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A method for the identification of glycoproteins from human serum by a combination of lectin affinity chromatography along with anion exchange and Cu-IMAC selection of tryptic peptides

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Abstract

This paper reports a method for identifying glycoproteins from human serum. Glycoproteins were selected with a concanavalin A (Con A) lectin column and then tryptically digested prior to sequential chromatographic selection of acidic and histidine containing peptides. Acidic peptides were selected with a strong anion exchange (SAX) column. Peptides captured by the SAX columns were then released and histidine-containing peptides in the mixture selected with a copper loaded immobilized metal affinity chromatography (Cu-IMAC) column. This serial chromatographic selection process reduced the complexity of proteolytic digests by more than an order of magnitude. Peptides selected by this serial process were then fractionated by reversed-phase chromatography (RPC) and identified by tandem mass spectrometry. The method was initially validated using human transferrin before application to human serum. The results show that all the peptides identified except one contained histidine and acidic amino acids.
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Keywords: Glycoprotein; Lectin affinity chromatography; Ion exchange; Cu-IMAC

1. Introduction

Glycoproteins on the cell surface and in the extracellular matrix are involved in a variety of cellular functions and intercellular interactions. One of the challenges in identifying these proteins is how to deal with the separation problem caused by the complexity of the attached oligosaccharides. This heterogeneity gives rise to a "train" of spots in 2D gel electrophoresis (2DE) [1,2], reflecting differences in both the isoelectric points and size of glycoproteins. It also complicates mass spectral (MS) identification, as has been described in a number of 2DE papers on the MS analysis of glycoproteins [3–7].

Based on the wide spread use of lectins for the isolation and analysis of glycoproteins during the past few decades [8], lectins are now being used in glycoproteomics [9,10]. Broad selectivity lectins such as concanavalin A (Con A), wheat germ agglutinin (WGA), and Jacalin are being used together to capture most glycoproteins [11]. This multi-lectin approach is often referred to as "glyco-catch method" [12,13].

When lectins are being used to capture proteins, any peptide from a captured protein can be used for identification of the parent glycoprotein. But tryptic digests are 30–50 times more complex than the mixtures of proteins from which they were derived. The fact that most tryptic peptides provide a unique signature for the parent protein from which they were derived is very useful. This means that a few peptides from a protein can be selected from a tryptic digest and used in the qualitative and quantitative analysis of parent proteins [14]. This has led to the development of methods that select peptides from digests on the basis of some specific amino acid [15]. Selection of histidine containing peptides with immobilized metal affinity chromatography (IMAC) is such a case [16]. Metal-chelating stationary phases loaded with copper have a high affinity for histidine containing peptides [15].

This paper examines the use of Cu-IMAC and strong anion exchange (SAX) chromatography to select peptides of specific

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amino acid composition from tryptic digests of lectin affinity selected proteins. Although the parent protein is glycosylated, these peptides in general will not carry a glycan. The objective is to reduce the complexity of tryptic digests through the selection of a small representative sample of peptides and aid in database searches by knowing one or more amino acids in the selected peptides through affinity selection.

2. Materials and methods

2.1. Materials

Human transferrin, human serum, tris[hydroxymethyl]-aminomethane (Tris base), tris[hydroxymethyl]aminomethane hydrochloride (Tris acid), L-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, iodoacetic acid, cysteine, dithiothreitol (DTT), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), manganese chloride, calcium chloride, *N*-hydroxysuccinimide were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN) was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Concanavalin A agarose, a HiTrap chelating HP column and a Hitrap Q XL column were purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Proteolysis

One milliliter of human serum (50–70 mg/ml protein in serum) was reduced with 10 mM DTT in 0.1 M phosphate buffer (pH 8.0) and 8 M urea. After incubation at 37 °C for 2 h, iodoacetic acid was added to a concentration of 25 mM and incubated in darkness on ice for an additional 2 h. The alkylation reaction was quenched for 30 min at room temperature by the addition of cysteine to a final concentration of 20 mM. After diluting the sample with 0.1 M phosphate buffer to a final concentration of 2 M urea, TPCK-treated trypsin was added to the sample at a 50:1 protein to trypsin mass ratio. The sample was incubated at 37 °C for 24 h. Proteolysis was terminated by adding TLCK protease inhibitor at a molar concentration exceeding that of trypsin by two-fold. The sample was then frozen in liquid nitrogen for 10 min.

