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Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography

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Abstract

Previously, we reported that the distribution of glycoproteins into the lectin displacement fractions of a multi-lectin affinity column was determined by the glycosylation patterns of the proteins. This distribution was observed by the sequential use of displacers specific to the lectins in the column. In this study we have evaluated the multi-lectin column (containing Concanavalin A, Wheat germ agglutinin and Jacalin lectin) to screen glycoproteins with known glycosylation pattern changes. The presence of a glycoprotein in a given displacer fraction was determined by LC–MS/MS analysis of a tryptic digest. We have used the enzyme neuraminidase to modify the oligosaccharide chains present in human transferrin, and used the enzymes, neuraminidase and fucosidase, to modify glycoproteins present in human serum. Then, by comparison with the untreated samples, we demonstrated a distribution shift of the enzyme-treated serum glycoproteins in the displacement fractions isolated from the multi-lectin column. The fractions were analyzed by a protein assay, Sequest rank comparison and peak area measurement from the extracted ion chromatogram. The results indicated that the multi-lectin affinity column (M-LAC) is sensitive to changes in the content of sialic acid and fucosyl residues present in serum glycoproteins, and has the potential to be used to screen serum proteins for glycosylation changes due to disease. In addition, the use of a glycosidase to induce specific structural changes in glycoproteins can support the development of multi-lectin column formats specific for detecting changes in the glycoproteome of certain diagnostic fluids and types of disease.

Keywords: Lectin; Neuraminidase; Fucosidase; Sialic acid; Fucose; Glycosylation pattern change; Human serum; Glycoproteins; Transferrin; Glycoproteome

1. Introduction

The carbohydrate moieties (glycan) of glycoproteins are synthesized in the endoplasmic reticulum (ER) and Golgi organelles in a cell. Recent advances in genomics have shown that glycan assembly is an elaborate process with 16 putative nucleotide sugar transporters, 14 enzymes in the N-glycan assembly line, 13 separate enzymes that can attach galactose to the glycan precursor, 200 sequences for glycosyltransferases and 23 mammalian sulfotransferases [1]. Dysregulation of cells, such as in cancer, will affect the biosynthesis of the carbohydrates present in glycoproteins [2]. Tumor cells show aberrant patterns of carbohydrates linked to cell surface proteins, with the presence of larger and more branched N-linked oligosaccharides [3]. To add to this complexity, increase in tumor cell sialylation is common for various tumors and could be due to an increase in sialyltransferase activity [4]. Also, the rate of clearance of sialylated glycoproteins is largely controlled by the interaction of the asialo form with the corresponding liver receptor. In other studies of well recognized plasma proteins, such as transferrin and haptoglobin, the structure of the oligosaccharide portion was shown to change in disease or metabolic derangement [5–10]. One can, therefore, conclude that the search for better biomarkers for disease detection is significantly hindered by the absence of information about global and specific changes in one of the major post-translational modifications (PTMs). This lack of knowledge is especially true for the plasma and serum glycoproteome where the bulk of proteins are released by a process of secretion from tissues, which, in tissues such as the liver, is usually linked to glycosylation.

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At present it is unknown how often a glycoprotein circulates in an altered state without a change in concentration, although a number of 2D gel studies provide evidence that this can be a common occurrence in certain diseases [11,12]. Current approaches to studying changes in glycoproteins are limited to the study of detailed glycosylation forms in a single target protein of biological or clinical interest, such as the comprehensive structure study of tissue plasminogen activator at Genentech Inc. [13–15]. There are at present no methods which can be used to screen glycoproteins for glycosylation pattern changes in complex biological samples, such as serum, apart from 2D gels, which has a limited dynamic range.

Previously, we used multi-lectin affinity chromatography (M-LAC) to enrich glycoproteins from human serum [16]. The M-LAC column contained Con A, WGA and Jacalin lectin. With the sequential use of displacers specific for the lectins in M-LAC, we have concluded that the distribution of glycoproteins in the lectin displacement fractions was determined by the glycosylation patterns. This observation suggests that the distribution of a given glycoprotein in displacement fractions will shift if the glycosylation pattern is changed. Therefore, we have proposed that a shift in the distribution of a glycoprotein in the three displacement fractions in a comparison of a disease versus normal sample can indicate that a disease is associated with a change in the carbohydrate moieties of a putative disease biomarker.

