Glycoproteomic Reactor for Human Plasma

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We describe the development of a glycoproteomic reactor that combines multiple biochemical and chemical protein processing into a single device for the study of N-glycosylated proteins. The glycoproteins are first enriched by concanavalin A affinity chromatography and then transferred onto and efficiently processed in the glycoproteomic reactor. This glycoproteomic reactor combines protein concentration and purification, disulfide bond reduction, peptide-N-glycosidase-mediated ^{18}O-labeling and deglycosylation, alkylation, tryptic digestion and pH based fractionation in a device that has an interstitial volume (reaction volume) of $\sim$1 μL. We demonstrated the potential of the glycoproteomic reactor using human plasma. Under stringent criteria, 82 unique glycopeptides representing 41 unique glycoproteins were identified from as little as 5 μL of human plasma. Our glycoproteomic reactor reduces the sample processing time to less than 1.5 h, reduces the reagent consumption while providing over 1000-fold concentration of the sample, provides efficient removal of high concentration of glycan buffer, and, finally, allows both glycopeptides and nonglycosylated tryptic peptides to be analyzed by the mass spectrometer which provides much greater protein coverage and more reliable identifications.

Keywords: glycoproteins • mass spectrometry • proteomics • plasma • proteomic reactor • microscale

Introduction

Protein glycosylation plays a fundamental role in biological processes[1] such as in the immune system,[2] in cellular regulation processes,[3] and in protein secretion. Changes in protein glycosylation have also been associated with the progression of disease,[4–6] the development of various cancers,[7,8] and in other diseases.[2,9] Although there is still limited knowledge of protein glycosylation, the Swiss-Prot database predicts that over 50% of all proteins should be glycosylated.[10] Protein glycosylation can be divided into two main groups based on the type of linkage to the protein: the N-glycosylation and O-glycosylation.[11,12] In N-glycosylation, the oligosaccharides are covalently attached to asparagine residues via an N-acetylglucosamine (GlcNAc) residue at specific regions of the protein that have the consensus sequence Asn-Xxx-Ser(Thr) where Xxx can be any amino acid except proline.[12,13] The N-linked glycosylation is prevalent in protein sorting and secretion into extracellular environments[14] and has been touted as a potential source of biomarkers, while O-glycosylated proteins are rarely present in the blood.

Human plasma is one of the most widely used biological fluids for biomarker assays. Despite its extensive use in clinical laboratories, human plasma is a challenge for the discovery of novel candidate biomarkers because of the order of magnitude 10 in protein concentrations. Essentially, a small number of proteins constitute the bulk of the plasma protein mass; for example, albumin constitutes 57–71% of total serum protein.[15] This is further complicated by the heterogeneity of many plasma proteins caused by different modifications including glycosylation.[16] One strategy for identifying potential biomarkers is to use the presence of post-translational modifications, such as glycosylation, as tethers to purify a subgroup of proteins.

Although the term 'glycoproteomics'[17] has been coined for the study of the ensemble of glycoproteins, this area of research is limited by a lack of technology for characterization of glycoproteins. Currently, there are two major methods available for identifying the site of N-linked glycosylation. One is based on conjugating glycoproteins on a solid support using hydrazide chemistry, followed by the specific cleavage of the peptide from the sugar using peptide-N-glycosidase F (PNGase F).[18–20] The cleavage of the glycoprotein/peptide by Peptide-N-glycosidase F converts N-linked glycosylated asparagines (Asn) residues into aspartic acids (Asp). This change in mass of 0.9840 Da can be detected by mass spectrometry.[22] Unfortunately, the same results can occur due to the frequent and random nonenzymatic deamidation of asparagines to aspartic

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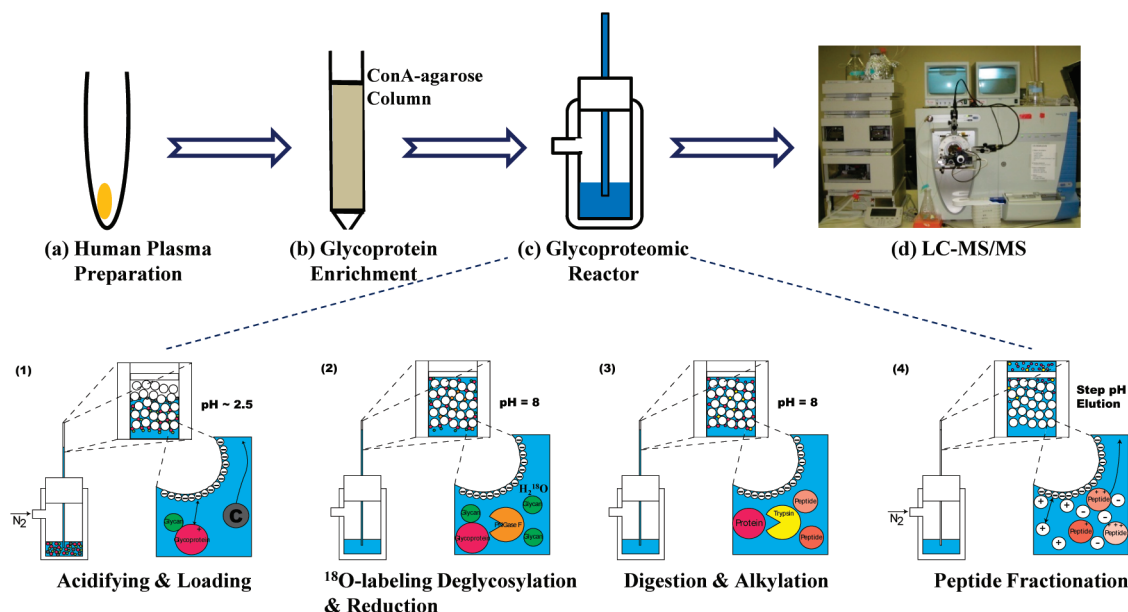|| Dalian Institute of Chemical Physics.

Figure 1. Flowchart of glycoproteomic reactor for plasma sample. (a) Human plasma sample preparation: human plasma was filtered through 0.2 μm filter, then diluted in equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$ and 1 mM CaCl$_2$, pH 7.4). (b) Glycoprotein enrichment: the diluted plasma sample was loaded onto the ConA-agarose column. After washing by equilibration buffer and water, the glycoproteins were eluted by elution buffer (1 M α-D-mannopyranoside). (c) Glycoproteomic reactor: (1) the eluant from ConA column was acidified by adding formic acid and loaded onto the glycoproteomic reactor; (2) after washing and drying, the deglycosylation buffer (50 mM dithiothreitol, 20 mM ammonium bicarbonate and 2 unit/μL PNGase F in ^{18}O water, pH 8.0) was introduced onto the reactor, and the proteins present on the reactor were subjected to ^{18}O-labeling deglycosylation and disulfide bond reduction; (3) after washing and drying, the digestion buffer (10 mM iodoacetamide, 20 mM ammonium bicarbonate and 2 μg/μL trypsin, pH 8.0) was introduced onto the reactor to perform alkylation and tryptic digestion; (4) the resulting peptides were then fractionated with step pH buffers (pH 3.0, 5.0, 6.0, 8.0 and 12, 100 μL, respectively). (d) The peptide fractions from the glycoproteomic reactor were analyzed by LC-MS/MS.

