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Research Article

EVALUATION OF GLYCAN PROFILES OF TAMM-HORSFALL GLYCOPROTEIN AND UROMODULIN

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ABSTRACT

Uromodulin is well-known to be the more potent immunosuppressive glycoprotein isolated from pregnant women urine, compared to its counterpart, Tamm-Horsfall glycoprotein (THP) isolated from non-pregnant women/male urine. However, structural profiles of these glycoproteins were controversial until today. Hence, this paper aims to identify the pregnancy associated glycosylation changes of THP and uromodulin. THP and uromodulin isolated with diatomaceous earth filtration were subjected for structural characterization using MALDI-TOF tandem mass spectrometry (MS) analyses. Both THP and uromodulin expressed high-mannose and complex-type *N*-glycan carrying Sd^a, LacNAc, LacdiNAc sequence as the capping antennae. In comparison to THP, uromodulin expressed higher abundance of LacdiNAc sequence and heavily sialylated complex type *N*-glycans, where up to four sialic acid residues were observed in its tetra-antennary glycans. Both THP and uromodulin expressed Core 1 and Core 2 *O*-glycans. These findings suggested that the higher level of LacdiNAc sequence and sialylation in uromodulin might facilitate its lymphocyte suppressive response more effectively than that of THP.

Keywords: Tamm-Horsfall glycoprotein (THP), Uromodulin, Structural, Glycosylation, Immunosuppressive, MALDI-TOF MS.

INTRODUCTION

Uromodulin was rediscovered by Muchmore and Decker [1] in human pregnancy urine 35 years after the discovery of Tamm-Horsfall glycoprotein (THP) by Tamm and Horsfall in non-pregnant women/male urine in 1950 [2]. Uromodulin was found to express immunosuppressive properties when low concentration of uromodulin was reported to inhibit T cell proliferation induced by tetanus toxoid [1] or by IL-1 [3]. Comparison study showed that uromodulin was a 13-fold more active inhibitor in suppressing the antigen-specific T cell proliferation i*n vitro* than THP [4]. Beside, uromodulin was also reported by Muchmore and colleagues that it could bind to recombinant IL-1, recombinant IL-2 and recombinant TNF. These cytokine binding activities have been suggested to contribute to uromodulin's immunosuppressive properties [5-7]. Unfortunately, there were limited literatures reporting on THP's cytokine binding activities.

Amino acid analysis showed that THP and uromodulin have similar protein backbone [4; 8] but differentially glycosylated, suggesting that uromodulin represented THP during pregnancy. Subsequent glycomics studies revealed that carbohydrates accounted for 30% of the weight of THP and uromodulin, consisting mainly of di-, tri- and tetra-antennary *N*-glycans. Many of contradictory results have been reported so far regarding the glycoform profiles of THP and uromodulin. There were reports on pregnancy-associated decreased in the $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ in uromodulin while $Man_7GlcNAc_2$ remained unchanged [9]. van Rooijen *et al.* [10] reported that no changes in the molar ratio of $Man_6GlcNAc_2$ in the course of pregnancy and the sialic acid and sulfate residues of complex type glycans remained the same in both glycoproteins [10]. Afonso *et al.* [11] and Rosenfeld *et al.* [12] failed to detect *O*-glycans in THP and uromodulin, while recently, Easton *et al.* [13] have identified the presence of *O*-glycans in these glycoproteins, in which uromodulin was shown to express unusual Core 2 *O*-glycans compared to Core 1 *O*-glycans in THP [13]. It is controversial until today on the glycan profiles of THP and uromodulin. Hence, this study was carried out to identify the *N*- and *O*-glycome of THP and uromodulin by using ultra-sensitive mass spectrometry (MS) approach, and to compare the differential glycosylation between them. We hypothesized that pregnancy-associated glycosylation changes in uromodulin contribute to its enhanced immunosuppressive properties.

MATERIALS AND METHODS

Chemicals and solvents

Sodium chloride (NaCl), diatomaceous earth, SigmaMarker™ (Cat. No. S8445), sodium hydroxide (NaOH) were bought from Sigma Chemical Co. (St. Loius, MO). Bradford reagent, ammonium bicarbonate, ready-mixed acrylamide-bis-acrylamide solution were from Merck. Dithiothreitol (DTT), ioacetic acid (IAA), trypsin and 2,5-dihydrobenzoic acid (DHB) were from Sigma-Aldrich. Peptide *N*-glycosidase F (PNGase F) was from Roche. Sep-Pak C_{18} column was from Waters Corporation. Methanol, propan-1-ol, chloroform, acetic acid, acetonitrile (ultra purity solvent) and dimethyl sulfoxide (DMSO) were from Romil. Methyl iodide was from Lancaster.