In order to explore the above hypothesis, we have used the enzyme neuraminidase (releases sialic acid from oligosaccharides) to modify the oligosaccharide chains present in human transferrin and analyzed the distribution of glycoforms in the displacement fractions generated from an M-LAC column (Con A, WGA and Jacalin lectin). We then used the enzymes, neuraminidase and fucosidase (releases fucosyl residues from oligosaccharides), to modify glycoproteins present in human serum, and compared the distribution of glycoproteins in displacement fractions to that in untreated serum. The presence of a glycoprotein in a given displacement fraction was determined by LC–MS/MS analysis of a tryptic digest. We used the Sequest rank, which represents the confidence of assignment of a protein in the sample, to screen for significant changes in the abundance of a given glycoprotein in a displacement fraction. The change in ranking was then established by peak area measurements of selected peptides from representative proteins. We were able to demonstrate that the glycosidase treatment resulted in a significant change in distribution of certain glycoproteins to the M-LAC displacement fractions, and that the changes were related to both the nature of the oligosaccharide chain and the specificity of the glycosidase digestion. These results indicated that the M-LAC column is sensitive to the loss of sialic acid and fucosyl residues present in serum glycoproteins, and that this approach can be used to screen serum proteins for glycosylation change due to disease. In addition, the use of a glycosidase to induce specific changes of glycoproteins can support the development of M-LAC columns containing various groups of lectins specific for certain diagnostic fluids and types of disease.

2. Experimental

2.1. Materials

Agarose bound Concanavalin A (Con A) with a protein concentration of 6 mg lectin/mL gel and binding capacity of more than 4 mg ovalbumin/mL gel, agarose bound Wheat germ agglutinin (WGA) with a protein concentration of 7 mg lectin/mL gel and binding capacity of 8 mg NGA/mL gel and agarose bound Jacalin lectin with a protein concentration of 4 mg lectin/mL gel and a binding capacity of more than 4 mg monomeric IgA/mL gel were obtained from Vector Laboratories (Burlingame, CA). Trypsin (sequence grade) was purchased from Promega (Madison, WI). Human serum, bovine serum albumin (BSA), transferrin, α (2 → 3,6,8,9) neuraminidase Arthrobacter ureafaciens (proteomics grade, EC number: 3.2.1.18) and α-1,6-fucosidase solution (recombinant, EC number was not provided by the supplier, and it is possibly 3.2.1.127) were purchased from Sigma–Aldrich. Novex pH 3–10 IEF gel and SimpleBlue SafeStain were purchased from Invitrogen (Philadelphia, PA). Coomassie Plus protein assay reagent was purchased from Pierce (Rockford, IL).

2.2. Treatment of transferrin with neuraminidase

Human transferrin at a concentration of 10 mg/mL was dissolved in a 100 mM phosphate buffer at pH 7.4. A sample of transferrin solution, 30 μL, was diluted using reaction buffer (5×), then incubated with 4 units of neuraminidase at 37 °C for 3 h (one unit enzyme will release 1 nmol of 4-methylumbelliferone from 2-(4-methylumbelliferyl) α-D-*N*-acetylneuraminic acid per minute at pH 5.5 at 37 °C). The sample, asialotransferrin, was immediately chromatographed on the M-LAC column.

2.3. Treatment of human serum with neuraminidase

Human serum (100 μL) was incubated with 25 units of neuraminidase in the reaction buffer (pH 5.5) at 37 °C for overnight.

2.4. Treatment of human serum with fucosidase

Human serum (100 μL) was incubated with 0.038 unit of fucosidase in the reaction buffer (pH 5.0) at 37 °C for 12 h (one unit enzyme will release 1.0 μmol of methylumbelliferone from 4-methylumbelliferyl-α-L-fucoside per min at pH 5.0 at 37 °C).