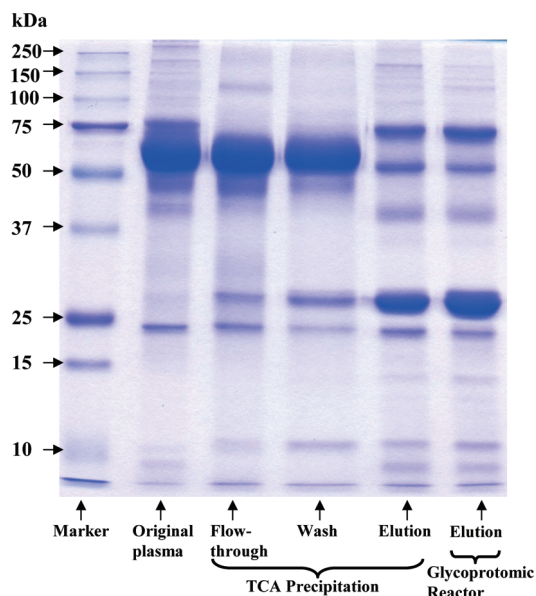**Figure 2.** Colloidal blue-stained gel of original plasma sample and sample from ConA affinity chromatography. Plasma sample (10 μg), flow-through, wash and elution fractions from ConA column using TCA/acetone precipitation (10 μg each) and the elution fraction from ConA column using the glycoproteomic reactor (10 μg) were loaded on the 7-cm 12% SDS-PAGE, then the gel was staining by Colloidal blue.

acid during sample handling.[21] Therefore, the observation of a mass shift of 0.9840 Da does not conclusively identify a site of N-glycosylation.[21,22] The second method uses lectin column-mediated affinity to capture a set of glycopeptides generated by tryptic digestion of the protein mixtures. This is then followed by a PNGase F treatment in the presence of ^{18}O water which leads to a mass shift of +3.0121 Da from the incorporation of the ^{18}O isotope into the newly formed Asp.[21] This mass shift is more readily detected by mass spectrometry and can be distinguished from the naturally occurring deamidation at the glycosylation sites.[21] Immobilized lectins have been extensively used for glycoproteomic research for the capture of glycoproteins.[9,17,23,24] The most common lectin method uses Concavalin A (ConA), which binds very tightly to high-mannose-type N-glycans.[25] ConA affinity chromatography has been used in human plasma, but to date, few glycopeptides or glycosites have been identified.[17,26,27]

In our previous studies, we reported the development of a single microfluidic device, termed the *proteomic reactor*, for the rapid preconcentration, derivatization, and enzymatic digestion of minute amounts of protein prior to mass spectrometry analysis.[28] Furthermore, we established a 96-well plate version of the proteomic reactor that allows multiplexed processing of minute amounts of protein sample,[29] and we also demonstrated that the proteomic reactor is a microfluidic processing device which efficiently analyzes affinity-purified ubiquitinated proteins by LC-MS/MS.[30] In the following study, we discuss the development of our glycoproteomic reactor used specifically for processing and identifying glycoproteins. Briefly, the glycoproteomic reactor is composed of a short length of fused silica capillary tubing packed with strong cation exchange (SCX) resin. Glycoprotein samples prepurified by ConA are acidified and loaded on the glycoproteomic reactor. At low pH, proteins are positively charged and absorbed onto the SCX resin, but the uncharged glycans (such as α-D-mannopyrano-

Table 1. N-Glycopeptides Identified from Standard Glycoproteins: Fetuin A and Ovalbumin

glycoprotein	identified glycopeptides	Swiss-Prot annotated glycosite[a]	instrument	
			LTQ	Qstar
Fetuin A	RPTGEVYDIEIDTLETTCHVLDPTPLA**N#CS**VR	99	√	√
	LCPDCPLLAPL**N#DS**R	156		√
	VVHAVEVALATFNAES**N#GS**YLQLVEISR	176	√	√
	LCPDCPLLAPL**N#DS**RVVHAVEVALATFNAES**N#GS**YLQLVEISR	156,176	√	
Ovalbumin	Y**N#LT**SVLMAMGITDVFSSSANLSGISSAESLK	293	√	

[a] All of the three N-glycosites of fetuin A and one N-glycosite of ovalbumin annotated by Swiss-Prot database are identified using the glycoproteomic reactor.

side) are readily washed away leaving the sample free of contaminants. After increasing the pH, proteins are subjected to ^{18}O-labeling deglycosylation via PNGase F and disulfide bond reduction in the reactor. Then, trypsin is loaded onto the reactor where an in-reactor digestion is performed, and the resulting peptides are eluted using 5-step MS-compatible pH buffers. Thus, when this method is used, both glycosylated peptides and nonglycosylated peptides can be identified simultaneously, leading to a high sequence coverage and a comprehensive characterization of the N-linked glycosylation sites in proteins present in human plasma. The use of the glycoproteomic reactor enabled 82 N-linked glycopeptides (including 63 unique N-linked glycosylation sites) to be precisely mapped on 41 proteins from only 5 μL of human plasma.

Experimental Procedures

Material. Water used in the experiments was prepared from a Milli-Q system. Ammonium bicarbonate, dithiothreitol (DTT) and sodium carbonate were purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Acetonitrile with 0.1% formic acid and water with 0.1% formic acid were purchased from J.T. Baker (Phillipsburg, NJ). Trypsin was purchased from Promega (Madison, WI). Concanavalin A-agarose (C7555), methyl-α-D-mannopyranoside (M6882), Iodoacetamide (IAA), Fetuin A (F3004) and Ovalbumin (A7641) were obtained from Sigma-Aldrich (Saint Louis, MO). Strong cation exchange beads were obtained from Polymer Laboratories, Varian, Inc. *N*-Glycosidase F (11 365 185 001) was purchased from Roche Applied Science (Indianapolis, IN). ^{18}O water (^{18}O, 97%, OLM-240-97-1) was purchased from Cambridge Isotopic Laboratories, Inc. (Andover, MA). The pH buffer kits were obtained from Column Technology, Inc. (Fremont, CA).

Human Plasma Preparation. The human plasma from a single donor, collected in sodium citrate, was purchased from Innovative Research (MI). An initial protein concentration of ~63 μg/μL of plasma was determined using the Bradford method. A total of 180 μL of the plasma sample was diluted with 5 mL of 20 mM Tris-HCl buffer (pH 7.4), and then filtered through 0.22 μm filters (Corning, NY). The sample was further diluted in 45 mL of equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$ and 1 mM CaCl$_2$, pH 7.4), with a resulting protein concentration of 0.17 μg/μL.

Concanavalin A Affinity Chromatography. Concanavalin A (ConA)-agarose was prepared by adding the ConA solution to an empty column (1.5 cm i.d., bed volume 6 mL, Pierce, Rockford, IL). The self-packing ConA-agarose column was prewashed with 5-column vol of buffer (1 M NaCl, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 5 mM CaCl$_2$) and equilibrated with 10-column vol of equilibration buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM MgCl$_2$, 1 mM MnCl$_2$ and 1 mM CaCl$_2$, pH 7.4). Then, the diluted plasma sample was loaded onto the ConA-agarose column. The solution flowed twice through the ConA-agarose column by gravity. The collected solution was called the *flow-through fraction*. The column was then washed twice with 18 mL of equilibration buffer to remove unbound proteins followed by 2 mL of milli-Q water to remove the salt in the column. The 20-mL fraction was called the *wash fraction*. Finally, the ConA bound glycoproteins were eluted twice with 18 mL of elution buffer (1 M α-D-mannopyranoside), and the resulting 18-mL solution was called the *eluate fraction*.

