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Separation of isomeric 2-aminopyridine derivatized N-glycans and N-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography[1]

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Keywords: Zwitterion-interaction column; hydrophilic-interaction chromatography; oligosaccharides; glycopeptides; mass spectrometry.

Abstract

Isomeric oligosaccharides and isomeric glycopeptides are sometimes difficult to separate on normal-phase (NP) and reversed-phase (RP) columns. A zwitterionic type of hydrophilic-interaction chromatography column with sulfobetaine groups (called ZIC-HILIC column) was first applied to the separation of 2-aminopyridine derivatized (PA) N-glycans and tryptic peptides of human serum immunoglobulin G (IgG). It is shown that the ZIC-HILIC column has high capability for structural recognition of isomeric N-glycans as well as high selectivity for glycopeptides. The former feature (i.e., structural recognition) was proven by sufficient separation of neutral PA N-glycan isomers, which are usually difficult to separate on NP and RP columns. In addition, it is noteworthy that IgG glycopeptides consisting of isomeric N-glycans and the same peptide sequences can be sufficiently separated on a ZIC-HILIC column. The latter feature (i.e., selectivity) was also demonstrated by easily separating two peptide groups with/without N-glycans. Thus, we note that the ZIC-HILIC column is highly promising for a simple analysis of N-glycans and N-glycopeptide samples.

1. Introduction

Glycosylation is one of the most common post-translational modifications of proteins. It is related to numerous biological processes [1]. To better understand the roles of glycoproteins, it is important to elucidate oligosaccharide (glycan) structures [2]. However, oligosaccharides make up some of the most complex and challenging samples for chromatography because of the structural diversity of oligosaccharides (composition, sequence, anomeric character, linkage position, and branching pattern) and, as a result, the existence of many isomers. Thus far, several types (reversed-phase (RP), normal-phase (NP), ion exchange, size exclusion) of HPLC have been used extensively in the separation of glycans and glycopeptides [3]. Among these methods, RP-HPLC and NP-HPLC have been used most often for the separation of fluorescent labeled (e.g., 2-aminopyridine (PA) [4,5], 2-aminobenzamide (AB) [6-8]), and permethylated glycans [9]. The HPLC methods based on both the RP- and NP-column separations are referred to as the two-dimensional (2D) mapping method [4,5,10]. Recently, several attempts involving the use of new types of columns have been reported for the separation of non-derivatized/derivatized glycans. One is the RP-HPLC method, which uses a porous graphitized carbon (PGC) column [11-16]. The other is hydrophilic-interaction chromatography (HILIC) using silica, amino, amide, cellulose, cyclodextrin [17-24]. In addition, it has been reported that these PGC [15, 25], cellulose [26], and ZIC-HILIC [27] columns can be used for selective purification/enrichment of glycopeptides from a large amount of peptides and also, the HILIC method can separate sialylated glycopeptide isomers from recombinant human interferon-γ [28].

In this study, we apply a commercially available ZIC-HILIC column with sulfobetaine groups to the separation of PA N-glycans and tryptic peptides of human serum immunoglobulin G (IgG).

Although several types of ZIC columns for HILIC chromatography have been developed, their chief target was simultaneous analysis of small cations, anions, and polar compounds in aqueous solution [29-34], and exceptionally, the separation of large molecules such as proteins and peptides [35]. Here, we report on the two most useful features of the ZIC-HILIC column: (1) high structural recognition capability for N-glycan isomers and (2) high selectivity for glycopeptides. To the best of our knowledge, this is the first report of the ZIC-HILIC separation of PA N-glycans and tryptic glycopeptides of human serum IgG.

2. Experimental

2.1. Materials

Acetonitrile (HPLC/MS grade), ammonium acetate, and trypsin were purchased from Wako (Osaka, Japan). Water was purified by Milli-Q (Millipore, Milford, MA, USA). IgG from human serum (I4506) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A ZIC-HILIC (150 mm x 2.1 mm I. D., particle size 3.5 μm) column produced by SeQuant (Umeå, Sweden) was purchased from Nomura Chemical (Aichi, Japan).

