Journal of Proteomics 187 (2018) 47–58

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Reductive chemical release of *N*-glycans as 1-amino-alditols and subsequent 9-fluorenylmethyloxycarbonyl labeling for MS and LC/MS analysis

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ARTICLE INFO

Keywords:
N-glycans
Reductive chemical release
Fmoc
Permethylation
LC/MS

ABSTRACT

Glycoproteins play pivotal roles in a series of biological processes and their glycosylation patterns need to be structurally and functionally characterized. However, the lack of versatile methods to release *N*-glycans as functionalized forms has been undermining glycomics studies. Here a novel method is developed for dissociation of *N*-linked glycans from glycoproteins for analysis by MS and online LC/MS. This new method employs aqueous ammonia solution containing $NaBH_3CN$ as the reaction medium to release glycans from glycoproteins as 1-amino-alditol forms. The released glycans are conveniently labeled with 9-fluorenylmethyloxycarbonyl (Fmoc) and analyzed by ESI-MS and online LC/MS. Using the method, the neutral and acidic *N*-glycans were successfully released without peeling degradation of the core α-1,3-fucosylated structure or detectable de-*N*-acetylation, revealing its general applicability to various types of *N*-glycans. The Fmoc-derivatized *N*-glycans derived from chicken ovalbumin, *Fagopyrum esculentum* Moench Pollen and FBS were successfully analyzed by online LC/MS to distinguish isomers. The 1-amino-alditols were also permethylated to form quaternary ammonium cations at the reducing end, which enhance the MS sensitivity and are compatible with sequential multi-stage mass spectrometry (MS^n) fragmentation for glycan sequencing. The Fmoc-labeled *N*-glycans were further permethylated to produce methylated carbamates for determination of branches and linkages by sequential MS^n fragmentation.

Significance of the study: *N*-Glycosylation represents one of the most common post-translational modification forms and plays pivotal roles in the structural and functional regulation of proteins in various biological activities, relating closely to human health and diseases. As a type of informational molecule, the *N*-glycans of glycoproteins participate directly in the molecular interactions between glycan epitopes and their corresponding protein receptors. Detailed structural and functional characterization of different types of *N*-glycans is essential for understanding the functional mechanisms of many biological activities and the pathologies of many diseases. Here we describe a simple, versatile method to indistinguishably release all types of *N*-glycans as functionalized forms without remarkable side reactions, enabling convenient, rapid analysis and preparation of released *N*-glycans from various complex biological samples. It is very valuable for studies on the complicated structure-function relationship of *N*-glycans, as well as for the search of *N*-glycan biomarkers of some major diseases and *N*-glycan related targets of some drugs.

1. Introduction

N-Glycosylation of proteins is one of the important post-translational modification forms. It has been revealed that glycan moieties of glycoproteins have essential roles in a variety of biological processes, such as cell adhesion, signal transduction, immune recognition as well as cell proliferation and differentiation [1–4]. Moreover, abnormal alterations in glycan structures are associated with the etiology of many diseases such as cancer, inflammation and congenital disorders of glycosylation (CDG) [5, 6].

Abbreviations: Fmoc-Cl, 9-fluorenylmethyloxycarbonyl chloroformate; PMP, 1-phenyl-3-methyl-5-pyrazolone; CDG, congenital disorders of glycosylation; HILIC, hydrophilic interaction liquid chromatography; UV, ultraviolet; MS^n, multi-stage mass spectrometry

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https://doi.org/10.1016/j.jprot.2018.06.002
Received 3 March 2018; Received in revised form 3 June 2018; Accepted 4 June 2018
Available online 06 June 2018
1874-3919/

To investigate the complex structures and functions of *N*-glycans, they normally need to be released from the protein backbone. At present, enzymatic approaches are mainly used for the release of *N*-glycans from glycoproteins. Peptide-*N*-glycanase F (PNGase F) is commonly used for the release of most *N*-glycans except core α-1,3-fucosylated *N*-glycans that often exist in plants, insects and other lower organisms [7, 8]. Peptide-*N*-glycanase A (PNGase A) is only applicable to short glycopeptides and inefficient for sialylated glycans [9–11]. Endoglycosidases (Endos), such as Endo D [12], Endo H [13] and Endo F [14] can cleave the glycosidic bond between the two *N*-acetylglucosamine (GlcNAc) residues at the *N*-glycan chitobiosyl core. However, each endoglycosidase acts on only certain types of *N*-glycans. In addition, exhaustive Pronase E digestion can release *N*-linked glycans as asparagine-linked forms. The obtained glycosylated asparagine can be further labeled with 9-fluorenylmethyl chloroformate (Fmoc-Cl) for chromatographic separation and characterization, providing a useful tool for functional glycomics studies [15]. However, the complete digestion of proteins by Pronase E often relies on specific protein and is difficult to control. Despite the high specificity and efficiency, the enzymes are costly and have poor versatility, limiting their application to high-throughput analysis and preparation of complex *N*-glycans.

Some chemical methods have also been explored to release various *N*-glycans during the past several decades. In the presence of NaOH and sodium borohydride, *N*-glycans are released as reduced alditols [16, 17]. The lack of hemiacetal reducing end prevents further derivatization and high-sensitivity chromatography analysis. Hydrazinolysis [18, 19] allows nonreductive chemical release of *N*-glycans from glycoproteins, but anhydrous hydrazine is highly toxic and potentially explosive and leads to serious de-*N*-acetylation and peeling reaction. In recent years, Huang et al. [20] developed a method for releasing *O*-glycans in the presence of saturated ammonium carbonate in ammonia. It is worth noting that *N*-glycans were also partially released during this reaction. However, this method causes considerable peeling degradation of core α-1,3-fucosylated *N*-glycans. Yuan et al. reported a novel strategy for non-reductive chemical release of *N*-glycans with simultaneous 1-phenyl-3-methyl-5-pyrazolone (PMP) labeling to avoid peeling reaction [21]. However, the end product cannot be further functionalized, such as in microarray preparation.

Furthermore, released *N*-glycans are derivatized to enhance the detection sensitivity during analysis by mass spectrometry (MS), high-performance liquid chromatography (HPLC), glycan microarrays et al. [21]. The biosynthesis of glycans is not template-driven, producing glycans with complex branches and isomers [22]. Therefore, detailed characterization of glycan structures is still challenging. Liquid chromatography coupling with mass spectrometry (LC/MS) has become the main technique to separate and analyze glycan isomers [23]. In addition, permethylation of glycans has been used to improve their MS detection sensitivity and characterize their detailed structures by tandem mass spectrometry (MS^n), including linkages and branches [24, 25].

In the present study, we report a novel method for the reductive chemical release of *N*-glycans without detectable peeling degradation of core α-1,3-fucosylated *N*-glycans and deacetylated by-products, enabling generation of 1-amino-alditols with reactive amino groups at the reducing end. The *N*-glycans released from different types of glycoproteins were labeled with Fmoc and analyzed by ESI-MS and online LC/MS to distinguish glycan isomers. The detailed *N*-glycan structures were characterized by MS^n after permethylation. The results demonstrate that the method represents a simple and rapid protocol for high throughput preparation and analysis of complex *N*-glycans for glycomics studies.

