STRUCTURAL ANALYSIS OF THE N-LINKED OLIGOSACCHARIDES FROM HUMAN URINARY TRYPSIN INHIBITOR

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The N-linked oligosaccharides from human urinary trypsin inhibitor were purified and their structures were investigated by compositional analysis, the two-dimensional sugar map method and 500 MHz ^{1}H-NMR. The results revealed that they were composed of disialosyl, monosialosyl and asialosyl oligosaccharides, which have the common biantennary core structure; Gal1-4GlcNAc1-2Man1-3(Gal1-4GlcNAc1-2Man1-6)Man1-4GlcNAc1-4GlcNAc.

KEYWORDS trypsin inhibitor; N-linked oligosaccharide; glycoprotein; proteoglycan

Urinary trypsin inhibitor(UTI) contains an N-linked oligosaccharide chain and a low-sulfated chondroitin 4-sulfate(LSC)chain [1,2] attached to Asn-45 and Ser-10, respectively.[3] Inter-α-trypsin inhibitor(ITI), which is currently considered the precursor of UTI, is a glycoprotein and/or proteoglycan. It has been established that the LSC chain acts as the cross-link between two fragments consisting of ITI. [4-6] We studied the structure of the N-oligosaccharides in this experiment.

UTI (500 μg) was purified from normal human urine by the successive column chromatographic method described previously [2] with further purification by affinity chromatography [7] using a trypsin-Sepharose column. Then it was subjected to reduction, carboxymetylation, thermolysin digestion (80 °C, 60 min) and N-glycopeptidase treatment [8] (about 75 % of N-linked oligosaccharide was released as glucosamine). After the released oligosaccharides were reductively labeled with 2-aminopyridine,[9] Sephadex G-25 gel chromatography (eluent: 10 mM $NaHCO_3$) was performed for removal of excessive 2-aminopyridine. Then the pyridylamino (PA)-oligosaccharides were separated into two fractions (tentatively named G1 and G2 in eluting order, Fig.1) on a Sephadex G-25 column (eluent: H_2O). Analysis of each PA-oligosaccharide in fraction G1 and G2 by ODS-silica column gave the patterns shown in Fig. 2. When the fractions G1 and G2 were treated with 0.025 N HCl at 80 °C, 60 min for hydrolysis of sialic acid, the unaltered peak was observed in the fraction G2, while only one peak was detected in the fraction G1 after acid hydrolysis (Fig. 2). Peak B in Fig.2 showed the same chromatographic behavior on a two-dimensional sugar map[10] as a standard PA-oligosaccharide shown in Fig. 3-B.

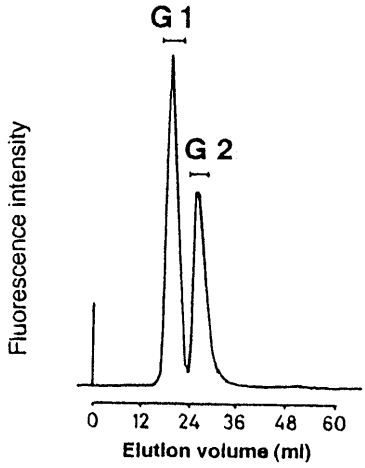

Fig. 1. Elution Profile of PA-Oligosaccharides on Sephadex G-25

The column (1.0 cm i.d. X 50 cm) was eluted with H_2O. Detection, fluorescence (Ex. 320 nm, Em. 400 nm).