2.2. Sample preparation

Desialylated PA N-glycans from IgG, shown in Table 1, were prepared following the method previously described [36]. Tryptic peptides of IgG, shown in Table 1, were prepared as follows. Human serum IgG (1 mg) was digested with 20 µg of trypsin at 37°C overnight in 100 µL of 50 mM ammonium bicarbonate buffer (pH 7.8). To terminate the digestion and to release the sialic acids, the digested mixture was heated at 90°C for 1 hour with 0.01 M HCl (pH 2.0). The reaction was then quenched by adding 1.0 M ammonium bicarbonate buffer. After evaporation, the sample was dissolved in 20 µL of water and centrifuged (20 400g, 15 min, 4°C). A part of the supernatant liquid (4 µL) was diluted with acetonitrile (16 µL), and then injected into the ZIC-HILIC/ESI-IT MS system.

2.3. ZIC-HILIC/ESI-IT MS analysis

Experiments were performed by using a HPLC/MS system consisting of a Hitachi LaChrom HPLC system with a fluorescence (FL) and ultraviolet detector (UV) and a Hitachi M-8000 3DQ (ion trap: IT) MS with an electrospray ionization (ESI) source. Separations were performed with the SeQuant ZIC-HILIC column in a column oven at 40°C. A gradient elution was applied at a flow rate of 200 µL/min using 50% acetonitrile (solvent A), acetonitrile (solvent B), and 100 mM ammonium acetate buffer (solvent C) (A/B/C = 36/59/5 (0 min) →64/31/5 (120 min)). The ESI-MS conditions were: desolvation temperature, 200°C; desolvation gas (nitrogen) pressure, 300 kPa; capillary voltage, +4 kV; drift voltage, 30 V; scan range, *m/z* 200-2000; scan time, 500 ms; and low-mass cutoff *m/z*, 105. Excitation (Ex) and emission (Em) wavelengths of the FL detector were set at 320 nm and 400 nm, respectively. A value of 220 nm was used for UV detection.

3. Results and discussion

3.1. ZIC-HILIC separation of PA N-glycans of human serum IgG

We first applied the ZIC-HILIC column to the separation of desialylated PA N-glycans of human serum IgG to investigate the structural recognition capability of isomeric N-glycans. Human serum IgG contains two dominant pairs of complex-type N-glycan isomers, $Hex_4HexNAc_4Fuc_1$ (b and c) and $Hex_4HexNAc_5Fuc_1$ (f and g) [36,37] (see Table 1). In particular, the latter isomers (f and g) are known to be difficult to separate on both the RP (C18 and C30) and NP (amide) columns (i.e., difference between their Glucose Units: 0.1(NP), 0.2(RP)) [10,36]. ZIC-HILIC separation was performed using a high concentration of volatile organic solvent (60-80% acetonitrile) and a low concentration of volatile electrolytes (5 mM ammonium acetate), which are compatible with ESI-MS. Figure 1A and 1B show ZIC-HILIC/ESI-MS chromatograms of PA N-glycans of human serum IgG observed by fluorescence (FL) detection and mass chromatograms (MCs) corresponding to the molecular ions $[M+zH]^{z+}$ (z=1, 2) of the major N-glycans a-h, respectively. The FL chromatograms in sequential 10 runs are overlaid in Figure 1A. The retention time RSD(%) values of the peaks a-h are 0.37-0.71, which show a good repeatability of this ZIC-HILIC method. It should be noted that both pairs of isomeric PA N-glycans (b/c and f/g) are completely separated in the present method (see Figure 1B). We also note that these isomers derivatized with AB have been partially separated on NP-HPLC [7].

From the results shown in Figure 1, it can be seen that the ZIC-HILIC method has superior structural recognition capability of N-glycan isomers. Although the particular detailed retention mechanism of ZIC-HILIC separation of large molecules has not yet been completely elucidated in the stationary phase, the structural recognition capability of N-glycans seems to be primarily based on the hydrophilicity of N-glycans and the electrostatic interaction between N-glycans and the sulfobetaine groups on the surface.

3.2. ZIC-HILIC separation of tryptic peptides of human serum IgG

Next, we applied the ZIC-HILIC method to tryptic peptides of human serum IgG. Tryptic peptides of IgG were directly analyzed by ZIC-HILIC/ESI-IT MS. Figure 2A shows the UV chromatogram of tryptic peptides of IgG detected at 220 nm. Figure 2B and 2C show accumulated mass spectra over 0-60 min and over 60-100 min, respectively. It is likely that a large number of hydrophobic peptides are observed at the earlier retention time of 0-60 min and that relatively hydrophilic N-glycopeptides are observed at the later retention time of 60-100 min. This result clearly indicates that the

ZIC-HILIC method has a high degree of selectivity for the two peptide groups with/without N-glycans. Figure 2D shows MCs of the major N-glycopeptides (see Table 1).