Glycoproteomic Reactor. The glycoproteomic reactor was constructed by first creating a frit at one end of a fused silica tubing (700-μm i.d.) using a mixture of potassium silicate (KASIL 1, PQ Corporation, Valley Forge, PA) and formamide (11/2 volume ratio). Briefly, 88 μL of KASIL 1 solution was added into a 0.6 mL Eppendorf tube, then 16 μL of formamide (EMD chemicals, Inc., Darmstadt, Germany) was added. The solution was fully mixed by vortex for 1 min, and one end of a fused silica tubing was dipped into the solution until 0.5 cm of tubing was filled with the solution. Then, the silica tubing was suspended vertically overnight at room temperature. Once the frit was solidified, an 8 cm long reactor was created in the capillary by pressure packing (400 psi) a slurry of strong cation exchange (SCX) beads (10 μm, Polymer Laboratories, Varian, Inc.) from the open end of the capillary tubing. Next, the reactor was washed with washing buffer (20 mM citric acid, pH 2.5) prior to its use. As shown in Figure 1, standard glycoprotein samples or eluants from the Con-A agarose column were acidified and loaded on the 200- and 700-μm i.d. glycoproteomic reactor using a pressurized vessel. The reactor was then washed with washing buffer (20 mM citric acid, pH 2.5, 20 μL) and dried. The proteins present on the reactor were subjected to ^{18}O-labeling deglycosylation and disulfide bond reduction by introducing the deglycosylation buffer (50 mM dithiothreitol, 20 mM ammonium bicarbonate and 2 units/μL PNGase F in ^{18}O water, pH 8.0) in the reactor for 1.5 h at 37 °C. The reactor was again washed with washing buffer (20 mM citric acid, pH 2.5, 20 μL) to quench the reaction and dried. Proteins were subjected to simultaneous alkylation and trypsin digestions by introducing the digestion buffer (10 mM iodoacetamide, 20 mM ammonium bicarbonate and 2 μg/μL trypsin, pH 8.0) in the reactor for 1.5 h at 37 °C. For standard glycoprotein samples, the resulting peptides were eluted with 3 N ammonium hydroxide (pH ~12). For plasma glycoproteins, the resulting peptides were then eluted with step pH buffers (pH 3.0, 5.0, 6.0, 8.0 and 12, 100 μL, respectively).

Comparison of the Protein Recovery from Standard TCA/Acetone Precipitation and SCX Proteomic Reactor by SDS-PAGE. TCA/acetone precipitation was performed according to the previous publishedthe protocol with some modifica-
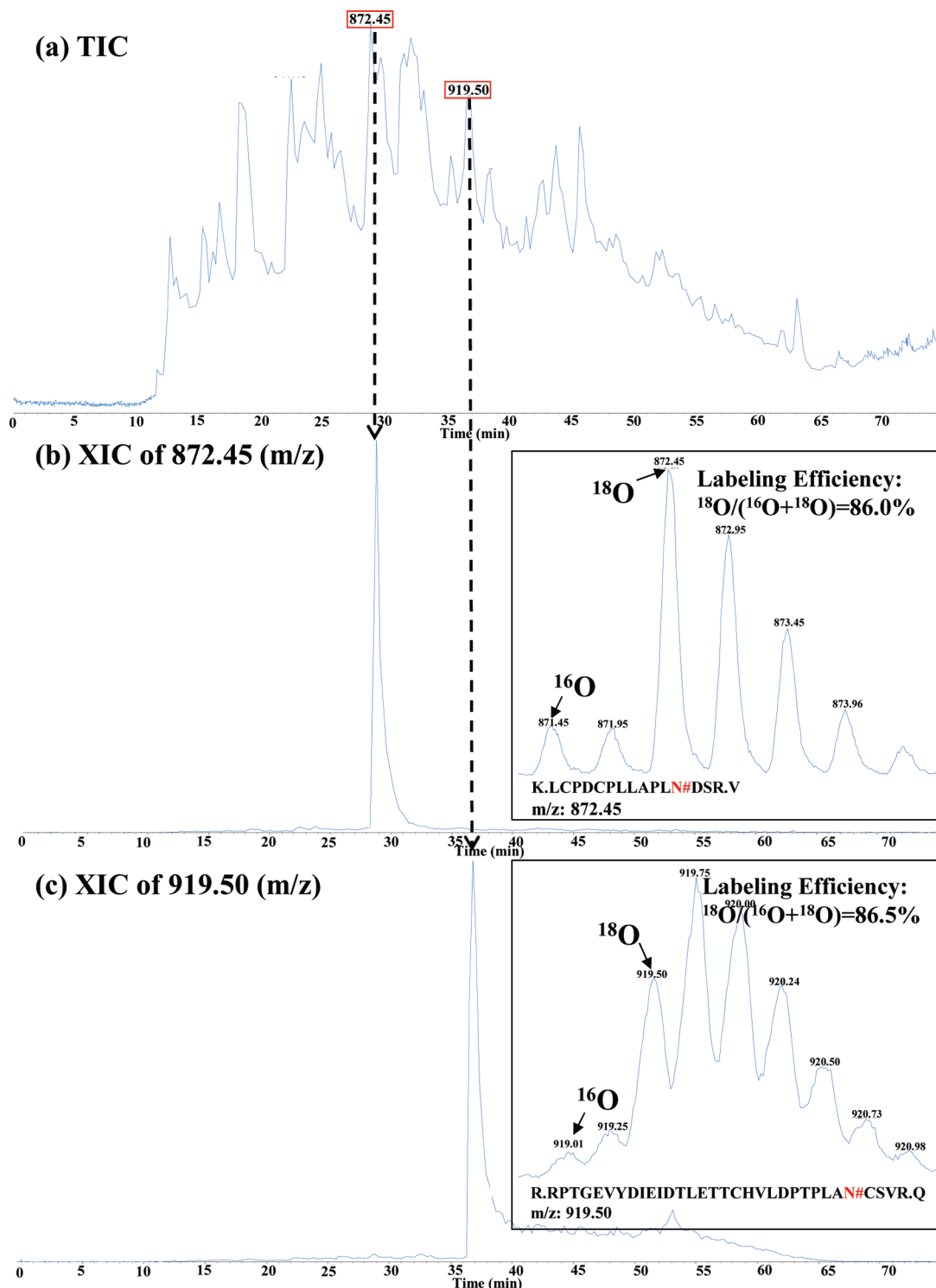
Figure 3. ^{18}O-labeling efficiency of identified N-glycopeptides. (a) The total ion chromatography (TIC) of standard glycoprotein from the Qstar MS, and the peaks of 872.45 (*m/z*) and 919.50 (*m/z*) were significant; (b) the extract ion chromatography and the isotopic cluster peaks of 872.45 (*m/z*); (c) the extract ion chromatography and the isotopic cluster peaks of 919.50 (*m/z*).

tions.[30] Trichloroacetic acid was added to the different ConA fractions up to a final concentration of 25%, and the fractions were placed on ice for 30 min with occasional mixing. The fractions were then centrifuged for 30 min at 15 000 rpm (4 °C), after which the supernatants were removed, and the protein pellets were washed with chilled (−20 °C) acetone, incubated on ice for 10 min, and centrifuged for another 20

min at 15 000 rpm (4 °C). Once the acetone was removed, the fractions were air-dried at room temperature, and the protein pellets were reconstituted in 20 mM Tris-HCl (pH 7.4).

The protein recovery from the reactor was tested using the protocol described in Glycoproteomic Reactor. However, the proteins were only captured in the reactor and then eluted with 3 N ammonium hydroxide (Fisher Scientific, Nepean, Ontario,

Table 2. Peptides, Glycopeptides, Unique Peptides and Unique Glycopeptides Identified in Glycoproteomic Reactor by pH Fractionation

	pH 3.0	pH 5.0	pH 6.0	pH 8.0	pH 12	total
Peptide	736	430	642	526	499	2833
Glycopeptide	49	41	96	60	77	323
Unique peptide	380	284	394	341	307	922[a]
Unique glycopeptide	39	31	42	33	25	82[a]

[a] Unique ones, not the simple sum of the number from each pH step.