2. Materials and methods

2.1. Materials and reagents

Maltodextrins, chicken ovalbumin, 9-fluorenylmethyl chloroformate (Fmoc-Cl), sodium cyanoborohydride ($NaBH_3CN$), sodium borohydride ($NaBH_4$), borane-ammonia complex ($NH_3{\cdot}BH_3$), sodium hydroxide (Small Beads), dimethyl sulfoxide (DMSO) and ribonuclease B (RNase B) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Peptide *N*-glycanase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA, USA). Sep-Pak C18 (100 mg/1 mL) solid phase extraction (SPE) columns and nonporous graphitized carbon (Carbograph) SPE columns (150 mg/4 mL) were purchased from Waters (Milford, MA, USA) and Alltech Associates (Deerfield, IL, USA), respectively. HPLC-grade acetonitrile and methanol were products of Fisher Scientific (Fairlawn, NJ, USA). The plants used were grown in a green house. Fetal bovine serum (FBS) was purchased from Thermo Scientific Co. Ltd. (Beijing, China). MD34 (8000–14,000) dialysis membrane was the product of Union Carbide Co. (Danbury, CT, USA). The water used was purified through a Milli-Q purification system (Millipore, Milford, MA, USA). Other reagents and solvents were of the highest grade commercially available.

2.2. Reductive amination of reducing oligosaccharides and Fmoc derivatization

10 mg of maltodextrins was dissolved in 1 mL of 1 M ammonium acetate solution and mixed with 200 μL of 0.1 M $NaBH_3CN$ solution prepared in 10% glacial acetic acid, followed by incubation at 70 °C for 1 h. The sample was purified using a graphitized carbon SPE column. After washing the column with 3 mL of water to remove salts, the glycans were eluted with 5 mL of 25% acetonitrile (ACN) and dried under a stream of nitrogen. The dried sample was labeled with Fmoc based on the procedure described by Song et al. [15]. Briefly, the glycans were dissolved in 200 μL of water. Equal volume of sodium bicarbonate solution (50 mg/mL), water, and a solution of Fmoc-Cl in tetrahydrofuran (20 mg/mL) were sequentially added. The mixture was then shaken vigorously for 30 min, followed by extraction of excess Fmoc reagents using 3 volumes of ethyl acetate. The obtained aqueous phase was desalted using a C18 Sep-Pak cartridge, and the Fmoc labeled glycans were eluted with 50% acetonitrile for HPLC or ESI-MS analysis.

2.3. Extraction of total proteins from biological samples

Total protein of *Fagopyrum esculentum* Moench Pollen was extracted by ammonium sulfate precipitation [21]. Briefly, 4 g of *Fagopyrum esculentum* Moench Pollen was frozen in liquid nitrogen and pulverized using a sample grinder. The obtained powder was dissolved in 50 mL of 0.1 M sodium phosphate buffer (pH 7.5), and the supernatant was obtained by centrifugation (1500 *g*, 10 min, 4 °C). Subsequently, 28 g of ammonium sulfate was added, and the mixture was kept at 4 °C overnight to precipitate proteins. The precipitate was collected by centrifugation (10,000 *g*, 10 min, 4 °C) and then dissolved in 10 mL of water for exhaustive dialysis against water at 4 °C for 48 h. The obtained sample was lyophilized for use. 5 mL of FBS was dialyzed against 2 L of Milli-Q water at 4 °C for 72 h, during which water was refreshed once every 12 h. The dialyzed sample was lyophilized for further use.

2.4. Reductive release and purification of N-glycans

For the neutral *N*-glycan, the chicken ovalbumin or pollen protein sample (10 mg) was dissolved in 28% aqueous ammonia solution (1 mL) containing 1 M $NaBH_3CN$ and 0.3 M NaOH, and incubated at 40 °C for 16 h. For the acidic *N*-glycan, 0.3 M NaOH were not added to the reaction solution. To remove ammonia, the sample was dried by an RE-52A rotation evaporator (Shanghai Yarong Biochemistry Instrument

Factory, China) at 40 °C. The dried sample was redissolved in 1 mL of water and then neutralized with glacial acetic acid, followed by repeated evaporation. Subsequently, the sample was purified using Sep-Pak C18 SPE cartridge and nonporous graphitized carbon SPE column. Briefly, the dried sample was redissolved in 1 mL of water and then loaded onto Sep-Pak C18 SPE column preconditioned with 5 mL of ACN and 10 mL of water, prior to elution with 10 mL of water. The water fraction containing *N*-glycans was loaded into a graphitized carbon SPE column prewashed with 3 mL of ACN and 3 mL of water. Then the column was washed with 20 mL of water for desalting. The neutral *N*-glycans of chicken ovalbumin and *Fagopyrum esculentum* Moench Pollen were eluted with 3 mL of 25% ACN, while the sialylated *N*-glycans of FBS were eluted with 3 mL of 25% ACN containing 0.05% TFA (vol/vol). Then the eluates were collected and dried by a Savant Speed-Vac (Thermo Scientific, Asheville, NC) for Fmoc derivatization and MS analysis.

2.5. Permethylation

Released *N*-glycans with or without Fmoc-label were dried and then permethylated according to reported procedures [26]. Briefly, a dried sample was treated with a DMSO-NaOH slurry (300–400 μL) and methyl iodide (75–100 μL) for 30 min. The supernatant was then partitioned between water (500 μL) and chloroform (500 μL). The organic layer was washed with water (500 μL) to remove salts. The samples were finally dried under a stream of nitrogen and redissolved in methanol for MS and MS^n analysis.

2.6. HPLC separation

An HPLC LC-2010A HT system (Shimadzu) coupled with a UV detector (SPD-20AV) was used for HPLC analysis of Fmoc labeled *N*-glycans. UV absorption at 254 nm was used to detect the Fmoc derivatives of *N*-glycans. A 4.6 mm × 250 mm TSK-GEL Amide-80 column (Tosoh Corporation, Tokyo, Japan) and a 4.6 mm × 250 mm SinoChrom C8 column were employed for HILIC and RP-HPLC analysis, respectively. Sample injection volume is 20 μL. The flow rate was at 1.0 mL/min, and the temperature was at 25 °C. The mobile phases were acetonitrile (solvent A), 100 mM ammonium acetate (pH 6.0, solvent B) and 0.05% aqueous acetic acid solution (vol/vol, solvent C). For HILIC analysis, the column was initially equilibrated with a mobile phase containing 80% A and 20% B for 10 min, and then the mobile phase composition was changed to 55% A with 45% B over 150 min via a linear gradient. For RP-HPLC analysis, a linear elution gradient was performed from 12% A, 88% C to 27% A, 73% C over 60 min.

2.7. ESI–MS and MS^n analysis

The MS analysis was performed with an LTQ XL ion-trap mass spectrometer equipped with an electrospray ion (ESI) source and an HPLC system (Thermo Scientific, USA). The samples were infused via a 2-μL Rheodyne loop and brought into the electrospray ion source by a stream of 50% methanol at a flow rate of 20 μL/min. The spray voltage was set at 4 kV, with a sheath gas (nitrogengas) flow rate of 20 arb, an auxiliary gas (nitrogen gas) flow rate of 5.0 arb, a capillary voltage of 37 V, a tube lens voltage of 250 V, and a capillary temperature of 300 °C. MS^n analysis was carried out using helium (He) as the collision gas, a normalized collision energy degree of 35–45% and an isotope width of *m/z* 3.00. The MS and MS^n data were acquired with LTQ Tune software (Thermo). The other parameters are acquiescent.

