Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 850 (2007) 109–119
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous profiling of *N*-glycans and proteins from human serum using a parallel-column system directly coupled to mass spectrometry

Yun-Gon Kim [a], Kyoung-Soon Jang [a], Hwang-Soo Joo [b], Hyun-Ki Kim [a], Chang-Soo Lee [c], Byung-Gee Kim [a,b,*]

[a] *Interdisciplinary Program of Biochemical Engineering and Biotechnology, Seoul National University, Seoul, Republic of Korea*
[b] *School of Chemical and Biological Engineering and Institute of Molecular Biology and Genetics, Seoul National University, 151-742 Seoul, Republic of Korea*
[c] *Department of Chemical and Biological Engineering, Chungnam National University, 305-364 Daejeon, Republic of Korea*

Received 2 October 2006; accepted 9 November 2006
Available online 4 December 2006

Abstract

A method for the rapid identification of proteins and their *N*-glycans was developed through the use of two parallel columns directly connected to a mass spectrometer. Both porous graphitic carbon (PGC) and C18 capillary columns were connected in parallel with two switching valves for the simultaneous analysis of glycans and peptides, respectively. To verify the efficiency of the analytical system, profiling of *N*-glycans and proteins from human serum was demonstrated. This method is suitable for high-throughput analysis and automation, is contamination-free for the identification of *N*-glycans and proteins in a complex biological sample, and can be applied to glycomics and proteomics.

Keywords: Human serum; C18; Porous graphite carbon; Electrospray mass spectrometry

1. Introduction

The majority of proteins in eukaryotic cells are post-translationally modified by glycosylation. The glycosylation of a protein often changes its biological activity, folding, degradation kinetics and pathways of signal transduction by competing with Ser/Thr for phosphorylation [1]. In addition, glycosylation provides another degree of freedom to proteins in their molecular interactions, such as protein–protein and cell–cell interactions. The protein glycoforms are highly diverse due to the number of branches in the sugar tree and the different types of glycosidic linkages (α or β configurations) [2–4], which can be heterogeneous even in a single protein. There are numerous analytical methods for investigating the structure of glycans, including X-ray crystallography [4], nuclear magnetic resonance (NMR) [5], frontal affinity chromatography (FAC) [6], high-performance liquid chromatography (HPLC) coupled with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [7], exoglycosidase array by using mass spectrometry (MS) [8], and fragmentation of glycans by tandem mass spectrometric (MS/MS) methods in combination with matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) [9]. However, in recent proteomics and glycomics, simultaneous analyses of the structure of unknown proteins and their glycosylation patterns from samples of biological mixtures is essential for substantial savings in time and labor.

In-gel deglycosylation followed by HPLC and MALDI-MS was not only able to elucidate *N*-glycan structure, but also identify all glycoproteins simultaneously [7,10]. In this method, *N*-glycans from proteins separated by one-dimensional (1D) or two-dimensional (2D) gel electrophoresis were directly obtained by in-gel deglycosylation using *N*-glycosidase F (PNGase F), and the deglycosylated proteins were identified by in-gel trypsin treatment followed by MALDI-MS mapping. However, this analysis requires at least 3 days to complete and is labor-intensive. Moreover, there is a high risk of contamination by other proteins and sugars during this complicated procedure. More recently, Itoh et al. [11] studied mass spectrometric peptide/glycopeptide mapping by LC–MS^n and SDS-PAGE. Proteins isolated by electrophoresis were digested with trypsin

* Corresponding author. Tel.: +82 2 880 6774; fax: +82 2 874 1206.
E-mail address: byungkim@snu.ac.kr (B.-G. Kim).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.11.012

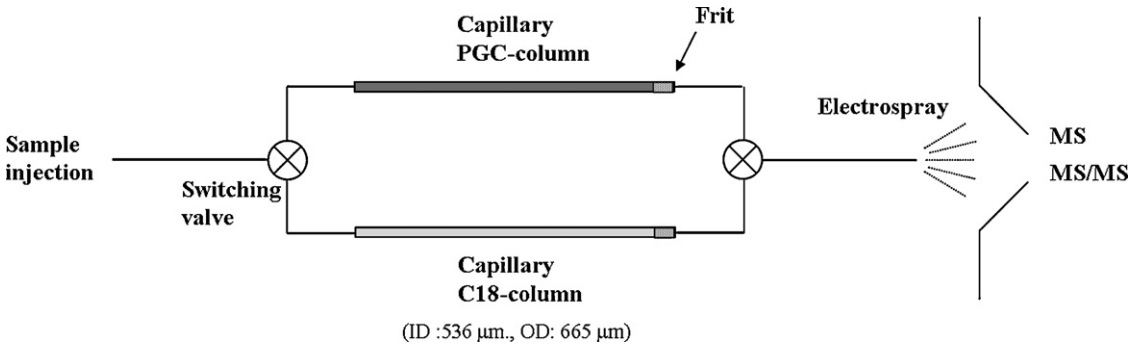


Fig. 1. Schematic diagram of the parallel C18 and porous graphitic carbon column directly connected on to ion trap electrospray mass spectrometry.

and subsequently introduced to collision-induced dissociation (CID)-MS/MS. This analytical method is suitable for the identification of unknown proteins and their site-specific glycosylation, but it might be difficult to get total *N*-glycans from CID-MS/MS method without prior PNGase F treatment.

Herein, a high-throughput method for simultaneously profiling both *N*-glycan and protein was developed with a parallel-column system containing porous graphitic carbon (PGC) [12] and C18 capillary columns, which were directly connected to a mass spectrometric system (Fig. 1). The PGC column, which can separate and clearly characterize heterogeneous oligosaccharides through electron donor–acceptor interactions, is applied to the structural analysis of *N*-glycans in glycoproteins [11,13–17]. In addition, the reverse-phase C18 column is applied to the determination of the structure and sequence of proteins, an application for which it is used extensively. To analyze both *N*-glycans and proteins in a single analysis, we established an automated parallel-column system coupled with ESI-MS and MS/MS and validated the system with a comprehensive analysis of human serum. Total human serum *N*-glycans show the global glycosylation of all serum glycoproteins. Its abnormal glycosylation, which might reflect changes in the glycosylation mechanism, has been studied for the diagnosis or prognosis of various diseases [15,16,18]. Using this high-speed analytical system, 41 structures of *N*-glycans in human serum, as well as serum proteins, were identified and characterized after the removal of abundant albumin and clotting factors.

