

Development and Application of High Performance Liquid Chromatography Map of Glucuronyl *N*-glycans

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Abstract: Although the multi-dimensional HPLC maps of neutral, sialyl, and sulfated *N*-glycans have been reported and widely used for glycosylation profiling, those of glucuronyl oligosaccharides have not yet been available. In the present study, by *in vitro* enzymatic reactions, we prepared 55 different glucuronyl PA-oligosaccharides that include 6 kinds of HNK-1-containing *N*-glycans, and established their HPLC map. Furthermore, we applied this map to the characterization of branch specificity in glucuronylation reaction catalyzed by human GlcAT-S, revealing that this enzyme transfers the glucuronyl residues preferentially onto the Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4Man α 1 \rightarrow 3 and Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3 branches of a galactose-terminated tri-antennary oligosaccharide. The HPLC map developed in the present study will be a useful glycomics tool for identification and profiling of glucuronyl *N*-glycans expressed in the neural and other biological systems.

Keywords: Glucuronyl oligosaccharides/*N*-glycans/HPLC map/GlcAT-S/branch specificity.

INTRODUCTION

Cell-cell recognition and adhesion are mediated by a variety of anionic oligosaccharides possessing sialyl, glucuronyl, and/or sulfated groups [1,2]. HNK-1 carbohydrate epitope [3], which is a trisaccharide containing sulfoglucuronyl residue, HSO₃-3GlcA β 1-3Gal β 1-4GlcNAc-, was originally reported as a specific antigenic determinant for human natural killer cells but is now known to be expressed predominantly in the neural system, where it plays roles in intercellular adhesion, cell migration and synaptic plasticity [4-6]. Based on the specific antibody binding and mass spectrometric (MS) analyses, this determinant has been identified on several glycoproteins such as neural cell adhesion molecule [7,8], myelin-associated glycoprotein [9], tenascin-C and -R [10], and tissue plasminogen activator [11]. However, there are only a few reports that describe detailed structures of HNK-1-containing glycans expressed on proteins [12] primarily due to the lack of a conventional method for identification of glucuronyl oligosaccharides.

We have been developing the multi-dimensional HPLC mapping method for quantitative *N*-glycosylation profiling at molecular, cellular, and tissue levels, distinguishing isomeric *N*-glycan structures [13-15]. In this method, identifications of individual *N*-glycans are based on their elution positions on the three kinds of HPLC columns. The accumulated HPLC data of approximately 500 different *N*-glycans are now available in the web application GALAXY (<http://www.glycoanalysis.info/>) [16], and the applicability of this method has been extended to sialyl [14] and sulfated oligosaccharides [15]. However, the widespread application of the HPLC map has been hampered by a lack of data concerning glucuronyl oligosaccharides.

In view of this situation, we attempted to develop an HPLC-based method for structural analysis of the glucuronyl *N*-glycans. On the basis of the previously established HPLC map, we successfully made a map for 55 different glucuronyl *N*-glycans including 6 kinds of HNK-1-containing oligosaccharides, which were prepared by *in vitro* enzymatic reactions using recombinant enzymes. By using this extended HPLC map, we characterized a branch specificity in glucuronylation catalyzed by human second β 1,3-glucuronyltransferase (GlcAT-S).

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Materials and Methodology

Materials

Glycoamidase A from sweet almond, β -galactosidase and β -*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo Co. α -Fucosidase from bovine kidney was from Boehringer-Mannheim. Trypsin and chymotrypsin were from Sigma. Pronase protease from *Streptomyces griseus* was from Calbiochem. The pyridylamino (PA) derivatives of isomalto-oligosaccharides 4-20 (indicating the degree of polymerization of glucose residues) and reference PA-oligosaccharides (Code Nos. 100.1, 100.2, 110.1, 110.2, 200.1, 200.2, 200.3, 200.4, 210.1, 210.2, 210.3, 210.4, 300.8, 310.2, 310.3, 310.4, 310.8 and 400.16) were purchased from GLYENCE Co. and Seikagaku Kogyo Co.