Canda) before being dried with a SpeedVac. The resulting proteins were dissolved in 20 mM Tris-HCl (pH 7.4), and the protein concentrations were determined using a Bradford protein assay kit (Bio-Rad).

The protein samples from the standard TCA/acetone protocol and the glycoproteomic reactor were separated by the 12% SDS-PAGE. The separation was conducted at a constant voltage setting at 80 V for 30 min, and then at 120 V thereafter until the bromophenol blue dye marker reached the end of the gel. Following the SDS-PAGE, Colloidal blue staining was performed according to manufacturer's guidelines (Invitrogen, Carlsbad, CA).

LC-MS/MS. Peptide samples from the glycoproteomic reactor were acidified with formic acid and loaded on a 200 μm × 50 mm fused silica precolumn packed in-house with 5 cm of 5-μm YMC ODS-A C18 beads (Waters Co., Milford, MA) using an 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA). Following a desalting step, the flow was split, and peptides were eluted through a second 75 μm × 50 mm column packed with the same beads at approximately 200 nL/min. The peptides were eluted using a 2-h gradient (5−80% acetonitrile with 0.1% formic acid) into an ESI LTQ linear ion trap mass spectrometer (Thermo Electron, Waltham, MA). MS/MS spectra were acquired in a data-dependent acquisition mode that automatically selected and fragmented the ten most intense peaks from each MS spectrum generated. For standard glycoproteins, the LC effluent was also electrosprayed into a QSTAR Pulsar quadrupole-TOF mass spectrometer (ABI/MDS Sciex). MS and MS/MS spectra were acquired in a data-dependent mode, with one MS scan followed by four MS/MS scans.

Data Analysis. The acquired MS/MS spectra from standard glycoproteins were searched against the NCBI yeast protein sequence database (6298 protein entries, released April 2007) to which 6 standard protein sequences were added. Mascot 2.2.02 (Matrix Science) was used to search the protein sequence database using the following criteria: carbamidomethyl (C) was set as a fixed modification, while oxidation (M, +15.99492 Da) and Delta:H(1)O(−1)18O(1) (N, +3.01207 Da) for the deamidation of asparagines incorporating ^{18}O were set as variable modifications. The acquired MS/MS spectra from human plasma were subsequently searched against the human International Protein Index (IPI) protein sequence database (version 3.47, 72 079 protein entries; European Bioinformatics Institute, www.ebi.ac.uk/IPI/). A decoy database was also searched by Mascot. For the Qstar data, precursor and fragment ion mass tolerances were set at 100 ppm and 0.2 Da, respectively. For LTQ data, precursor and fragment mass tolerances were set at 2.0 and 0.8 Da, respectively. Mascot cutoff scores were set to 40. Furthermore, the peptides should be ranked first, and the probability-based Mowse (expect) scores of candidate peptides should have a *P*-value smaller than 0.05. Finally, the stringency was further increased by rejecting all the Delta:H(1)O(−1)18O(1)

(N)-containing peptides that could not be assigned to the consensus tripeptide sequence for N-linked glycosylation (N-X-S/T) regardless of the Mascot score.

Results and Discussion

Glycoproteomic Reactor Development. We developed a glycoproteomic reactor that rapidly and efficiently processes glycoproteins when combined with ConA based glycoprotein purification. The glycoproteomic reactor greatly simplifies the processing of glycoproteins by combining, eliminating, and simplifying many biochemical steps that were previously required (see Figure 1). One of the challenges with ConA purification has always been the presence of high-concentration glycan buffer (1 M α-D-mannopyranoside) in the sample which is not compatible with downstream processes, especially mass spectrometry. The first advantage of the glycoproteomic reactor is that it is compatible with high-concentrations of glycan buffer. The glycoproteins are efficiently captured by the glycoproteomic reactor, while the glycan buffer is washed away.

One standard protocol for the removal of α-D-mannopyranoside is to perform the trichloroacetic acid precipitation[21] or acetone precipitation[31] after ConA affinity chromatography. Alternatively, Xu et al. dialyzed the eluted fraction from ConA column using Milli Q water at 4 °C to remove the α-D-mannopyranoside.[24] As well, a repeated buffer exchange during repeated ultrafiltration has been performed to remove α-mannopyranoside by Lewandrowski et al.[32] However, dialysis, ultrafiltration and TCA, or acetone precipitations are all multistep and time-consuming processes which result in sample variation, loss of sample, contamination, and so forth. We compared the performance of the glycoproteomic reactor for capturing glycoproteins in the presence of α-D-mannopyranoside to the standard TCA/acetone precipitation for removing α-mannopyranoside. Bradford assays indicated that the amount of protein recovered using the glycoproteomic reactor is identical to the TCA/acetone precipitation method for protein fractioning from ConA column (ratio 1.03 for the reactor recovery over the TCA/acetone recovery). Furthermore, the glycoproteomic reactor does not seem to introduce any protein biases in the glycoprotein patterns as compared to the TCA/acetone precipitation method (Figure 2). Therefore, the glycoproteomic reactor provides an efficient and rapid alternative that only requires 45 min to load a 2-mL protein fraction from a ConA column, wash it, and then elute it.

The second advantage of the glycoproteomic reactor is that enzymatic deglycosylation can be performed quickly and directly while the glycoproteins are concentrated on the reactor. The calculated interstitial volume of the 700-μm i.d. × 8 cm glycoproteomic reactor is ∼1 μL. This represents more than a 1000-fold concentration effect when 1 mL of glycoprotein sample is loaded. A typical enzymatic deglycosylation by PNGase F takes 4−12 h to perform.[18−21] However, the glycoproteomic reactor reduces the total time for complete deglycosylation to less than 1.5 h. Therefore, the catalysis and labeling in the glycoproteomic reactor can be performed much faster due to the increased concentration of glycoproteins. Furthermore, tryptic digestion on the reactor can also be done and it yields a better performance than solution based digestion as we previously described.[28−30]

The third advantage of the glycoproteomic reactor is that it drastically reduces the amount of reagent needed for labeling and deglycosylation. The total available volume of the glycoproteomic reactor, taking into account the void volume and