2.8. Online HILIC-MS/MS analysis

Online HILIC-MS analysis was also performed on the HPLC-ESI-MS system (Thermo scientific, USA), using a TSK-GEL amide-80 column (4.6 mm × 250 mm, 5 μm) (Tosoh Corporation, Tokyo, Japan). The glycan sample was dissolved in 20 μL of deionized water, and 10 μL of the sample solution was injected by an auto sampler. The elution gradient was as follows: solvent A, ACN; solvent B, 100 mM aqueous ammonium acetate (pH 6.0); time = 0 min (t = 0 min), 80% A, 20% B, 1 mL·min^{-1}; t = 120 or 150 min, 60 or 55% A, 40 or 45% B, 1 mL·min^{-1}. The fractions eluted from the chromatographic column were directly imported into the ESI-MS system for detection through a T-branch splitter. The absorption wavelength of the PDA detector was set at 254 nm. The parameters of MS analysis were the same as those described above. Data acquisition was performed using Xcalibur software (Thermo). The obtained data were manually interpreted, and the proposed *N*-glycan compositions and sequences were checked using GlycoWorkbench software [27].

3. Results and discussion

3.1. Principle of the method

It is well known that the amide bond undergoes hydrolysis under alkaline conditions [21, 28], including the *N*-glycan-peptide linkage. In this study, *N*-glycans are released from glycoproteins in the presence of aqueous ammonia solution and reduced in situ by $NaBH_3CN$ (Fig. 1). Under the alkaline condition in 28% aqueous ammonia solution, the aspartamide amide bond where *N*-glycans attach to protein backbone can be cleaved to produce a labile glycosylamine (substance 1), which is in equilibrium with an open-ring form (substance 2) in the reaction system. Substance 2 is reduced to an open-ring form 1-amino alditol

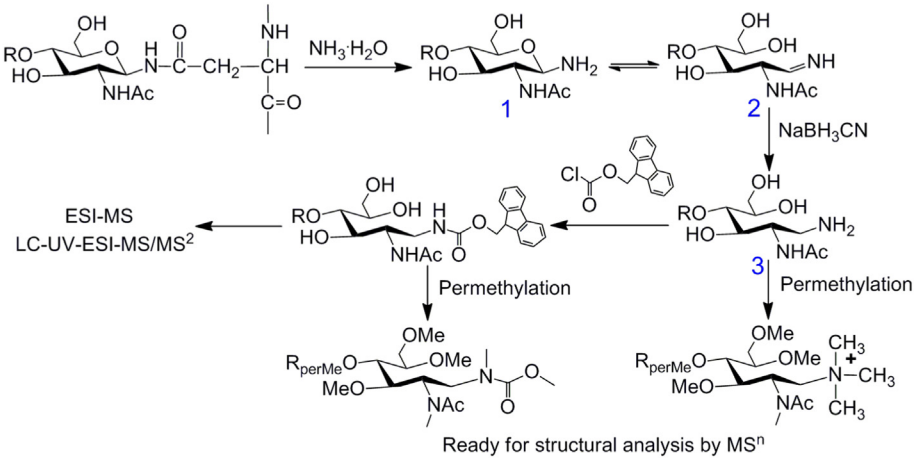

Fig. 1. Reductive chemical release and derivatization of *N*-glycans. The strategy allows for the reductive release of *N*-glycans from glycoproteins based on aqueous ammonia solution catalysis and in-situ reduction by $NaBH_3CN$. Fmoc derivatives of released *N*-glycans are quite suitable for analysis by HPLC and online LC/MS. Permethylation products allows for high-sensitivity MS detection and detailed structural identification by MS^n.