2.3. Chromatography

All chromatographic separations were performed using a Biocad 60 workstation from Applied Biosystems (Framingham, MA).

2.4. Con A affinity selection

Concanavalin A agarose beads (Amersham Biosciences) were packed into a 50 mm × 10 mm I.D. PEEK column. The sample was applied to a Con A column after it had been equilibrated with 0.2 M Tris (pH 7.5) loading buffer containing 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$. After the unbound components were washed away, 200 mM methyl-α-D-mannopyranoside (α-MM) in loading buffer was used to elute the bound glycoproteins.

2.5. Anion exchange chromatography

A HiTrap Q XL 1 ml column was prepared by washing with five columns of starting buffer (20 mM bis–tris, pH 7.0). The flow rate was 1 ml/min. The UV absorbance was monitored at 280 nm. After the sample was applied, the column was washed with 10 column volumes of starting buffer until the UV absorbance baseline was stable. The adsorbed peptides were eluted with 10 column volumes of 1 M NaCl in starting buffer.

2.6. Cu-IMAC affinity chromatography

A 1 ml HiTrap chelating HP column was washed with 10 column volumes of the following solutions sequentially before selection: deionized water, 50 mM EDTA with 1 M NaCl at pH 8.0, deionized water, 50 mM $CuSO_4$, and elution buffer (0.1 M acetate, 0.5 M NaCl, pH 4.0). Then, the column was equilibrated with 10 column volumes of binding buffer (50 mM phosphate, with 0.5 M NaCl, pH 7.2). The flow rate was 1 ml/min, and the UV absorbance was monitored at 280 nm. After the sample was loaded, the column was washed with 10 column volumes of binding buffer until the UV absorbance was stable. The bound peptides were then eluted with 10 column volumes of elution buffer.

2.7. HPLC fractionation

Fractionation of peptides on a 250 mm × 4.6 mm I.D. Vydac C_{18} column was achieved with a 90 min gradient from 100% buffer A (5% ACN, 95% H_2O containing 0.1% TFA) to 60% buffer B (95% ACN, 5% H_2O containing 0.1% TFA) at a flow rate of 1 ml/min. Eluted peptides were monitored at 215 nm. Fractions were manually collected and vacuum-dried in a Savant Speed-Vac centrifuge before ESI-MS analysis.

2.8. ESI-MS analysis

Electrospray mass spectra were obtained on a QSTAR quadrupole time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA) equipped with an API ion source. The instrument was operated in the positive ion TOF mode (*m*/*z* 300–2000). Samples were dissolved in 100 μl MeOH/H_2O (50/50, v/v) containing 1% (v/v) formic acid and infused into the instrument at 8 μl/min. The ion spray voltage was 5500 V.

2.9. Tandem mass spectrometry

Tandem MS analysis was performed by transmitting the appropriate parent ion from the quadrupole to the collision cell. Collision gas used was nitrogen at a pressure of $4–6 \times 10^{-6}$ Torr. Peptides were fragmented with appropriate collision energy (25–60 eV). Proteins were identified by searching the MS/MS spectra against the NCBInr database (release date 26 November 2004) using the Mascot search engine (Matrix Science, London,

UK) (version 1.9). The following search parameters were used: trypsin and chymotrypsin were used as cutting enzymes, mass tolerance for monoisotopic peptide window was set to ±1.2 Da, the MS/MS tolerance window was set to ±0.8 Da, two missed cleavages were allowed, carboxymethylation of cysteine was set as fixed modification and methionine oxidation was set as variable modification. A positive identification was obtained from the statistically significant identification given by MASCOT.