2.5. Fractionation of glycoproteins by sequential use of three displacers on a M-LAC column

The multi-lectin column was prepared by mixing 0.5 mL of agarose bound Con A, 0.5 ml of agarose bound WGA, and 0.5 ml of agarose bound Jacalin lectin in an empty PD-10 disposable column. In a series of experiments the following samples: transferrin (300 μg), asialotransferrin (300 μg), untreated serum (100 μL), neuraminidase-treated serum (100 μL) and fucosidase-treated serum (100 μL), were diluted with the M-LAC equilibration buffer (20 mM Tris, 0.15 M NaCl, 1 mM Mn^{2+} and 1 mM Ca^{2+}, pH 7.4) to a volume of 1 mL, and then loaded individually on separate M-LAC columns. In order to avoid cross contamination, separate multi-lectin columns were used for each study. After 15 min reaction, the unbound proteins were eluted with 10 mL of equilibration buffer. To fractionate the captured proteins based on glycosylation motifs, the sample was eluted with the following displacers. Proteins bound to Jacalin lectin were first released with 4 mL of 0.8 M galactose in 20 mM Tris buffer (pH 7.4) containing 0.5 M NaCl. Then Con A selected proteins were released with 4 mL of 0.5 M methyl-α-D-mannopyranoside in a 20 mM Tris buffer (pH 7.4) containing 0.5 M NaCl. Finally, the WGA selected proteins were released with 4 mL of 0.5 M *N*-acetyl-glucosamine in 20 mM Tris buffer, pH 7.4 containing 0.5 M NaCl. The three fractions were concentrated with a 10 kD Amicon filter (Millipore, Billerica, MA). All studies were performed in duplicate to test reproducibility of the enzymatic digestion.

2.6. Protein assay

The amount of proteins in M-LAC displacement fractions of transferrin and asialotransferrin were measured using Bradford protein assay. BSA solutions in a series of concentrations, 200, 400, 600, 800, and 1000 μg/mL, were used as standards. Standards and samples with 10 μL each were pipetted on a 96-well flat bottom plate (Corning Inc. Corning, NY), and 300 μL Coomassie Plus protein assay reagent was added. After 10 min incubation at ambient temperature, the samples were measured with a UV detector at a wavelength of 595 nm using a SPECTRA max plate-reader (Molecular Devices, Sunnyvale, CA).

2.7. IEF separation

Transferrin, asialotransferrin, and the corresponding displacement fractions collected from the M-LAC were loaded on a Novex IEF gel (1.0 mm, 10 well, pH 3–10), 5 μg proteins in each sample. The proteins were focused on the gel with a voltage gradient. The voltage was first kept at 100 V for 1 h, changed to 200 V and kept for another hour, and then finished at 500 V (30 min). The gel was then stained using SimpleBlue SafeStain. The samples were analyzed in the absence of detergent and reducing agents.

2.8. LC–MS/MS

The glycoprotein fractions were digested with trypsin, using a procedure described previously [17]. The trypsin digested peptides were then separated and analyzed on a Ettan MDLC system (GE Healthcare, Piscataway, NJ) coupled to a LTQ linear ion trap (ThermoElectron, San Jose, CA). About 2 μg of each sample was injected onto a Peptide Captrap column (Michrom Bioresources Inc. Auburn, CA) using the autosampler of the MDLC system. To desalt the sample, the trap column was washed with H_2O with 0.1% formic acid at a flow rate of 10 μL/min for 4 min. The flow was directed to the solvent waste through a 10-port valve. After desalting, the valve was switched to directing flow to the separation column. Then, the desalted peptides were released and separated on a C18 capillary column (packed in house, Magic C18, 150 mm × 0.075 mm). The flow rate was maintained at 400 μL/min monitored with a flow-meter. The gradient was started at 0% acetonitrile (ACN) with 0.1% formic acid and linearly increased to 35% ACN in 60 min, then to 60% ACN in 15 min, and to 90% ACN in another 5 min, then kept at 90% ACN for 10 min. The Ettan MDLC was operated from UNICORN^{TM} control software (GE Healthcare, Piscataway, NJ). The resolved peptides were analyzed on an LTQ linear ion trap mass spectrometer with a nano-ESI ion source. The temperature of the ion transfer tube was controlled at 200 °C and the spray voltage was 2.0 kV. The normalized collision energy was set at 35% for MS/MS. Data dependent ion selection was monitored to select the most abundant seven ions from a MS scan for MS/MS analysis. Dynamic exclusion was continued for duration of 3 min.

2.9. Bioinformatics

Peptide sequences were identified using Sequest algorithm (Version C1) incorporated in BioWorks software (Version 3.1 SR) (ThermoElectron, San Jose, CA). Only peptides resulting from tryptic cleavages were searched. The Sequest results were filtered by Xcorr versus charge state. Xcorr was used for a match with 1.5 for singly charged ions, 2.0 for doubly charged ions, and 2.5 for triply charged ions. The proteins with more than one peptide detected were considered.

