CHARACTERIZATION OF A MANNOSE-CONTAINING GLYCOASPARAGINE ISOLATED FROM URINE OF A PATIENT WITH ASPARTYLGLYCOSYLAMINURIA (AGU)

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1. Introduction

Patients with AGU, which is caused by very low activity of a lysosomal enzyme, 4-L-aspartylglycosylamine amidohydrolase (EC 3.5.1.26), excrete large amounts of a glycoasparagine, 2-acetamido-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (GlcNAc-Asn), in their urine [1]. A number of glycoasparagines other than GlcNAc-Asn have also been found in the urine of such patients [2]. Pollitt and Pretty [3] postulated the presence of 13 glycoasparagines.

We have set up a simple procedure to screen a number of suspected AGU urines [4]. Meanwhile, we have been fractionating AGU urine and characterizing structures of some of the isolated glycoasparagines [5,6]. These glycoasparagines have a common internal structure, represented by β-Gal-(1→4)-β-GlcNAc-Asn, which has so far not been found in glycoproteins.

The present paper reports the isolation of a glycoasparagine which is composed of diacetylchitobiose as the internal structure and of dimannoside as the peripheral structure. A similar type of structure has been found in many glycoproteins [7].

2. Materials and methods

Urine was obtained from a Finish patient with AGU. α-Mannosidase (EC 3.2.1.24) was prepared from hog kidney by the method of Okumura and Yamashina [8], and β-mannosidase (EC 3.2.1.25) from snails, *Achatina fulica*, by the method of Sugahara et al. [9]. β-*N*-Acetylhexosaminidase (EC 3.2.1.30) was prepared from jack bean meal according to the method of Li and Li [10]. One unit of these enzymes is defined as the amount of enzyme which hydrolyzes 1 μmol of the substrate per min.

Descending paper chromatography was carried out using the following solvents; (a) the upper layer of butan-1-ol/ethanol/water (4 : 1 : 5, by vol); (b) ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, by vol) and (c) propan-1-ol/ethyl acetate/water (6 : 1 : 3, by vol).

Dansylated (DNS-) glycoasparagine was prepared as described previously [5], and its location on paper was detected with an ultraviolet lamp.

Neutral sugar was determined by the orcinol-H_2SO_4 method of Hewitt [11]. Identification of mannose was carried out by gas-liquid chromatography of the trimethylsilyl ether derivative of the methyl mannoside according to Miyajima et al. [12]. Glucosamine was determined with a Hitachi amino acid analyzer, KLA 3B, after hydrolysis in 2 N HCl at 100°C for 16 h. Sialic acid was determined by the resorcinol method of Jourdian et al. [13]. GlcNAc-Asn was hydrolyzed enzymatically with aspartylglycosylamine amidohydrolase, then the released *N*-acetylglucosamine was determined by the Morgan-Elson reaction [14].

Hydrolyses of DNS-glycoasparagine by α- and β-mannosidases and β-*N*-acetylhexosaminidase were carried out at 37°C, followed by determination of the increase in reducing power using the method of Park and Johnson [15]. Other conditions for the enzyme reactions were as follows. α-Mannosidase treatment: the substrate corresponding to 180 nmol of mannose was incubated with 3.4 units of the enzyme in 1.64 ml of 0.05 M acetate buffer, pH 4.5.

The reaction was complete after 10 h. β-Mannosidase treatment: the substrate corresponding to 70 nmol of mannose was incubated with 16 munits of the enzyme in 0.26 ml of the same buffer. The reaction was complete after 10 h. β-*N*-Acetylhexosaminidase treatment: the substrate corresponding to 120 nmol of *N*-acetylglucosamine was incubated with 0.92 unit of the enzyme in 0.5 ml of 0.05 M acetate buffer, pH 5.0. The reaction was complete after 5 h.

After the enzymatic hydrolyses, the digests were directly applied to a column of Bio-Gel P-2 (0.9 × 104 cm) equilibrated with 2% acetic acid. The column was developed with 2% acetic acid and 3.5 ml fractions were collected. DNS-glycoasparagines were monitored by a spot fluorescence test. By this column treatment, DNS-glycoasparagines could be separated from released monosaccharides and buffers. The fractions containing DNS-glycoasparagines were evaporated to dryness and used in subsequent experiments.