Sample collections

First morning urine specimens were collected weekly from a healthy non-pregnant woman and a healthy pregnant woman over a period of 8 weeks. Urine volume was measured and was neutralized with 1 M NaOH to pH 7.0.

Isolation and purification of THP and uromodulin

THP and uromodulin were isolated from non-pregnant and pregnant woman urine, respectively using the diatomaceous earth filtration derived by Serafini-Cessi *et al.* [14]. Briefly, diatomaceous earth suspended in deionized water was poured into Büchner funnel, lined with no.1 Whatman filter paper. Layer of diatomaceous earth was washed with deionized water and 0.02 M sodium phosphate buffer containing 0.14 M NaCl (PBS) accordingly. Urine was poured carefully into the funnel and filtered through the diatomaceous earth layer. At the end of filtration, diatomaceous earth layer was washed with PBS exhaustively. Then, the layer was scrapped off from the filter paper and suspended in deionized water, with occasional stirring for 30 minutes. Suspension was centrifuged at 20000 x *g* for 20 minutes and pellet was discarded. The supernatant was brought to the salt concentration of PBS and left for 20 minutes in room temperature. Then, the mixture was filtered once again through a new diatomaceous earth layer. The layer was washed exhaustively with PBS and suspended in deionized water to extract the protein. Finally, supernatant from centrifugation was collected, dialyzed against water at 4°C for 48 hours and freeze-dried. Protein content of THP and uromodulin was determined by Bradford assay. Crude proteins were purified with Sephadex G-100 chromatography using 50 mM ammonium bicarbonate (AmBic), pH 8.5 as the eluting buffer.

Electrophoresis

Molecular size and purity of protein was determined by SDS-PAGE, under reducing condition with 10% separating and 4% stacking gels. SigmaMarker™ (Cat. No. S8445) was used as the protein marker. Gels were stained with Coomassie brilliant blue to detect protein bands.

Reduction and carboxymethylation

THP and uromodulin was subjected to reduction and carboxymethylation to break down the disulfide bridges and then cap the cysteine residues to prevent them from reforming disulfide bridges. For reduction, dithiothreitol (DTT) in 50 mM Tris buffer, pH 8.5was added to samples in a 4-fold molar excess over the number of disulfide bridges and incubated for 1 hour at 37°C. For carboxymethylation, iodoacetic acid (IAA) in 50 mM Tris buffer, pH 8.5 was added in a 5-fold molar excess over DTT and incubated in the dark at room temperature for 2 hours. The reaction was terminated by dialysis against 50 mM Ambic buffer at 4°C for 48 hours.

Tryptic digestion

THP and uromodulin was digested with trypsin using a 1:8 w/w ratio of trypsin to THP/ uromodulin in 50 mM Ambic buffer, pH 8.4. The reaction was terminated by placement in boiling water for 3 minutes and lyophilized.

Release of *N*-glycans

THP and uromodulin were digested with 5 units of PNGase F in 50 mM Ambic buffer, pH 8.4 and incubated for 24 hours at 37°C. Samples were lyophilized and *N*-glycans were separated from the mixture using the Sep-Pak C_{18} propan-1-ol/ 5% acetic acid system. *N*-glycans were recovered in the 5% acetic acid portion.

Release of *O*-glycans

Propanol fractions after the PNGase F digestion containing *O*-glycopeptides were subjected to the reductive elimination to release the *O*-glycans. Samples were incubated with 55 mg/ml sodium borohydride ($NaBH_4$) in 0.1 M NaOH at 45°C for 16 hours. The reaction was terminated by adding 5 drops of 5% acetic acid. Dowex 50 x 8 chromatography was used to desalt and separate the released *O*-glycans and remaining peptides.

NaOH Permethylation

Released *N*-glycans were permethylated to improve the glycan structural analysis. Briefly, dried *N*-glycans were added into freshly NaOH-DMSO slurry and incubated for 25 minutes at room temperature on an automatic shaker. The reaction was terminated with slow drop-wise addition of ultra-pure water (~ 1 ml) with constant shaking. Then, 2 ml of chloroform was added and the mixture was made up to 5 ml with ultra-pure water. The mixture was mixed thoroughly and allowed to settle into two layers. Upper aqueous later was discarded and lower chloroform layer was washed with ultra-pure water (4 x 5 ml). Finally, the chloroform later was dried down under a gentle stream of nitrogen and purified with Sep-Pak C_{18} column.