2. Experimental

2.1. Sample preparation for mass spectrometry

The albumin in human serum (obtained from a healthy male donor, 41 ages) was removed by using SwellGel Blue Albumin Removal Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. Each ovalbumin (45 μl, 30 mg protein/ml, Sigma) as a control glycoprotein and the albumin depleted human serum (5 μl, 52.2 mg protein/ml) were denatured at 90 °C for 10 min. After cooling at room temperature, the protein sample was digested with trypsin (10 μl, 1 μg/μl, sequencing grade, Promega, Madison, WI) at 37 °C for 16 h, and then the reaction was stopped at 90 °C for 10 min. Subsequently, PNGase F (5 unit, 1 unit/μl in 50 mM sodium phosphate buffer, pH 7.4, Roche, Germany) digestion was carried out at 37 °C for 16 h, and the reaction mixture was directly lyophilized. This lyophilized mixture of peptides and glycans was dissolved in double distilled water and used for further mass spectrometric analysis.

2.2. Packing capillary column

The capillary PGC and C18 columns were made in-house by a modified procedure similar to that described by Ackermann et al. [19]. Each slurry (i.e., 30 mg/500 μl) of PGC (<70 μm, Alltech, Deerfield, IL) and C18 (<70 μm, Alltech, Deerfield, IL) in acetonitrile was introduced into a 15 cm-long fused silica capillary tubing (536 μm i.d., 665 μm o.d., Polymicro Technology, Phoenix, AZ, USA) by pressurized N_2 gas, and the final length of the packed column was 10 cm. Prior to the packing, a sintered frit was prepared by soaking the capillary into a mixed solution of potassium silicate (150 μl, KASIL-1, PQ Corp., Valley Forge, PA, USA) and formamide solution (50 μl). The outside of the capillary was wiped out with Kimwipes and baked at 90 °C for at least 2 h. Using the same pressurizing vessel, the capillary with the frit was sequentially washed for three times with 1 M HCl, distilled water and acetonitrile.

2.3. ESI-MS and MS/MS analysis conditions

Mass spectrometric analysis was performed using LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) with atmospheric pressure electrospray source using positive and negative-ion mode at 5 kV of spray voltage. The heated capillary was maintained at 200 °C, and nitrogen was used as a sheath gas at a flow rate of 20 arbitrary units. The ion entrance capillary voltage and tube lens offset were +15 and +30 V for the positive-ion mode and −15 and −30 V for the negative-ion mode, respectively. For the MS^n fragmentation, 35–37% of normalized collision energy and 2.5–3 Da of isolation width were used. The maximum ion collection time was set to 50 ms and three microscans were averaged per scan. Among the mixtures of peptides and *N*-glycans, the *N*-glycans were mainly loaded onto home-made PGC column coupled to an Agilent 1100 Series Capillary LC-System (Agilent Technologies, Palo Alto, CA) by feeding the mixture for 30 min, and the capillary column was washed with deionized water at the flow rate of 5 μl/min for 30 min. The loaded *N*-glycans were eluted isocratically with acetonitrile/water 40:60 (v/v) for 30 min and analyzed

by MS. When switched to C18 capillary column coupled to the Agilent 1100 system, the peptides from the mixtures were selectively loaded to C18 column for 50 min. The peptides were eluted with acetonitrile gradient from 0 to 95% for 60 min after 30 min of washing with deionized water. The expected *N*-glycan structures were determined by both matching intact *N*-glycan masses in GlycoMod (http://us.expasy.org/tools/glycomod/) and sequencing with ESI-MS/MS, and the proteins were identified using the SEQUEST software on human protein database from NCBI. Precursor ion tolerance (1.6 Da), group scan (25), minimum group (1), and minimum ion (25) were used as search parameters for SEQUEST software. Cross correlation score (XCorr) criteria for confidential match used for peptide analysis were 1.6, 2.2, 3.0 for singly-, doubly-, and triply-charged peaks, respectively.

3. Results and discussion

3.1. Design of parallel column system for N-glycan and protein analyses

The primary goal of this study was the simultaneous analyses of both *N*-glycans and proteins in biological samples using the parallel-column system in a single analysis. Compared with separate identification of the *N*-glycan and protein, there are three main advantages for the proposed system: First, once the parallel-column system is directly connected to ESI-MS and MS/MS via two switching valves, the rest of the operation can proceed automatically. Consequently, the total analytical time for simultaneous profiling of both *N*-glycan and protein is reduced remarkably, and the entire procedure is also simplified. Second, the on-line system prevents sample contamination by the external addition of sugars and proteins from our hands or hairs, which might occur during the operation of conventional single column analysis. Finally, this system can be established inexpensively by in-house production of each column. However, a determination of glycosylation sites, which are also important in glycoprotein functional study could not be performed, as a drawback in our system. Thus, the switching parallel-column system is superior for profiling glycosylation patterns of whole mixture proteins, as well as for determining unknown proteins in biological samples.

3.2. Profiling the N-glycans and peptides in a model glycoprotein

To validate the feasibility of the switching parallel-column system for the simultaneous analysis of *N*-glycans and proteins, chicken ovalbumin was chosen as a model glycoprotein because its glycan content is well-known. The mixture of *N*-glycans and peptides from ovalbumin was prepared by serial treatment with PNGase F and trypsin. A sample of the resulting mixture was directly introduced to the ESI-MS system through the parallel-column system, i.e., (1) PGC capillary column for *N*-glycan profiling and (2) C18 capillary column for peptide analysis.