3. Results and discussion

3.1. Analytical strategy

The analytical strategy for isolation and identification of glycoproteins from human serum was to use Con A lectin affinity chromatography to select N-linked glycoproteins first and after proteolysis to select peptides with specific amino acids using a combination of strong anion exchange and IMAC chromatography. Passage through a strong anion exchange chromatography column selected peptides with two or more acidic amino acid residues. These acidic peptides were then passed through a Cu-IMAC column to select peptides that also contained histidine.

The rationale for selecting acidic peptides is based on the fact that aspartic and glutamic acid are sufficiently abundant in nature that substantial numbers of peptides will be found in tryptic digests that contain multiple acidic amino acids. These acidic peptides will be selectable with a strong cation exchange chromatography column using neutral to slightly basic mobile phases. Desorption is most easily achieved by increasing the ionic strength of the mobile phase.

Selection of histidine peptides is based on the fact that they are adsorbed on copper loaded IMAC columns and can be desorbed by either an acidic mobile phase or addition of imidazole to the mobile phase. High ionic strength mobile phases used to elute the SAX column do not interfere with binding to the Cu-IMAC column. Since the pK of the imidazole group lies below neutral pH, Cu-IMAC selection was executed at pH 7.2 [17].

In general the eluent from the anion exchange column was directly applied to the Cu-IMAC column, but the order can be reversed. After anion exchange/Cu-IMAC selection, the peptides collected will contain both acidic amino acids and histidine and have been derived from a glycoprotein. Selected peptides were further fractionated by reversed-phase chromatography (RPC). Fractions from the RPC column were collected manually and analyzed by electrospray ionization tandem mass spectrometry. In this method, the peptides used to identify the glycoproteins are predominantly non-glycopeptides.

In principle, target glycans are fully recognized by the lectin, even after proteolysis. However, when, lectins are used to select glycopeptides, immobilized lectin could be damaged by exposure to highly processive protease. Furukawa et al. [18] obtained evidence for the multivalent nature of the interaction between WGA-Sepharose and sialoglycoconjugates. It was shown that whereas glycophorin bound to WGA-Sepharse, the disialytetrasaccharide of glycophorin did not bind. Similarly, fetuin and alpha-1-acid glycoprotein bound, but the glycopeptides derived from them did not. The strategy described here is an alternative method in the identification of glycoproteins in which their glycopeptides are not lectin selectable.

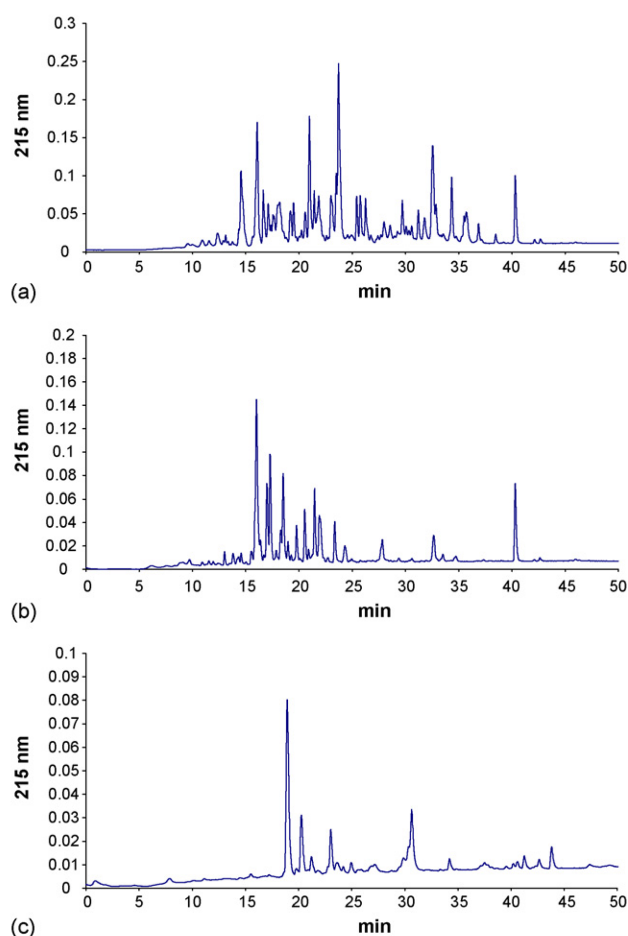

Fig. 1. Reversed-phase chromatograms of a tryptic digest of human transferrin. (a) Total tryptic digest; (b) after Cu-IMAC selection; (c) after anion exchange/Cu-IMAC affinity selections. Elution protocols are described in Section 2.

