PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2014, 41(4): 403~408
www.pibb.ac.cn

Enrichment and Characterization of Total N-linked Glycans From Glycoproteins by Ultrafiltration Units and Mass Spectrometry*

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Abstract Glycosylation is one of the most common post-translational modifications in proteins. Current methods for glycan analysis are generally based on multiple preparation processes to separate glycans. However, glycans are continuously lost and the difficulty for accurate quantitative analysis is increased in the procedure. Here, a filter aided sample preparation-based total N-linked glycans from the glycoproteins enrichment and separation method (N-glycan-FASP-T) was developed using ultrafiltration units according to the molecular mass difference among the glycans, the impurities and proteins. The enriched glycans were characterized and confirmed by the MALDI-TOF/TOF-MS. A total of 23 distinctive N-linked glycans were characterized from human serum.

Key words N-linked glycans, filter aided sample preparation-based total N-linked glycans from glycoproteins enrichment and separation method, ultrafiltration unit, glycoproteins, MALDI-TOF/TOF-MS
DOI: 10.3724/SP.J.1206.2013.00103

Glycosylation is one of the most common post-translational modifications in proteins. It has been estimated that over 50% of human proteins are glycosylated [1]. The glycans play important structural roles in protein folding, attaching proteins to cell membranes, protecting proteins from degradation, and mediating many important biological functions [2-4]. Therefore, characterization of the glycan structures of the glycoproteins is useful to elucidate the biological function of the glycan and glycoprotein. Moreover, comparing the glycan chains between the various pathological states and the normal controls may provide useful information for the diagnosis, prognosis, and the development of therapeutic strategies[5-8].

Many approaches for analysis of glycans from glycoproteins have been described [9-10]. Typically, N-linked glycans are first released from glycoproteins or glycopeptides by enzymes such as PNGase F and PNGase A. After release, glycans are desalted and purified from enzymes, chemicals, and their concatenate peptides for mass spectrometry analysis[11]. Currently, the general methods such as affinity column, the graphite guard column, reverse-phase high-performance liquid chromatography, capillary electrophoresis, hydrophilic interaction chromatography, multidimensional separations, and hydrazide beads are used to separate the glycans from a complex mixture [12]. The major obstacle of these methods is their time-consuming and incapability to completely separate glycans from the peptides and other nonglycan molecules, especially from the hydrophilic

*This work was supported by grants from Doctoral Fund of Ministry of Education of China (20106101110012) and The International S&T Cooperation Program (2009DFA32730) from The Chinese Ministry of Science and Technology.
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Received: March 14, 2013 Accepted: May 8, 2013

peptides or salts. Moreover, glycans are continuously lost and the procedure also increases the difficulty for accurate quantitative analysis of glycans. To overcome the drawbacks, a filter aided sample preparation-based total N-linked glycans enrichment and separation method (N-glycan-FASP-T) was introduced using ultrafiltration unit that allows rapidly enrichment of the total N-linked glycans from the glycoproteins in this study.

1 Materials and methods

1.1 Reagents

Amicon Ultra-0.5 10 kD devices were obtained from Millipore (Billerica, USA). Sepharose 4B, 2, 5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich(St. Louis, USA). Peptide-N-glycosidase F (PNGase F) was from New England Biolabs Inc. 1-Butanol, ethanol, and typical chemical reagents were purchased from Merck (Darmstadt, Germany). Other chemical reagents were obtained from commercial suppliers and used without further purification. All of the solutions were prepared with ultra-pure water obtained from a Milli-Q 50 SP Reagent Water System (Millipore Corporation, USA) and were filtered by 0.2 μm Minisart® syringe filters(Sartorius, Germany).

1.2 Collection of the serum

The human sera were obtained after the bloods were clotted for 30 min at room temperature, centrifuged and mixed. Aliquots from the sera were frozen at –80℃ immediately after processing. The sera were thawed in a water bath at 37℃ when used.