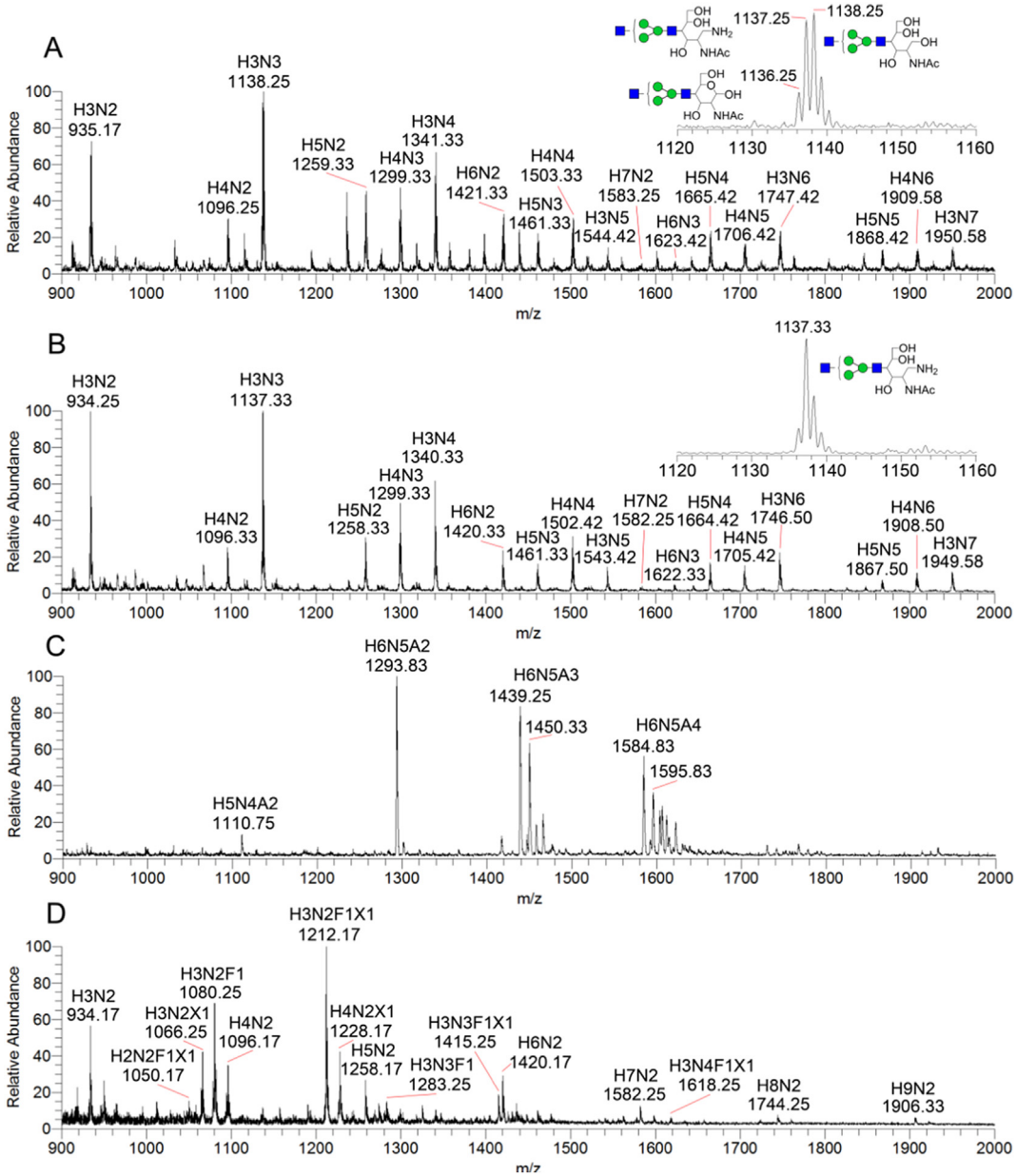

Fig. 2. ESI-MS profiles of *N*-glycans released from different samples. (A) ESI-MS profile of *N*-glycans released from chicken ovalbumin under unoptimized reaction conditions: 0.3 M aqueous NaOH solution containing 1 M $NaBH_4$ at 50 °C for 16 h. (B) ESI-MS profile of *N*-glycans released from chicken ovalbumin under optimized reaction conditions: 28% aqueous ammonia solution containing 1 M $NaBH_3CN$ and 0.3 M NaOH at 40 °C for 16 h (C) ESI-MS profile of *N*-glycans released from FBS under optimized reaction conditions: 28% aqueous ammonia solution containing 1 M $NaBH_3CN$ at 40 °C for 16 h. (D) ESI-MS profiles of *N*-glycans released from *Fagopyru mesculentum* Moench pollen. The MS profiles of (A), (B) and (D) are in the positive ion mode and all of the corresponding glycan signals are assigned to $[M + Na]^+$ type ions, while the MS profile of (C) is in the negative ion mode and all of the corresponding glycan signals are assigned to $[M - 2H]^{2-}$ or $[M - 3H + Na]^{2-}$ type ions. Structure formulas: blue square, *N*-acetylglucosamine; green circle, mannose. H: hexose; N: *N*-hexosamine; F: fucose; X: xylose; A, acetylneuraminic acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(substance 3) by the reducing agent $NaBH_3CN$.

The 1-amino alditol form *N*-glycans thus released can be derivatized with Fmoc-Cl and analyzed by ESI-MS and LC-UV-MS/MS. The online LC/MS technique allows for the separation and analysis of various naturally occurring glycan isomers. With or without Fmoc labeling, the 1-amino alditols can be permethylated for detailed structural analysis by sequential MS^n fragmentation. After permethylation, 1-amino alditol gives a quaternary ammonium cation with a permanent positive charge to enhance the MS detection sensitivity, while the Fmoc labeled 1-amino alditol produces methylated carbamate. The linkages and branches of *N*-glycans can be determined in detail by sequential MS^n fragmentation.

3.2. Investigation of reaction conditions

Previous studies have shown that *N*-glycans can be partially released as aldose-forms from glycoproteins in $NaOH/NaBH_4$ system [16]. Yuan et al. developed a nonreductive chemical method to release *N*-glycans without core α-1,3-linked fucose from glycoproteins in 0.5 M NaOH solution [20]. Based on this information, we performed a series of tests on the reaction conditions of our reductive release method using chicken ovalbumin as a model glycoprotein. We initially incubated the glycoprotein in 0.3 M aqueous NaOH solution containing 1 M $NaBH_4$ at 50 °C for 16 h, in consideration of the weak alkaline nature of $NaBH_4$. However, the released glycans were a mixture of aldose-forms (*m/z*

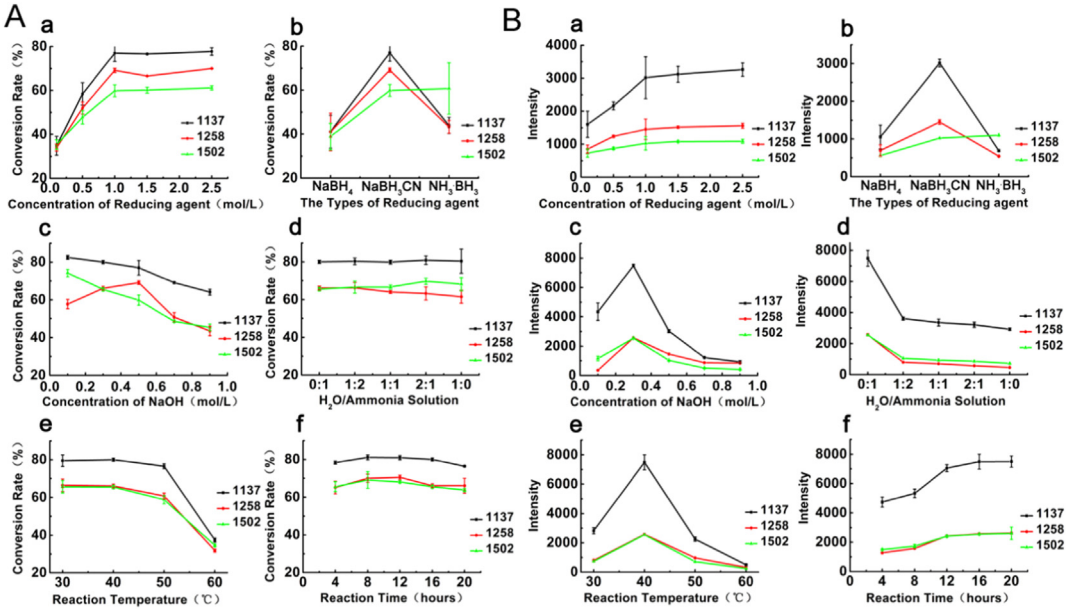

Fig. 3. Optimization of reaction conditions for the reductive release of *N*-glycans. The optimized conditions were obtained according to the yield (A) and intensity (B) of three different types of 1-amino-alditol form *N*-glycans derived from ovalbumin, including the complex type at *m/z* 1137 (black line), the high-mannose type at m/z 1258 (green line) and the hybrid type at m/z 1502 (red line). Panel a-f: optimization of reducing agent concentration, reducing agent type, NaOH concentration, ammonia concentration, reaction temperature and reaction time, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1136.25) [21], 1-amino alditol-forms (*m/z* 1137.25) and alditol-forms (*m/z* 1138.25) [16] (Fig. 2A). Therefore, the reaction conditions need to be optimized to improve yields of 1-amino alditol form *N*-glycans.

Based on the conversion rate (Fig. 3A) and MS signal intensity (Fig. 3B) of *N*-glycans, the reaction conditions were successively varied and optimized, including the concentration of reducing agent (Fig. 3A-a and B-a), the types of reducing agent (Fig. 3A-b and B-b), the concentration of NaOH (Fig. 3A-c and B-c), aqueous ammonia solution (Fig. 3A-d and B-d), reaction temperature (Fig. 3A-e and B-e) and reaction time (Fig. 3A-f and B-f). During this process, three different types of glycans were selected as model glycans, such as the complex type glycan at *m/z* 1137, the high-mannose type at *m/z* 1258 and the hybrid type at *m/z* 1502. To investigate the reproducibility of these results, each condition was repeated three times. As a result, a set of optimal reaction conditions were obtained for the reductive release of *N*-glycans, including $NaBH_3CN$ as the reducing agent, a concentration of reducing agent of 1 M, a NaOH concentration of 0.3 M, a reaction solution of aqueous ammonia, a reaction temperature at 40 °C and a reaction time of 16 h. The conversion rate of 1-amino alditol form *N*-glycans is up to 75%, while the proportion of the corresponding aldose-forms and alditol-forms is reduced to 25% (Fig. 2B), indicating that $NaBH_3CN$ is more efficient with the −C═NH group than with the aldehyde group of reducing glycans and enabling the protection of core α-1,3-fucosylated *N*-glycans from peeling degradation. Moreover, when testing the optimized method on *Fagopyrum esculentum* Moench pollen, we found intact core α-1,3-fucosylated *N*-glycans and core non-1,3-fucosylated *N*-glycans were simultaneously released, without any detectable de-*N*-acetylation or peeling products (Fig. 2D). These results indicate the good reliability of the method for various neutral *N*-glycans.