MALDI-TOF MS/ tandem MS analysis

Permethylated samples were dissolved in 10 µl of methanol, and then 1 µl of dissolved sample was premixed with 1 µl of 2,5-dihydrobenzoic acid (DHB) (20 mg/ml in 70% methanol in water) as matrix before spotted onto a MALDI plate. For MALDI-TOF profiling, permethylated *N*-glycans were analyzed in positive ion mode $[M+Na]^+$ with a Voyager STR MALDI-TOF mass spectrometer in the reflectron mode with delayed extraction; while collision-activated decomposition (CAD) tandem MS data were acquired using a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The collision energy was set to 1 kV and argon was used as collision gas.

RESULTS

Isolation of THP and uromodulin

Crude THP and uromodulin isolated by diatomaceous earth filtration was further purified on Sephadex G-100 size exclusion column equilibrated in 50 mM AmBic buffer. An average urine volume of 204.63 ml and 215.67 ml have yielded protein recovery of 82.80 ± 6.29% and 75.44 ± 5.34% for THP and uromodulin, respectively. SDS-PAGE analysis showed that both THP and uromodulin had a major band approximately at 97 kDa.

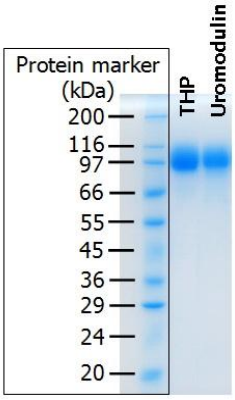

Fig. 1: SDS-PAGE of THP (Lane 2), uromodulin (Lane 3) and protein marker (Lane 1, SigmaMarker™ S8445).

MALDI-TOF MS profiling of *N*-glycans released from THP and uromodulin

N-glycans of THP and uromodulin were released by PNGase F enzyme, permethylated and subjected to MALDI-TOF analysis to elucidate the *N*-glycans profile. Table 1 shows the signals and assignments of all *N*-glycans obtained from THP and uromodulin. The assignments were assigned from compositional information provided by the MS and tandem MS data together with the aid of GlycoWorkbench glycoinfomatics tool [15].

MALDI-TOF analysis of the THP and uromodulin indicated that they were rich in *N*-glycans. 19 oligosaccharides were identified in THP *N*-glycans, with three high-mannose ($Hex_{5-7}HexNac_2$) and the remainder being complex *N*-glycans ($NeuAc_{0-1}Fuc_{0-1}Hex_{5-8}HexNAc_{4-7}$) (Table 1). Compositions $Hex_6HexNAc_2$ and $Fuc_1Hex_7HexNAc_6$ were the most abundant glycans for high-mannose and complex *N*-glycan category, at *m/z* 1784 and 3142, respectively. On the other hand, uromodulin expressed 22 *N*-glycans structures (Table 1). Three high-mannose *N*-glycans were observed as well with $Hex_6HexNAc_2$ at *m/z* 1784 as the most abundant composition among all of the glycans, followed by the signal at *m/z* 4587, corresponding to tetra-antennary glycan $NeuAc_4Fuc_1Hex_7HexNAc_6$ as the second most abundant glycan. Both THP and uromodulin expressed a few glycans carrying Sd^a [NeuAc-(GalNAc)-Gal-GlcNAc] antennae (*m/z* 3749 and 4356 in THP; *m/z* 4560 in uromodulin) or LacdiNAc epitope (*m/z* 2111and 2285 in THP; *m/z* 2152 and 2326 in uromodulin).