1.3 The separation of the glycans

Human serum (25 μl) were added into a size-exclusion spin ultrafiltration unit (Amicon Ultra-0.5 10 ku device, Millipore)supplemented with 40 mmol/L NH_4HCO_3 (200 μl) and centrifuged the ultrafiltration unit at 12 000 *g* for 15 min. All following centrifugation steps were performed applying the same conditions allowing maximal concentration. 200 μl of 40 mmol/L NH_4HCO_3 were added and centrifuged. This step was repeated 2 times. The effluents in the collection tube were discarded. 8 mol/L urea in 40 mmol/L NH_4HCO_3 (200 μl) were added to the ultrafiltration unit and centrifuged. This step was repeated 2 times and the effluents were also discarded. 200 μl of 40 mmol/L NH_4HCO_3 was added to the ultrafiltration unit and centrifuged. This step was repeated 2 times and the effluents were discarded. 10×Denaturation Buffer (20 μl) (PNGase F kit, New England BioLabs) and 40 mmol/L NH_4HCO_3 (180 μl) were added, and the ultrafiltration unit was boiled in water bath for 5 min. Then, it was centrifuged and the effluents were discarded. The ultrafiltration unit was transfer to a new collection tube. 10×Denaturation Buffer (20 μl) and 10% NP-40 (20 μl) (PNGase F kit, New England BioLabs) were added and mixed in rocking device for 2 min. PNGase F (3 μl) (PNGase F kit, New England BioLabs) and 157 μl of 40 mmol/L NH_4HCO_3 were added to the ultrafiltration unit and incubated at 37℃ in wet chamber overnight to release the N-linked glycans from the human serum glycoproteins. The digests were collected by centrifugation, and the ultrafiltration unit was rinsed with 200 μl of 40 mmol/L NH_4HCO_3 and centrifuged. This step was repeated 2 times. The filtrates were collected and lyophilized using Alpha 2-4 freeze dryer(Martin Christ, Germany).

1.4 Clean-up of the N-linked glycans

Sepharose 4B was used for further purification and desalting[13]. Sepharose 4B (Sigma) was used for further purification and desalting. The hydrophilic resin (100 μl) in the microtube was washed with eluting solution (ethanol/H_2O (1∶1, *v*/*v*)) and washing solution (1-butanol/ethanol/H_2O (5∶1∶1, *v*/*v*)) by centrifugation. The glycans were dissolved in 500 μl of washing solution containing 1 mmol/L $MnCl_2$ and mixed with the sepharose resins. After gently shaking for 45 min, the resins were washed three times by washing solution. The glycans bound to the resins were eluted with 1 ml of eluting solution and collected.

1.5 The characterization of the N-linked glycans by the MALDI-TOF/TOF-MS

To determine the enriched N-linked glycans from the glycoproteins, the obtained glycans were characterized by MALDI-TOF/TOF-MS(UltrafleXtreme, Bruker Daltonics). The glycan mixture was dissolved in 10 μl of 50% (*v*/*v*) methanol, and 1 μl was spotted directly on a MTP AnchorChip var/384 sample target and dried. Then an equal volume 20 g/L DHB in 50% (*v*/*v*) methanol solution was spotted to re-crystallize the glycans. The target was introduced in the mass spectrometry. Ionisation was performed in MS and MS/MS by irradiation of a nitrogen laser (337 nm) operating at 1 kHz. Data were acquired at a maximum accelerating potential of 25 kV in the positive and reflection modes. Mass calibration was done using the peptide calibration standards 250 calibration points from Bruker Daltonics. 1500 laser shots per pixel (200 laser shots per position of a random walk within each

pixel) were collected and the data were acquired using the Flex software suite (FlexControl 3.3, FlexAnalysis 3.3)[14]. The glycan compositions were further confirmed by MS/MS analysis. The data of *m/z* were analyzed and annotated by Glycoworkbench software[15].

2 Results and discussions

The principle for the enrichment and characterization of the N-linked glycans was illustrated in Figure 1. The key feature of the N-glycan-FASP-T is the ability of the ultrafiltration membrane to allow through low-molecular-mass substances (impurities) before the PNGase F digestion and block high-molecular-mass substances (proteins) after the glycan were released[16–17]. Here, it is essential to select a filter with the desired separation properties. Because the molecular mass (m) of the proteins were all greater than 10 ku and the longest N-linked glycan chain was 16 monosaccharides[18], the m of the largest N-linked glycan was smaller than 5 ku. Hence, the 10 ku ultrafiltration unit was chosen to isolate the glycans from the glycoproteins as well as deplete the chaotropic agent.