3.2. Analysis of human transferrin

The method described above was tested using human transferrin as a model protein. Human transferrin is a glycoprotein containing two *N*-glycosylation sites. Fig. 1 shows the reversed-phase chromatography separation of a tryptic digest of human transferrin. Fig. 1a is the RPC separation of the whole human transferrin tryptic digest whereas Fig. 1b is the chromatogram of transferrin peptides after Cu-IMAC affinity selection. It can be seen that the chromatographic profile in Fig. 1b is simpler than that in Fig. 1a. This indicates that Cu-IMAC affinity selection simplifies the sample. Fig. 1c is the chromatogram of transferrin peptides after double selection with anion exchange and Cu-IMAC columns. The chromatogram is even simpler than that in Fig. 1b.

Fractions from the RPC separation in Fig. 1c were collected and analyzed by electrospray tandem mass spectrometry. Table 1 shows all the identified peptides from human transferrin, together with the number of negatively charged aspartic acid

Table 1
Peptides identified from tryptic digest of human transferrin

gi no.	Protein name	Peptide sequence	Number of D/E	Charge (pH 7.0)
4557871	Transferrin	WCAVSEHEATK	2	−2.65
		KPVEEYANCHLAR	2	−2.65
		SVEEYANCHLAR	2	−2.95
		KPVDEYKDCHLAQVPSHTVVAR	3	−2.56
		EDLIWELLNQAQEHFGK	4	−3.15
		HSTIFENLANKADRDQYELLCLDNTR	5	−3.73
		SMGGKEDLIWELLNQAQEHFGK	4	−3.44
34810822	Trypsin	SIVHPSYNSNTLNNDIMLIK		

(D) or glutamic acid (E) residues in each peptide and the calculated peptide charge [19]. Histidine residues are underlined. The results show that all of the seven identified peptides contained at least one histidine residue and at least two D or E residues. The calculated charge of these peptides was less than −2 in all cases. Only one non-specifically bound peptide from trypsin was found. These results show that anion exchange/Cu-IMAC double selection is an effective approach to simplify tryptic digests by selecting only negatively charged histidine-containing peptides. Moreover, it shows that the analytical protocol can be used to identify glycoproteins.

3.3. Analysis of glycoproteins from human blood serum

Glycoproteins from 1 ml of human serum were selected by a Con A column and tryptically digested in the manner described above. The tryptic digest was then applied to an anion exchange column and the bound peptides were eluted with 1 M NaCl. The eluted peptides were then applied to the Cu-IMAC column and the peptides retained on the column were eluted and collected as a single fraction (Fig. 2). The peptides collected from the Cu-IMAC column were then applied to a C_{18} column. The reversed-phase chromatogram is shown in Fig. 3. All fractions from the RPC column were manually collected and analyzed by electrospray tandem mass spectrometry.

3.4. The peptides and proteins identified

Table 2 lists the peptides and proteins identified, together with the number of D or E residues in each peptide and the calculated charge of each peptide. The results show that all the peptides are negatively charged and contain histidine, except one peptide from alpha-1-antitrypsin, which is a non-specifically bound peptide. Thirty glycoproteins were identified. Twenty-two of these glycoproteins were also identified by the lectin affinity chromatography method alone [20]. The other eight glycoproteins not seen by the lectin affinity chromatography method are marked with an asterisk (*) in Table 2. On the other hand, fifteen glycoproteins were identified by lectin affinity chromatography method, but not by the method described in this paper. This is attributed to the different selection mechanisms used in these two methods. Generally one or two peptides are selected and used to identify proteins in the lectin affinity chromatography method. Matrix suppression of ionization can cause single peptides to be missed and result in failure to identify proteins present in mixtures. Use of larger numbers of peptides for identification in this method led to the identification of these proteins.