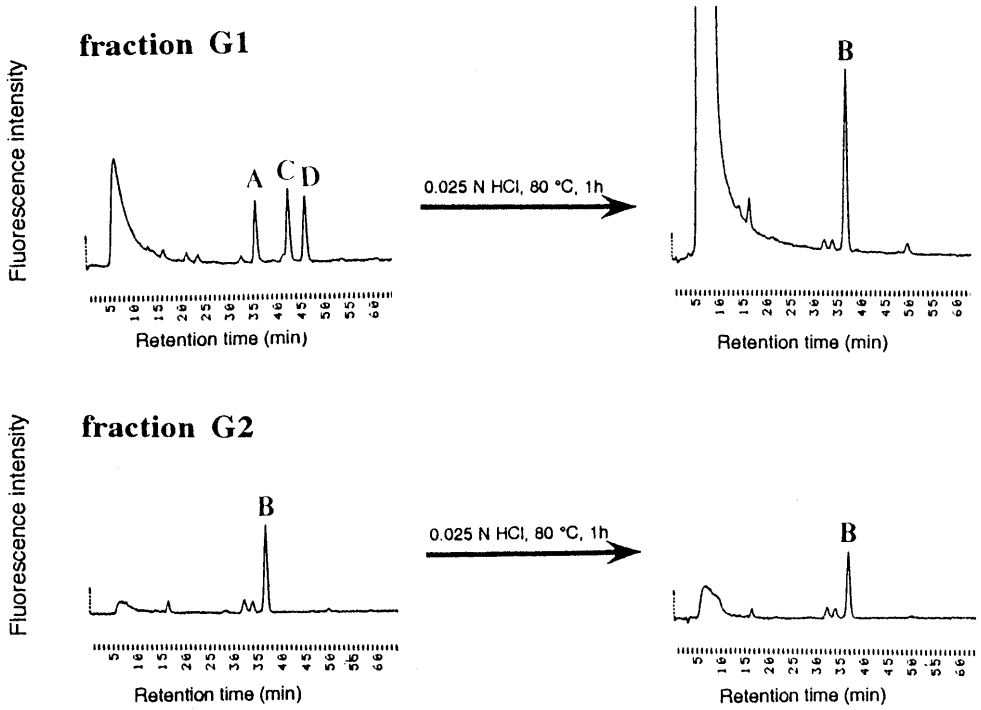

Fig. 2. HPLC Profiles of PA-Oligosaccharides Obtained from UTI

Column: Shimpack CLC-ODS (6 mm I.D. X 150 mm).
Solvent A: 10 mM sodium phosphate buffer (pH 3.8).
Solvent B: solvent A containing 0.5 % 1-BuOH.
Gradient: 0-80 min, 20-60 % B.
Flow rate: 1.0 ml/min.
Column temperature: 40 °C.
Detection: fluorescence (Ex. 320 nm, 400 nm).
Sample size: 10 μl.

The ^{1}H-NMR spectrum of the PA-oligosaccharide B in Fig. 2 at 303 K is presented in Fig. 4. Comparison of the spectral data with those of the reference compound, which was isolated from human transferrin and characterized by Vliegenthart et al.,[11] allows an assignment of the signals of the N-acetyl signals, at 2.002 ppm (GlcNAc-1), 2.052 ppm (GlcNAc-5'), 2.061 ppm (GlcNAc-5) and 2.086 ppm (GlcNAc-2), respectively. The signals of Gal, Man and GlcNAc are in perfect agreement with their positions, though absence of signals corresponding to the anomeric proton of GlcNAc-1 is observed because pyridylamination was carried out. Especially, the substitution pattern of the Man residues 4 and 4' is reflected in the chemical shifts of their H-1 atoms. The H-1 signals of Gal-6 and -6' are fairly distinct from each other, whereas the H-1 signals of GlcNAc-5 and -5' coincide at 4.590 ppm at 333 K (they can't be observed in Fig. 4 obtained at 303 K, because of overlapping with the HOD signal). Furthermore, structure B in Fig. 3 was supported by HPLC compositional analysis (GlcNAc: Gal: Man: Fuc: NeuAc , 4: 2: 3: 0: 0).

Consequently, PA-oligosaccharide B was identified to be as shown in Fig. 3-B from its behavior on the two-dimensional sugar map method, by HPLC compositional analysis and by ^{1}H-NMR study. The other PA-oligosaccharides A, C and D were

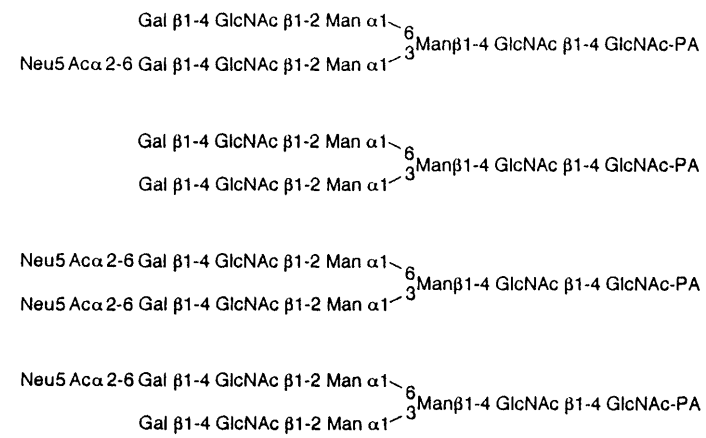

Fig. 3. Structure of PA-Oligosaccharides

determined as shown in Figs. 3-A, C and D, respectively, by comparison with each standard PA-oligosaccharide on a two-dimensional sugar map, compositional analysis of sialic acid (1:2:1, respectively) and ^{1}H-NMR (axial and equatorial C3H signals of the Neu5Ac residues were observed at 1.688 ppm and 2.686 ppm[11]).