3. Results and discussion

3.1. The distribution of transferrin and asialotransferrin into the three displacement fractions of M-LAC

Transferrin was treated with neuraminidase to generate asialotransferrin and loaded on an M-LAC column. The same amount of an untreated transferrin sample was loaded on to a separate fresh multi-lectin column. By sequentially using different displacers to release the protein captured by each

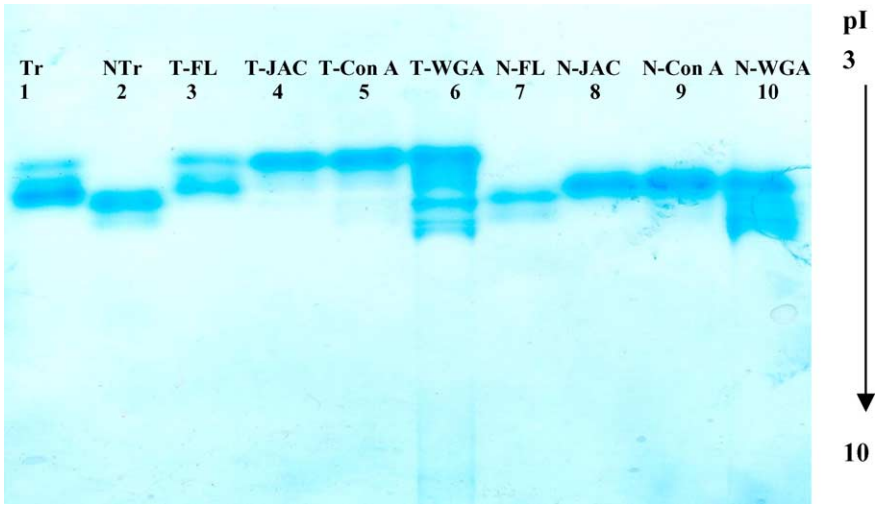

Fig. 1. IEF gel separation profile of transferrin, asialotransferrin, and their fractions collected from M-LAC. Novex IEF gel, pH 3–10; loaded 5 μg protein in each well (native conditions); voltage: 100 V, 1 h; 200 V, 1 h; 500 V, 30 min. Transferrin (Tr) and asialotransferrin (NTr) were loaded on two M-LAC columns. The unbound proteins were collected in the flow-through (lanes 3 and 7, respectively). By sequentially using three displacers, the proteins captured by Jacalin, Con A, and WGA were separated and collected as three displacer fractions. Lanes 4–6 were the fractions from transferrin, and lanes 8–10 were the three fractions from asialotransferrin. In this profile, the displacement fractions from asialotransferrin showed a higher p*I* value than those from the original transferrin, which indicated the success of removing sialic acid from transferrin by the use of neuraminidase.

lectin present in the column, the glycoproteins were separated into three displacement fractions. Meanwhile, the unbound protein was collected in the flow-through fraction, and this portion of the transferrin sample was expected to contain mainly the non-glycosylated fraction of the protein. Fig. 1 shows the IEF separation of all the fractions, including intact transferrin and asialotransferrin. The higher p*I* of the components in the asialotransferrin relative to the untreated sample indicated the successful release of sialic acid residues from the glycan structures of transferrin by neuraminidase. With the same amount of proteins loaded in each lane, the WGA fraction showed multiple bands, which indicated that sialic acid variants present in low levels in the transferrin sample were enriched by this lectin. In addition, the amount of proteins in each fraction was measured using the Bradford assay. The assay showed similar recoveries between the intact protein and asialo protein after M-LAC separation, 86 and 85%, respectively. These are typical results for this procedure and indicate good performance. Compared with the M-LAC results for transferrin, the amount of asialotransferrin captured by WGA was reduced by about 40%, while the amount of protein captured by Jacalin and Con A lectin were correspondingly increased about 6 and 13%. This distribution shift was reproducible, and was attributed to a decreased affinity of the asialo protein to WGA, which has a specific affinity to sialic acid. Also, in the glycan structures of transferrin, more galactosyl residues are exposed after removal of sialic acid residues, which increased the affinity of this protein to the Jacalin lectin. In a similar manner, Con A exhibited increased affinity to asialotransferrin in relation to WGA.