Human serum IgG consists of the major two subclasses, IgG-1 and IgG-2. Their tryptic peptide sequences containing N-glycosylation sites are EEQYNSTYR for IgG-1 and EEQFNSTFR for IgG-2. The retention times of the IgG-1 N-glycopeptides (a-1–h-1) are always longer than are those of the IgG-2 N-glycopeptides (a-2–h-2). The reason may be that the IgG-1 glycopeptide containing tyrosine (Y) is more hydrophilic than is the IgG-2 glycopeptide containing phenylalanine (F) because tyrosine has an additional hydroxyl group. In addition, it should be noted that the N-glycopeptide isomers (b-1/c-1, b-2/c-2 and f-2/g-2) were also sufficiently separated from each other. Thus, the results in Figure 2 likely indicate that the ZIC-HILIC separation of N-glycopeptides is primarily based on the hydrophilicity of both the peptide and the neutral N-glycan residues.

Finally, we note that this method capable to separate N-glycopeptide isomers is particularly useful for a LC/MS based direct analysis of peptide amino-acid sequence and isomeric N-glycan structures of N-glycopeptides.

Conclusion

The HPLC method using a ZIC-HILIC column with sulfobetaine groups showed high structural recognition capability for isomeric neutral PA N-glycans, which are known to be difficult to separate on both normal-phase and reversed-phase columns. In addition, this method demonstrated high selectivity for N-glycopeptides obtained by trypsin digestion of human serum IgG. In particular, it is noteworthy that IgG N-glycopeptides consisting of isomeric N-glycans and the same peptide sequence could be separated sufficiently by the ZIC-HILIC method of separation. Studies are underway to examine its usefulness for other types of glycans, glycopeptides, and glycolipids, according to a recommendation of the anonymous reviewers.

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CAPTIONS:

Figure 1. ZIC-HILIC separation of PA N-glycans of human serum IgG.

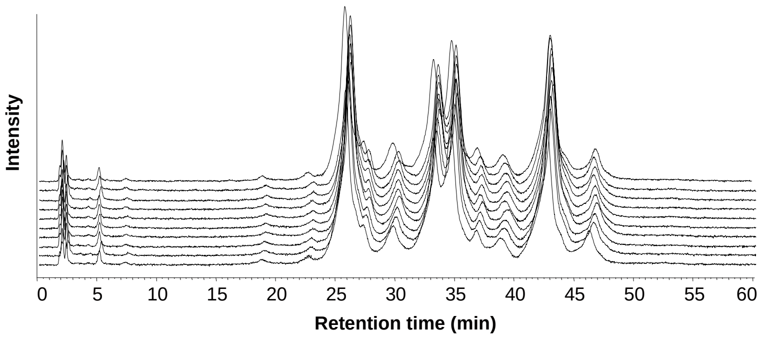
(A) Fluorescence (FL) chromatograms (Em, 400 nm; Ex, 320 nm) in sequential 10 runs. Retention time RSD(%) values of peaks a-h are 0.37-0.71. (B) Mass chromatograms (MCs) of molecular ions $[M+zH]^{z+}$ ($z=1, 2$; *m/z* ± 1(tolerance)) of major eight peaks (a-h). Mass chromatograms of d, e, f, and g are a sum of MCs for their singly- and doubly- protonated ions which are relatively abundant. The inconsistencies between the *m/z* integer values and those expected from the exact mass values in Table 1 are due to both the rounding error of integer calculations and the mass calibration error in a range of higher m/z values.

Figure 2. ZIC-HILIC separation of tryptic peptides of human serum IgG.

(A) UV chromatograms (detection at 220 nm). (B) Accumulated mass spectrum over 0-60 min. (C) Accumulated mass spectrum over 60-100 min. (D) Mass chromatograms of molecular ions $[M+zH]^{n+}$ ($z=2$; m/z ± 1(tolerance)) of major N-glycopeptides of IgG-1 (a-1–h-1) and IgG-2 (a-2–h-2).