The native structure of N-linked oligosaccharide in UTI is presumed to be the disialosyl oligosaccharide shown in Fig. 3-C, which is the same structure found in human ceruloplasmin.[12] It is very interesting that UTI has an N-linked oligosaccharide together with a LSC. Further investigations are necessary to elucidate biosynthetic and biodegradative pathways and to clarify the biological importance of the biantennary complex type carbohydrate chain in UTI.

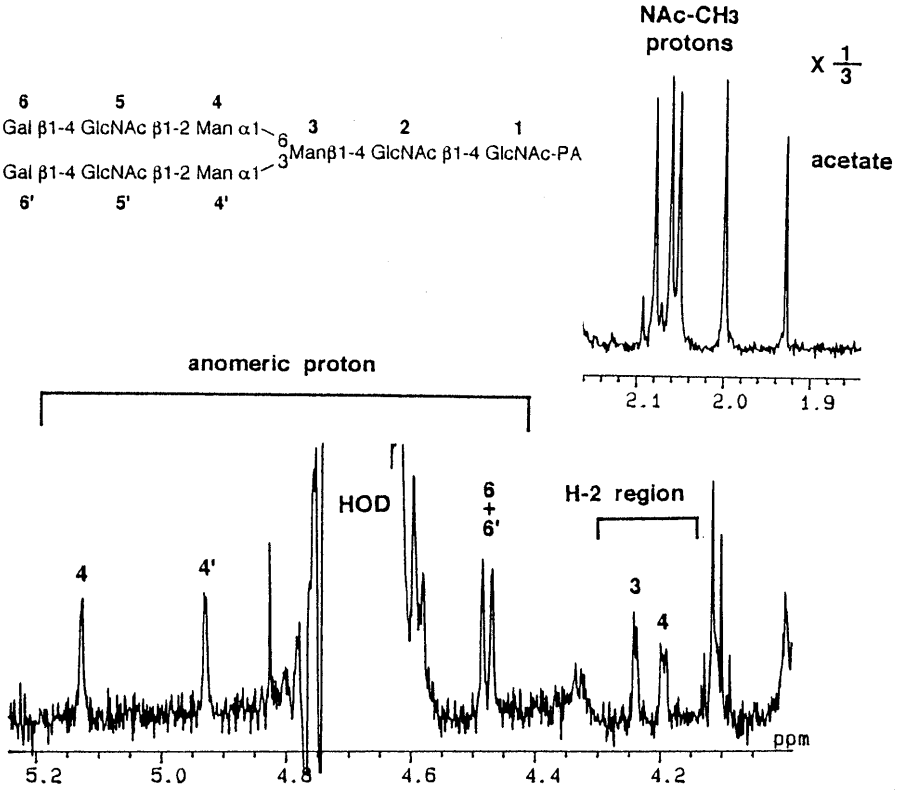

Fig. 4. Structural Reporter-Group Region of 500 MHz ^{1}H-NMR Spectrum of PA-Oligosaccharide B

REFERENCES

1) M. Balduyck, C. Mizon, H. Loutfi, C.Richet, P. Roussel and J. Mizon, *Eur. J. Biochem.*, **158**, 417(1986).

2) H. Ochiai, H. Toyoda, M. Onodera, A. Shinbo, K. Shinomiya and T. Imanari, *Chem. Pharm. Bull.*, **36**, 3726(1988).

3) K. Hochstrasser, O. L. Schonberger, I. Rossmanith and E. Wachter, *Hoppe-Seyler's Z. Physiol. Chem.*, **362**, 1357(1981).

4) T. Imanari, A. Shinbo, H. Ochiai, Tatsuo I, I. Koshiishi and H. Toyoda, *J. Pharmacobio-Dyn.*, **15**, 231(1992).

5) T. E. Jessen, K. L. Faarvang and M. Ploug, *FEBS Lett.*, **230**, 195(1988).

6) J. J. Enghild, G. Salvesen, S. A. Hefta, I. B. Thogersen, S. Rutherfurd and S. V. Pizzo, *J. Biol. Chem.*, **266**, 747(1991).

7) H. Sumi, M. Maruyama, H. Mihara, S. Maehara and N. Toki, *Acta Hematol. JPN.*, **44**, 146(1981).

8) N. Takahashi, *Biochem. Biophys. Res. Commun.*, **76**, 1194(1977).

9) A. Kondo, *Trends Glycosci. Glycotechnol.*, **3**, 130(1991).

10) N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata and N. Takahashi, *Anal. Biochem.*, **171**, 73(1988).

11) J. F. G. Vliegenthart, L. Dorland and H. V. Halbeek, *Adv. Carbohydr. Chem. Biochem.*, **41**, 209(1983).

12) K. Yamashita, C. Liang, S. Funakoshi and A. Kobata, *J. Biol. Chem.*, **256**, 1283(1981).

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