Protein Expression and Purification of GlcAT-S

The DNA fragment encoding the amino acids residues 79-323 of human GlcAT-S was amplified by the polymerase chain reaction from the cDNA template cloned in the previous study [17], and was cloned into the pET-28a(+) plasmid vector (Novagen). The expression and purification of GlcAT-S (79-323) were performed as described previously [18] with slight modifications. The protein was expressed in *E. coli* BL21 (DE3) pLysS strain (Stratagene) grown in an LB broth containing 0.4 % (w/v) glucose and 20 μ g/ml kanamycin at 30 °C by vigorous aeration upon induction with 0.1 mM isopropyl β -D-thiogalactopyranoside (WAKO). The *E. coli* cells were harvested by centrifugation and sonicated in 20 mM Tris-HCl (pH 7.4) containing 5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 0.15 M NaCl, and 3 % (v/v) protease inhibitor cocktail (Sigma). After centrifugation of the sonicated cell suspension, the cell lysate was applied onto an open column filled with a Hi-trap Heparin HP resin (Amersham Biosciences). After washing with 0.4 M NaCl, the fraction eluted by 0.75 M NaCl was collected, diluted with 20 mM Tris-HCl (pH 7.4) so as to adjust the NaCl concentration to 0.45 M, and then loaded onto the Hi-Trap Heparin HP column. The fractions containing GlcAT-S were collected by applying a NaCl gradient (0.45-0.75 M), pH-adjusted to 8.0 with Tris-HCl, and then applied onto a Hi-Trap Chelating column (Amersham Biosciences), which had been equilibrated with 0.1 M CuSO₄ and subsequently with 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The GlcAT-S-containing fractions were collected by applying a gradient of glycine concentration (0-150 mM), diluted so as to adjust the NaCl concentration to 0.25 M, and then loaded onto the Hi-Trap Heparin HP column equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.25 M NaCl. After washing with 0.25 M NaCl and 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 6.0), GlcAT-S was eluted with 0.7 M NaCl and 50 mM MES (pH 6.0) and then concentrated by ultrafiltration using Amicon Ultra YM-10 (Amicon). The yield of the GlcAT-S protein was 1 mg per 2 liters of culture.

Purification of HNK-1sulfotransferase Fused with Protein A

COS7 cells grown in a 10-cm-diameter tissue culture dish (Corning) were transfected with 4 μ g of the relevant plasmid, pEF-BOS-HNK-1sulfotransferase (ST) [19] using LipofectAMINE PLUS (Invitrogen) according to manufacturer instructions. After 24 h of culture in Dulbecco's modified Ea-

gle's medium containing 10 % (v/v) fetal calf serum, the medium was replaced with Dulbecco's modified Eagle's medium containing 2 % (v/v) IgG-free fetal calf serum. The cells were cultured for an additional 96 h. Subsequently, the culture medium was collected and concentrated to 1 ml by Amicon Ultra-15 (Millipore Co.). The protein A-fused HNK-1ST recombinant protein expressed in the medium was adsorbed to IgG-Sepharose (20 μ l resin/1 ml of culture medium) at 4 °C for 3 h. The resin was collected by centrifugation and washed three times with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. Consequently, the resin was suspended in 20 μ l of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and used as the enzyme source.

Enzymatic Reactions

Glucuronylation of the PA-glycans were carried out in a reaction mixture containing 50 mM MES, pH 6.5, 1 mM MnCl₂, 0.12-1.0 μ M of PA-oligosaccharides, 1 mM uridine-5'-diphosphoglucuronic acid, 0.2 % (v/v) Nonidet P-40, and 0.04 mg/ml GlcAT-S in a final volume of 50 μ l. After incubation at 37 °C for 5-180 min, the reaction was terminated by heating at 100 °C for 10 min.

The resultant glucuronylated PA-glycans were used as acceptors in the sulfation reactions by protein A-fused HNK-1ST in solution containing 25 mM Tris-HCl (pH 7.5), 10 mM MnCl₂, 0.25 μ M PA-oligosaccharide, 7.5 μ M adenosine 3'-phosphate 5'-phosphosulfate, 0.1 % TritonX-100. After incubation at 37 °C overnight, aliquots of 5 μ l of the reaction mixture were subjected to HPLC analysis (*vide infra*).