Since the number of glycopeptides is smaller than the number of negatively charged histidine containing peptides in a tryptic digest from a protein, one advantage of the method described in this paper is that for most glycoproteins, more than one peptide will be selected and used to identify the source proteins. This increases the certainty of protein identification. But the disadvantages are that glycosylation site information is harder to obtain.

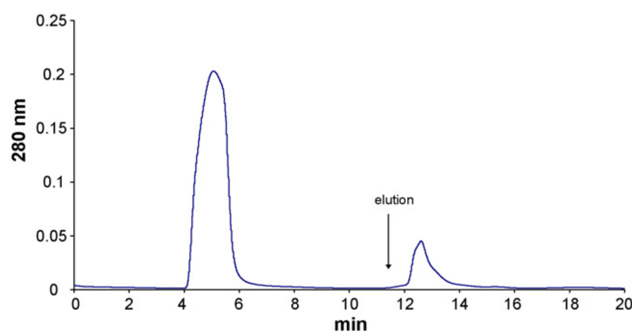

Fig. 2. Cu-IMAC affinity chromatogram of tryptic peptides from a digest of human serum after anion exchange selection. Elution protocols are described in Section 2.

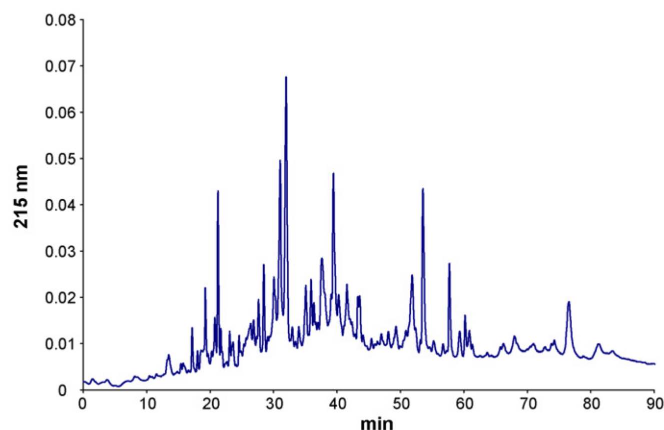

Fig. 3. Reversed-phase chromatogram of peptides from human serum glycoproteins captured by Con A affinity chromatography. Following lectin selection the captured proteins were tryptic digested and then applied sequentially to anion exchange chromatography and Cu-IMAC affinity selections. Peptides thus selected were fractionated by a C_{18} column as shown above.