3.2. An investigation of the distribution shift of serum glycoproteins after neuraminidase treatment

Human serum was treated with neuraminidase to cleave sialic acid residues from the glycan forms present in serum glycoproteins, and the product was loaded on a M-LAC column. The glycoproteins captured by each lectin immobilized in the M-LAC column were sequentially released with the three specific lectin displacers. After trypsin digestion, each fraction was analyzed by LC–MS/MS, and the proteins were identified using the Sequest algorithm. In order to investigate the relative abundance change of the glycoproteins in each lectin fraction, we compared the Sequest rank, a parameter representing the quality of assignment of a protein in a sample, of the lectin fractions before and after enzyme treatment. While Sequest ranking (smaller the number, generally higher the concentration) is not a direct measure of protein concentration, it can be used to describe trends in changes of relative protein concentration due to dependence of the algorithm on parameters such as number of peptides detected and quality of spectra in a given peptide identification. The average Sequest rank change, which represents the summation of protein level changes (in terms of absolute magnitude) between the two samples, is listed in Table 1. The average of Sequest rank differences between duplicate serum samples was used as a control. Compared with the control value, the neuraminidase treated serum sample had an obvious Sequest rank shift in all three displacer fraction samples, i.e., 7, 18, and 31 for the Jacalin, Con A, and WGA fractions, respectively (Table 1). This result indicated that neuraminidase digestion did indeed cause a shift in the distribution of the proteins in the three

Table 1
The average Sequest rank[a] shift of serum glycoproteins in each lectin displacement fraction from the M-LAC column after enzymatic treatment

Average rank shift[b]	Neu. Serum[c]	Fuc. Serum[d]
Jacalin fraction	7	9
Con A fraction	18	6
WGA fraction	31	18

[a] The Sequest rank represents the probability of assignment of a given protein.
[b] The average of the Sequest rank differences of proteins between an enzyme-treated sample and the intact serum sample in each lectin displacement fraction. The average of Sequest rank differences between duplicate analyses of untreated serum samples was used as a control.
[c] Neuraminidase-treated serum.
[d] Fucosidase-treated serum.

displacer fractions. Also, this result showed that the multi-lectin column is sensitive to the changes of sialic acid content in the glycan forms.

In addition, we specifically investigated the distribution of five different glycoproteins in this complex sample, human serum, with and without neuraminidase treatment. These proteins, transferrin, haptoglobin, alpha-2HS-glycoprotein, beta-2-glycoprotein, and alpha-1-acid glycoprotein, have been reported as containing sialic acid residues in their carbohydrate moieties [18–20]. Some of these structures have shown a correlation to diseases in terms of sialic acid-related glycosylation pattern changes [21–23]. The Sequest rank of the above set of glycoproteins from untreated serum and neuraminidase-treated serum were compared for each displacement fraction (Table 2). According to the Sequest rank comparison, the abundance of these proteins was reduced in the WGA fraction after neuraminidase treatment, although some trends were more substantial than the others. In the case of transferrin, alpha-2HS-glycoprotein and beta-2-glycoprotein, the proteins could not be identified at all in the WGA fraction after neuraminidase digestion. Instead, the levels of transferrin and alpha-2HS-glycoprotein were raised in the Jacalin fraction.

However, the Sequest rank is at best a semi-quantitative parameter to evaluate the relative abundance of a protein in a complex sample. And thus, to further investigate the distribution shift of this set of proteins between the displacer fractions, we compared peak areas of selected peptides for quantification. Specifically, the extracted ion chromatogram peaks were selected if they were within 1 amu of the expected mass, ±0.5 min of the observed retention time, and above a S/N (signal to noise) of 5:1. We arrived at this criterion by verifying that the standard deviation for retention time over multiple runs was 0.5 min. Fig. 2 shows the examples of extracted ion chromatograms of the peptide of transferrin, "KPVEETANCHLAP" (M/Z signal 530.2) from the M-LAC displacer fractions of serum and neuraminidase-treated serum samples. Within the acceptable retention time window, a small peak area of 1.6 (E6 counts) was integrated from the WGA fraction of the neuraminidase-treated serum sample (Fig. 2d).

A comparison of the peak areas shows that the distribution of transferrin in serum, after treatment with neuraminidase, shifted from the WGA fraction to the Jacalin and Con A fractions. This result agreed with both the Sequest rank comparison and the study of a transferrin standard (See Section 3.1). In addition, this result was confirmed by the measurement of peak areas of a second selected transferrin peptide, FDEFFSEGCAPGSK, (Table 3). Also, another comparison of peak areas (Table 3) indicated that haptoglobin exhibited a similar shift from the WGA fraction to the Jacalin and Con A fractions after the serum sample was treated with neuraminidase. The distribution changes observed for beta-2-glycoprotein and alpha-1-acid glycoprotein in Jacalin and Con A fractions were similar to the other two proteins but could only be detected by peak area measurement and not the Sequest rank comparisons. For each protein, multiple peptides, if detected, were selected for peak area measurements, and the peak area comparison between different runs showed the similar trend as shown in Table 3.