Multi-Dimensional HPLC and MALDI-TOF-MS Analysis

All analytical procedures used in this work, including the chromatographic conditions, glycosidase treatments, and matrix-assisted laser desorption / ionization time of flight (MALDI-TOF)-MS analysis have been reported previously [14,15,20]. The reaction mixtures after the GlcAT treatments were applied onto a Shim-pack HRC-octadecyl silica (ODS) column (Shimadzu). The individual PA-oligosaccharide fractions were further separated on a TSK-gel Amide-80 column (Tosoh). The elution volumes of each glucuronyl PA-*N*-glycan both on the ODS and the amide columns were recorded and expressed as the glucose units values, GU(ODS) and GU(amide), respectively. Unit contribution values were calculated by a linear multiple regression analysis as described previously [21,22].

RESULTS AND DISCUSSION

Structural Identification and Characterization of Glucuronyl Oligosaccharides

Glucuronyl PA-oligosaccharides were prepared through the reaction catalyzed by human GlcAT-S using bi-, tri-, and tetra-antennary PA-oligosaccharides as starting materials. The products were separated by the HPLC methods. The procedure of structural analyses of the glucuronyl PA-oligosaccharides would be exemplified by the determination of structures of oligosaccharides resulting from the glucuronylation reaction using a bi-antennary PA-glycan, Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 6(Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 2Man α 1 \rightarrow 3)Man β 1 \rightarrow 4GlcNAc β 1 \rightarrow 4(Fuc α 1 \rightarrow 6)GlcNAc-PA (code no. 210.4 in GALAXY), as a precursor.

Figs. (1a and 1b) compare the elution profiles on the ODS column of the acceptor glycan and reaction products, respectively, indicating that the reaction mixture contained the three expected products A, B, and C. MALDI-TOF-MS analysis revealed that product A was di-glucuronyl oligosaccharides while B and C were mono-glucuronyl products. According to substrate specificity of GlcAT-S, the di-glucuronyl PA-oligosaccharide A was determined as $\text{GlcA}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{GlcA}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 (\text{Fuc}\alpha 1 \rightarrow 6) \text{GlcNAc-PA}$. Next, we attempted to identify the structures of the mono-glucuronyl PA-oligosaccharides B and C by comparison of the HPLC data of their degalactosyl derivatives with those of the glucuronylation products of the bi-antennary monogalactosyl PA-oligosaccharides as standard materials. Upon the galactosidase treatment, B and C were converted to D and E, which correspond to the products resulting from the GlcAT-S-catalyzed glucuronylation of $\text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 (\text{Fuc}\alpha 1 \rightarrow 6) \text{GlcNAc-PA}$ and $\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 (\text{Fuc}\alpha 1 \rightarrow 6) \text{GlcNAc-PA}$, respectively (Fig. 1c). On the basis of these data, we concluded that the mono-glucuronyl PA-oligosaccharides B and C are $\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{GlcA}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 4 (\text{Fuc}\alpha 1 \rightarrow 6) \text{GlcNAc-PA}$ and $\text{GlcA}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 6 (\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 2 \text{Man}\alpha 1 \rightarrow 3) \text{Man}\beta 1 \rightarrow 4$

$\text{GlcNAc}\beta 1 \rightarrow 4 (\text{Fuc}\alpha 1 \rightarrow 6) \text{GlcNAc-PA}$, respectively. In this way, we identified 49 kinds of glucuronyl PA-oligosaccharides as products of enzymatic reactions of bi-, tri-, and tetra-antennary complex type glycans (Table 1). Furthermore, we carried out *in vitro* sulfation by recombinant HNK-1ST using some isolated glucuronyl PA-oligosaccharides as substrates and thereby prepared 6 kinds of HNK-1-containing PA-oligosaccharides. The HPLC data of these oligosaccharides are summarized in Table 2.

It has been shown that the elution position of a given PA-glycan could be represented by the sum of the contributions of individual monosaccharides unit at specific positions, i.e. unit contribution (UC) [21,22]. By a linear multiple regression analysis using the HPLC data obtained in this study, the UC values of the four glucuronyl units were diagrammatically obtained for GU(ODS) (Fig. 2). These calculated UC values would be useful in predicting GU(ODS) values for putative glucuronyl PA-N-glycans.

Branch Specificity of GlcAT-S

The HPLC map thus established will facilitate structural identification of glucuronyl N-glycans distinguishing isomeric structures. Here we demonstrate the utility of this HPLC map by revealing branch specificity of human GlcAT-S. Although this enzyme has been reported to exhibit the highest activity toward triantennary N-glycan [23], its branch specificity remains to be addressed.