Methylation analysis of the DNS-glycoasparagine was carried out according to the method of Hakomori [16] using a LKB 9000 gas chromatograph mass spectrometer. The amount of the sample used was 0.2 μmol. Conditions for gas-liquid chromatography are shown in the legend to fig.2, and those for mass spectrometry were as follows: ionizing potential, 20 eV; accelerating voltage, 3.5 kV; ion source temperature, 210°C; and separator temperature, 190°C. Identification of methylated mannitols and 2-*N*-methylacetamido-2-deoxyglucitol were made mainly according to the data of Lindberg [17] and of Tai et al. [18], respectively. Further, ovalbumin glycopeptides prepared according to Tai et al. [19] were used as reference substances for methylation analysis. However, molar proportions of the methylated sugars could not be determined as their response factors are unknown under the conditions used in the present study.

3. Results and discussion

A lyophilized sample of the urine (200 ml) obtained from an AGU patient was dissolved in 40 ml of 0.05 M pyridine–acetic acid buffer, pH 5.0. The solution was applied to a column of Sephadex G-25 (4 × 120 cm), then eluted with the same buffer. The eluates were analyzed for neutral sugars, sialic acid and GlcNAc-Asn (fig.1). Fractions of the largest sialic acid peak (Fraction C), which seemed to be characteristic for AGU urine, were collected and evaporated to dryness. The dried material was dissolved in 2.5 ml of water and applied to a column of Dowex 50X8 (20–50 mesh, H^+ form). The column was eluted initially with water, then with a linear gradient from water to 1 M pyridine–acetic acid buffer, pH 5.0. Glycoasparagines (C-I) which passed through the column are two isomeric α-NANA-(2→3 and 4)-β-Gal-(1→4)-β-GlcNAc-Asn, as reported previously [5,6]. The fractions eluted as a broad peak (C-II), which were positive in the orcinol-H_2SO_4 reaction but contained no sialic acid, were

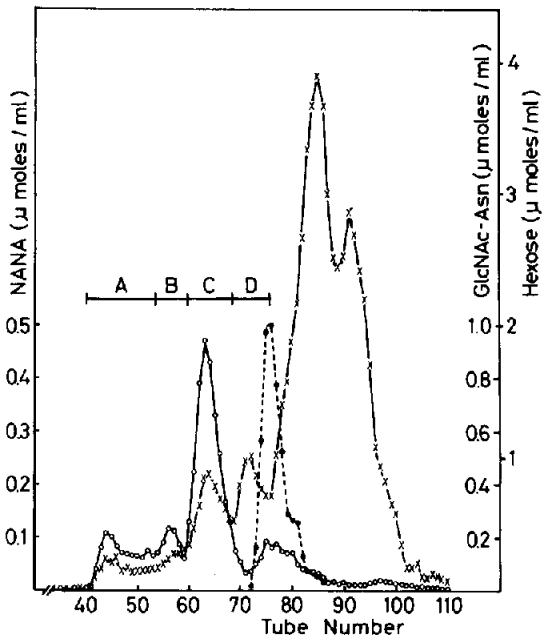

Fig.1. Gel filtration on Sephadex G-25 of urine from an AGU patient. A lyophilizate from 200 ml of urine was dissolved in 40 ml of 0.05 M pyridine–acetic acid buffer, pH 5.0, then applied to a column (4 × 120 cm) of Sephadex G-25 equilibrated with the same buffer. The column was developed with the buffer at 60 ml per hr, and 15 ml fractions were collected. Portions were analyzed for neutral sugars (×—×), sialic acid (○—○) and GlcNAc-Asn (●- - -●). Galactose (Hexose) and *N*-acetylneuraminic acid (NANA) were used as standards for the neutral sugar and sialic acid determinations, respectively. Glycoasparagine fractions other than GlcNAc-Asn which appeared to be characteristic of AGU urine compared with the pattern for normal urine (see ref. [5]) were pooled and designated as A, B, C and D.

Table 1
Sequential release of monosaccharides from DNS-glycoasparagine

Treatment	Remaining monosaccharide residues	
	Mannose	*N*-acetylglucosamine
None	2.28	2.00[a]
α-Mannosidase	1.14	2.00
then β-mannosidase	0	2.00
then β-*N*-acetylhexosaminidase	0	1.00

[a] Based on the structure determined finally, the value was taken as 2.00. Since no correction was made for destruction of glucosamine during hydrolysis, the molar ratio of glucosamine to mannose was less than theoretical value.

collected and evaporated to dryness. The dried material was dansylated to facilitate further investigation.