These results showed that the M-LAC approach, with the sequential use of displacers can be used to investigate a shift in the distribution of glycoproteins in a complex sample, which will enable the search for proteins with differential glycosylation patterns that can be related to disease status.

3.3. An investigation of the distribution shift of serum glycoproteins after using fucosidase treatment

A change in the level of fucosylation has been shown to occur in a significant number of diseases. The studies of fucosyltransferases have suggested important changes in fucose metabolism in cancer [24]. Abnormally-fucosylated haptoglobin was found to be elevated in serum in patients with active rheumatoid arthritis [25] and carcinoma of the

Table 2
The Sequest rank of selected glycoproteins identified in three M-LAC displacer fractions from serum and neuraminidase-treated serum samples

Sample displacer fraction	Human serum			Neuraminidase-treated serum		
	JAC	Con A	WGA	JAC	Con A	WGA
Transferrin	2	2	19	1	2	X[a]
Haptoglobin	9	7	4	9	7	2
Alpha-2HS-glycoprotein	14	32	81	7	31	X
Beta-2-glycoprotein	20	20	52	23	24	X
Alpha-1-acid-glycoprotein	21	33	7	27	96	23

[a] The protein was not identified in the sample. In the case of the WGA fraction from transferrin and alpha-2HS-glycoprotein, the peptide could be identified by extracted ion monitoring.