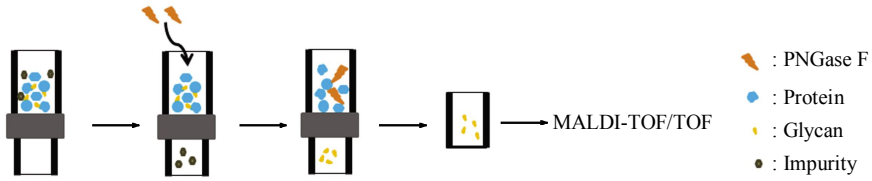

Fig. 1 Schematic view to isolate and characterize the total N-linked glycans of glycoproteins

For glycome analysis, mass-profile procedures are often employed to characterize the oligosaccharides. Figure 2 showed the positive mode MALDI-TOF-MS profile of the obtained N-linked glycans in the human sera. A total of 23 distinctive N-linked glycans were characterized and the sodiated ions were predominately in the profile. The annotated N-linked glycans were no acidic oligosaccharides due to suppression of abundant neutral oligosaccharides [19]. The *m/z* 1485.533, 1647.897, 1663.581, 1809.639 and 1850.665 were the major glycans in the serum glycoproteins, which were annotated as $(Fuc)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2$, $(Fuc)_1(Gal)_1(GlcNAc)_2+(Man)_3(GlcNAc)_2$, $(Gal)_2(GlcNAc)_2+(Man)_3(GlcNAc)_2$, $(Fuc)_1(Gal)_2(GlcNAc)_2+(Man)_3(GlcNAc)_2$, and $(Fuc)_1(Gal)_1(GalNAc)_2(GlcNAc)_2+(Man)_3(GlcNAc)_2$, respectively. The MALDI MS spectrum confirmed that the oligosaccharides were successfully released and isolated from the impurities and proteins.

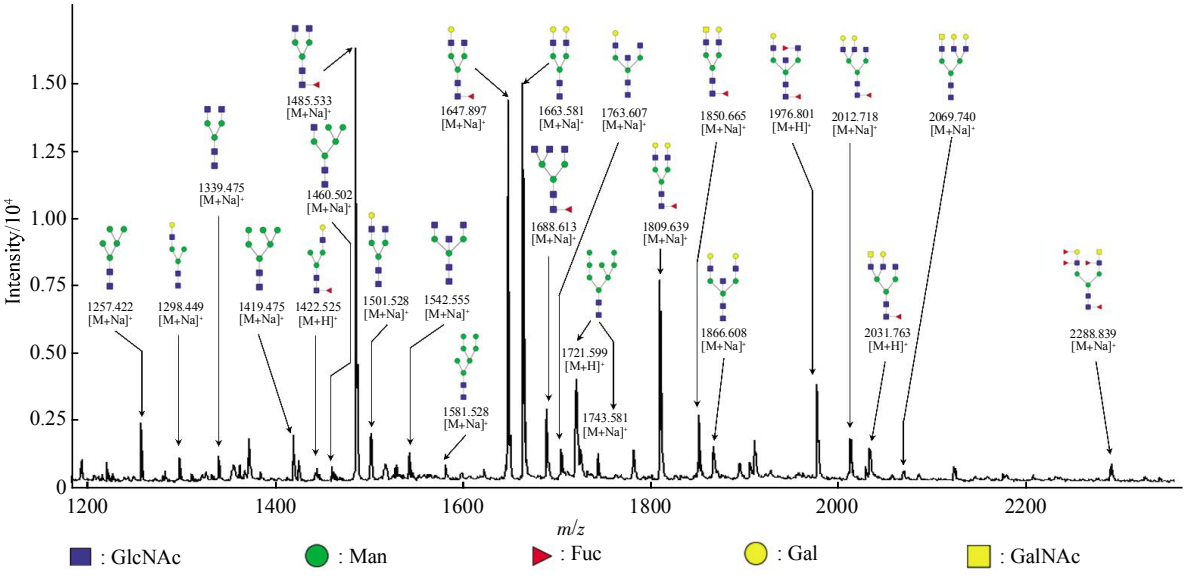

Fig. 2 One MALDI-TOF-MS spectra of the enriched total N-linked glycans from the serum glycoproteins