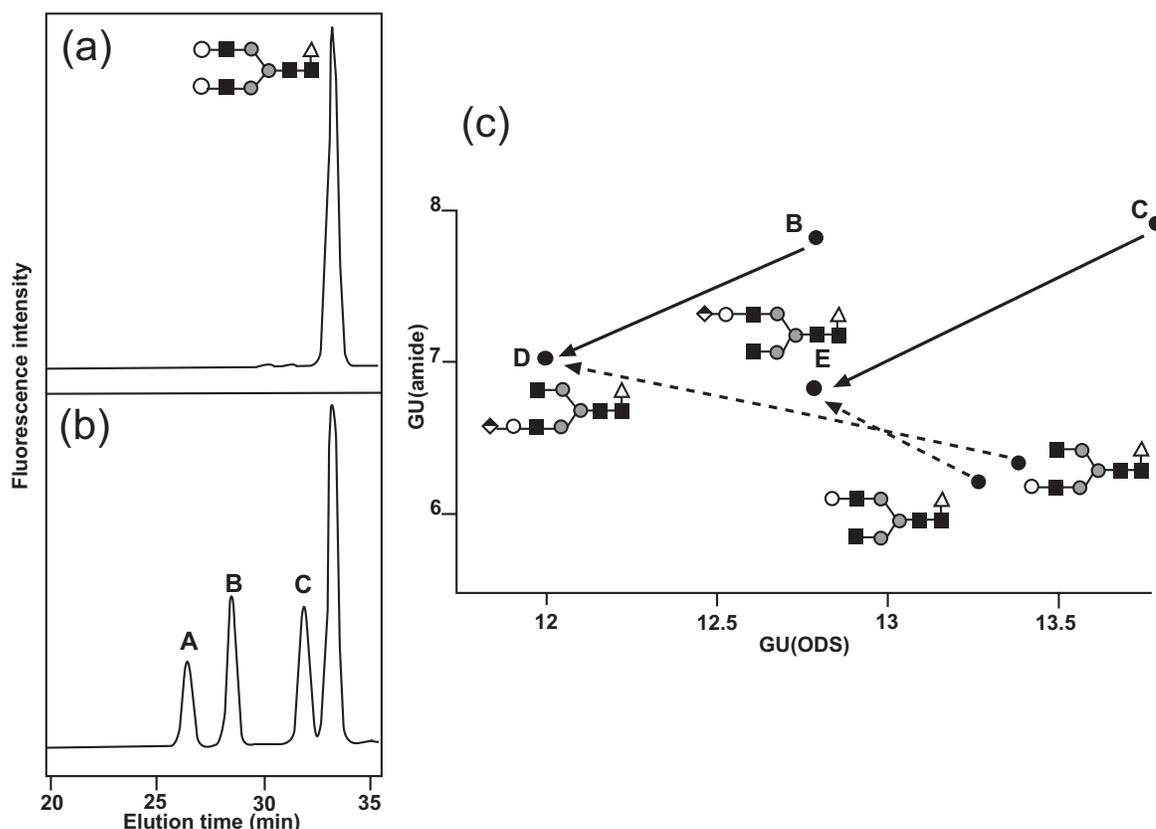


Fig. (1). Identification of the bi-antennary glucuronyl PA-oligosaccharides produced by the reaction catalyzed by GlcAT-S.

Chromatograms of the precursor (a) and reaction mixture (b) on an ODS column. c: Schemes of identification of the mono-glucuronyl glycans by using enzymatic treatments. On this map, the horizontal and vertical axes correspond to GU(ODS) and GU(amide), respectively. Trajectories for galactosidase digestion and glucuronylation were indicated by arrows with solid and dashed lines, respectively. Symbol: gray circle, mannose; white circle, galactose; black square, N-acetylglucosamine; diamond, glucuronic acid.

(Table 1). Contd.....