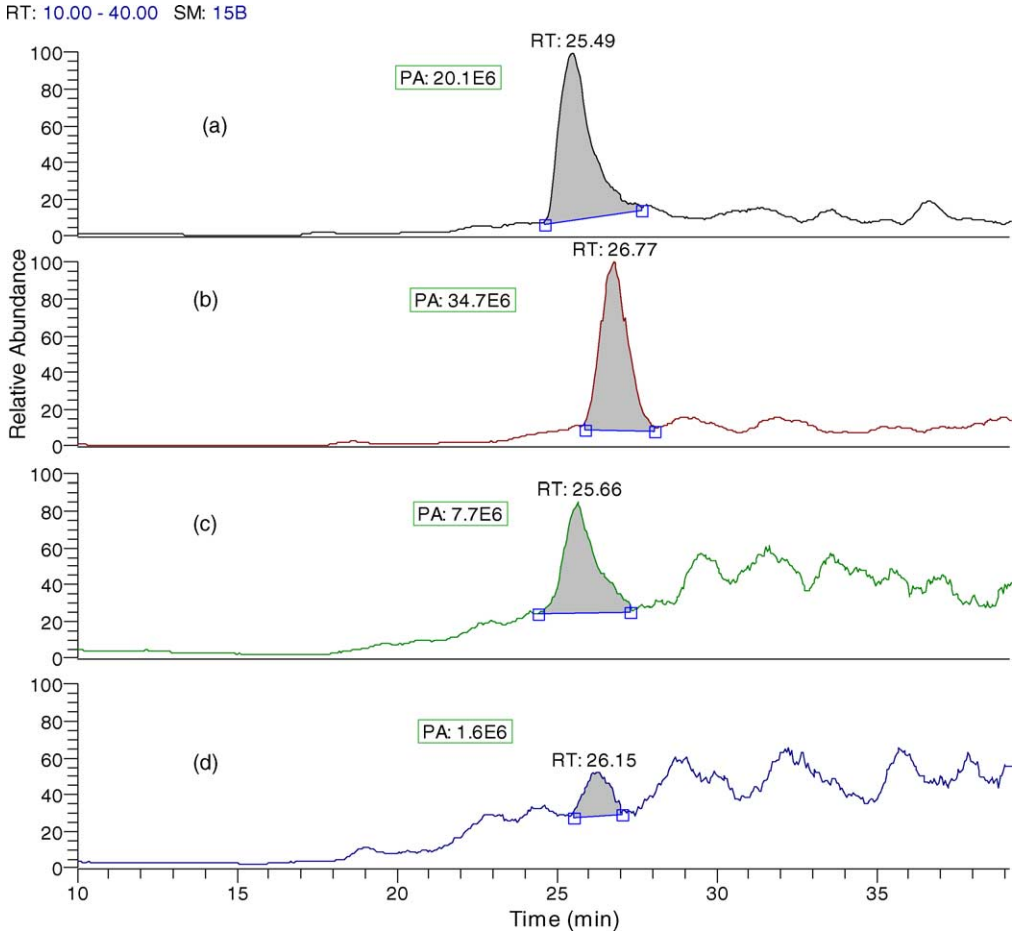

Fig. 2. Some examples of extracted ion chromatograms of the transferrin peptide, "KPVEETANCHLAP" (M/Z signal 530.2), for M-LAC displacer fractions from serum and neuraminidase-treated serum samples. The figure shows extracted ion chromatograms of the transferrin peptide, PVEETANCHLAP (M/Z signal 530.2), in the following samples: Con A and WGA fractions from serum (a and c, respectively), and Con A and WGA fractions from neuraminidase treated serum (b and d, respectively). Within the acceptable retention time window, a small peak area of 1.6 was integrated from the WGA fraction of the neuraminidase-treated serum sample (d).

ovary or breast [24]. The level of fucosylation of alpha-1-acid glycoprotein is significantly higher in patients with liver disease [26].

In this research study, human serum was treated with a fucosidase to release fucosyl residues from oligosaccharides present in glycoproteins, and then fractionated on a M-LAC column with a sequential use of displacers specific for each lectin. The changes in the Sequest rank of the displacement fractions of the enzyme-treated sample relative to the original serum sample were shown in Table 1. These changes indicated that the glycosylation pattern change induced by fucosidase digestion resulted in a distribution shift

Table 3
Selected peptide peak areas (×E6 counts) of targeted glycoproteins identified in three M-LAC displacer fractions from serum and neuraminidase-treated serum samples

Protein	Selected peptide[a]	M/Z signal[b]	Human serum			Neuraminidase-treated serum		
			JAC	Con A	WGA	JAC	Con A	WGA
Transferrin	KPVEEYANCHLAR	530.2	19	20	7.7	24	35	1.6
Transferrin	FDEFFSEGCAPGSK	790.1	7.0	7.8	5.0	8.3	11	0
Haptoglobin	YVMLPVADQDQCIR	854.6	10	39	236	13	47	163
Alpha-2HS-glycoprotein	AQLVPLPPSTYVEFTVSGTDCVAK	1290.32	4	2.6	1.7	7.4	2.6	0.9
Beta-2-glycoprotein	ATVVYQGER	512.3	2.6	3.5	1.8	3.8	8.4	0
Alpha-1-acid-glycoprotein	EQLGEFYEALDCLR	872.3	9.7	9.2	129	12	11	0

[a] The selected peptide was identified by data-dependent MS/MS in LTQ, and the peak area of the extracted ion chromatogram was measured at expected retention time (±0.5 min).
[b] The peak area of a selected peptide of the protein was extracted at the M/Z signal where the peptide was detected.

Table 4
The Sequest rank of selected glycoproteins identified in three M-LAC displacer fractions from serum and fucosidase-treated serum samples

Sample displacer fraction	Human serum			Fucosidase-treated serum		
	JAC	Con A	WGA	JAC	Con A	WGA
Haptoglobin	9	7	4	7	5	2
Alpha-1-acid-glycoprotein	21	33	7	19	38	9
Apo A	4	19	10	5	12	24
Ceruloplasmin	24	14	8	6	8	28
Inter-alpha-trypsin inhibitor	6	22	9	31	13	30
Pregnancy zone protein	×	34	7	6	25	17

Table 5
The selected peptide peak areas (×E6 counts) of the given glycoproteins identified in three M-LAC displacer fractions from serum and fucosidase-treated serum samples

Protein	Selected peptide[a]	M/Z signal[b]	Human serum			Fucosidase-treated serum		
			JAC	Con A	WGA	JAC	Con A	WGA
Haptoglobin	YVMLPVADQDQCIR	854.6	10	40	236	22	51	82
Alpha-1-acid-glycoprotein	EQLGEFYEALDCLR	872.3	9.7	9.2	129	17	10	50
Apolipoprotein A	VSFLSALEEYTK	694.0	40	12	60	43	12	16
Ceruloplasmin	MFTTAPDQVDKEDEDFQESNK	826.0	2.0	7.8	9.1	6.2	9.8	11
Inter-alpha-trypsin inhibitor	IYGNQDTSSQLK	678.5	0.56	0.5	0.74	1.5	0	0
Pregnancy zone protein	ATVVYQGER	512.3	0	6.6	11	0	16	5.2