The resulting *N*-glycan profile from the ovalbumin is shown in Fig. 2, including the expected structure of each mass. The 18 intact masses of neutral *N*-glycans released from ovalbumin exactly matched those reported in previous studies [20,21]. Most terminal sites of the antennary structures of the *N*-glycan

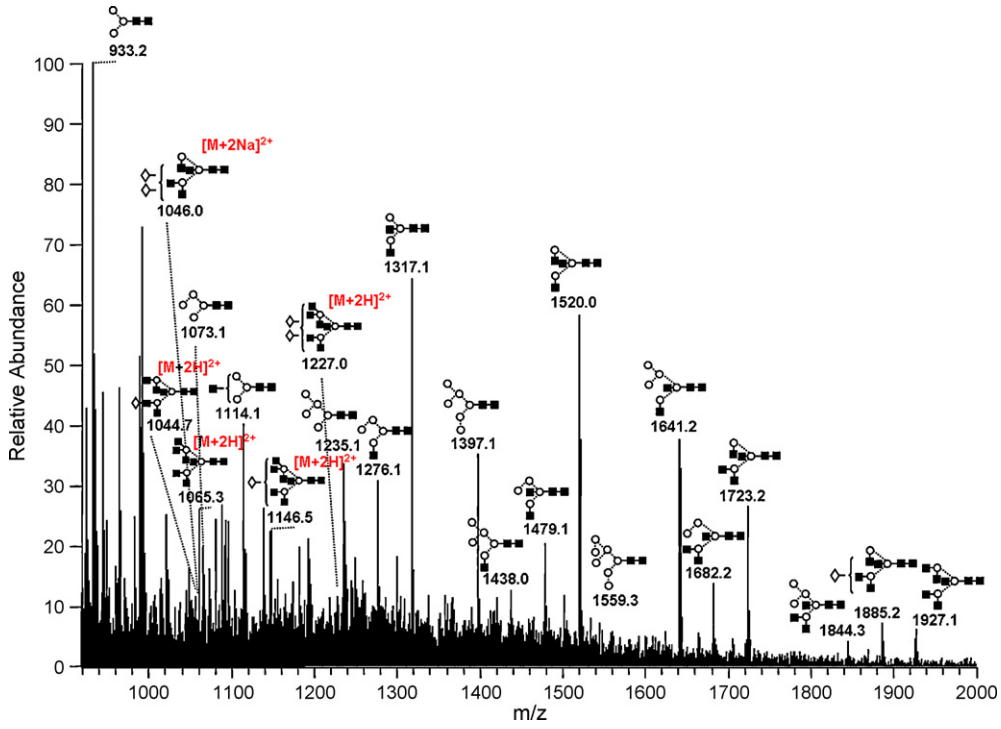


Fig. 2. Positive-ion ESI-MS spectra of the chicken ovalbumin *N*-glycans using PGC capillary column. Key to symbols: filled square (■) GlcNAc; open circle (◯) mannose; open diamond (◇) galactose; open diamond with spot (◈) fucose; solid connecting line β-linkage; broken connecting line α-linkage. Linkage positions are shown by the lines connecting the symbols thus: |: 2-linkage; /: 3-linkage; -: 4-linkage; \: 6-linkage.

Table 1
Summary structures of neutral and sialic acid glycans from human serum by using porous graphitic carbon (PGC) capillary column and ESI-MS and MS/MS

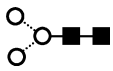
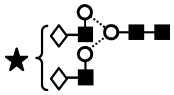
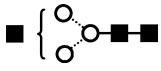
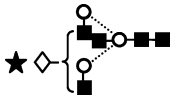
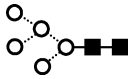
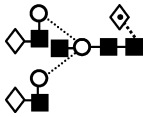
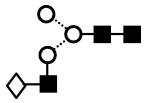
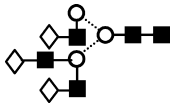
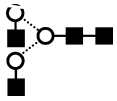
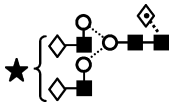
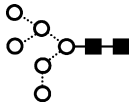
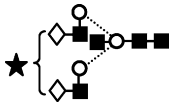
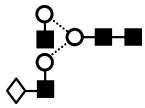
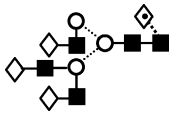
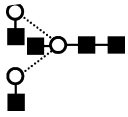
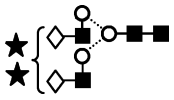
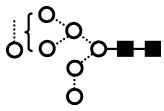
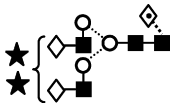
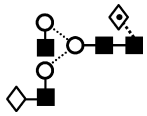
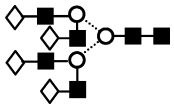
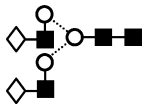
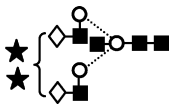
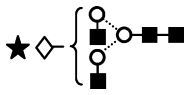
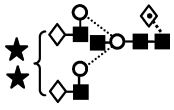
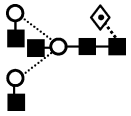
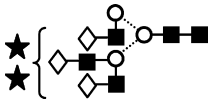
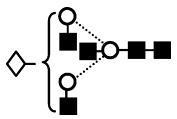
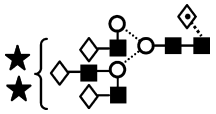
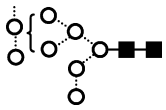
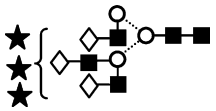
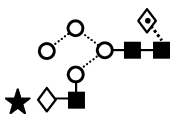
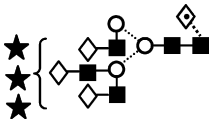
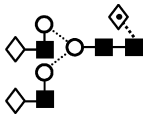
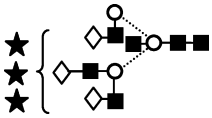
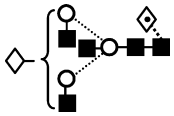
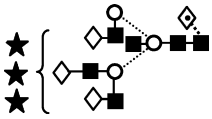
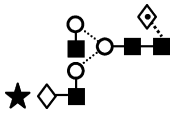
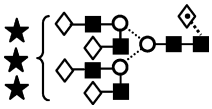
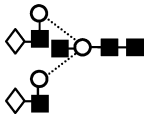
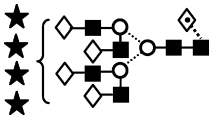
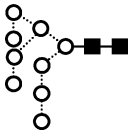
Proposed structure	Mass			Proposed structure	Mass		
	Calc.	Found	Charge		Calc.	Found	Charge
	911.3	911.0	$[M+H]^+$		1952.7	1952.4	$[M+Na-2H]^-$
	1114.4	1114.7	$[M+H]^+$		1993.7	1993.9	$[M+Na-2H]^-$
	1235.4	1235.6	$[M+H]^+$		1017.9	1017.6	$[M+2Na]^{2+}$
	638.7	638.3	$[M+2H]^{2+}$		1025.9	1025.5	$[M+2Na]^{2+}$
	659.2	659.0	$[M+2H]^{2+}$		1037.9	1037.7	$[M-2H]^{2-}$
	721.2	721.3	$[M+2Na]^{2+}$		1066.4	1066.7	$[M-2H]^{2-}$
	1501.5	1501.5	$[M+Na]^+$		1076.9	1077.2	$[M+2H]^{2+}$
	1520.6	1521.0	$[M+H]^+$		1110.4	1110.7	$[M-2H]^{2-}$
	780.3	780.4	$[M+2H]^{2+}$		1183.4	1183.6	$[M-2H]^{2-}$
	835.3	835.2	$[M+2Na]^{2+}$		1186.4	1186.6	$[M+2H]^{2+}$
	821.3	821.5	$[M+2H]^{2+}$		1211.9	1211.6	$[M-2H]^{2-}$
	883.8	883.5	$[M-2H]^{2-}$		1285.0	1285.2	$[M-2H]^{2-}$
	855.8	856.3	$[M+2Na]^{2+}$		1293.0	1293.1	$[M-2H]^{2-}$
	841.8	841.8	$[M+2H]^{2+}$		1366.0	1366.2	$[M-2H]^{2-}$
	1743.6	1743.9	$[M+Na]^+$		1438.5	1438.3	$[M-2H]^{2-}$