Table 2
Peptides and their glycoprotein parents identified in human serum

gi no.	Protein name	Peptide sequence	Number of D/E	Charge (pH 7.0)
4501987	Afamin precursor	ESLLNHFLYEVAR	2	−1.15
		HELTDEELQSLFTNFANVVDK	5	−5.23
177831	Alpha-1-antitrypsin	TDTSHHDQDHPTFNK	3	−3.34
		DTEEEDFHVDQVTTVK	6	−5.41
69990	Alpha-1-B-glycoprotein	NGVAQEPVHLDSPAIK	2	−2.15
		SWVPHTFESELSDPVELLVAES	4	−5.44
2521981	Alpha-2-HS glycoprotein	HTLNQIDEVK	2	−2.23
224053	Macroglobulin alpha 2	HYDGSYSTFGER	2	−2.24
		MCPQLQQYEMHGPEGLR	2	−2.91
		SLFTDLEAENDVLHCVAFAVPK	4	−4.94
		KYSDASDCHGEDSQAFCEK	5	−6.14
		YSDASDCHGEDSQAFCEK	3	−6.14
		LVHVEEPHTETVR	3	−3.06
		TEVSSNHVLIYLDK	2	−2.51
		VVSMDENFHPLNELIPLVYIQDPK	4	−4.17
179151	Antithrombin III	TSDQIHFF	1	−1.51
4557327	Beta-2-glycoprotein I precursor	ATFGCHDGYSLDGPEEIECTK	5	−6.11
4503635	Coagulation factor II	KPVAFSDYIHPVCLPDR	2	−2.65
		HQDFNSAVQLVENFCR	2	−2.74
4557385	Complement component 3 precursor	HLIVTPSGCGEQNMIGMTPTVIAVHYLDETEQWEK	5	−5.64
		VELLHNPAFCSLATTK	1	−1.68
		LESEETMVLEAHDAQGDVPVTVTVHDFPGK	7	−7.05
		DTWVEHWPEEDECQDEENQK	7	−8.63
*20141171	Complement C4	YVSHFETEGPHVLLYFDSVPTSR	3	−3.06
		STQDTVIALDALSAYWIASHTTEER	4	−4.45
*29565	c4b-binding protein alpha chain	GSSVIHCDADSK	2	−2.65
2258128	Complement 9	AEQCCEETASSISLHGK	3	−4.11
180498	Complement H factor	TKEEYGHSEVVEYYCNPR	3	−4.01
		EGWIHTVCINGR	1	−0.65
		SSNLIILEEHLK	2	−2.45
		SITCIHGVWTQLPQCVAIDK	1	−2.45
*2347133	Complement factor B	LEDSVTYHCSR	2	−2.65
		HVIILMTDGLHNMGGDPITVIDEIR	4	−4.15
1070458	Ferroxidase	AEEEHLGILGPQLHADVGDK	5	−5.02
		KAEEEHLGILGPQLHADVGDK	5	−5.05
		ERGPEEEHLGILGPVIWAEVGDTIR	6	−4.14
		IGIIETTWDYASDHGEK	5	−4.15
		GPEEEHLGILGPVIWAEVGDTIR	5	−5.14
223170	Fibrinogen gamma	YEASILTHDSSIR	2	−2.15
67586	Haptoglobin precursor	LPECEADDGCPKPPEIAHGYVEHSVR	6	−6.05
		AVGDKLPECEADDGCPKPPEIAHGYVEHSVR	7	−6.02
		SPVGVQPILNEHTFCAGMSK	1	−1.95
		SPVGVQPILNEHTF	1	−1.45
		YVMLPVADQYDCITHYEGSTCPK	3	−4.15
		YQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDK	8	−8.64
		HYEGSTVPEK	2	−2.24
386789	Hemopexin precursor	SLGPNSCSANGPGLYLIHGPNLYCYSDVEK	2	−3.45
		LLQDEFPGIPSPLDAAVECHR	3	−4.64
		SGAQATWTELPWPHEK	2	−2.45
		QDEFPGIPSPLDAAVECHR	4	−4.64
		EVGTPHGIILDSVDAAFICPGSSR	3	−2.65

Table 2 (*Continued*)

gi no.	Protein name	Peptide sequence	Number of D/E	Charge (pH 7.0)
*2144705	Ig alpha-1 chain C region	DLCGCYSVSSVLPGCAEPWNHGK	2	−2.65
		KGDTFSCMVGHEALPLAFTQK	2	−2.65
		GDTFSCMVGHEALPL	2	−2.65
		SGNTFRPEVHLLPPPSEELALNELVTLTCLAR	4	−3.94
		KGDTFSCMVGHEALPL	2	−2.65
*70028	Ig gamma-1 chain C region	TPEVTCVVVDVSHEDPEVK	4	−6
		VVSVLTVLHQDWLDGK	2	−2.18
321150	Ig gamma-3 chain C region	WYVDGVEVHNAK	2	−2.15
		TPEVTCVVVDVSHEDPEVQFK	5	−6
69716	Ig J chain	FVYHLSDLCK	1	−1.65
127514	Ig mu chain C region	DVMQGTDEHVVCK	3	−2.65
		FTCTVTHTDLPSPLK	1	−1.65
		TVSEEEWNTGETYTCVVAHEALPNR	4	−6
4504783	Inter-alpha (globulin) inhibitor H2	AEDHFSVIDFNQNIR	3	−3.11
*68785	Kininogen, HMW precursor	DIPTNSPELEETLTHTITK	4	−3.14
73986398	*N*-Acetylmuramoyl-L-alanine amidase precursor	GSQTQSHPDLGTEGCWDQLSAPR	3	−3.65
*387031	Plasminogen	VQSTELCAGHLAGGTDSCQGDSGGPLVCFEK	4	−5.67
*2780174	Thyroxine-binding globulin	GTEAAAVPEVELSDQPENTFLHPIIQIDR	6	−6.14
4557871	Transferrin	WCAVSEHEATK	2	−2.65
		KPVEEYANCHLAR	2	−2.65
		SVEEYANCHLAR	2	−2.95
		KPVDEYKDCHLAQVPSHTVVAR	3	−2.56
		EDLIWELLNQAQEHFGK	4	−3.15
		EDLIWELLNQAQEHFGKDK	4	−3.15
38026	Zn-alpha 2-glycoprotein	HVEDVPAFQALGSLNDLQFFR	3	−3.23