Table 1: Summary of all the *N*-glycans obtained from THP and uromodulin

THP			Uromodulin		
Mass (*m/z*)	**Assignment**	**Remarks**	**Mass (*m/z*)**	**Assignment**	**Remarks**
1580	Hex_2 +$Man_3GlcNAc_2$	High-mannose	1580	Hex_2 +$Man_3GlcNAc_2$	High-mannose
1784	Hex_3 +$Man_3GlcNAc_2$	High-mannose; ¶	1784	Hex_3 +$Man_3GlcNAc_2$	High-mannose; ¶
1988	Hex_4 +$Man_3GlcNAc_2$	High-mannose	1988	Hex_4 +$Man_3GlcNAc_2$	High-mannose
2070	$Hex_2HexNAc_2$ +$Man_3GlcNAc_2$		2040	$HexHexNAc_2Fuc$ +$Man_3GlcNAc_2$	
2111	$HexHexNAc_3$ +$Man_3GlcNAc_2$	LacdiNAc	2152	$HexNAc_4$ +$Man_3GlcNAc_2$	LacdiNAc
2244	$Hex_2HexNAc_2Fuc$ +$Man_3GlcNAc_2$		2244	$Hex_2HexNAc_2Fuc$ +$Man_3GlcNAc_2$	
2285	$HexHexNAc_3Fuc$ +$Man_3GlcNAc_2$	LacdiNAc	2326	$HexNAc_4Fuc$ +$Man_3GlcNAc_2$	LacdiNAc

Table 1: Summary of all the *N*-glycans obtained from THP and uromodulin (continue)

THP			Uromodulin		
Mass(*m/z*)	**Assignment**	**Remarks**	**Mass (*m/z*)**	**Assignment**	**Remarks**
2519	$Hex_3HexNAc_3$ +$Man_3GlcNAc_2$		2605	$Hex_2HexNAc_2Fuc$-NeuAc +$Man_3GlcNAc_2$	
2693	$Hex_3HexNAc_3Fuc$ +$Man_3GlcNAc_2$		2966	$Hex_2HexNAc_2Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	
2968	$Hex_4HexNAc_4$ +$Man_3GlcNAc_2$		3416	$Hex_3HexNAc_3Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	
3142	$Hex_4HexNAc_4Fuc$ +$Man_3GlcNAc_2$	†	3504	$Hex_4HexNAc_4Fuc$-NeuAc +$Man_3GlcNAc_2$	
3300	$Hex_3HexNAc_4Fuc$-NeuAc +$Man_3GlcNAc_2$		3777	$Hex_3HexNAc_3Fuc$-$NeuAc_3$ +$Man_3GlcNAc_2$	Heavily sialylated
3418	$Hex_5HexNAc_5$ +$Man_3GlcNAc_2$		4140	$Hex_5HexNAc_5NeuAc_2$ +$Man_3GlcNAc_2$	
3504	$Hex_4HexNAc_4Fuc$-NeuAc +$Man_3GlcNAc_2$		4213	$Hex_4HexNAc_4Fuc_3$-$NeuAc_2$ +$Man_3GlcNAc_2$	
3592	$Hex_5HexNAc_5Fuc$ +$Man_3GlcNAc_2$		4226	$Hex_4HexNAc_4Fuc$-$NeuAc_3$ +$Man_3GlcNAc_2$	Heavily sialylated
3749	$Hex_4HexNAc_5Fuc$-NeuAc +$Man_3GlcNAc_2$	Sd^a epitope	4314	$Hex_5HexNAc_5Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	
4042	$Hex_6HexNAc_6Fuc$ +$Man_3GlcNAc_2$	Poly-LacNAc	4413	$Hex_4HexNAc_4NeuAc_4$ +$Man_3GlcNAc_2$	Heavily sialylated
4199	$Hex_5HexNAc_6Fuc$-NeuAc +$Man_3GlcNAc_2$		4560	$Hex_5HexNAc_6Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	Sd^a epitope
4356	$Hex_4HexNAc_6Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	Sd^a epitope	4587	$Hex_4HexNAc_4Fuc$-$NeuAc_4$ +$Man_3GlcNAc_2$	Heavily sialylated; †
			4765	$Hex_6HexNAc_6Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	
			4806	$Hex_5HexNAc_7Fuc$-$NeuAc_2$ +$Man_3GlcNAc_2$	
			4833	$Hex_4HexNAc_5Fuc$-$NeuAc_4$ +$Man_3GlcNAc_2$	Heavily sialylated