Table 1 (*Continued*)

Proposed structure	Mass			Proposed structure	Mass		
	Calc.	Found	Charge		Calc.	Found	Charge
	1874.7	1874.2	$[M-H]^-$		1511.5	1511.4	$[M-2H]^{2-}$
	894.3	894.8	$[M+2H]^{2+}$		1540.0	1540.0	$[M-2H]^{2-}$
	914.8	914.7	$[M+2H]^{2+}$		1613.1	1613.2	$[M-2H]^{2-}$
	1936.7	1936.9	$[M+Na-2H]^-$		1694.1	1694.0	$[M-2H]^{2-}$
	922.8	923.2	$[M+2H]^{2+}$		1839.7	1839.7	$[M-2H]^{2-}$
	942.3	942.0	$[M+2H]^{2+}$				

originating from avian protein are *N*-acetyl-galactosamine (GlcNAc) residues, which correlated well with our findings as shown in Fig. 2. In addition, none of the detected *N*-glycan peaks were interrupted by any peptides from ovalbumin, which was attributed to the different retention times for peptides and glycans in the PGC column. The PGC capillary column appears to selectively retain most of the *N*-glycans of ovalbumin, as well as to purify possible contaminants from the sample mixture. In addition, the glycoprotein itself was successfully identified by peptide mapping and using the SEQUEST database with high cross-correlation scores between a predicted peptide spectrum and an experimental spectrum (XCorr > 1.6, 2.2, 3.0 for singly-, doubly- and triply-charged peaks, respectively). In the positive mode of ESI-MS/MS, the following doubly-charged peaks were representatively identified with high XCorr: *m/z* 2010.1 (REVVGSAEAGVDAASVSEEFRA), 1860.1 (RELINSWVESQTNGIIRN), 1774.9 (KISQAVHAAHAEINEAGRE), 1688.8 (RGGLEPINFQTAADQARE) (data not shown). The results support that this parallel-column system is sensitive and convenient for protein identification, as well as profiling of glycosylation.

3.3. Profiling of N-glycans from human serum by PGC capillary column connected to ESI-MS and MS/MS

N-glycan profiling of the albumin-depleted human serum was carried out with a PGC capillary column directly connected to ESI-MS and MS/MS. First, the mixture of peptides and *N*-glycans was introduced into the PGC capillary column for the glycosylation profiling of human serum proteins. During the loading and washing stages for the sample, we did not detect any *N*-glycan peaks for 30 min; they were subsequently released for 60 min by 40% acetonitrile. In order to sequence both neutral and sialylated *N*-glycans, positive- and negative-mode ESI-MS and MS/MS were performed without additional derivatization. Table 1 shows all of the proposed neutral and sialylated glycans. Recently, Callewaert et al. reported 11 desialylated oligosaccharides using DNA sequencer-based total serum protein glycomics, and Morelle et al. identified a total of 26 *N*-glycan structures from total serum based on direct release of serum *N*-glycans by solid-phase extraction on graphitized carbon adsorbent and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis [15,16]. In this study, we elucidated 41 *N*-glycans, including high-mannose-types, desialylated fucosyl complex-types, sialylated complex-types and sialylated fucosyl complex-types, from total human serum with the PGC capillary column portion of our parallel-column system. Interestingly, high-mannose-type glycans were not reported in the previous studies with total human serum. Thus, our MS system based on the positive and negative MS analysis is well qualified for sensitive and rapid *N*-glycan profiling. In addition, most of the *N*-glycans from human serum were doubly-charged forms. Although no specific (α- or β-) linkage of the *N*-glycans was identified by mass spectrometry, this method could provide information on *N*-glycan composition in human serum.