(*): Proteins that were not seen in the previous study [20].

Table 3
Peptides from non-specifically selected serum proteins and trypsin

gi no.	Protein name	Peptide sequence
178775	Proapolipoprotein	ATEHLSTLSEK
106529	Ig kappa chain C region	ACEVTHQGLSSPVTK
		YACEVTHQGLSSPVTK
		LYACEVTHQGLSSPVTK
33395	Immunoglobulin lambda light chain	SYSCQVTHEGSTVEK
5174411	CD5 antigen-like protein	CSGEEQSLEQCQHR
4502493	Complement component 1	HSCQAECSSELYTEASGYISSLEYPR
4557393	Complement component 8, gamma polypeptide	YGFCEAADQFHVLDEVR
28549	Alpha globin	VGAHAGEYGAEALER
34810822	Trypsin	SIVHPSYNSNTLNNDIMLIK
28592	Serum albumin	CCAAADPHECYAK
		NECFLQHK
		EFNAETFTFHADICTLSEK
		LVRPEVDVMCTAFHDNEETFLKK
		ALVLIAFAQYLQQCPFEDHVK
		MPCAEDYLSVVLNQLCVLHEK
		HADICTLSEK

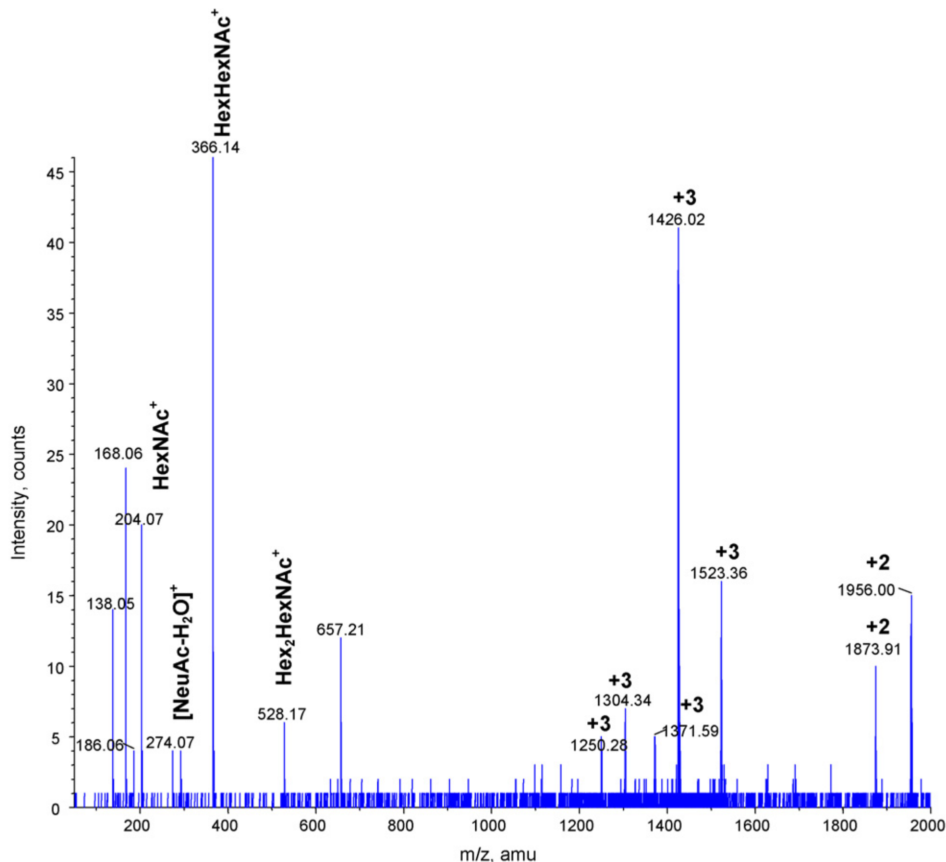

Fig. 4. MS/MS spectrum of the glycopeptide at *m/z* 1523.3 $[M+3H]^{3+}$. The singly charged ions at *m/z* 204.07 $(HexNAc)^+$, *m/z* 366.14 $(HexHexNAc)^+$, *m/z* 274.07 $(NeuAc\text{-}H_2O)^+$, and *m/z* 528.17 $(Hex_2HexNAc)^+$ are non-reducing terminal fragments.

3.5. Tandem mass spectrum of a glycopeptide

Although infrequent, it is possible that a glycopeptide can also contain histidine and acidic amino acid residues. Fig. 4 shows the MS/MS spectrum of such a peptide. The spectrum shows an ion at *m/z* 1523.3 $[M+3H]^{3+}$ and definitive evidence of glycosylation via the oxonium ion (B ion) signals at *m/z* 204 (HexNAc), 366 (HexHexNAc), 274 (NeuAc-H_2O), and 528 (Hex_2HexNAc) in the low mass region. These ions were derived from glycosidic cleavages at the non-reducing end of the carbohydrate chain. In the high mass region, the spectrum contains predominately Y type fragment ions (peptide + sugar). The spectrum shows the formation of a number of triply charged ions at *m/z* 1523.3, 1426.0, 1371.5, 1304.3 and 1250.2. These ions are separated by sugar mass differences of NeuAc, Hex, HexNAc, and Hex, respectively, suggesting a stepwise stripping of these sugars from a linear oligosaccharide attached to the glycopeptide. No peptide sequence information was obtained in this spectrum. The fact that more abundant ions