Code No. ^a	PA-Oligosaccharides	GU		Molecular mass ^b (Da)
		ODS	Amide	
1H1-110.4	$\begin{array}{c} \text{Man}\alpha 6 \quad \text{Fuc}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 3 \end{array}$	10.4	6.8	1677
1H1-100.3	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{Man}\alpha 3 \end{array}$	10.2	5.6	1530
1H1-100.9	$\begin{array}{c} \text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \end{array}$	8.9	7.7	1530
1H1-100.4	$\begin{array}{c} \text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 3 \end{array}$	7.5	6.4	1530
3H1-310.8	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \quad \text{Fuc}\alpha 6 \\ \diagdown \quad \diagup \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	15.3	1.0	2759
3H1-300.8	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	11.0	10.6	2613
2H2-310.8	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \quad \text{Fuc}\alpha 6 \\ \diagdown \quad \diagup \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	17.7	10.3	2583
2H3-310.8	$\begin{array}{c} \text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \quad \text{Fuc}\alpha 6 \\ \diagdown \quad \diagup \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	15.8	10.0	1734
2H2-300.8	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	12.7	9.9	2437
2H1-300.8	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	8.9	7.7	2437
2H3-300.8	$\begin{array}{c} \text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \\ \diagdown \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2 \end{array}$	11.4	10.0	2437
2H1-310.6	$\begin{array}{c} \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 2\text{Man}\alpha 6 \quad \text{Fuc}\alpha 6 \\ \diagdown \quad \diagup \quad \diagup \\ \text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc} \\ \diagup \quad \diagdown \\ \text{GlcA}\beta 3\text{Gal}\beta 4\text{GlcNAc}\beta 4 \\ \text{Man}\alpha 3 \\ \text{GlcNAc}\beta 2 \end{array}$	15.7	9.1	2421

(Table 1). Contd.....

Code No. ^a	PA-Oligosaccharides	GU		Molecular mass ^b (Da)
		ODS	Amide	
2H1-310.5		13.9	9.1	2421
2H1-310.9		15.5	9.4	2421
2H1-300.6		12.9	8.7	2275
2H1-300.9		12.3	8.9	2275
1H2-310.8		17.6	9.5	2407
1H3-310.8		18.2	9.6	2407
1H1-310.8		16.3	9.6	2407
1H2-300.8		12.6	9.2	2261
1H3-300.8		13.0	9.2	2261
1H1-300.8		11.7	9.2	2261
1H2-310.5		15.2	8.2	2245

(Table 1). Contd.....

Code No. ^a	PA-Oligosaccharides	GU		Molecular mass ^b (Da)
		ODS	Amide	
1H1-400.16		10.0	10.4	2626
1H1-400.4		9.6	7.6	2140
1H1-400.2		8.8	7.6	2140
1H1-400.3		9.6	7.7	2140
1H1-400.5		9.1	7.5	2140

^aThe PA-oligosaccharides are coded according to the literature [13,16].^bAverage mass calculated from the *m/z* values of [M-H]⁻, [M+Na-2H]⁻, or [M+2Na-3H]⁻ ions for glucuronyl PA-oligosaccharides.

Fig (3a) shows time-dependent transition of HPLC elution profiles of products of the *in vitro* glucuronylation catalyzed by the recombinant GlcAT-S enzyme using a triantennary PA-glycan, Galβ1→4GlcNAcβ1→2Manα1→6 (Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-PA (code no. 300.8), as an acceptor. The HPLC map enabled us to identify the individual products and to monitor their appearance and disappearance in a quantitative manner during the reaction (Fig. 3b). This result clearly indicates that the GlcAT-S-catalyzed glucuronylation preferentially occurs at the Galβ1→4GlcNAcβ1→4Manα1→3 and Galβ1→4GlcNAcβ1→2Manα1→3 branches, while the intermediate products possessing the glucuronyl residue at the Galβ1→

4GlcNAcβ1→2Manα1→6 branch were barely detected during the reaction.

The biosynthesis of the HNK-1 carbohydrate moieties of glycoprotein is mainly regulated by GlcAT-P as well as GlcAT-S. It has been reported that GlcAT-P preferentially transfers the glucuronyl group to the terminal galactose residue at the Galβ1→4GlcNAcβ1→4 Manα1→3 branch in tetra-antennary oligosaccharides [24]. The branch specificity of GlcAT-S revealed in the present study will provide an additional insight into the enzyme functions of the glucuronyltransferases.