For acidic *N*-glycans, however, we found that there were deacetylated by-products generated under these conditions, such as those observed at *m/z* 1090.17, *m/z* 1272.67, *m/z* 1418.17 and *m/z* 1563.67 (Supporting information Fig. S1B). This indicated that the acetyl group was removed from sialic acids of acidic *N*-glycans. Therefore, we then reduced the alkali concentration by removing NaOH from the reaction system, to obtain a set of reaction conditions for sialylated *N*-glycans. With reference to the MS profile of FBS *N*-glycans released by PNGase F (Supporting information Fig. S1A), we found no desialylation or deacetylation products occurring in the MS profile of glycans released using the modified method (Fig. 2C). These results indicated that the optimal reaction conditions for acidic *N*-glycans were 28% aqueous ammonia solution containing 1 M $NaBH_3CN$ at 40 °C for 16 h. In addition, we found *O*-glycans can also be released from glycoproteins under these conditions, but their high peeling degradation rates (about 83%) hinder the application of the current method to *O*-glycan analysis (Fig. S2). Moreover, the co-released larger *O*-glycans and their peeling degradation products may contaminate the samples and complicate the analysis of *N*-glycans. To distinguish target *N*-glycans from co-released *O*-glycans and their degradation products, online LC/MS analysis is usually needed to perform clear structural differentiation.

The lowest detection limit of glycoprotein of this method was determined using RNase B as a model glycoprotein (Supporting information Fig. S3). Different amounts of RNase B were treated using the method and the obtained glycans were detected by ESI-MS. As a result, we clearly observed the major typical *N*-glycans even in the sample from 1 μg of RNase B. Therefore, the method is highly sensitive for *N*-glycan detection of glycoprotein samples.

3.3. Profiling of Fmoc derivatives of N-glycans by ESI-MS

Because *N*-glycans are released as 1-amino alditols, their amino groups can be chemoselectively labeled with Fmoc under mild conditions. This reaction can be utilized for the specific detection of 1-amino alditol form N-glycans, to further confirm the reliability of the method. In this study, *N*-glycans released from chicken ovalbumin, *Fagopyrum esculentum* Moench pollen and FBS were derivatized with Fmoc and analyzed by ESI-MS. For the ovalbumin sample, a total of 25 molecular ion peaks of the Fmoc derivatives of 1-amino alditol form *N*-glycans were observed in the positive-ion-mode MS profile, including 5 high-mannose type, 12 complex type and 8 hybrid type *N*-glycans (Fig. 4A). These glycan species are well consistent with those reported previously [21, 29]. Obviously, these Fmoc derivatives are 222 Da larger than the corresponding 1-amino alditol form *N*-glycans in terms of molecular

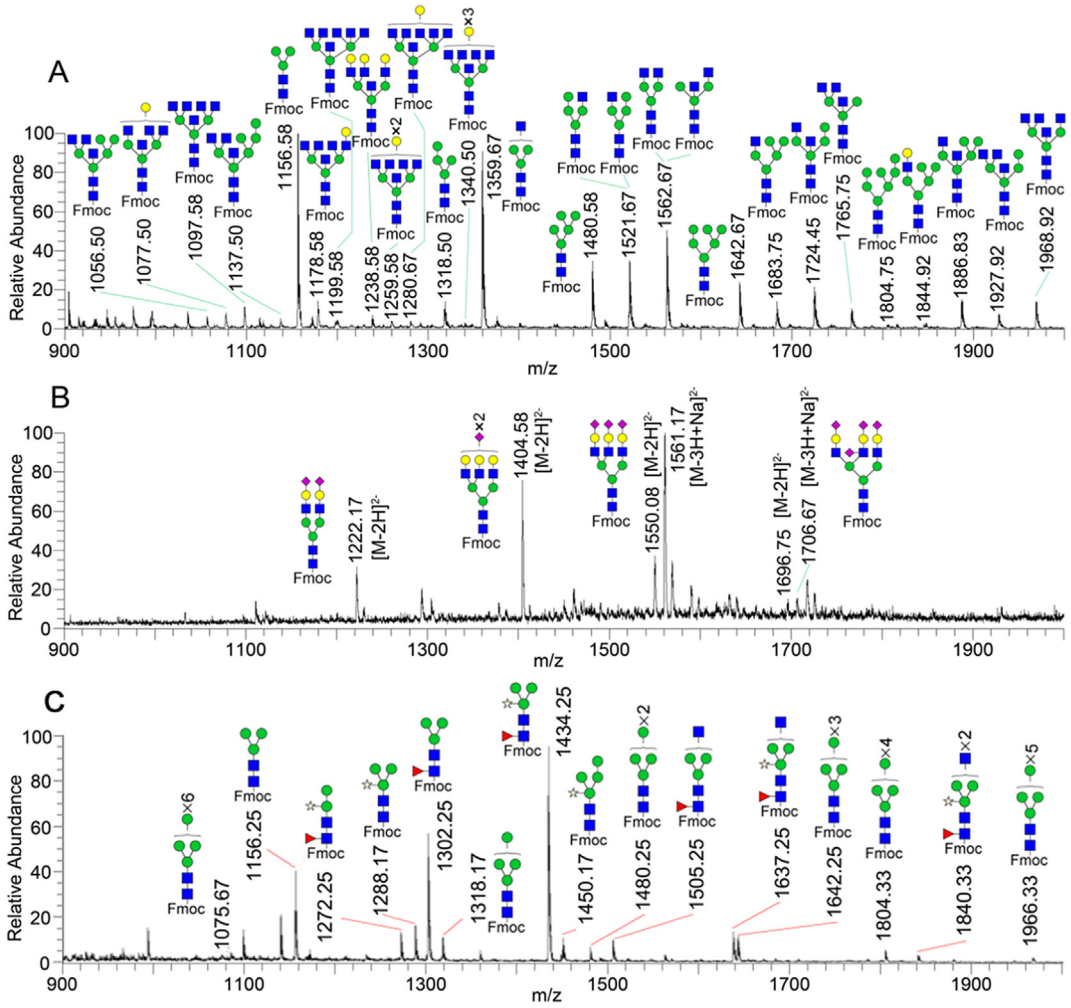

Fig. 4. ESI-MS profiles of the Fmoc-derivatized 1-amino-alditols of *N*-glycans released from chicken ovalbumin (A), FBS (B) and *Fagopyrum esculentum* Moench pollen (C). The MS profiles of (A) and (C) are in the positive ion mode and all of the corresponding glycan signals are assigned to $[M + Na]^+$ or $[M + 2Na]^{2+}$ type ions, while the MS profile of (B) is in the negative ion mode and all of the corresponding glycan signals are assigned to $[M - 2H]^{2-}$ or $[M - 3H + Na]^{2-}$ type ions. Structure formulas: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, *N*-acetylneuraminic acid; red triangle, fucose; gray five-pointed star, xylose. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

weights (Fig. 2B). Moreover, these Fmoc derivatives are more than the corresponding 1-amino alditol form *N*-glycans when detected by ESI-MS, indicating an improvement of glycan detection sensitivity caused by Fmoc tagging. These results have demonstrated the feasibility and the good reaction efficiency of the Fmoc derivatization method.

