pooled and lyophilized. (B) Elution profile of oligosaccharides obtained from F_2-P-6 glycopeptides after treatment with the almond-emulsin preparation.

these results, a sequence β-D-Man*p*→β-D-Glc*p*NAc→β-D-Glc*p*NAc→L-Asn is proposed.

Treatment with exoglycosidases. — The sequence and anomeric configuration of the peripheral monosaccharides, in the glycopeptides obtained from the major hexose-containing fraction yielded by Bio-Gel P-4 chromatography, were determined by sequential treatment with specific jack-bean glycosidases. Direct enzymic degradation by β-D-galactosidase and *N*-acetyl-β-D-hexosaminidase led to a rapid release of 30% of the D-galactose, but failed to release 2-acetamido-2-deoxy-D-glucose. These data are in good agreement with the observation that all the 2-acetamido-2-deoxy-D-glucose residues are substituted, and that most of the D-galactose are probably substituted by L-fucose residues (see below).

In order to perform a sequential, enzymic degradation of the oligosaccharide

chain, the first step was an acid hydrolysis to remove L-fucose residues, as α-L-fucosidase from guinea pig and rat epididymis failed to release any L-fucose residues. After 80% of the L-fucose residues had been released, >95% of the D-galactose residues were liberated by β-D-galactosidase. Subsequent treatment with *N*-acetyl-β-D-hexosaminidase hydrolyzed 47% of the now-terminal 2-acetamido-2-deoxy-D-glucose residues. Finally, 42% of the D-mannose residues was released by α-D-mannosidase, thus establishing the sequence of the peripheral carbohydrate chains as α-L-Fuc*p*→β-D-Gal*p*→β-D-Glc*p*NAc→α-D-Man*p*. The anomeric configuration of the L-fucose residue was further established, as α-L-fucosidase from almond emulsin was able to release almost all the external L-fucose residues.

Production of oligosaccharides from the glycopeptides. — Oligosaccharides were released with a preparation from almond emulsin containing a β-aspartylacetylglucosaminidase (glycopeptidase) that cleaves the β-asparaginyl–*N*-acetylglucosamine linkage in glycopeptides. This preparation also contains the following exoglycosidases: α-L-fucosidase, β-D-galactosidase, and *N*-acetyl-β-D-hexosaminidase. Each glycopeptide fraction, F_1-P-6 and F_2-P-6, was treated with the enzymic preparation and then chromatographed on the same column of Bio-Gel P-6. Five phenol–sulfuric acid-positive fractions were obtained in both cases (Fig. 2), each one corresponding to oligosaccharides having a residue of 2-acetamido-2-deoxy-D-glucose in terminal reducing position. The presence of approximately one residue of 2-acetamido-2-deoxy-D-glucitol after borohydride reduction provided evidence that the

TABLE II

CARBOHYDRATE COMPOSITION OF THE OLIGOSACCHARIDE FRACTIONS OBTAINED FROM F_1-P-6 AND F_2-P-6 GLYCOPEPTIDES

Compounds	*Components*[a]				
	Fuc	*Man*	*Gal*	*GlcNAc*	*GlcNAcol*[b]
Glycopeptide F_1-P-6	4.6	3.0	3.5	4.5	
oligosaccharides					
1	3.7	3.0	2.4	4.3	(0.9)
2	2.1	3.0	1.4	4.4	(1.0)
3	1.8	3.0	0.9	3.6	(0.9)
4	1.1	3.0	0.4	3.1	(0.9)
5	1.0	3.0		2.5	(0.9)
Glycopeptide F_2-P-6	3.5	3.0	2.4	3.6	
oligosaccharides					
1	3.5	3.0	1.9	3.5	(0.9)
2	2.2	3.0	1.2	3.4	(0.9)
3	1.8	3.0	0.8	2.8	(0.9)
4	1.1	3.0	0.2	2.3	(0.9)
5	1.0	3.0		1.7	(1.0)

[a]Molar ratio relative to D-mannose. [b]Number of 2-acetamido-2-deoxy-D-glucitol residues relative to D-mannose residues after sodium borohydride reduction.