To allow further characterization of the *N*-glycan structures, collision-induced MS/MS fragmentation was performed in the negative mode for structural identification of the glycans. Daughter spectra of doubly-charged parent ions in the form of $[M-2H]^{2-}$ of disialylated *N*-glycans showed the detailed sequences and confirmed the intact mass data (Fig. 3). All of the spectra of the disialylated *N*-glycans contain B_3 and B_4 fragment ions (nomenclature according to that proposed by Domon and Costello [22]) at *m/z* 665 and 817, having the composition $NeuAc_1$-Hex_1-$HexNAc_1$, which represents sialylated *N*-glycans. The major peak at *m/z* 1133.2 in Fig. 3(b), rather than the peak at *m/z* 1060.2 in Fig. 3(a), was interpreted as an ion of the core-fucosylated biantennary *N*-glycan with sialic acid residues. In addition, the existence of the B_6 ion at *m/z* 1101.8 and the $^{0,2}A_7$ ion at *m/z* 1234.8, as shown in Fig. 3(c), indicated that the *N*-glycan was bisected by GlcNAc and core-fucosylated by fucose. The fucose residue was not contained in the B_6 ion, but was in $^{0,2}A_7$, confirming that it was located on the core GlcNAc linked to asparagine residues. Therefore, the fragmentation profile of the sialylated biantennary glycans was established by the PGC capillary column connected to ESI-MS/MS and provided trustworthy structural information of *N*-glycans from human serum.

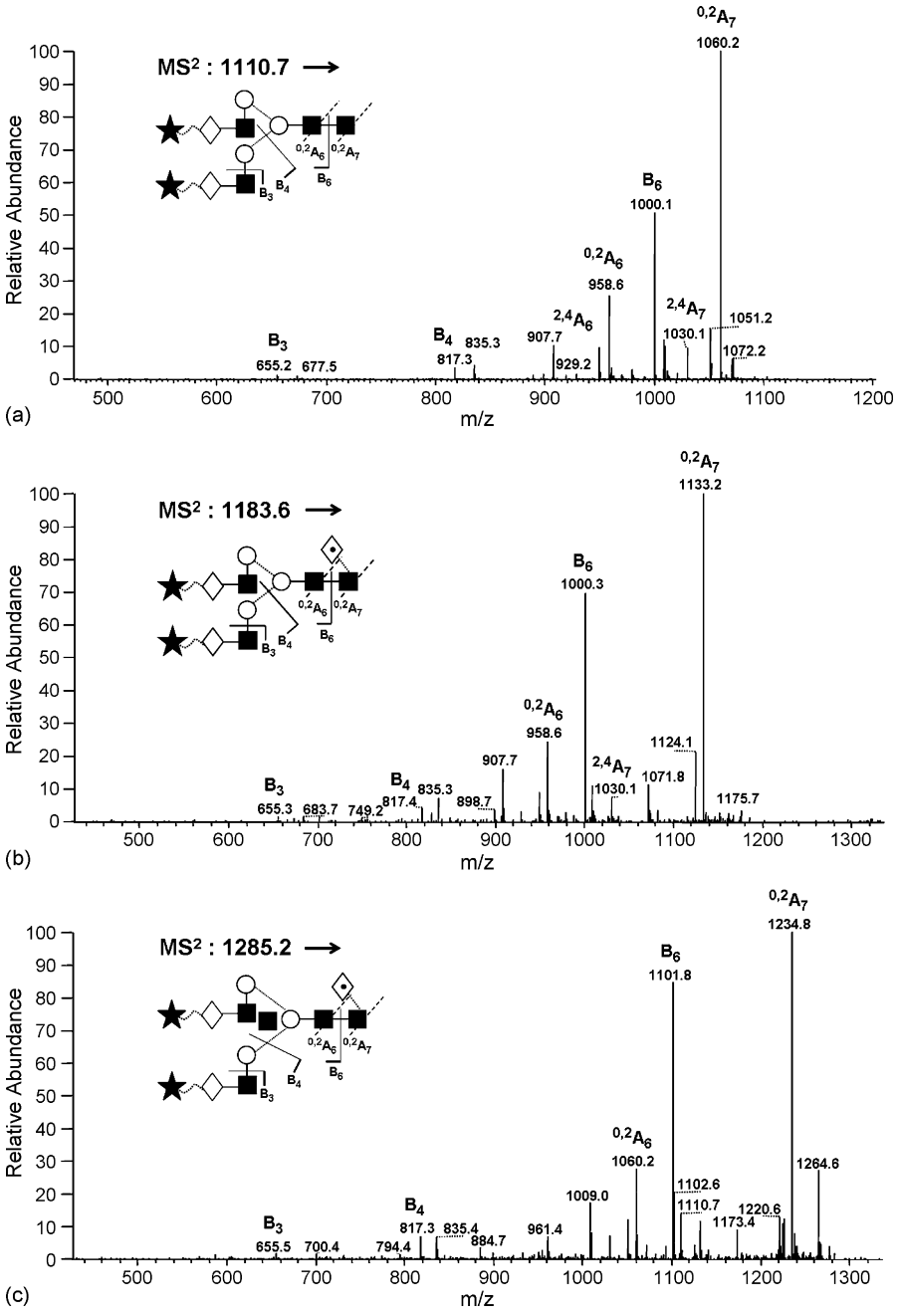


Fig. 3. Negative ion MS/MS spectra of the sialylated biantennary *N*-glycans ($[M-2H]^{2-}$) obtained from human serum. (a) MS^2 of *m/z* 1110.7; (b) MS^2 of *m/z* 1183.6; (c) MS^2 of *m/z* 1285.2.