Note: ¶ most abundant signal; † second most abundant signal

MALDI-TOF MS profiling of *O*-glycans released from THP and uromodulin

MALDI-TOF analyses of *O*-glycans showed that both THP and uromodulin expressed Core 1 and Core 2 *O*-glycans (Table 2). THP expressed several low molecular weight Core 1 glycans, such as at *m/z* 534 (HexHexNAc-ol) and 896 (NeuAcHexHexNAc-ol); and Core 2 glycans at *m/z* 984 ($Hex_2HexNAc_2$-ol), 1157 ($FucHex_2HexNAc_2$-ol), 1331 ($Fuc_2Hex_2HexNAc_2$-ol) and 1881 ($NeuAc_2FucHex_2HexNAc_2$-ol), with the most abundant signal being the HexHexNAc-ol. On the other hand, uromodulin expressed lesser type of *O*-glycans than THP. Only four signals were detected at *m/z* 896 (Core 1), 1141 ($NeuAcHexHexNAc_2$-ol), 1677 ($Hex_3HexNAc_4$-ol) and 1881 (Core 2), with the NeuAcHexHexNAc-ol being the most abundant signal.

Table 2: Summary of all the *O*-glycans obtained from THP and uromodulin

THP			Uromodulin		
Mass (*m/z*)	**Assignment**	**Remarks**	**Mass (*m/z*)**	**Assignment**	**Remarks**
534	HexHexNAc-ol	Core 1; ¶	896	NeuAcHexHexNAc-ol	Core 1; ¶
896	NeuAcHexHexNAc-ol	Core 1	1141	$NeuAcHexHexNAc_2$-ol	Core 2
984	$Hex_2HexNAc_2$-ol	Core 2	1677	$Hex_3HexNAc_4$-ol	Core 2
1157	$FucHex_2HexNAc_2$-ol	Core 2, isomers	1881	$NeuAc_2FucHex_2$-$HexNAc_2$-ol	Core 2
1331	$Fuc_2Hex_2HexNAc_2$-ol	Core 2, isomers			
1881	$NeuAc_2FucHex_2$-$HexNAc_2$-ol	Core 2			

Note: ¶ most abundant signal

MALDI-TOF tandem MS analysis of *N*- and *O*-glycans released from THP and uromodulin

Tandem MS is a simple and sensitive bio-analytical method to detect or validate biomolecules [16]. Similarly, in glycomics study, collision-activated decomposition (CAD) MALDI-TOF/TOF tandem MS of molecular ions observed in the MALDI experiment yielded fragment ions that are useful to define antenna sequence, thus distinguishing the minute differences in glycoforms of THP and uromodulin. Representative data from analysis of *m/z* 4042 (*N*-glycan from THP), 4587 (*N*-glycan from uromodulin), 1157 and 1331 (*O*-glycans from THP) are shown in Figure 2 (A), (B), (C) and (D), respectively. Assignments of key signals are given in the insets.

Predicted composition of THP *N*-glycan, the *m/z* 4042 ($FucHex_9HexNAc_8$) suggested the presence of repeating LacNAc units on this tetra-antennary structure. Sequential loss of one, two and three LacNAc units gave the signals at *m/z* 3578, 3129 and 2679, respectively. The expression of three repeating LacNAc units as antenna was confirmed by the fragment ions *m/z* 1385 (LacNAc-LAcNAc-LacNAc) and 2679 ($FucHex_6HexNAc_5$). The loss of one LacNAc unit from either two antenna could be observed at *m/z* 3114; while the loss of one LacNAc and two LacNAc units from either two arms could be seen at *m/z* 2665.

For uromodulin *N*-glycan, the *m/z* 4587 (Figure 2B), MS/MS analysis confirmed this assignment where the fragment ions at *m/z* 847 and 3263 were observed, indicating the loss of any non-reducing sialylated LacNAc and the remaining structure, respectively. Signal at *m/z* 4136 (loss of reducing-end fucosylated HexNAc) indicated that this glycan was core-fucosylated rather than expression of sialylated $Lewis^{x/a}$ antenna.

THP *O*-glycan, the $FucHex_2HexNAc_2$-ol with *m/z* 1157 was actually composed of two isomer structures. Dominant signal at *m/z* 921 represented the β-elimination of Hex (*m/z* 259). Fragment ion at *m/z* 520 was observed in structure A only, corresponding to a single β-cleavage of FucHexHexNAc (*m/z* 660, a $Lewis^x$ epitope) from $FucHex_2HexNAc_2$-ol without water loss. On the other hand, fragment ion at *m/z* 694, which can be observed in structure B only represented a single β-cleavage of HexHexNAc (*m/z* 486) from $FucHex_2HexNAc_2$-ol without water loss. Structure A was the major isomer as the fragment ions at *m/z* 520 and 660 specific in it had higher abundance compared to fragment ion *m/z* 486 and 694 specific in structure B.