[a] The selected peptide was identified by data dependent MS/MS in LTQ, and the peak area of the extracted ion chromatogram was measured at expected retention time (±0.5 min).
[b] The peak area of a selected peptide of the protein was extracted at the M/Z signal where the peptide was detected.

between the three displacement fractions. Although none of the three lectins in the M-LAC column have a absolute affinity to fucose, the loss of fucosyl residues from the glycan influences the environment of other sugar residues (e.g. *N*-acetylglucosamine) which also have an affinity to the lectins (e.g. WGA). These results indicate that the M-LAC column containing Jacalin, Con A, and WGA can detect a glycosylation pattern change as a result of the gain or loss of fucosyl residues in the glycan forms. Another implication of this study is that the more broad specificity of lectins versus antibodies are better suited to detect subtle structural changes in glycosylation motifs.

The distribution shift in the M-LAC column caused by fucosidase digestion was not as significant as neuraminidase digestion. There are several possible explanations for this including the properties of a specific lectin, such as WGA, which has affinity for sialic acid. Also, sialic acid is more common than fucosyl residues in glycan moieties and is charged at physiological pH values. Thus, the loss of sialic acid in the samples resulted in a more dramatic change in distribution between the three displacer fractions. Therefore, by optimizing the combination of lectins immobilized in M-LAC, such as including a combination of lectins with specificity to fucose, M-LAC will be more sensitive to glycosylation changes at fucosyl residues.

Although the distribution shift induced by the use of fucosidase was not as great in terms of the average Sequest rank change of the serum glycoproteins, some individual fucosylated glycoproteins showed a detectable distribution shift. For example, according to the Sequest rank and the peak area comparisons (Tables 4 and 5), after the treatment of fucosidase, a significant fraction of haptoglobin and alpha-1-acid-glycoprotein glycoforms shifted from the WGA fraction to the Jacalin fraction, and some haptoglobin shifted to the Con A fraction. The distribution of ceruloplasmin, in which variable fucosylation was shown to be a cause of micro-heterogeneity observed in ceruloplasmin samples [27], was shifted to the Jacalin fraction after fucosidase treatment. In addition, other glycoproteins, such as apolipoprotein A-I, inter-alpha-trypsin inhibitor, and pregnancy zone glycoprotein, also had altered distribution between the three displacement fractions after the treatment with fucosidase (Tables 4 and 5). The amount of apolipoprotein A-I observed in the WGA displacement fraction decreased, which was shown in both Sequest rank comparison and selected peptide peak area comparison. Inter-alpha-trypsin inhibitor and pregnancy zone protein also had a reduction in the WGA displacement fraction after treatment with fucosidase, with a corresponding increase in the Con A fraction. These results indicated that a change in fucosylation in a sample can be detected by a change in the distribution of the glycoproteins in the three displacement fractions from an M-LAC study. Such analysis can be a valuable tool to screen on a more global basis for subtle changes in the glycosylation of biomarkers in diseases, such as cancer and liver diseases.

4. Conclusions

We have described an application of multi-lectin affinity chromatography (M-LAC) which is a sequential use of different displacers to release glycoproteins captured by each

immobilized-lectin to discover proteins with changes in glycosylation. One example, asialotransferrin compared with the normal sialyated variant, showed an obvious distribution shift among the three displacer fractions of M-LAC. This shift in transferrin isoforms in different displacement fractions was also detected in a complex serum sample. Additional glycoproteins in serum were also observed by distribution shifts in the lectin displacement fractions after the treatment with specific glycosidases, such as neuraminidase and fucosidase. These results indicate that by searching the distribution shift in M-LAC of glycoproteins one can identify proteins having differential glycosylation patterns and discriminate this change from the other complexities of biological samples. As we know, a change in glycosylation patterns is common in many diseases, such as cancer and liver abnormalities, therefore, we propose that the use of this method for comparing normal and disease samples is a feasible approach to search for biomarkers with differential glycosylation related to disease status. In addition, using a specific glycosidase to induce a glycosylation change in a targeted protein may also be a means to optimize the combination of lectins in an M-LAC system for specific diagnostic purposes.

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