Table 1. Summary of structures, annotations, exact mass values, and observed integer mass values of PA N-glycans and major N-glycopeptides from human serum IgG in Figures 1 and 2.

(A) FL Chromatograms



(B) Mass Chromatograms

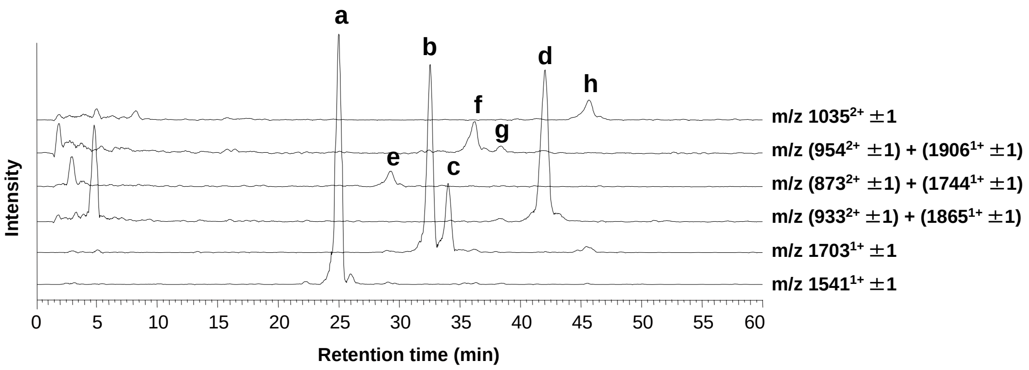
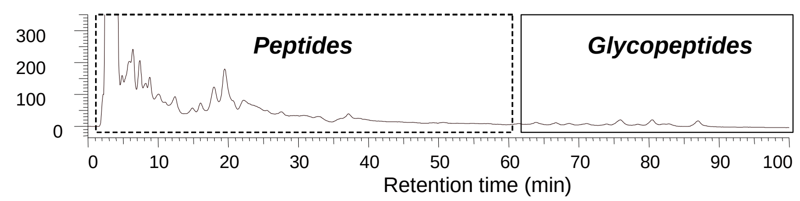
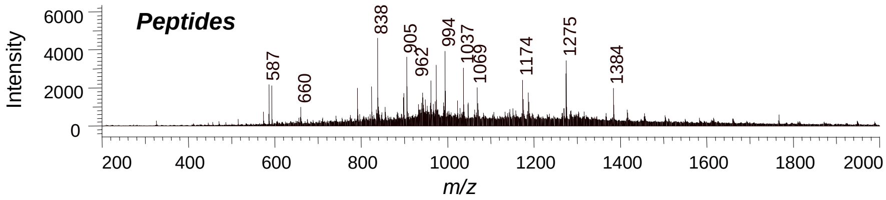


Figure 1.

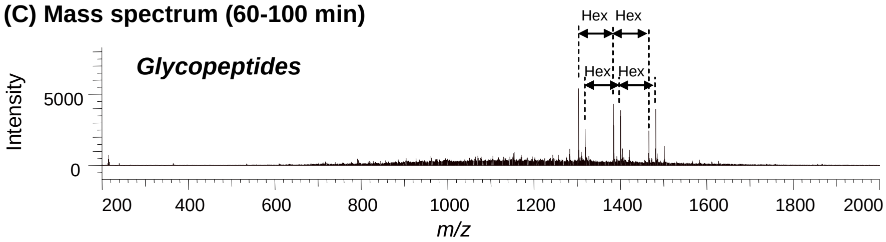
(A) UV chromatogram (220 nm)



(B) Mass spectrum (0-60 min)



(C) Mass spectrum (60-100 min)



