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Note

Structural analysis of the preponderant high-mannose oligosaccharide of human Tamm–Horsfall glycoprotein*,†

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Human Tamm–Horsfall (T–H) glycoprotein is produced by, and localized in, the plasma membrane of kidney cells of the ascending limb of the loop of Henle, and is excreted in the urine in quantities of ~50 mg a day[1]. The oligosaccharide component, which accounts for 30% of the weight of the glycoprotein, comprises several oligosaccharides *N*-linked to asparagine. The majority of these oligosaccharides have a polybranched structure with three or four *N*-acetyllactosamine-containing chains bound to the mannotriose branching core[2–5]. Morgan *et al.*[6], first, described that the Sd^a blood group determinant, also present in the urine of 95% of the Caucasian population, is associated with the carbohydrate portion of T–H glycoprotein. Subsequently, Morgan and co-workers demonstrated that 2-acetamido-2-deoxy-D-galactose in β-anomeric configuration is the immunodominant sugar of the Sd(a+) phenotype[7,8], and isolated a Sd^a active pentasaccharide from a preparation of Sd(a+) T–H glycoprotein[9]. Different lines of evidence[3,4] indicate that the Sd^a determinant is the outermost portion of polybranched oligosaccharides of T–H glycoprotein. Previously, we have shown[10] that the T–H glycoprotein carries, besides polybranched oligosaccharides, a minor carbohydrate component of high-mannose type. On the basis of various exoglycosidase treatments and t.l.c. analysis, the composition $(Man)_6GlcNAc$ was assigned to the preponderant oligosaccharide released by endo-*N*-acetyl-β-D-glucosaminidase H. We describe herein the purification, on a preparative scale, of

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TABLE I

YIELD AND RECOVERY OF PRONASE GLYCOPEPTIDES FROM T–H GLYCOPROTEIN AFTER FRACTIONATION ON BIO-GEL P-10

Compound	*Yield (mg/g of glycoprotein)*	*Recovery of neutral sugars[a] (mg/g of glycoprotein)*
T–H glycoprotein		122.5
Polybranched glycopeptide	229	93.9
High-mannose glycopeptide	56	19.2

[a]Neutral sugars were determined by the phenol–H_2SO_4 assay[17].

the $(Man)_6GlcNAc$ oligosaccharide by h.p.l.c. and its structural analysis by ^1H-n.m.r. spectroscopy.

The high-mannose glycopeptide was separated from the polybranched component by Bio-Gel P-10 filtration after exhaustive Pronase digestion of T–H glycoprotein. Table I reports the yield of the two glycopeptide fractions. The recoveries of neutral sugars of T–H glycoprotein were 15.6% and 76.6% in high-mannose and polybranched glycopeptide fractions, respectively. It has been reported[2,4] that the T–H glycoprotein carries five polybranched chains. We found that the yield of neutral sugars from the high-mannose glycopeptides was ~one fifth the content of neutral sugars of the polybranched fractions. Assuming that the oligosaccharide chains of the two types of glycopeptides have a similar hexose content, it may be suggested that, in T–H glycoprotein, only one individual chain is present as an oligomannoside.

In order to characterize the oligomannoside chains, the high-mannose glycopeptide was digested with endo-*N*-acetyl-β-D-glucosaminidase H, and the released oligosaccharides fractionated by l.c. Three peaks were well resolved (Fig. 1a);

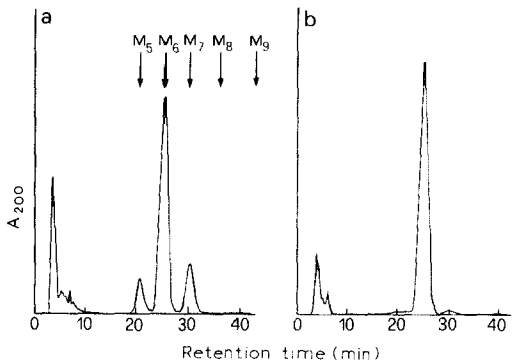


Fig. 1. (a) L.c. elution profile of oligomannosides released by endo-*N*-acetyl-β-D-glucosaminidase H from high-mannose glycopeptide of T–H glycoprotein. M_5, M_6, M_7, M_8, and M_9 are abbreviations for $Man_5GlcNAc$–$Man_9GlcNAc$ oligosaccharides, and the arrows indicate the corresponding retention time. (b) Elution profile of $Man_6GlcNAc$ from T–H glycoprotein purified by a double l.c. separation performed as illustrated in (a).