Table 2
Mass spectrometric analysis of the albumin depleted human serum proteome

Protein	Max. XCorr	Del CN	Charge	Filtered IDs for Sequence
Ig gamma (-1, -2, -3, -4) chain C region HLA class I histocompatibility antigen				
KGFYPSDIAVEWESNGQPENNYKT	5.88	0.68	2	5
KFNWYVDGVEVHNAKT	4.83	0.64	2	5
KTTPPVLDSDGSFFLYSKL	3.79	0.63	2	4
KGPSVFPLAPSSKS	2.81	0.52	1	4
KDTLMISRT	2.33	0.39	2	2
REPQVYTLPPSRDELTKN	2.34	0.41	2	1
KALPAPIEKT	1.72	0.40	1	1
Human apolipoprotein A				
KVSFLSALEEYTKK	4.05	0.56	2	6
REQLGPVTQEFWDNLEKE	4.08	0.63	2	2
KLLDNWDSVTSTFSKL	3.30	0.56	2	2
KATEHLSTLSEKA	2.10	0.38	1	2
RAHVDALRT	1.76	0.29	1	2
KLREQLGPVTQEFWDNLEKE	3.35	0.62	2	1
KVQPYLDDFQKK	1.63	0.12	1	1
RAELQEGARQ	2.67	0.23	2	1
KLHELQEKL	2.11	0.18	1	1
Transferrin				
KIMNGEADAMSLDGGFVYIAGKC	5.34	0.65	2	4
RTAGWNIPMGLLYNKI	3.83	0.52	2	3
KNGAGDVAFVKH	2.79	0.52	2	2
KYLGEEYVKA	1.68	0.29	1	1
KGDVAFVKH	1.69	0.37	1	1
Paraoxonase/arylesterase				
RIQNILTEEPKV	3.45	0.35	2	10
Alpha-2HS-glycoprotein				
RVKDLATVYVDVLKD	5.44	0.70	2	10
Complement component 3				
KVQLSNDFDEYIMAIEQTIKS	4.70	0.61	2	4
RVPVAVQGEDTVQSLTQGDGVAKL	5.20	0.74	2	3
RILLQGTPVAQMTEDAVDAERL	3.93	0.52	2	1
RSVQLTEKR	1.80	0.26	1	1
Ig kappa light chain VLJ region				
RTVAAPSVFIFPPSNEQLKS	3.02	0.48	2	8
Ig mu chain				
KQVGSGVTTDQVQAEAKE	3.96	0.64	2	8
Hypothetical protein				
RTPGAAANLELIFVGPQHAGNYRC	3.41	0.61	2	6
RSWVPHTFESELSDPVELLVAES	2.80	0.47	2	1
Serine (or cysteine) proteinase inhibitor				
KYLGNATAIFFLPDEGKL	4.88	0.69	2	3
KVFSNGADLSGVTEEAPLKL	5.70	0.64	2	2
RTLNQPDSQLQLTTGNGLFLSEGLKL	5.22	0.72	2	1
KGTEAAGAMFLEAIPMSIPPEVKF	4.33	0.61	2	1
Human apolipoprotein B-100				
RNNALDFVTKS	2.74	0.50	2	3
RLTLDIQNKK	2.46	0.38	2	1
KVNWEEEAASGLLTSLKD	4.18	0.66	2	1
Clusterin				
KTLLSNLEEAKK	2.85	0.48	2	2
RVTTVASHTSDSDVPSGVTEVVVKL	2.40	0.50	2	1
RASSIIDELFQDRF	4.01	0.58	2	1
KLFDSDPITVTVPVEVSRK	2.52	0.53	2	1
Human apolipoprotein C-2				
KSTAAMSTYTGIFTDQVLSVLKGEE	3.98	0.67	2	5

Table 2 (*Continued*)

Protein	Max. XCorr	Del CN	Charge	Filtered IDs for Sequence
Human apolipoprotein A-4				
KLGEVNTYAGDLQKK	4.54	0.58	2	2
KALVQQMEQLRT	3.40	0.50	2	1
KSLAELGGHLDQQVEEFRR	5.51	0.67	2	1
Ig lambda light chain VLJ region				
KYAASSYLSLTPEQWKS	4.60	0.73	2	3
KVTVLGQPKA	1.83	0.30	1	1
Ig heavy chain variable region				
KTTPPMLDSDGSFFLYSKL	3.02	0.59	2	4
Ribosomal protein L24				
KAAPKQKIVKP	1.99	0.31	1	3
Hemoglobin beta subunit				
RFFESFGDLSTPDAVMGNPKV	3.45	0.56	2	3
Amyloid beta A4 precursor protein-binding family A				
KSLNLPPEVKH	1.92	0.36	1	3
Ig alpha-1 chain C region				
RQEPSQGTTTFAVTSILRV	2.95	0.57	2	2
Human apolipoprotein A-2				
KDLMEKV	1.62	0.30	1	1
KSPELQAEAKS	2.18	0.39	1	1
Keratin 2				
RFLEQQNQVLQTKW	4.61	0.49	2	2
Alpha 1B-glycoprotein				
KLLELTGPKS	2.17	0.30	1	1
RLHDNQNGWSGDSAPVELILSDETLPAPEFSPEPESGRA	3.43	0.52	3	1
Lumican				
RLKEDAVSAAFKG	3.13	0.44	2	2
Inter-alpha-trypsin inhibitor heavy chain				
RGPDVLTATVSGKL	3.09	0.51	2	1
KNVVFVIDKS	1.16	0.47	1	1
Ig light chain variable region				
NIQMTQSPSSLSASVGDRV	5.46	0.40	2	1
Inter-alpha (globulin) inhibitor H3				
KEHLVQATPENLQEART	3.49	0.58	2	1
Keratin 1				
RSLDLDSIIAEVKAQYEDIAQKS	3.21	0.58	2	1
Alpha-2 macroglobulin				
RSSGSLLNNAIKG	2.59	0.37	2	1
RSASNMAIVDVKM	3.49	0.41	2	1
Human apolipoprotein D				
KKAGTELVNFLSYFVELGTQPATQ	3.46	0.62	2	1
Human apolipoprotein A-1				
RDYVSQFEGSALGKQ	3.15	0.62	1	1
Human apolipoprotein C-3				
KDALSSVQESQVAQQARG	4.07	0.54	2	1
Haptoglobin				
KVVLHPNYSQVDIGLIKL	3.97	0.60	2	1
Ig kappa light chain variable region				
RFSGSGSGTNFTLKI	3.19	0.57	2	1
Phosphoinositide-3-kinase				
RTWYVGKINRTQAEEMLSGKR	3.02	0.16	2	1
Ig kappa chain V-IV region				
-DIVMTQSPNSLAVSLGERA	2.76	0.51	2	1

Table 2 (*Continued*)