(D) Mass chromatograms

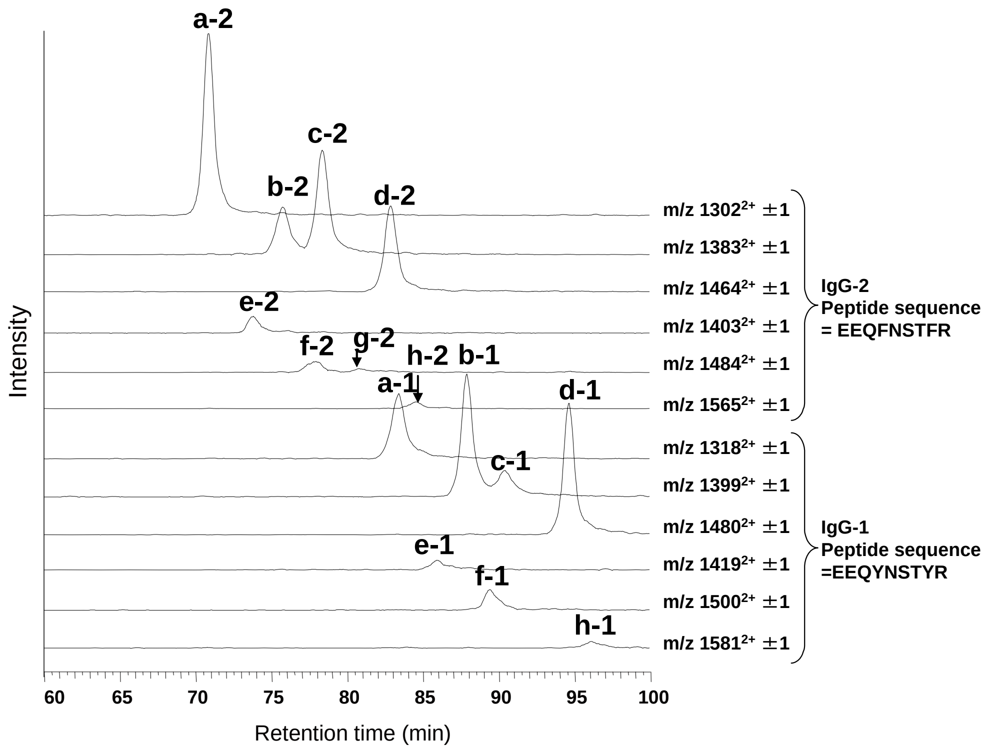
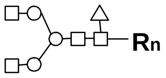
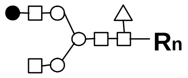
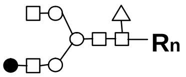
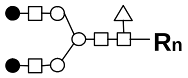
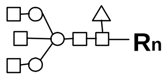
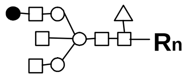
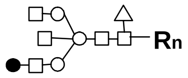
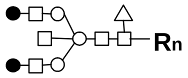


Figure 2.

Structures (n=1-3)	**PA *N*-glycans (R_1 = PA)**	**IgG-1 *N*-glycopeptides (R_2 = EEQYNSTYR)**	**IgG-2 *N*-glycopeptides (R_3= EEQFNSTFR)**
	**a** E.m. 1540.6 Obs. 1541(z=1)	**a-1** E.m. 2633.0 Obs. 1318(z=2)	**a-2** E.m. 2601.1 Obs. 1302(z=2)
 	**b / c** E.m. 1702.7 Obs. 1703(z=1)	**b-1 / c-1** E.m. 2795.1 Obs. 1399(z=2)	**b-2 / c-2** E.m. 2763.1 Obs. 1383(z=2)
	**d** E.m. 1864.7) Obs. 933(z=2),1865(z=1)	**d-1** E.m. 2957.1 Obs. 1480(z=2)	**d-2** E.m. 2925.2 Obs. 1464(z=2)
	**e** E.m. 1743.7 Obs. 873(z=2),1744(z=1)	**e-1** E.m. 2836.1 Obs. 1419(z=2)	**e-2** E.m. 2804.1 Obs. 1403(z=2)
 	**f / g** E.m. 1905.7 Obs. 954(z=2),1906(z=1)	**f-1** E.m. 2998.2 Obs. 1500(z=2)	**f-2 / g-2** E.m. 2966.2 Obs. 1484(z=2)
	**h** E.m. 2067.8 Obs. 1035(z=2)	**h-1** E.m. 3160.2 Obs. 1581(z=2)	**h-2** E.m. 3128.2 Obs. 1565(z=2)

○ : mannose (Man) **□ : *N*-acetyl-glucosamine (GlcNAc)** **Obs. : Observed mass**
● : galactose (Gal) **E.m. : exact mass** **z : number of charges**
△ : fucose (Fuc) **PA : 2-aminopyridine**

Table 1.