The MS profile of FBS *N*-glycans as Fmoc derivatives exhibited 4 groups of molecular ion peaks in the negative ion mode (Fig. 4B). These ion signals match doubly dehydrogenated ions of 4 typical sialylated *N*-glycans, including the disialylated biantennary glycan $Hex_5HexNAc_4NeuAc_2$ at *m/z* 1222.17 ($[M - 2H]^{2-}$), the disialylated triantennary glycan $Hex_6HexNAc_5NeuAc_2$ at *m/z* 1404.58 ($[M - 2H]^{2-}$), the trisialylated triantennary glycan $Hex_6HexNAc_5NeuAc_3$ at *m/z* 1550.08 ($[M - 2H]^{2-}$) and *m/z* 1561.17 ($[M - 3H + Na]^{2-}$), and the tetrasialylated triantennary glycan $Hex_6HexNAc_5NeuAc_4$ at *m/z* 1696.75 ($[M - 2H]^{2-}$) and ($[M - 3H + Na]^{2-}$). These glycan structures are in accordance with those reported previously [26], demonstrating the excellent applicability of the Fmoc labeling method to diverse sialylated *N*-glycans.

The MS profile of *Fagopyrum esculentum* Moench pollen *N*-glycans labeled with Fmoc was also obtained (Fig. 4C). We observed a total of 15 neutral *N*-glycans, in which 7 were high-mannose type, 5 were β-1,2-xylosylated, 6 were core fucosylated, and 1 was core pentasaccharide-truncated. With reference to the MS profile of the *Fagopyrum esculentum* Moench pollen *N*-glycans released by PNGase F (Supporting information Fig. S4), we propose that all of the 6 core-fucosylated glycans are core α-1,3-fucosylated type, including the glycan $Hex_2HexNAc_2Fuc_1Xyl_1$ at *m/z* 1272.25, $Hex_3HexNAc_2Fuc_1$ at *m/z* 1302.25, $Hex_3HexNAc_2Fuc_1Xyl_1$ at *m/z* 1434.25, $Hex_3HexNAc_3Fuc_1$ at *m/z* 1505.25, $Hex_3HexNAc_3Fuc_1Xyl_1$ at *m/z* 1637.25 and $Hex_3HexNAc_4Fuc_1Xyl_1$ at *m/z* 1840.33. These glycans are consistent with those reported in previously published articles [21]. Therefore, these results have demonstrated the great compatibility of the Fmoc labeling method with core α-1,3-fucosylated *N*-glycans.

3.4. HPLC separation and online LC-MS/MS analysis

The Fmoc-labeled glycans feature a chromogenic group and an increased hydrophobicity, enabling HPLC separation and high-sensitivity UV detection for glycan differentiation and quantification. In this study, the HILIC and RP-HPLC conditions of Fmoc-labeled *N*-glycans were optimized using Fmoc-labeled maltodextrin as a glycan standard. According to the principle of reductive amination, maltodextrin can be reduced in the presence of ammonium acetate and $NaBH_3CN$ to produce 1-amino alditols [22]. The obtained products were derivatized with Fmoc to provide glycan standards for optimization of HPLC separation conditions. We mainly optimized the elution gradients

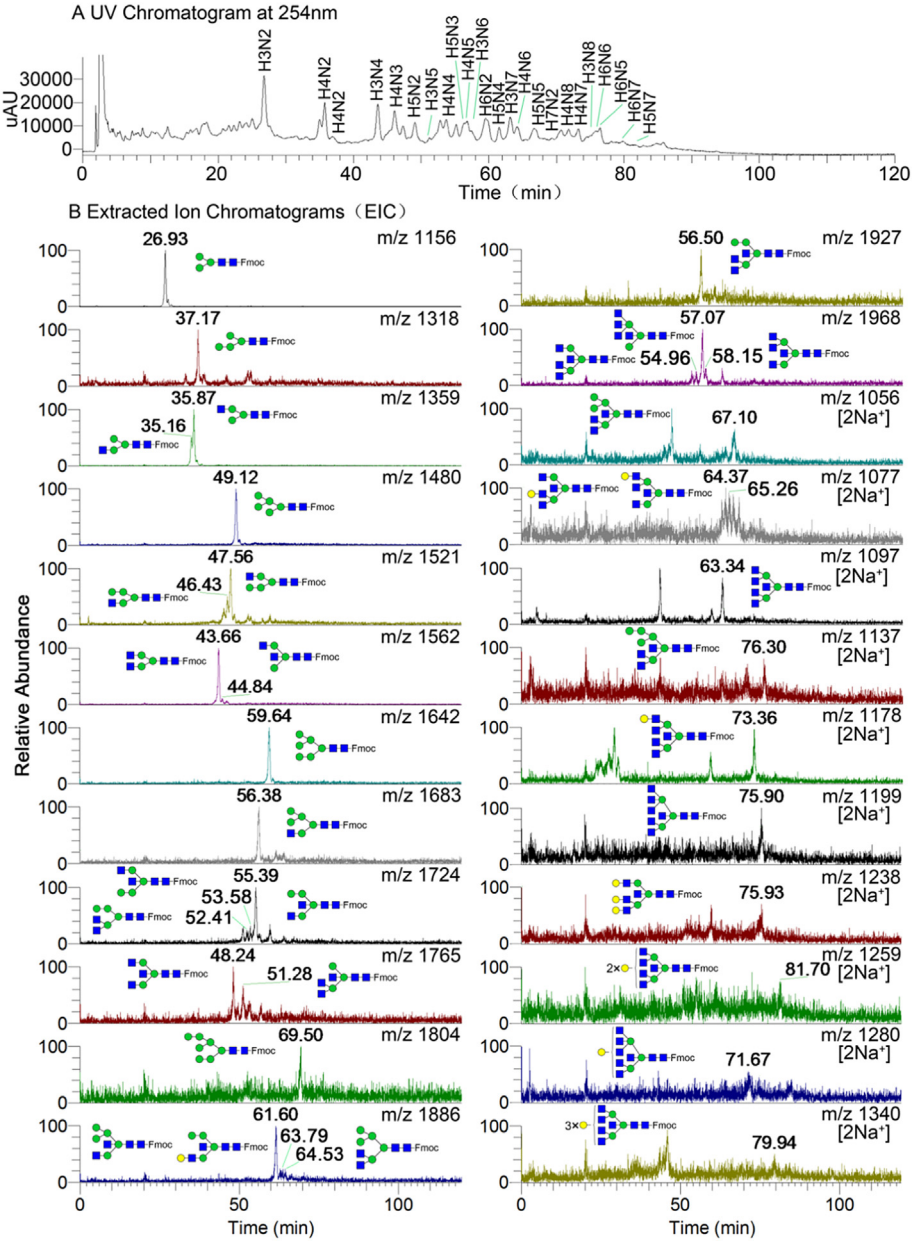

Fig. 5. Analysis of Fmoc-labeled *N*-glycans derived from chicken ovalbumin via online HILIC-MS. (A) The UV chromatogram at 254 nm. (B) Extracted ion chromatograms (EICs) in the positive ion mode. All of the *m/z* values are assigned to $[M + Na]^+$ or $[M + 2Na]^{2+}$ type ions.