TABLE II

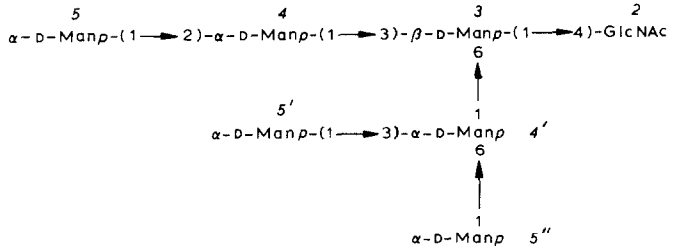
IDENTIFICATION OF $Man_6GlcNAc$ OLIGOSACCHARIDE BY 400-MHz ^1H-N.M.R. SPECTROSCOPY

Proton	*Residue or group*	*Anomer*	*Chemical shift (δ)*
H-1	*2*	α	5.247
		β	~4.71
	3	α,β	~4.78
	4	α,β	5.353
	4'	α,β	4.874
	5'	α	5.081
		β	5.105
	5''	α,β	4.912
	5	α,β	5.053
H-2	*3*	α	4.242
		β	4.232
	4	α,β	4.118
	4'	α,β	4.143
	5'	α	4.069
		β	4.050
	5''	α,β	3.991
	5	α,β	4.069
NAc	*2*	α,β	2.043

the major one was eluted at the retention time of the $(Man)_6GlcNAc$ standard and accounted for 70% of the total absorbance, whereas fractions showing 12 and 18% of the absorbance were eluted as $Man_5GlcNAc$ and $Man_7GlcNAc$, respectively. These results are consistent with previous data[10] on the relative distribution of radioactivity among the corresponding labeled alditols, obtained by borotritide reduction and separated by t.l.c. The purification of the preponderant oligomannoside was achieved by a double l.c. fractionation of the component eluted as $Man_6GlcNAc$. The chromatography of the pure sample is shown in Fig. 1b.

The primary structure of $Man_6GlcNAc$, purified as just described, was determined by 400-MHz ^1H-n.m.r. spectroscopy (see Table II). The first indication that the Man-*3* residue is disubstituted at O-3 and O-6 by Man-*4* and Man-*4'* residues, respectively, is given by the H-1 signal (δ 5.247) of the α-D-GlcNAc-*2* residue. This branching at the Man-*3* residue is confirmed by the characteristic downfield shift of the H-2 signal (δ 4.232) of the Man-*3* residue. According to Vliegenthart *et al.*[11], the presence of the first branching point [*4*→(*4'*)→*3*] in oligosaccharides of high-mannose type leads to the characteristic downfield shift from δ 4.08 to 4.23 of the H-2 signal of the Man-*3* residue. The presence in the oligosaccharide of the Man-*5'* and Man-*5''* groups, which are the more frequent substituents of the Man-*4'* residue, was ascertained by their respective H-1 and H-2 signals. Finally, the attachment of the sixth D-mannosyl group (Man-*5*) in an α-D-

(1→2)-linkage to the Man-*4* residue was proved by both the H-1 and H-2 signals of Man-*5*, and the observation that the H-1 and H-2 signals of Man-*4* were shifted to δ 5.353 and 4.118, respectively[11]. The absence of signals at δ 5.398, 5.407, 5.308, 4.106, 4.103, and 4.109 for H-1 and H-2 of the Man-*5* and Man-*5'* groups, which would indicate the substitution of these units by an α-D-(1→2)-linked mannopyranosyl group[11], showed that these groups occur in a terminal position, as expected for a $(Man)_6GlcNAc$ oligosaccharide that carries a Man-*5''* group. Signals of very low intensity might, however, be present at δ 5.142 and 4.025, indicating that a small fraction of Man-5'' may carry an α-D-(1→2)-linked mannosyl group. Together, these data indicate that more than 90% of the $(Man)_6GlcNAc$ oligosaccharide has structure **1**.