Similarly, $Fuc_2Hex_2HexNAc_2$-ol from THP with *m/z* 1331 was composed of isomer structures as well. Signals at *m/z* 921 and 1095 represented the β-elimination of FucHex and Hex residue, respectively. A β-cleavage of FucHexHexNAc without water loss was observed at *m/z* 694 in structure A only. This was ratified by the presence of non-reducing fragment at *m/z* 660 (FucHexHexNAc, a $Lewis^x$ epitope). Conversely, fragment ion at *m/z* 520 represented a single β-cleavage of $Fuc_2HexHexNAc$ (*m/z* 834, a $Lewis^y$ epitope)

from $Fuc_2Hex_2HexNAc_2$-ol without a water loss, which could be found in structure B only. Both isomers are equally expressed as the intensity of the fragment ions specific in each of them was not much different.

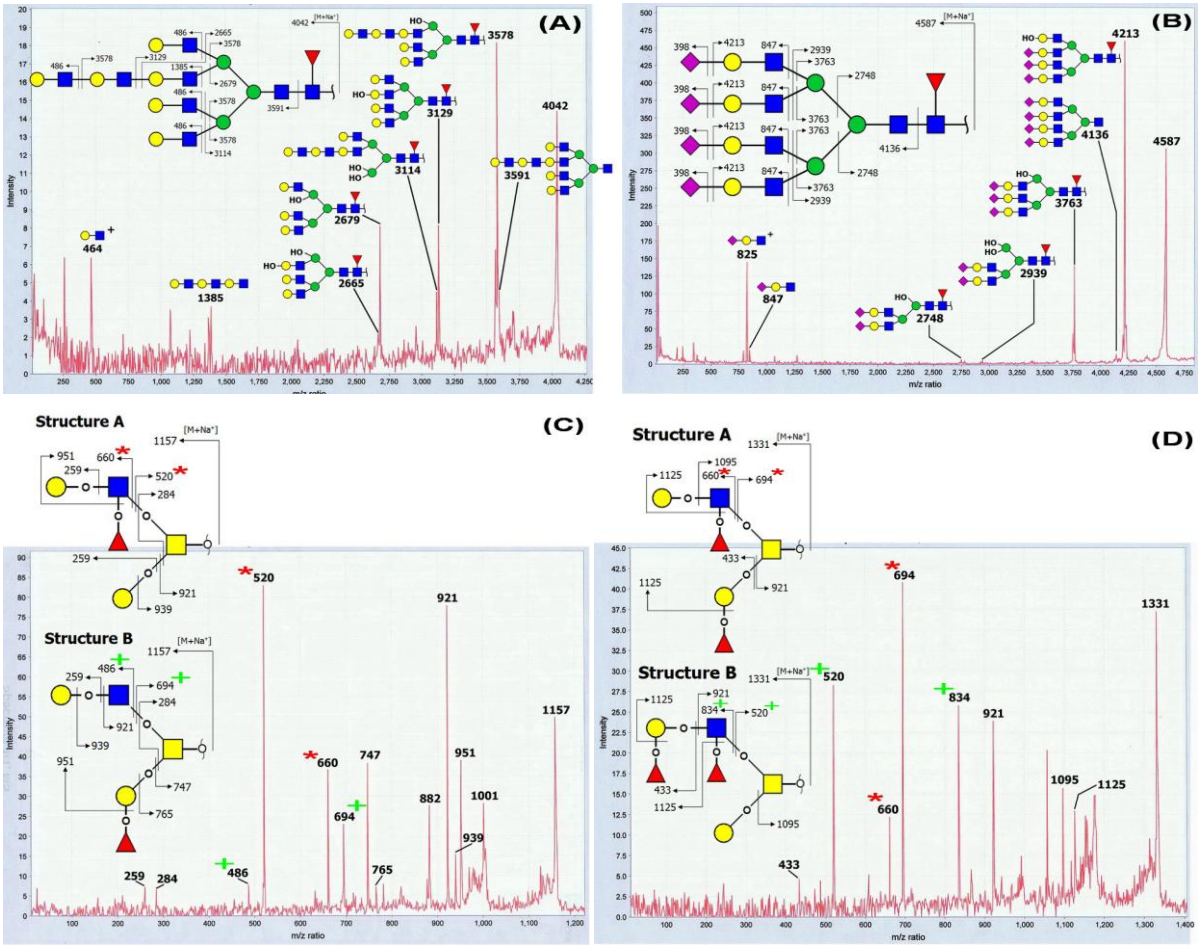

Fig 2: MALDI-TOF/TOF MS of selected *N*- and *O*-glycans provides additional structural data. Signals at *m/z* 4042 (*N*-glycan from THP), 4587 (*N*-glycan from uromodulin), 1157 and 1331 (*O*-glycans from THP) were subjected to tandem MS and the resulting MS/MS data are shown in A–D, respectively. Fragmentation is favoured on the reducing side of HexNAc residues. The fragment ions are consistent with the sequences shown in the insets.