(Supporting information Figs. S5 and S6), which influence retention time. The HILIC conditions were mainly based on an HILIC method used in our laboratory [30]. Considering analysis time and the total number of chromatographic peaks of Fmoc-labeled *N*-glycans, the optimized HILIC conditions for neutral *N*-glycan were as follows: solvent A, acetonitrile; solvent B, 100 mM ammonium acetate (pH 6.0); linear elution gradient, 80–60% acetonitrile within 120 min. Considering the longer retention time of acidic *N*-glycans compared with neutral ones on the HILIC column, we separated sialylated *N*-glycans using a long-time elution gradient: 80–55% acetonitrile within 150 min (Supporting information Fig. S7). For RP-HPLC, the optimized elution conditions were as follows: solvent A, acetonitrile; solvent B, 0.05% aqueous acetic acid solution; linear elution gradient, 12–27% acetonitrile within 60 min.

On this basis, we evaluated the quantification capability of the method and then performed quantitative preparation of individual *N*-glycans as Fmoc derivatives in a two-dimensional (2D) HPLC manner, which consists of the HILIC separation of Fmoc-labeled *N*-glycan mixtures as the first step and the RP-HPLC separation of glycan fractions from the HILIC separation as the second step. To evaluate the quantification ability of the method, maltohexaose was utilized as a model glycan for RP-HPLC analysis after transformation into the 1-amino-alditol form and derivatization with Fmoc. When the injection amount of the model glycan was varied, great standard quantification curves were obtained according to the relationship between its RP-HPLC peak area and injection amount, demonstrating the good quantification capability of the method (Figs. S8 and S9). Furthermore, detailed 2D-HPLC separation was successfully performed for the Fmoc derivatives of *N*-glycans released from chicken ovalbumin, *Fagopyrum esculentum* Moench pollen and FBS (Figs. S10–S15), and the amount of the obtained individual glycans was determined based on their RP-HPLC peak areas and the standard quantification curves (Table S1-S3). This provides a versatile method for the preparation of different types of natural *N*-glycans, which may be available for further functional glycomics

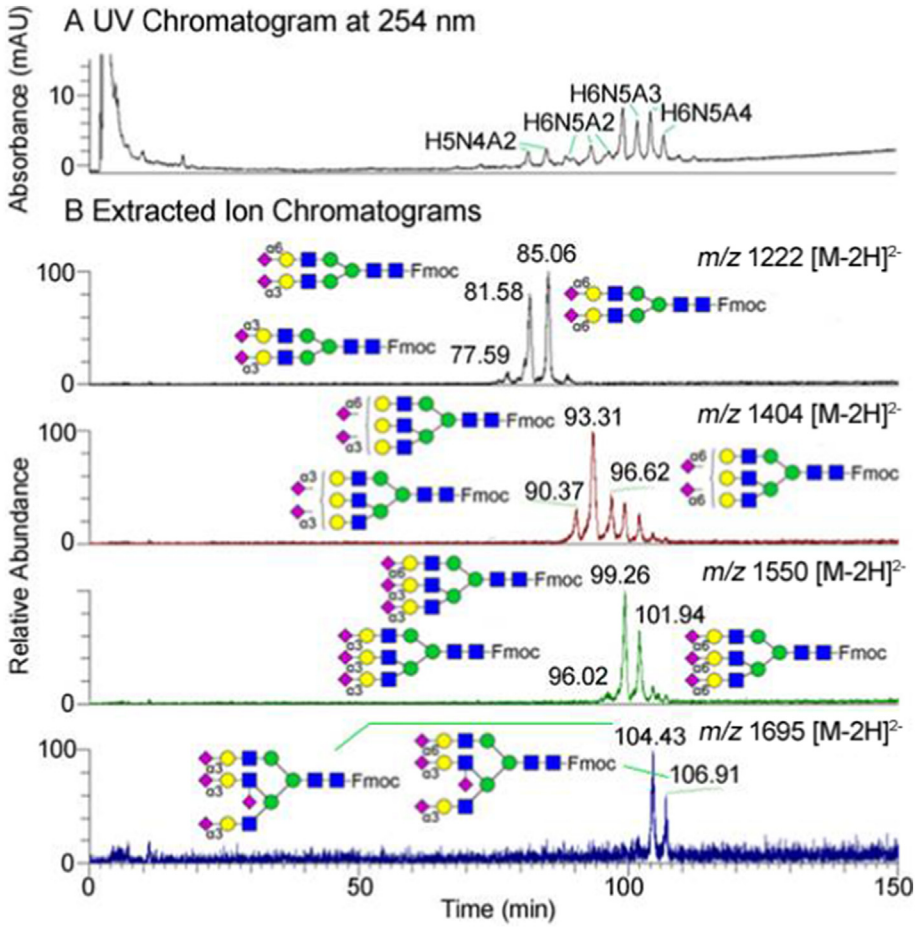

Fig. 6. Analysis of Fmoc-labeled N-glycans derived from FBS by online HILIC-MS. (A) The UV chromatogram at 254 nm. (B) Extracted ion chromatograms (EICs) in the negative ion mode. All of the *m/z* values are assigned to $[M - 2H]^{2-}$ type ions.

studies.