To provide insight into the substitution and branching pattern of the monosaccharide constituents, the majority of the N-linked glycan peaks observed in the MS spectrum were assigned based on tandem MS. The spectrums of the $[M+Na]^+$ ion of the five *m/z* were shown in Figure 3. The most abundant fragment ions were B and Y ions that were derived from glycosidic cleavages, which could reveal the composition of the oligosaccharide. For example, B_4 (*m/z* 1118.664), $Y_{1\beta}Y_{5\alpha}$ (*m/z* 1136.396) for the *m/z* 1485.789 (Figure 3a)

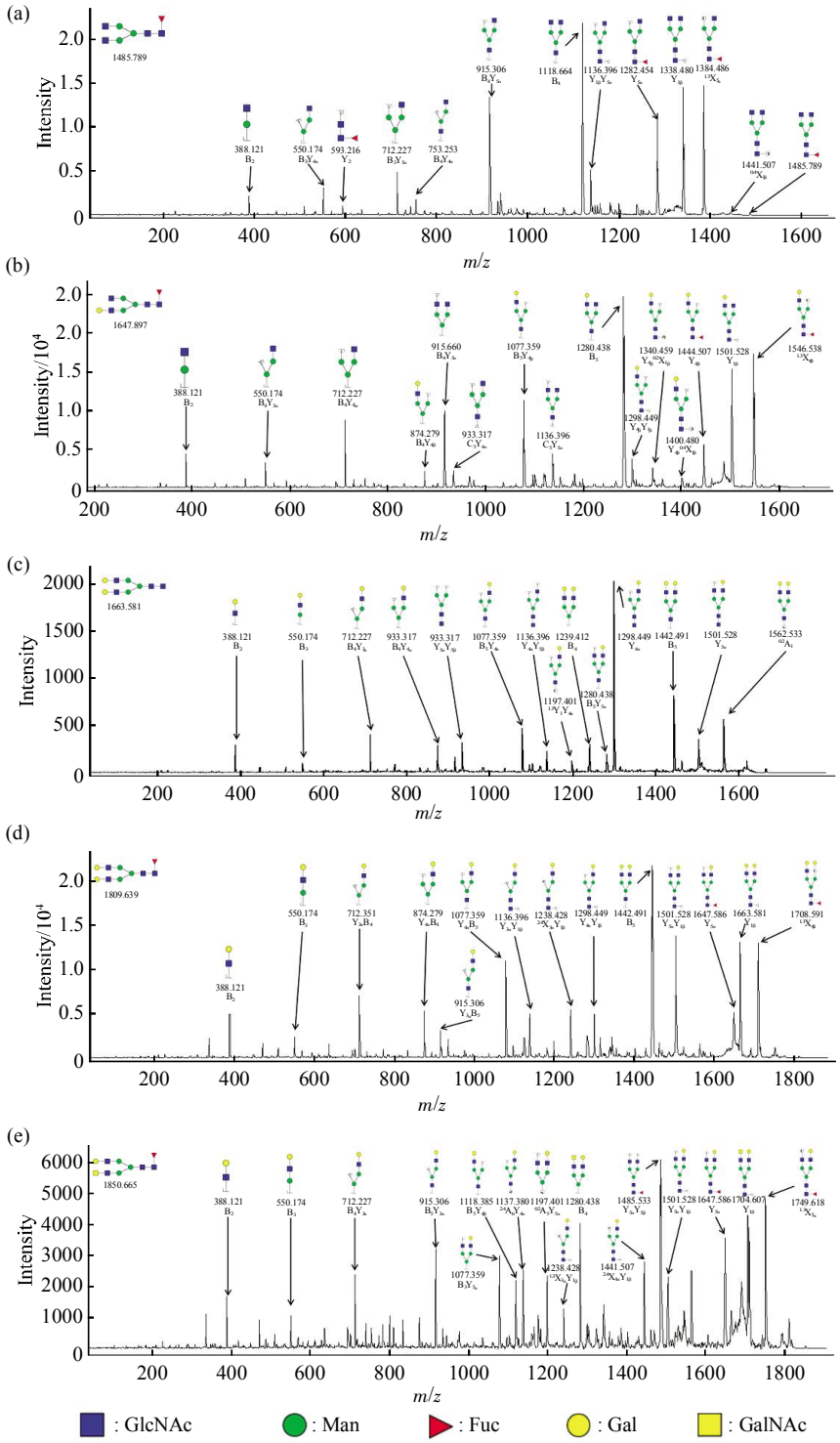

Fig. 3　MALDI-TOF/TOF tandem mass spectrometry sequencing and analyzing the N-linked glycan precusor ion from MS spectra

The five major N-glycan peaks (a) *m/z* 1485.789, (b) 1647.897, (c) 1663.581, (d) 1809.639 and (e) 1850.665 subjected to MS/MS analysis were indicated.

and B_2 (*m*/*z* 1280.638), $B_5Y_{4\beta}$ (1077.359) for the *m*/*z* 1647.595 (Figure 3b) were the glycosidic cleavage fragmentation patterns and showed the oligosaccharide composition. In addition, cross-ring fragments were also detected in MS/MS spectrum with lower abundance (most of them were not indicated in the figure), and consequently, linkage information could be obtained. For example, $^{0,2}A_1$ (*m*/*z* 1562.533), $^{1,3}X_1Y_{4\alpha}$ (*m*/*z* 1197.401) for the *m*/*z* 1663.581 (Figure 3c) indicated the reducing terminal N-acetylglucosamine residue was substituted