Table 3. N-Glycopeptides Identified from 5 μL of Human Plasma

protein name	IPI number	coverage	peptide sequence	peptide score	P-value	position	glycosites (ID'ed glycosites)
Ceruloplasmin	IPI00017601	42%	EN#LTAPGSDSAVFFEQGTTR	100	1.3×10^{-7}	397	6(5)
			ELHHLQEQN#VSNAFLDK	76	3.4×10^{-5}	762	
			EHEGAIYPDN#TTDFQR	70	1.1×10^{-4}	138	
			AGLQAFFQVQECN#K	52	7.1×10^{-3}	358	
			DVDKEFYLFPTVFDEN#ESLLLEDNIR	50	1.2×10^{-2}	588	
Keratin, type I cytoskeletal 9	IPI00019359	37%	N#YSPYYNTIDDLKDQIVDLTVGNNKTLLDIDNTR	85	3.2×10^{-6}	200	0(1)
Prothrombin	IPI00019568	26%	N#FTENDLLVR	47	2.0×10^{-2}	416	3(1)
Complement factor B	IPI00019591	27%	SPYYN#VSDEISFHCYDGYTLR	62	8.2×10^{-4}	122	4(1)
Alpha-1-acid glycoprotein 2	IPI00020091	24%	QNQCFYN#SSYLNVQR	71	8.4×10^{-5}	93	5(1)
Fibrinogen gamma chain	IPI00021891	37%	DLQSLEDILHQVEN#K	46	3.4×10^{-2}	78	1(1)
			VDKDLQSLEDILHQVEN#K	69	1.7×10^{-4}	78	
Fibronectin	IPI00022418	9%	HEEGHMLN#CTCFGQGR	50	1.2×10^{-2}	542	6(1)
Alpha-1-acid glycoprotein 1	IPI00022429	58%	QDQCIYN#TTYLNVQREN#GTISR	100	1.2×10^{-7}	93, 103	5(4)
			QDQCIYN#TTYLNVQR	99	1.6×10^{-7}	93	
			NEEYN#KSVQEIQATFFYFTPN#KTEDTIFLR	92	6.6×10^{-7}	56, 72	
Alpha-2-HS-glycoprotein	IPI00022431	45%	AALAAFNAQNN#GSNFQLEEISR	120	1.4×10^{-9}	176	2(2)
			VCQDCPLLAPLN#DTR	101	9.8×10^{-8}	156	
Serotransferrin	IPI00022463	82%	CGLVPVLAENYN#KSDNCEDTPEAGYFAVAVVKK	124	4.0×10^{-10}	432	2(2)
			CGLVPVLAENYN#KSDNCEDTPEAGYFAVAVVK	116	3.0×10^{-9}	432	
			IMNGEADAM*SLDGGFVYIAGKCGLVPVLAENYN#K	105	3.7×10^{-8}	432	
			QQQHLFGSN#VTDCSGNFCLFR	104	5.0×10^{-8}	630	
			CGLVPVLAENYN#K	66	2.6×10^{-4}	432	
Hemopexin	IPI00022488	58%	ALPQPQN#VTSLLGCTH	94	4.8×10^{-7}	453	5(3)
			SWPAVGN#CSSALR	50	1.2×10^{-2}	187	
			CSDGWSFDATTLDDN#GTMLFFK	44	4.9×10^{-2}	64	
Alpha-1B-glycoprotein	IPI00022895	42%	FQSPAGTEALFELHN#ISVADSAN#YSCVYVDLKPPFGGSAPSER	62	6.0×10^{-4}	363, 371	4(3)
			EGDHEFLEVPEAQEDVEATFPVHQPGN#YSCSYR	116	2.9×10^{-9}	179	
Corticosteroid-binding globulin	IPI00027482	16%	AQLLQGLGFN#LTER	75	3.5×10^{-5}	96	6(1)
Complement factor H	00029739	32%	MDGASN#VTCINSR	84	4.6×10^{-6}	1029	8(3)
			ISEEN#ETTCYMGK	78	1.6×10^{-5}	911	
			IPCSQPPQIEHGTIN#SSR	81	9.9×10^{-6}	882	
Antithrombin-III	IPI00032179	60%	LFGDKSLTFN#ETYQDISELVYGAK	105	3.7×10^{-8}	187	4(2)
			LGACN#DTLQQLMEVFK	95	4.1×10^{-7}	128	
			SLTFN#ETYQDISELVYGAK	81	8.9×10^{-6}	187	
			SLTFN#ETYQDISELVYGAKLQPLDFK	52	8.0×10^{-3}	187	
Complement C4-A	IPI00032258	9%	GLN#VTLSSTGR	82	6.3×10^{-6}	1328	4(1)
Kininogen-1	IPI00032328	14%	YNSQN#QSNNQFVLYR	45	3.7×10^{-2}	48	4(1)
IGHA1 protein	IPI00061977	27%	LAGKPTHVN#VSVVMAEVDGTCY	65	4.1×10^{-4}	487	0(2)
			LSLHRPALEDLLLGSEAN#LTCTLTGLR	55	3.4×10^{-3}	291	
Zinc-alpha-2-glycoprotein	IPI00166729	32%	DIVEYYNDSN#GSHVLQGR	46	2.7×10^{-2}	109	4(2)
			DIVEYYN#DSNGSHVLQGR	67	2.6×10^{-4}	106	
Immunoglobulin J chain	IPI00178926	21%	IIVPLNNREN#ISDPTSPLR	48	1.8×10^{-2}	49	1(1)
			EN#ISDPTSPLR	53	5.7×10^{-3}	49	
Clusterin	IPI00291262	7%	LAN#LTQGEDQYYLR	46	2.9×10^{-2}	374	6(1)
Plasma protease C1 inhibitor	IPI00291866	15%	GVTSVSQIFHSPDLAIRDTFVN#ASR	74	4.4×10^{-5}	238	6(1)
Fibrinogen beta chain	IPI00298497	43%	GTAGNALMDGASQLM*GEN#R	62	8.2×10^{-4}	394	1(1)
			GTAGNALM*DGASQLM*GEN#R	100	1.3×10^{-7}	394	
			GTAGNALMDGASQLMGEN#R	113	5.9×10^{-9}	394	
Beta-2-glycoprotein 1	IPI00298828	28%	LGN#WSAMPSCK	60	1.0×10^{-3}	253	4(1)
Kallistatin	IPI00328609	3%	FLN#DTMAVYEAK	62	7.7×10^{-4}	157	4(1)
Putative uncharacterized protein DKFZp686K04218	IPI00384952	21%	LAGKPTHVN#VSVVMAEVDGTCY	65	4.1×10^{-4}	465	0(2)
			LSLHRPALEDLLLGSEAN#LTCTLTGLR	55	3.4×10^{-3}	269	
Fibronectin 1 isoform 4 preproprotein	IPI00414283	11%	GGNSNGALCHFPFLYNNHN#YTDCTSEGR	79	1.5×10^{-5}	430	6(1)
			GGNSNGALCHFPFLYNNHN#YTDCTSEGRR	79	1.4×10^{-5}	430	
Putative uncharacterized protein DKFZp686J11235	IPI00426060	46%	LSLHRPALEDLLLGSEAN#LTCTLTGLR	158	1.9×10^{-13}	297	0(2)
			LAGKPTHVN#VSVVMAEVDGTCY	86	3.2×10^{-6}	493	
			TIDRLAGKPTHVN#VSVVMAEVDGTCY	62	7.6×10^{-4}	493	
IGHM protein	IPI00477090	30%	GLTFQQN#ASSMCVPDQDTAIR	102	7.1×10^{-8}	348	0(1)
			GLTFQQN#ASSM*CVPDQDTAIR	75	4.3×10^{-5}	348	
Haptoglobin-related protein	IPI00477597	51%	MVSHHN#LTTGATLINEQWLLTAK	131	9.8×10^{-11}	126	0(3)
			NLFLN#HSEN#ATAKDIAPTLTLYVGK	112	7.2×10^{-9}	149, 153	
			M*VSHHN#LTTGATLINEQWLLTAK	110	1.2×10^{-8}	126	
			NLFLN#HSEN#ATAK	100	1.0×10^{-7}	149, 153	
			NLFLN#HSEN#ATAKDIAPTLTLYVGKK	91	1.0×10^{-6}	149, 153	
			NLFLNHSEN#ATAK	55	3.7×10^{-3}	153	
Alpha-2-macroglobulin	IPI00478003	67%	SLGNVN#FTVSAEALESQELCGTEVPSVPEHGR	172	7.2×10^{-15}	869	8(4)
			SLGNVN#FTVSAEALESQELCGTEVPSVPEHGRK	163	5.0×10^{-14}	869	
			GCVLLSYLN#ETVTVSASLESVR	54	4.6×10^{-3}	55	
			TEVSSNHVLIYLDKVSN#QTLSLFFTVLQDVPVR	54	4.3×10^{-3}	1424	
			IITILEEEMN#VSVCGLYTYGKPVPGHVTVSICR	47	2.1×10^{-2}	247	