Protein	Max. XCorr	Del CN	Charge	Filtered IDs for Sequence
Leucine-rich alpha-2 glycoprotein RTLDLGENQLETLPPDLLRG	2.63	0.46	2	1
Human apolipoprotein E KVQAAVGTSAAPVPSDNH-	2.61	0.63	2	1
Vitamin D-binding protein KLPEATPTELAKL	2.28	0.47	2	1
Heparin cofactor II KGGETAQSADPQWEQLNNKN	2.63	0.57	2	1
Ig kappa light chain V-II region MIL RFSGSGSGTBFTLKI	2.26	0.49	2	1
Mannosyltransferase KKNVTQEAENRW	2.25	0.28	2	1
Delta globin KVLGAFSDGLAHLDNLKG	2.59	0.44	2	1
ALL-1 protein RTKGHIHDKK	2.01	0.32	1	1
Proteasome 26S ATPase subunit 2 RGNIKVLMATNRP	1.88	0.12	1	1
Myosin heavy chain nonmuscle form A KSVHELEKS	1.87	0.16	1	1
Interferon regulatory factor-7H KAWAVARG	1.78	0.32	1	1
ATP-binding cassette transporter 1 KQMKKHLKL	1.75	0.26	1	1
Myosin heavy polypeptide 13, skeletal muscle RAKIEKQRS	1.69	0.09	1	1
Single-strand DNA endonuclease KYIEYLNL-	1.69	0.24	1	1
Antithrombin RFATTFYQHLADSKNDNDNIFLSPLSISTAFAMTKL	4.98	0.63	3	1
Synapsin-3 KFPLVEQTFFPNHKPMVTAPHFPVVVKLGHAHAGMGKI	3.35	0.41	3	1
Complement C4-B RTLEIPGNSDPNMIPDGDFNSYVRV	2	3.23	2	1
Probable G-protein coupled receptor 125 RSLEFQTEYLLCDCNILWMHRWVKEKN	3.17	0.32	3	1
Angiotensionogen RTIHLTMPQLVLQGSYDLQDLLAQAELPAILHTELNLQKL	3.10	0.50	3	1
Insulin-like growth factor II receptor RYASACQMKYEKDQGSFTEVVSISNLGMAKT	3.04	0.28	3	1
Serine/threonine-protein kinase RIO3 KLNSEEMKEAYYQTLHLMRQ	2.71	0.26	2	1
Elongin C (transcription elongation factor B) KAMLSGPGQFAENETNEVNFRE	2.45	0.36	2	1
Alpha-1-antitrypsin KITPNLAEFAFSLYRQ	2.56	0.53	2	1
Fibrinogen KTFPGFFSPMLGEFVSETESRG	2.39	0.64	2	1
Beta platelet-derived growth factor receptor KIMSHLGPHLNVVNLLGACTKG	2.35	0.31	2	1
SHBG related protein (AA 1-32) KGSPAVLFKLTYAVITCFSLRL	2.32	0.19	2	1

Table 2 (*Continued*)

Protein	Max. XCorr	Del CN	Charge	Filtered IDs for Sequence
Unnamed protein product_1 RPFITLLPATTLMSLTDSKQ	2.30	0.40	2	1
Nebulin KPMLDFETPTYITAKESQQMQSGKE	2.30	0.31	2	1
Tripartite motif protein 22 RRLTGSSVEMLQDVIDVMKRS	2.26	0.38	2	1
Cytochrome P450 2C8 KEFPNPNIFDPGLFLDKNGNFKKS	2.25	0.26	2	1
Ig heavy chain V-I region V35 KISCKPSGYTFTEYTIHWVKL	2.22	0.21	2	1
Protein transport protein Sec61 alpha subunit isoform 2 RGTLMELGISPIVTSGLIMQLLAGAKI	2.22	0.29	2	1
Unnamed protein product_2 KNPSIKSAEQLTTFLKH	2.21	0.30	2	1
Ceruloplamin KNNEGTYYSPNYNPQSRS	3.76	0.59	2	1
Keratin 10 RNVQALEIELQSQLALKQ	3.15	0.62	2	1
Cytochrome c oxidase polypeptide Vb RLLRGAGTLAAQALRARG	1.80	0.31	1	1
Vacuolar sorting protein 4a/4b KEALKEAVILPVKF	1.75	0.39	1	1
TP53-target gene 5 protein RTVLKNLSLLKL	1.74	0.21	1	1
NKG2D ligand 4 RDLRMLLCDIKP	1.90	0.36	1	1
Nyctalopin RLAELRLAHNGDLRY	1.66	0.28	1	1
Myosin-9B REPPARR	1.62	0.25	1	1
Hydroxymethylglutaryl-CoA lyase RVKIVEVGPRD	1.73	0.22	1	1
EPSTI 1 protein KLQKMKDEQHQKS	1.86	0.29	1	1

Note. Max. XCorr is the highest XCorr value of the identified peptide. DelCN is the difference in magnitude between the peptide fit with the highest Xcorr and the peptide fit with the second best Xcorr. The number of identification (IDs) for sequence was filtered by criteria of XCorr > 1.6, 2.2, 3.0 for singly-, doubly-, and triply-charged peaks, respectively.

3.4. Characterization of various proteins from human serum by C18 capillary column connected to ESI-MS and MS/MS

Using the C18 capillary column coupled with ion-trap mass spectrometry, we identified 84 proteins in the albumin-depleted human serum; these proteins are listed in Table 2. Most of the immunoglobulins, transferrin, and apolipoproteins in human serum were identified by this system and ranked as high-abundance proteins according to Xcorr value. In addition, numerous low-abundance proteins that are critical for signaling cascades and regulatory events were effectively identified without any keratin contamination, which is often a major concern in proteomic analysis. Therefore, the results proved that the parallel-column system can extensively identify and characterize high- and low-abundance proteins from real biological samples in a contamination-free system.

In previous investigations, proteomic characterization of human plasma containing clotting factors has mainly been performed by 2D PAGE, and only 58 proteins have been reported [23]. The 2D PAGE methods are time- and labor-intensive processes that have exhibited limited recoveries from highly complex mixtures of proteins. More recently, Adkins et al. [24] have reported the immunoglobulin-depleted human serum proteome by on-line reversed-phase microcapillary LC coupled with ion-trap MS; the removal of immunoglobulins resulted in better recovery of low-abundance proteins that exist in human serum. However, the immunoglobulin is essential to better understanding total human serum glycosylation. The importance of the immunoglobulin glycosylation has already been demonstrated

in studies of rheumatoid arthritis and other immune reactions [25,26]. Therefore, in the present study, we depleted the serum of only human serum albumin, which is a globular nonglycosylated serum protein that comprises about one-half of the total serum protein. Consequently, we identified many more *N*-glycans from total human serum, and a large number of proteins were detected compared to the previous 2D PAGE method [27]. As the next step in our research, the optimization of the analytical procedure and sample preparation is now in progress to increase the number of low-abundance proteins identified. As a next step of our research, the optimization of the analytical procedure for identifying low-abundance proteins without loss of *N*-glycan list is now under way by using depletion kits of the high-abundance proteins.