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The accumulation in T–H glycoprotein of a high-mannose chain having structure **1** is very likely due to an incomplete processing of the $Glc_3Man_9(GlcNAc)_2$ precursor. This accumulation may occur, since the folding of the peptide chain close to an individual *N*-glycosylation site of the glycoprotein hinders the action of the α-D-mannosidase involved in the cleavage of the innermost α-(1→2)-linked D-mannose residues. There is evidence that a mannosidase located in the Golgi apparatus (Golgi mannosidase I) is responsible for the removal of the α-(1→2)-linked D-mannose residues and converts $(Man)_8(GlcNAc)_2$ into $(Man)_5(GlcNAc)_2$ (see ref. 12). Studies on the biosynthesis of chinese hamster ovary cell glycoproteins[13] and on the structure of the oligomannoside isomers carried by human IgM[14] and hen ovalbumin[15] support the notion that the Golgi mannosidase I acts in an ordered processing sequence. According to this sequence, the last processing step would involve the cleavage of the α-(1→2)-linked D-mannosyl group (Man-*5*) attached to the pentamannosyl intermediate, as in $Man_6GlcNAc$ **1**. The large preponderance of this Man_6-isomer in T–H glycoprotein strongly suggests that, also in human kidney cells, the Golgi mannosidase I follows the ordered processing sequence postulated for other cell systems.

EXPERIMENTAL

Separation of Pronase glycopeptides. — T–H glycoprotein was prepared from

pooled urine of healthy persons with the procedure originally described by Tamm and Horsfall[16]. A sample (1 g) of glycoprotein in 10mM Tris·HCl (30 mL), pH 8, was digested with Pronase for 72 h at 37°. Pronase, in 0.2M Tris·HCl, pH 7.5, had been autoincubated for 1 h at 37° to inactivate possible contaminating enzymes. The enzyme was added three times during the incubation period, achieving a final Pronase-to-glycoprotein ratio of 1:20. At the end of the incubation time, the sample was boiled for 3 min and centrifuged to eliminate the insoluble products. After lyophilization, the Pronase glycopeptides were chromatographed on a Bio-Gel P-10 column (200 mesh; 1 × 80 cm), equilibrated and eluted with 0.1M pyridinium–acetate buffer, pH 5. The column had been previously calibrated with fetuin glycopeptide and ovalbumin glycopeptide, in order to identify the elution volume of polybranched and high-mannose glycopeptides, respectively. Fractions (1 mL) were monitored for the neutral sugar content by the phenol–H_2SO_4 assay[17], and the fractions corresponding to the elution position of glycopeptide standards were separately lyophilized.

*Endo-*N*-acetyl-β-*D*-glucosaminidase H digestion.* — The high-mannose glycopeptide sample was dissolved in 0.1M sodium citrate buffer (200 μL; pH 5), and digested with the endoglycosidase under the conditions previously described[10]. At the end of the digestion period, the sample was boiled for 3 min and passed through coupled columns of Dowex 50 (H^+) and Dowex 1 (HCO_3^-) to obtain the salt-free oligomannosides. More than 90% of the oligosaccharides (evaluated as neutral sugars) were recovered in the flow-through fractions. They were pooled and the lyophilized sample used for l.c. fractionation.

Purification of major high-mannose oligosaccharides by l.c. — Oligosaccharides released by endo-*N*-acetyl-β-D-glucosaminidase H were fractionated in a h.p.l.c. apparatus consisting of a Waters 501 pump, a Waters U6K injector, and a Lambda-Max 481 Waters absorbance detector. A LiChrosorb Diol column (250 × 4 mm; Merck, Darmstadt, F.R.G.) was used with 7:3 (v/v) acetonitrile–water as eluent, at a flow rate of 0.5 mL/min. The oligosaccharides were detected by u.v. absorbance at 200 nm, and 0.5-mL fractions were collected. $Man_5GlcNAc$–$Man_9GlcNAc$ standards from unit A of thyroglobulin were prepared according to Godelaine *et al.*[18] and used to detect the retention time of each oligomannoside. To purify the preponderant oligomannoside unit of T–H glycoprotein, the fractions eluted at the retention time of the $Man_6GlcNAc$ standard were pooled, lyophilized, and rechromatographed twice in the same l.c. system.

^{1}H-N.m.r. spectroscopy. — The $Man_6GlcNAc$ oligosaccharide isolated by l.c. was repeatedly treated with D_2O at room temperature and lyophilized. The ^{1}H-n.m.r. spectrum was recorded at 400-MHz, with a Bruker MSL-400 n.m.r. spectrometer operating in the F.t. mode. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. The probe temperature was kept at 300 K. Chemical shifts are expressed downfield from the signal of internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.002 p.p.m.

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