Table 3a. Continued

protein name	IPI number	coverage	peptide sequence	peptide score	P-value	position	glycosites (ID'ed glycosites)
IGHM protein	IPI00549291	31%	TVDKSTGKPTLYN#VSLVMSDTAGTCY	116	3.2×10^{-9}	593	0(2)
			GLTFQQN#ASSM*CVPDQDTAIR	47	2.6×10^{-2}	362	
			STGKPTLYN#VSLVMSDTAGTCY	88	2.0×10^{-6}	593	
			GLTFQQN#ASSMCVPDQDTAIR	78	1.8×10^{-5}	362	
			STGKPTLYN#VSLVM*SDTAGTCY	72	8.0×10^{-5}	593	
Alpha-1-antichymotrypsin	IPI00550991	34%	TLN#QSSDELQLSMGNAMFVK	107	2.5×10^{-8}	127	6(2)
			TLN#QSSDELQLSM*GNAM*FVK	94	4.7×10^{-7}	127	
			YTGN#ASALFILPDQDK	56	2.8×10^{-3}	271	
Alpha-1-antitrypsin	IPI00553177	87%	ADTHDEILEGLNFN#LTEIPEAQIHEGFQELLR	138	1.6×10^{-11}	107	3(3)
			YLGN#ATAIFFLPDEGKLQHLENELTHDIITK	85	3.2×10^{-6}	271	
			YLGN#ATAIFFLPDEGK	72	7.0×10^{-5}	271	
			ITPNLAEFAFSLYRQLAHQSN#STNIFFSPVSIATAFAMLSLGTK	56	2.0×10^{-3}	70	
Ig alpha-2 chain C region	IPI00641229	62%	LSLHRPALEDLLLGSEAN#LTCTLTGLR	158	1.9×10^{-13}	131	4(2)
			LAGKPTHVN#VSVVMAEVDGTCY	86	3.2×10^{-6}	327	
			LSLHRPALEDLLLGSEAN#LTCTLTGLRDASGATFTWTPSSGK	85	3.0×10^{-6}	131	
			TIDRLAGKPTHVN#VSVVMAEVDGTCY	62	7.6×10^{-4}	327	
Haptoglobin	IPI00641737	67%	MVSHHN#LTTGATLINEQWLLTTAK	131	9.8×10^{-11}	184	4(4)
			NLFLN#HSEN#ATAKDIAPTLTLYVGK	112	7.2×10^{-9}	207, 211	
			M*VSHHN#LTTGATLINEQWLLTTAK	110	1.2×10^{-8}	184	
			NLFLN#HSEN#ATAK	100	1.0×10^{-7}	207, 211	
			NLFLN#HSEN#ATAKDIAPTLTLYVGKK	91	1.0×10^{-6}	207, 211	
			QLVEIEKVVLHPN#YSQVDIGLIK	89	1.5×10^{-6}	241	
			VVLHPN#YSQVDIGLIK	65	3.7×10^{-4}	241	
			NLFLNHSEN#ATAK	55	3.7×10^{-3}	211	
Ficolin (Collagen/fibrinogen domain containing) 3	IPI00643432	39%	VELEDFNGN#R	43	4.9×10^{-2}	67	0(1)
Complement C3	IPI00783987	31%	TVLTPATNHMGN#VTFTIPANR	71	1.0×10^{-4}	85	3(1)
Antithrombin	IPI00844156	24%	LGACN#DTLQQLMEVFK	95	4.1×10^{-7}	128	0(1)
Hypothetical protein, partial	IPI00887739	15%	TVLTPATNHMGN#VTFTIPANR	71	1.0×10^{-4}	85	0(1)
IGHA2 Protein	IPI00893853	33%	LSLHRPALEDLLLGSEAN#LTCTLTGLR	158	1.9×10^{-13}	22	0(1)
			LSLHRPALEDLLLGSEAN#LTCTLTGLRDASGATFTWTPSSGK	85	3.0×10^{-6}	22	
			WHPRLSLHRPALEDLLLGSEAN#LTCTLTGLR	84	4.4×10^{-6}	22	

the interstitial volume, amounts to ∼2 μL. Because of the limited volume, we only used 3 μL of PNGase F solubilized in ^{18}O water per experiment for labeling and deglycosylation.

N-Linked Glycosylation Sites Identification of Standard Glycoproteins Using Glycoproteomic Reactor. To evaluate the procedures for operating the glycoproteomic reactor and to optimize each step, we first used two standard glycoproteins (fetuin A from bovine and ovalbumin from chicken) to determine the specificity and efficiency of the glycoproteomic reactor. As shown in Table 1, all of the three N-linked glycosites of fetuin A and one N-linked glycosite of ovalbumin based on the Swiss-Prot database annotation were accurately identified using the glycoproteomic reactor. Two glycopeptides "RPTGEVYDIEIDTLETTCHVLDPTPLAN#CSVR" (glycosite: 99) and "VVHAVEVALATFNAESN#GSYLQLVEISR" (glycosite: 176) from fetuin A were identified by both the LTQ and the Qstar mass spectrometers. The glycopeptide "LCPDCPLLAPLN#DSR" (fetuin A, glycosite: 156) was only identified by the Qstar, and the glycopeptides "LCPDCPLLAPLN#DSRVVHAVEVALATFN-AESN#GSYLQLVEISR" (fetuin A, glycosite: 156, 176) and "YN#LTSVLMAMGITDVFSSSANLSGISSAESLK" (ovabumin, glycosite: 293) were only identified by the LTQ. The MS/MS spectra of these glycopeptides are shown in Supplementary Figure 1 in Supporting Information.

The efficiency of ^{18}O labeling incorporation was also calculated from the high-resolution MS spectra obtained on the Qstar MS using the following formula: Efficiency of ^{18}O labeling $= 100 \times I(\text{Asp-}^{18}\text{O})/[I(\text{Asp-}^{16}\text{O}) + I(\text{Asp-}^{18}\text{O})]$ (I means the intensity of the indicated monoisotopic peak).

As shown in Figure 3, the labeling efficiencies of "LCPD-CPLLAPLN#DSR" (m/z 872.45) and "RPTGEVYDIEIDTLETTCH-VLDPTPLAN#CSVR" (m/z 919.50) were 86.0% and 86.5%,

respectively. The patterns of isotopic clusters were similar to the mass spectra of glycopeptides by IGOT.[21] Water containing 97% of ^{18}O was used in our experiments, and as expected, the incorporation of ^{18}O to Asp was not fully completed. The use of ^{18}O labeling for identifying N-glycosylation sites can be limited by partial site occupancy.[33] This limitation could be a possible reason for the incomplete incorporation of ^{18}O in Asp. However, the labeling efficiency of enzymatic conversion of glycosylated asparagine to aspartic acid in glycoproteomic reactor was higher than 85%, which can facilitate the subsequent database searches.

Plasma Glycoprotein Enrichment Using Concanavalin A Affinity Chromatography. Glycoproteins are an important component of the plasma proteome,[34] and their level and type present in plasma can be affected by diseases occurring in different organs and tissues. Therefore, development of strong analytical tools to characterize these glycoproteins is of great importance. ConA lectin affinity chromatography has been used to enrich N-linked glycosylated proteins from plasma. It has been shown that ConA predominately recognizes alpha-mannose[35] which is very common in N-linked glycans.[25] Figure 2 shows a Colloidal blue-stained gel of original plasma sample along with samples after ConA purification. Of particular interest is the intensity of the gel bands for human serum albumin which nearly disappears when ConA affinity chromatography is performed. These results indicate that the ConA enrichment procedure is specific and about 13% of the total plasma protein mass is captured by ConA column.