DNS-C-II gave one major spot and one or two minor spots on paper chromatography using solvent a. By preparative paper chromatography and subsequent elution from paper with water, the major DNS-glycoasparagine was isolated. Its homogeneity was checked by paper chromatography using solvent b. The yield of this glycoasparagine was about 3 μmol from 200 ml of AGU urine. The minor spots were too little to be investigated further.

The isolated DNS-glycoasparagine contained equimolar amounts of hexose and glucosamine, as shown in table 1. The hexose was identified as mannose by gas-liquid chromatographic analysis.

To determine the monosaccharide sequence of the DNS-glycoasparagine, it was first treated with α-mannosidase from hog kidney, which released one half the total mannose, as shown in table 1. No reaction was observed on treatment of the DNS-glycoasparagine with β-mannosidase or β-*N*-acetylhexosaminidase. The degraded DNS-glycoasparagine was separated from the released mannose by gel filtration on a Bio-Gel P-2 column. The DNS-glycoasparagine now contained mannose and glucosamine in a molar ratio of 1.1 : 2.0. Thus, the parent DNS-glycoasparagine should consist of 2 mol each of mannose and glucosamine.

The degraded DNS-glycoasparagine was then treated with β-mannosidase from snails, upon which the remaining mannose was completely released as shown in table 1. The digest was chromatographed on a Bio-Gel P-2 column as for the α-mannosidase digest. The mannose-free DNS-glycoasparagine had an R_F value of 0.51 on paper chromatography using solvent a. This mannose-free DNS-glycoasparagine was incubated with β-*N*-acetylhexosaminidase from jack bean meal, with the results shown in table 1. The digest was chromatographed on Bio-Gel P-2 as for the mannosidase digests. The resulting DNS-glycoasparagine which was expected to be GlcNAc-Asn-DNS had the same R_F value as authentic GlcNAc-Asn-DNS on paper chromatography using solvents a, b and c. The R_F values were 0.65, 0.76 and 0.87, respectively.

Thus, the monosaccharide sequence of the parent DNS-glycoasparagine was deduced to be α-Man-β-Man-β-GlcNAc-β-GlcNAc-Asn.

To determine the positions of the glycosidic linkages, methylation analysis was carried out. As shown in fig.2, two peaks corresponding to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol (Peak I)

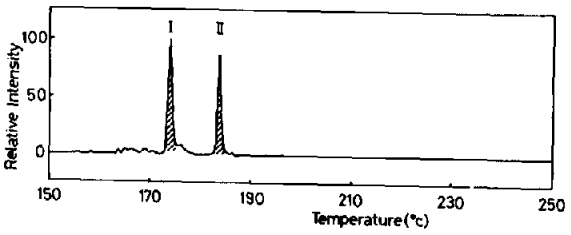

Fig.2. Gas-liquid chromatogram of methylated sugars from DNS-glycoasparagine. Column was 2% OV-17 Gas-Chrom Q (3 mm × 2 m). The column temperature was programmed from 150–250°C at a rate of 2°C/min. Flow rate of helium was 25 ml/min. The chromatogram was obtained by monitoring a single ion of *m/e* 117. Peak's I and II were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol, respectively.

and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol (Peak II) were obtained on gas-liquid chromatography by monitoring single ions of *m/e* 117 and *m/e* 161. These results indicate that the terminal mannose is linked to C-6 of the penultimate mannose. The positions of the linkages to *N*-acetylglucosamine were determined similarly by gas-liquid chromatography in which single ions of *m/e* 158 and 116 were monitored. On both these chromatograms, a single peak was obtained at the position corresponding to 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol. This excludes the possibility of substitution of *N*-acetylglucosamine at a position other than C-4. Thus, the structure of the parent glycoasparagine was deduced to be α-Man-(1→6)-β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-Asn. This type of carbohydrate structure has been demonstrated in many glycoproteins [7]. In these glycoproteins, however, the penultimate mannose residue is substituted at both C-6 and C-3.

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