TABLE III

O-METHYL DERIVATIVES OBTAINED FROM METHYLATED OLIGOSACCHARIDE 5 RELEASED FROM F_1-P-6 GLYCOPEPTIDES AFTER ALMOND-EMULSIN TREATMENT

O-Methyl derivatives[a]	*Molar ratio*[b]
L-Fucose	
2,3,4-	0.85
D-Mannose	
2,4-	1
2,3,4,6-	1.82
2-Acetamido-2-deoxy-D-glucose	
3,6-	0.69
2-Acetamido-2-deoxy-D-glucitol	
1,3,5-	0.73

[a]Determined as alditol acetates. [b]Molar ratio relative to 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-mannose.

carbohydrate–peptide linkage was cleaved by the almond-emulsin glycopeptidase. The sugar composition of these fractions is given in Table II. Each oligosaccharide fraction obtained from F_1-P-6 and F_2-P-6 glycopeptides may be considered as an intermediate step of monosaccharide release by the exoglycosidases of the almond-emulsin preparation. Fraction 6 corresponds, in both cases, to a mixture of monosaccharides: L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-glucose.

One of the principal feature of the carbohydrate chains of the proline-rich glycoprotein is shown by Oligosaccharide 5 obtained either from F_1-P-6 or F_2-P-6 glycopeptides, where the approximate molar ratios of 2-acetamido-2-deoxy-D-glucose to D-mannose to L-fucose are 2:3:1. These oligosaccharides correspond to the inner core of trimannosyl-di-*N*-acetylchitobiose involved in the carbohydrate–peptide linkage.

Methylation studies. — Oligosaccharide 5 (F_1-P-6) was methylated after sodium borohydride reduction and the resulting *O*-methylalditol acetates were identified by g.l.c.–m.s. (Table III).

On the basis of all the results described, structure **1** may be proposed for this oligosaccharide.

α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-GlcNAcol
6 6
↑ ↑
1 1
α-D-Man*p* α-L-Fuc*p*

1

DISCUSSION

Whereas there is no evidence for the number of carbohydrate chains per molecule of the human-parotid, proline-rich glycoprotein, this number is expected to be ~6, according to the mol. wt. (~36 000) of the glycoprotein, the sugar content (~40%), and the basic structural feature of one oligosaccharide chain. The principal drawback in structural studies of the carbohydrate chains of human-parotid saliva, proline-rich glycoprotein has been the difficulty in obtaining pure samples of each glycopeptide. Absence of whole molar ratios for the sugars (see Table I) is probably consistent with microheterogeneity of the carbohydrate chains. In spite of the elucidation of a common-core structure, results from enzymic and methylation studies of the glycopeptides did not permit the assignment of an unequivocal structure for the carbohydrate moiety. However, on the basis of the sugar composition, sequential enzymic degradation, and preliminary methylation studies, the tri-D-mannose core is probably substituted by two or three branches containing the sequence α-L-Fuc*p*→ β-D-Gal*p*→β-D-Glc*p*NAc. Thus, a basic structure having two carbohydrate chains (biantennary) similar to that described for several serum glycoproteins[15] would be expected.

Yamashita *et al.*[16] reported a similar basic structure for the carbohydrate chain of the human-parotid α-amylase A. This glycoenzyme contains one asparagine-linked carbohydrate chain and four different carbohydrate chains were described. All chains contain two or three L-fucose residues, and one chain contains one sialic acid residue. In the proline-rich glycoprotein, the sialic acid content reported[2] corresponds to approximatively one residue per molecule. Thus, it would be expected that one of the six carbohydrate chains contains one sialic acid residue. However, only in one of the two major glycopeptides fractions (Table I) was a trace of sialic acid determined, and no direct evidence for the presence or absence of one residue of sialic acid in the carbohydrate moiety of the proline-rich glycoprotein is available.

Misaki *et al.*[17] also reported a carbohydrate structure for the proline-rich glycoprotein of human-parotid saliva. Their results show some differences from the present ones, the main one being that Misaki *et al.*[17] obtained only one type of glycopeptide after Pronase treatment of the glycoprotein. Among other differences were the number of L-fucose residues (only two) and substitution at O-3 of the terminal reducing 2-acetamido-2-deoxy-D-glucosyl residue by an L-fucose residue. In addition, the presence of sialic acid was not reported.

ACKNOWLEDGMENTS

This work was supported by a Contrat de Recherches de l'U.E.R. 3 des Sciences Médicales de Lille and by La Fondation pour la Recherche Médicale Française. The authors thank Mrs. M. C. Houvenaghel and Mrs. D. Demeyer for skilled technical assistance.

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