Plasma N-Glycoprotein Identifications Using the Glycoproteomic Reactor. The performance of the glycoproteomic reactor for studying glycoproteins in human plasma was evaluated. Briefly, 2 mL of eluting fraction from a ConA-agarose
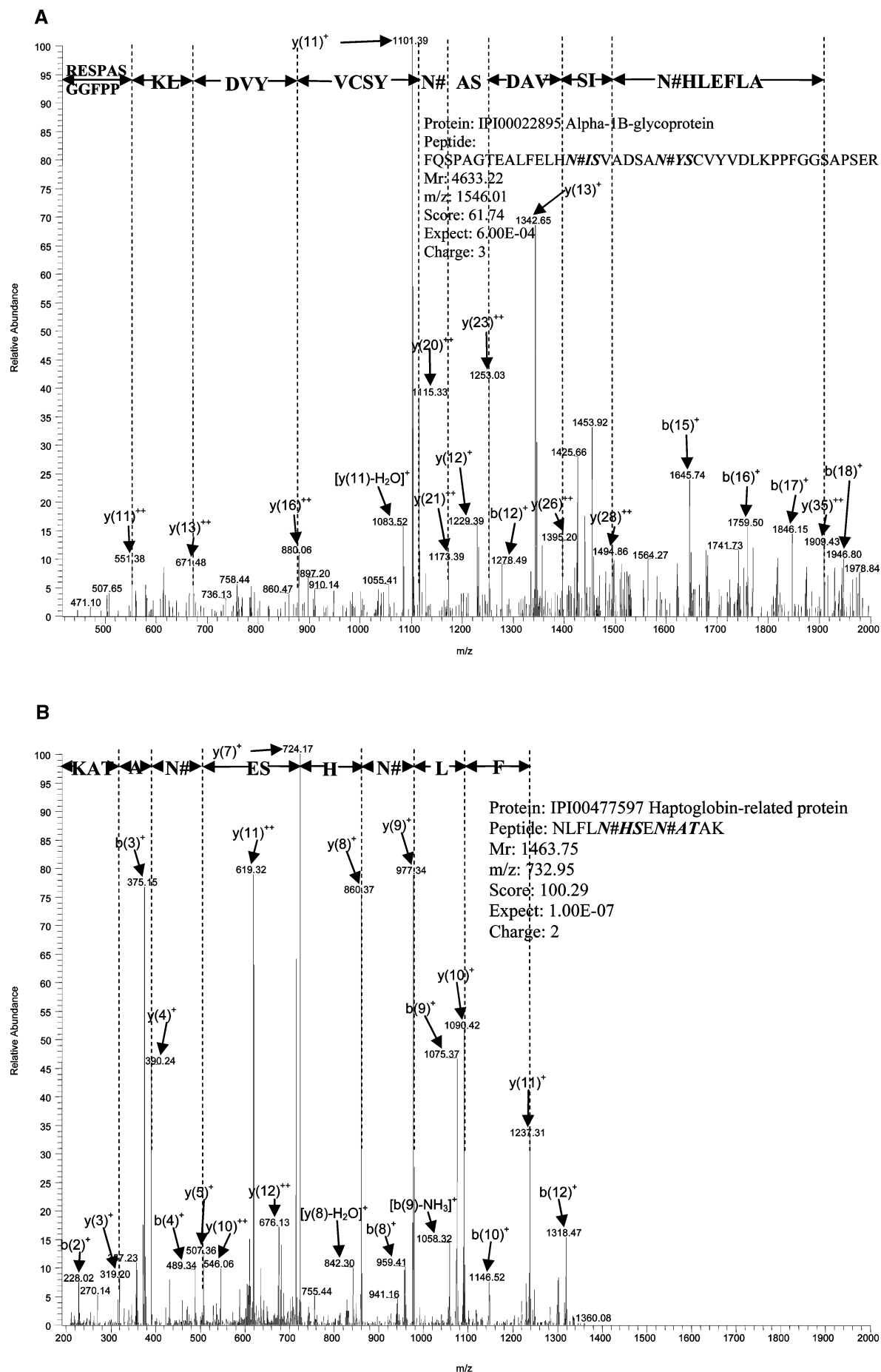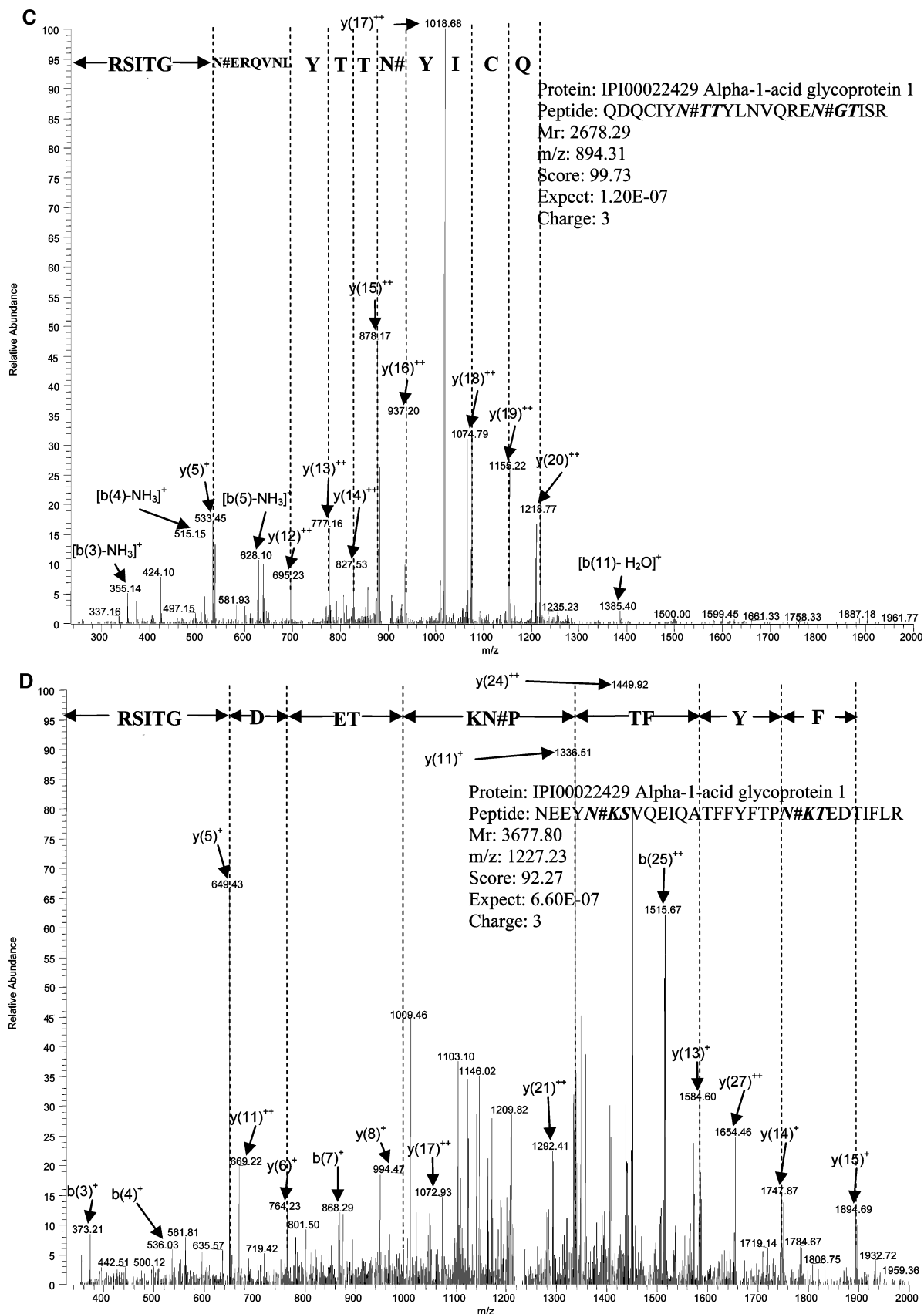
A