are obtained from oligosaccharide fragmentation than the peptide portion of the molecule points out the difficulty of identifying glycopeptides when both the glycan and peptide portions are unknown. However, a deglycosylation step could be added to the protocol to allow the identification of the peptide even in such cases.

3.6. Non-specific binding

It is frequently the case that non-glycosylated proteins are seen during the analysis of proteins captured by lectin columns. This phenomenon appears to have occurred in this study as well (Table 3). This could occur in several ways. One is that proteins can be non-enzymatically glycated and be selected with Con A. Con A is known to select glycated hemoglobin [21]. Moreover, glycation of serum albumin and immunoglobulin is well known. This technically is not a failure of the analytical method so much as a lack of specificity in the lectin selector [22]. The second possibility is that protein complexes containing associated non-glycosylated and glycosylated proteins are being captured. Evidence of this phenomenon can be found in the affinity chromatography literature [23,24]. Non-specific binding is a problem in this method because it leads to false positives, i.e. the identification of proteins as being glycosylated when they are not.

4. Conclusions

It can be concluded that lectin affinity selection combined with anion exchange/Cu-IMAC affinity selection is an effective technique for isolation and identification of glycoproteins from complex mixtures. The advantage of the method is that the cer-

tainty of protein identification is increased compared to lectin affinity selection alone because more peptides are used for protein identification and it is known that peptides with acidic amino acids and histidine are being selected. The method described here is of particular utility in the identification of glycoproteins in which their glycopeptides are not lectin selectable. The disadvantage of the method is that glycosylation sites are not identified and there is no mechanism to prevent false positives.

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