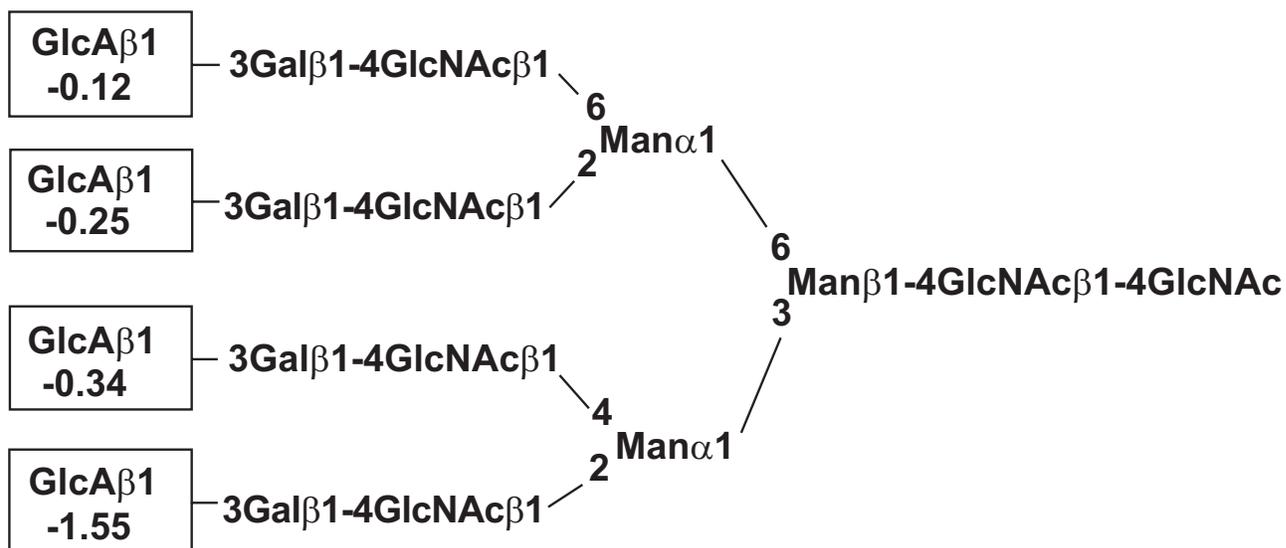
In conclusion, the HPLC map developed in the present study will be a useful glycomics tool for identification and

Table 2. HPLC and MS Data of the HNK-1-Containing *N*-Glycans

Code No. ^a	PA-Oligosaccharides	GU		Molecular mass ^b (Da)
		ODS	Amide	
2H1-1S1-200.4	$\begin{array}{c} \text{GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{3} \end{array}$	9.0	6.6	2378
2H1-2S1-200.4	$\begin{array}{c} \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{3} \end{array}$	11.0	5.2	2378
1H1-1S1-300.8	$\begin{array}{c} \text{Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{Gal}\beta\text{4GlcNAc}\beta\text{4} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2} \end{array}$	10.9	8.6	2693
1H3-1S1-300.8	$\begin{array}{c} \text{Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta \\ \text{Gal}\beta\text{4GlcNAc}\beta\text{2} \end{array}$	11.4	8.7	2693
3H1-1S1-300.8	$\begin{array}{c} \text{GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{4} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2} \end{array}$	11.6	7.1	2341
3H1-1S3-300.8	$\begin{array}{c} \text{GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2Man}\alpha\text{6} \\ \text{HSO}_3\text{-3GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta \\ \text{GlcA}\beta\text{3Gal}\beta\text{4GlcNAc}\beta\text{2} \end{array}$	13.4	7.2	2341

^aThe PA-oligosaccharides are coded according to the literature [13,16].

^bAverage mass calculated from the m/z values of $[M+Na-2H]^+$, or $[M+2Na-3H]^+$ ions for HNK-1-containing PA-oligosaccharides.