Protein: IPI00022895 Alpha-1B-glycoprotein
Peptide:
FQ$PAGTEALFELH*N#IS*VADSA*N#YS*CVYVDLKPPFGG$APSER
Mr: 4633.22
m/z: 1546.01
Score: 61.74
Expect: 6.00E-04
Charge: 3

B

Protein: IPI00477597 Haptoglobin-related protein
Peptide: NLFL*N#HS*E*N#AT*AK
Mr: 1463.75
m/z: 732.95
Score: 100.29
Expect: 1.00E-07
Charge: 2

Figure 4. (Continued)

Figure 4. MS/MS spectra of glycopeptides with two N-glycosites. MS/MS spectra of glycopeptides with two N-glycosites at *m/z* (a) 1456.01 (3+), (b) 732.95 (2+), (c) 894.31 (3+), and (d) 1227.23 (3+) matching to alpha-1B glycoprotein, haptoglobin-related protein and alpha-1-acid glycoprotein 1, respectively.
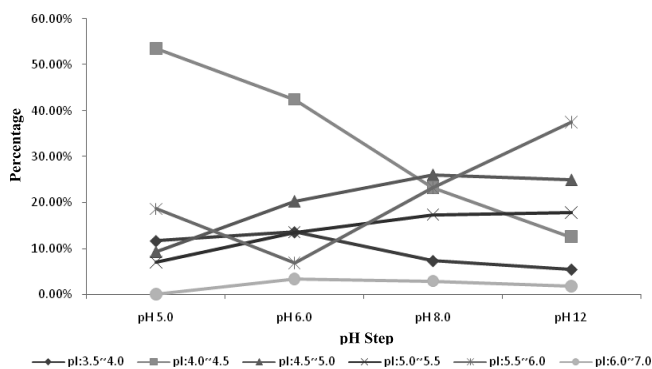
Figure 5. Distribution of glycopeptides with their pH fractions and theoretical p*I* values. Y-axis: percentage of glycopeptides with their theoretical p*I* values in a specific p*I* range in total glycopeptides by each pH fractions.

column (about 160 μg of proteins from 20 μL of human plasma) were acidified, loaded, and processed onto the glycoproteomic reactor (see Experimental Procedures). The resulting peptides were fractionated in the reactor using 5-step pH elution[36,37] (pH 3.0, 5.0, 6.0, 8.0, ~12). One-fourth of the volume of each pH eluted fraction was analyzed by LC-MS/MS (LTQ). The acquired MS/MS spectra were searched against human protein database using Mascot, and the results were filtered by the following criteria: (I) the Mascot cutoff ion score was set at 40, with false discovery rates of 0.66%, 1.74%, 1.66%, 0.55% and 0.62% from the respective pH fractions; (II) all peptides except the first rank were excluded; (III) peptides with expected *P*-values higher than 0.05 were excluded; and finally (IV) the ^{18}O-labeling deamidated asparagines-containing peptides, without the consensus tripeptide sequence (N-X-S/T) for N-linked glycosylation, were also excluded regardless of the match score. This procedure resulted in 2833 identified peptides out of which 922 were unique peptides (see Table 2). It also resulted in 323 identified glycopeptides out of which 82 were unique glycopeptides. This means that glycopeptides represented 11% of the total peptides and 9% of the unique peptides. A total of 122 unique proteins, among which 41 (34%) contain at least one N-linked glycosylation site, were identified using only 5 μL of human plasma (see Supplementary Table in Supporting Information). The remaining 81 proteins, for which no glycopeptides were identified, are annotated in the Swiss-Prot database as glycoproteins (47%), immunoglobulins or immunoglobulin-related proteins (36%), and others (17%). As shown in Table 3, 82 unique glycopeptides (including 63 unique N-linked glycosylation sites) representing 41 unique proteins were identified. Most of the glycopeptides had single N-glycosites, but some glycopeptides carried two N-glycosites (Figure 4). The MS/MS spectra of the 82 unique glycopeptides are provided in the Supplementary Figure 2 in Supporting Information. We compared the results obtained from our experiments with results reported in previous glycoproteomic studies of plasma[19,38,39] and the information available in the Swiss-Prot database. Unlike other glycopeptide-enrichment methods, our method can identify glycopeptides while still obtaining a good protein sequence coverage from the nonglycopeptides. For example, the sequence coverage of alpha-1-antitrypsin (IPI00553177) is 87%, and all of the three glycosites annotated by Swiss-Prot database were identified. Five of the six annotated glycosites of ceruloplasmin (IPI00017601) were identified with a protein sequence coverage of 42%. All of the

four annotated glycosites of Haptoglobin (IPI00641737) were also identified with 67% coverage.

Zhou et al.[19] identified 161 unique glycopeptides from mouse plasma and Liu et al.[39] claimed identification of 2053 unique glycopeptides. Considering the volume of plasma used, Zhou et al. identified 8 unique glycopeptides/μL of mouse plasma, whereas Liu et al. identified 2 unique glycopeptides/μL of human plasma. We identified 16 unique glycopeptides/μL of human plasma using the glycoproteomic reactor. Furthermore, we only enriched the glycoprotein that were specifically attached to the ConA-agarose column, while Zhou and Liu broadly enriched the N-glycoproteins and N-glycopeptides by hydrazide chemistry.[18]

Our procedure includes step pH elution which has not been used in other glycopeptides identification methods. The analysis of the theoretical p*I* distribution versus the pH of elution for identified glycopeptides is provided in Figure 5. The fraction eluting at pH 3.0 is not considered in this p*I* analysis because it is expected to have significant overlaps with other p*I* ranges due to the previous digestion step at pH 8.0. PNGase F-mediated deglycosylation converted asparagines to aspartic acid, so the calculated p*I* values for the identified glycopeptides were all less than 7. Figure 5 shows that there was also a positive correlation for the calculated p*I* of the converted glycopeptides and the elution pH. In particular, the percentage of converted glycopeptides with theoretical p*I* within 3.5–4.0 and 4.0–4.5 decreased gradually with the elution pH. Conversely, the percentage of the converted glycopeptides with higher theoretical p*I* values was more predominant in higher pH fractions. Therefore, the step pH elution from the glycoproteomic reactors provides efficient separations for glycopeptides.

Conclusion

In summary, the glycoproteomic reactor is a novel technology that can enrich glycoproteins and perform quick (1.5 h) and cost-efficient ^{18}O-labeling enzymatic deglycosylation (half the amount of [18]O water) and tryptic digestion. Using stringent criteria for filtering the results after database searching, 82 unique glycopeptides assigned to 41 unique glycoproteins were identified from only 5 μL of human plasma. This is the highest number of unique glycopeptides identified per microliter of sample.

Supporting Information Available: The mass spectra of identified glycopeptides from standard glycoproteins, MS/MS spectra of identified glycopeptides from human plasma, table of unique proteins identified using only 5 μL of human plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR800734R