DISCUSSION

THP is the most abundant protein in normal human urine, with the daily excretion ranging from 20-150 mg in humans [17]. The present study shows that protein contents of THP and uromodulin are different for each batch of samples. This is due to the fact that THP and uromodulin excretion is influenced by many factors such as urine volume, dietary and exercise [17], and donors in the present study had no experimental dietary or other restriction.

Although *N*-glycan profiling of THP has been studied in several NMR structural analysis [18-20], current study is employing high-sensitive MALDI-TOF instrumentation. As shown in Table 1, it is obvious that there are both similarity and difference in their glycomes. Both of the glycoproteins expressed high-mannose glycans complex type *N*-glycans, while most notable feature of the data was associated with the variation in sialic acid expression. Uromodulin was heavily sialylated but THP was poorly sialylated, where THP's most abundant complex-type *N*-glycans was non-sialylated. Up to four sialic acid residues were found to be substituted on uromodulin's tetra-antennary glycans ($NeuAc_{1-4}Fuc_{0-1}Hex_{5-7}HexNAc_{4-7}$), while THP only expressed maximum two sialic acid residues on its tetra-antennary glycan. Interestingly, most of the sialylated glycans in uromodulin were not observed in THP's *N*-glycan. The increased of sialylation in uromodulin might be pregnancy-associated, as elevation on the relative amount of sialic acid or sialic acid-containing glycoproteins has been reported in plasma, urine and amniotic fluid during pregnancy [21-23]. On the other hand, di-antennary glycans of uromodulin with *m/z* 2152 and 2326 had both antennae capped with LacdiNAc sequence, while only one antenna of THP *N*-glycans (*m/z* 2111 and 2285) is capped with this epitope. The reason behind the changes of LacdiNAc expression in THP and uromodulin remains unclear.

For *O*-glycosylation, both THP and uromodulin expressed Core 1 and Core 2 glycans, with THP expressing more *O*-glycans. As consistent with the FAB-MS analysis by Easton *et al*. [13], the present study also shows that THP expressed mainly Core 1 *O*-glycans, while uromodulin expressed Core 2 *O*-glycans. Uromodulin has been shown to express complex Core 2 *O*-glycans [13]. However, this unique feature of uromodulin could not be found in present study. We suggested that the different protein isolation methods (salt precipitation vs. diatomaceous earth filtration) would probably account for it.

THP and uromodulin are known to express immunosuppressive activity, such as inhibiting viral haemagglutination and suppressing antigen-specific lymphocyte proliferation, with uromodulin having

greater suppressive properties [2; 4; 24; 25]. We propose that uromodulin may suppress the lymphocytes response more effectively with its heavily sialylated glycans and higher expression of LacdiNAc sequence compared to that of THP during pregnancy. This is because B cell inhibitory receptor, CD22, can bind to sialylated LacNAc sequences [26], resulting in inhibition of B cell activation [27]. Glycodelin A, a potent immunosuppressive glycoprotein isolated from amniotic fluid has been suggested to employ the similar suppression mechanism through its sialylated LacNAc- and LacdiNAc-containing glycans [28]. Nowadays, therapeutic protein-based pharmaceuticals are emerging prominently [29]. Therefore, uromodulin might be a potential therapeutic protein for future to treat patients in transplantation and autoimmune disease.

CONCLUSION

This study using MALDI-MS/MS has allowed for detailed glycoform characterization. The complex and minute difference in the glycosylation profile for both THP and uromodulin was achieved using this technique, in which it is highly sensitive and accurate. It is clear from the present study that uromodulin expressed a different set of glycans compared to that of THP. Heavily sialylation and higher expression of LacdiNAc sequence of *N*-glycans in uromodulin might contribute to its higher immunosuppressive activities during pregnancy.

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