at C-4. Furthermore, the signal for $^{2,4}X_{3\alpha}Y_{1\beta}$ (*m*/*z* 1238.428) for the *m*/*z* 1809.639 (Figure 3d) indicated that α-mannose was substituted at C-3 or C-4. Other ions in the spectrum included those derived from multiple cleavages, which involved loss of fucose, for example, at *m*/*z* 1704.607 and 1501.528 for the *m*/*z* 1850.665 (Figure 3e).

3 Conclusion

In summary, this method simplified the procedure for the N-linked glycan preparation from a complex mixture and can be a powerful tool for the N-linked glycan analysis.

Acknowledgements The authors thank Dr. LIU Zhao-Yang and ZHANG Xin-Yi of the Bruker Daltonics for the MALDI-MS analysis.

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基于超滤膜辅助的糖蛋白全 N-连接糖链的富集和质谱解析 *

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摘要　糖基化作为一种常见的蛋白质翻译后修饰，对蛋白质的空间结构、生物功能等具有重要的影响．解析糖蛋白糖链结构有助于更清楚地认识糖蛋白及其功能．本研究建立了一种基于超滤膜富集血清中糖蛋白全 N- 连接糖链，并利用质谱技术对糖链结构进行分析的方法．根据糖蛋白及其糖链结构之间的分子质量差异，利用 Millipore 公司的 10 ku 超滤膜富集血清糖蛋白上酶解(PNGase F)释放的全 N- 连接糖链，并使用 MALDI-TOF/TOF-MS 解析糖链结构．通过该技术可以从血清中富集并鉴定到 23 种独特的 N- 连接的糖链结构，并且利用二级质谱进行了结构确认．该方法可以被用于从大量生物样本中富集糖蛋白全 N- 连接糖链，可以达到快速、高通量地解析糖蛋白 N- 连接糖链的目的．

关键词　N- 连接糖链，超滤膜辅助的糖蛋白全 N- 连接糖链富集法，超滤膜，糖蛋白，MALDI-TOF/TOF-MS
学科分类号　$Q513^{+}.2$

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* 教育部高等学校博士学科点专项科研基金(20106101110012)和科技部国际科技合作计划项目(2009DFA32730)资助.
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收稿日期：2013-03-14，接受日期：2013-05-08