**Fig. (2).** Diagram of the partial UC values of glucuronyl residues for GU(ODS).

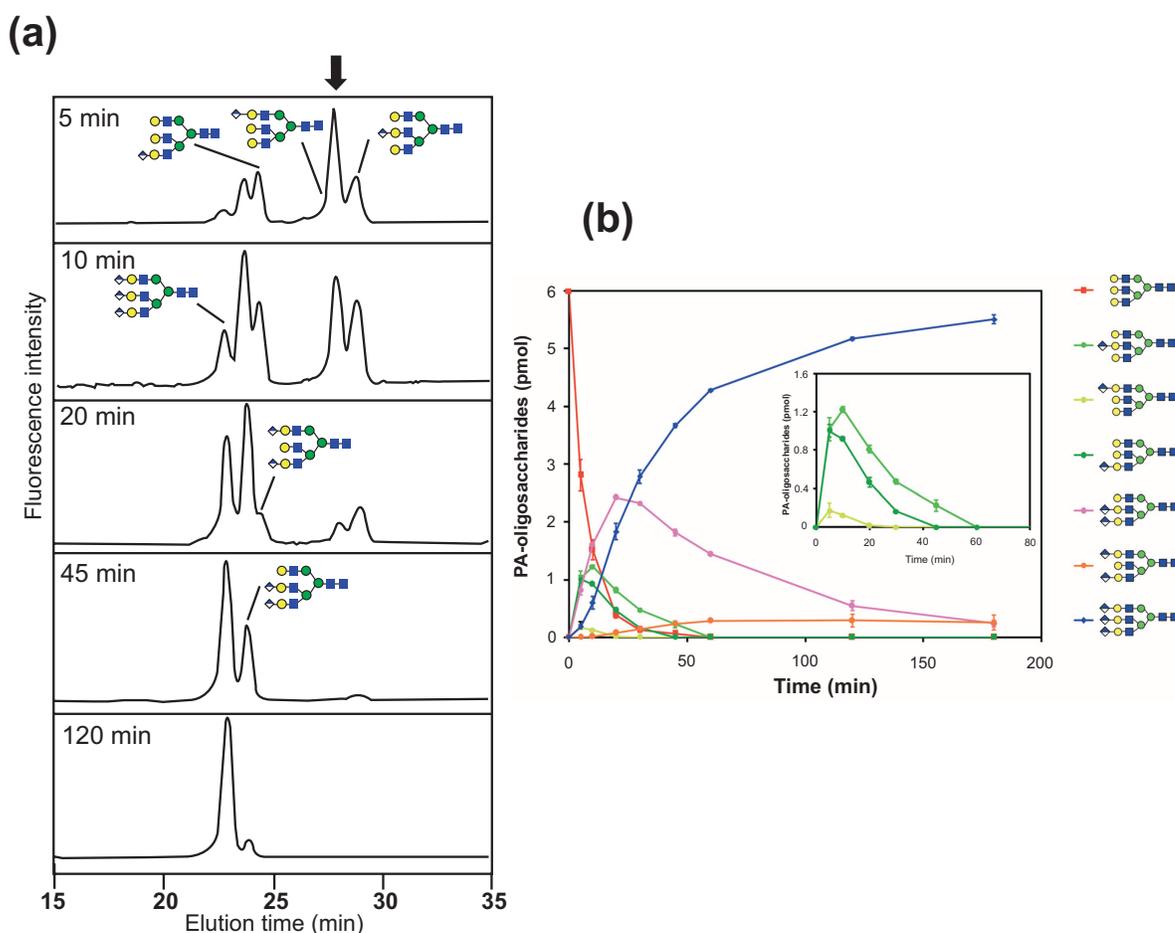


Fig. (3). HPLC-based characterization of branch specificity of GlcAT-S.

a: Time-dependent transition of the HPLC profiles on an ODS column of the products resulting from the galactose-terminated tri-antennary PA-oligosaccharide (indicated by arrow) during the glucuronylation reaction catalyzed by the recombinant human GlcAT-S enzyme. **b:** Time courses of the amounts of the starting material and resultant glycans. Data are presented as mean \pm S.D. of three independent experiments. Symbol: green circle, mannose; yellow circle, galactose; blue square, *N*-acetylglucosamine; diamond, glucuronic acid.

profiling of glucuronyl *N*-glycans expressed in the neuronal and other biological systems.

ABBREVIATIONS

Da	= dalton
GlcAT	= β 1,3-glucuronyltransferase
GU	= glucose unit
GU(amide)	= glucose unit value on the amide column
GU(ODS)	= glucose unit value on the ODS column
HNK-1	= human natural killer cell-1
HPLC	= high performance liquid chromatography
MALDI-TOF-MS	= matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
UC	= unit contribution
Fuc	= fucose
Gal	= galactose
Man	= mannose

GlcNAc = *N*-acetylglucosamine

Hex = hexose

HexNAc = *N*-acetylhexosamine

DeoxyHex = deoxyhexose

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