4. Conclusion

We have presented rapid profiling of both *N*-glycans and proteins in human serum using two analytical columns connected in parallel to ESI-MS. The mass spectra of 41 *N*-glycans and 84 proteins from human serum were interpreted, and we listed the identified proteins and the structures of *N*-glycans obtained from human serum. The use of the parallel-column system with on-line ESI-MS allows us to avoid contamination during the sample preparation and remarkably economizes the total analysis time and cost for analyzing a biological mixture. This system can be applied not only to the identification of unknown proteins, but also to the profiling of their post-translational modifications. Furthermore, this high-throughput analytical method can be utilized for disease monitoring, early diagnosis and biomarker discovery.

Acknowledgments

This work was supported by the ERC program of MOST/KOSEF (R11-2000-075-03001-0) and National R & D Program Grant of The Ministry of Science & Technology (M10417060004-04N1706-00410).

References

[1] A. Varki, R. Cummings, J.D. Esko, H. Freeze, G. Hart, J. Marth, Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999.
[2] R.A. Dwek, C.J. Edge, D.J. Harvey, M.R. Wormald, R.B. Parekh, Annu. Rev. Biochem. 62 (1993) 65.
[3] P.M. Rudd, R.A. Dwek, Crit. Rev. Biochem. Mol. Biol. 32 (1997) 1.
[4] R.J. Woods, Curr. Opin. Struct. Biol. 5 (1995) 591.
[5] E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson, H. Van Halbeek, J. Biol. Chem. 263 (1988) 18253.
[6] H. Kaji, H. Saito, Y. Yamauchi, T. Shinkawa, M. Taoka, J. Hirabayashi, K. Kasai, N. Takahashi, T. Isobe, Nat. Biotechnol 21 (2003) 667.
[7] B. Kuster, S.F. Wheeler, A.P. Hunter, R.A. Dwek, D.J. Harvey, Anal. Biochem. 250 (1997) 82.
[8] Y. Zhao, S.B. Kent, B.T. Chait, Proc. Natl. Acad. Sci. U S A 94 (1997) 1629.
[9] D.J. Harvey, Expert Rev. Proteomics 2 (2005) 87.
[10] P.M. Rudd, C. Colominas, L. Royle, N. Murphy, E. Hart, A.H. Merry, H.F. Hebestreit, R.A. Dwek, Proteomics 1 (2001) 285.
[11] S. Itoh, N. Kawasaki, A. Harazono, N. Hashii, Y. Matsuishi, T. Kawanishi, T. Hayakawa, J. Chromatogr. A 1094 (2005) 105.
[12] J.Q. Fan, A. Kondo, I. Kato, Y.C. Lee, Anal. Biochem. 219 (1994) 224.
[13] N. Kawasaki, M. Ohta, S. Hyuga, O. Hashimoto, T. Hayakawa, Anal. Biochem. 269 (1999) 297.
[14] N. Kawasaki, M. Ohta, S. Hyuga, M. Hyuga, T. Hayakawa, Anal. Biochem. 285 (2000) 82.
[15] N. Callewaert, H. Van Vlierberghe, A. Van Hecke, W. Laroy, J. Delanghe, R. Contreras, Nat. Med. 10 (2004) 429.
[16] W. Morelle, C. Flahaut, J.C. Michalski, A. Louvet, P. Mathurin, A. Klein, Glycobiology 16 (2006) 281.
[17] H.J. An, T.R. Peavy, J.L. Hedrick, C.B. Lebrilla, Anal. Chem. 75 (2003) 5628.
[18] T.M. Block, M.A. Comunale, M. Lowman, L.F. Steel, P.R. Romano, C. Fimmel, B.C. Tennant, W.T. London, A.A. Evans, B.S. Blumberg, R.A. Dwek, T.S. Mattu, A.S. Mehta, Proc. Natl. Acad. Sci. U S A 102 (2005) 779.
[19] B.L. Ackermann, T.A. Gillespie, B.T. Regg, K.F. Austin, J.E. Coutant, J. Mass Spectrom. 31 (1996) 681.
[20] D.J. Harvey, D.R. Wing, B. Kuster, I.B. Wilson, J. Am. Soc. Mass Spectrom. 11 (2000) 564.
[21] M.L. Corradi Da Silva, H.J. Stubbs, T. Tamura, K.G. Rice, Arch. Biochem. Biophys. 318 (1995) 465.
[22] B. Domon, C.E. Costello, Glycoconjug. J. 5 (1988) 397.
[23] L. Anderson, N.G. Anderson, Proc. Natl. Acad. Sci. U S A 74 (1977) 5421.
[24] J.N. Adkins, S.M. Varnum, K.J. Auberry, R.J. Moore, N.H. Angell, R.D. Smith, D.L. Springer, J.G. Pounds, Mol. Cell Proteomics 1 (2002) 947.
[25] R.B. Parekh, R.A. Dwek, B.J. Sutton, D.L. Fernandes, A. Leung, D. Stanworth, T.W. Rademacher, T. Mizuochi, T. Taniguchi, K. Matsuta, et al., Nature 316 (1985) 452.
[26] C. Basset, V. Durand, C. Jamin, J. Clement, Y. Pennec, P. Youinou, M. Dueymes, I.M. Roitt, Scand. J. Immunol. 51 (2000) 300.
[27] P.P. Minghetti, D.E. Ruffner, W.J. Kuang, O.E. Dennison, J.W. Hawkins, W.G. Beattie, A. Dugaiczyk, J. Biol. Chem. 261 (1986) 6747.