The *N*-glycans released from glycoproteins are rather complicated and contain multiple isomers. MS profiling of a mixture of glycans allows for assignment of monosaccharide compositions but cannot distinguish different isomers [22, 31]. In contrast, online LC/MS can be used to separate and identify Fmoc-labeled *N*-glycan isomers. Therefore, Fmoc-labeled *N*-glycans derived from chicken ovalbumin, FBS and *Fagopyrum esculentum* Moench pollen were analyzed by online LC/MS. As a result, all of the three *N*-glycan samples give a series of peaks with good resolution in the UV chromatograms at 254 nm and the extracted ion chromatograms (EICs), showing an efficient separation of the Fmoc-labeled *N*-glycans. As shown in Fig. 5, a total of 24 *N*-glycan structures of chicken ovalbumin were found when glycan isomers were taken into account. Each of the *N*-glycans with compositions of $Hex_3GlcNAc_3$ (35.16 min and 35.87 min), $Hex_4GlcNAc_3$ (46.43 min and 47.56 min), $Hex_3GlcNAc_4$ (43.66 min and 44.84 min), $Hex_3GlcNAc_5$ (48.24 min and 51.28 min) and $Hex_4GlcNAc_6$ (64.37 min and 65.26 min) has two isomers, while each of the *N*-glycans with compositions of $Hex_4GlcNAc_4$ (52.41 min, 53.58 min and 55.39 min), $Hex_5GlcNAc_4$ (61.60 min, 63.79 min and 64.53 min) and $Hex_3GlcNAc_6$ (54.96 min, 57.07 min and 58.15 min) has three isomers. The other *N*-glycan compositions have only single structures. These glycan isomers are consistent with literature reports [29, 32]. As shown in Fig. 6, 14 *N*-glycan isomers of FBS were discovered. The *N*-glycan $Hex_6HexNAc_5NeuAc_4$ (104.43 min and 106.91 min) has two isomers, and each of the *N*-glycans $Hex_5HexNAc_4NeuAc_2$ (77.59 min, 81.58 min and 85.06 min), $Hex_6HexNAc_5NeuAc_2$ (90.37 min, 93.31 min and 96.62 min) and $Hex_6HexNAc_5NeuAc_3$ (96.02 min, 99.26 min and 101.94 min) has three isomers. These sialylated isomeric structures were assigned according to those reported previously [6]. As shown in Fig. 7, 14 *N*-glycans of *Fagopyrum esculentum* Moench pollen were observed. The *N*-glycan $Hex_7GlcNAc_2$ (69.28 min and 71.16 min) has two isomers (Supporting information Fig. S16), and $Hex_8GlcNAc_2$ (77.73 min, 78.48 min and 79.89 min) has three isomers. The other *N*-glycan compositions have only single structures. These glycan structures are also consistent with the literature [21]. During these analytical processes, online LC-MS/MS data were also taken into account besides literature reports, to define glycan isomer structures. For example, the extracted ion chromatograms (EICs) of the Fmoc-labeled pollen glycan $Hex_7HexNAc_2$ at *m/z* 1804 exhibited two isomers (69.28 min and 71.16 min), which were individually identified by online MS/MS (Supporting information Fig. S6). The obtained MS/MS fragment ions, such as those at *m/z* 1097, *m/z* 814, *m/z* 654, *m/z* 1119, *m/z* 996, *m/z* 834 and *m/z* 611, show different fragmentation patterns, allowing for assignment and differentiation of isomeric structures.

3.5. Permethylation and structural analysis of released N-glycans and their Fomc derivatives

Permethylation of released *N*-glycans before and after Fmoc-labelling and sequential MS^n analysis of the permethylated products were investigated for detailed sequencing. As a result, the *N*-glycans and their Fmoc derivatives derived from chicken ovalbumin, FBS and *Fagopyrume sculentum* Moench pollen were all successfully permethylated (Fig. 8 and supporting information Figs. S17 and S18). Many doubly or triply charged ions of permethylated products were observed in MS profiles. The obtained MS spectra showed that 1-amino alditols form a quaternary ammonium cation, which features a permanent

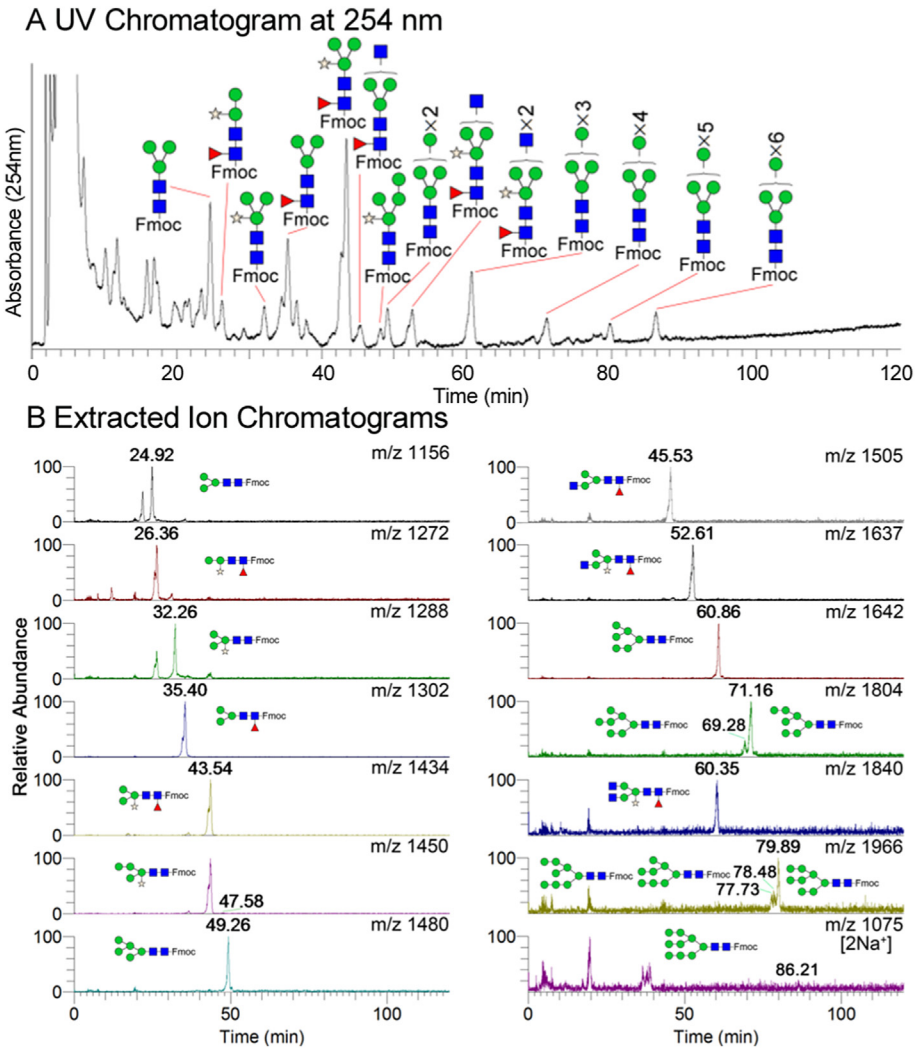

Fig. 7. Analysis of Fmoc-labeled *N*-glycans released from *Fagopyrum esculentum* Moench pollen by online LC/MS. (A) UV chromatogram at 254 nm. (B) Extracted ion chromatograms (EICs) in the positive ion mode. All of the *m/z* values are assigned to $[M + Na]^+$ or $[M + 2Na]^{2+}$ type ions.

positive charge and can improve the MS detection sensitivity, while Fmoc derivatives produce methylated carbamates.

Subsequently, we performed sequential MS^n analysis of these permethylation products of 1-amino alditols and their Fmoc derivatives. For example, the pollen *N*-glycan $Hex_3HexNAc_2Fuc_1Xyl_1$ at *m/z* 1526.25 is cleaved from the non-reducing end and the charge center is at the reducing end (Fig. 9). The characteristic fragment ions at *m/z* 493 ($Y_{1\alpha}$) in the MS^2 spectrum and at *m/z* 739 (Y_2) and 1279 ($Y_{1\beta}$) in the MS^3 spectrum indicate the core-linked fucose, while the cross-ring cleavage fragment at *m/z* 1203 ($^{0,3}X_2$) in the MS^2 spectrum can be used to illustrate the positions of branches and the xylose residue. It is noteworthy that only X, Y or Z type fragment ions that have reducing-end charge center can be detected, while the A, B or C type fragments cannot be detected due to the lack of charge center. Moreover, the detected fragments are generated from a single parent molecular ion in a sequential manner, allowing for sequence assignment of the parent glycan. The detailed structure of permethylated products of Fmoc-labeled *N*-glycans was also determined by MS^n analysis. As shown in Fig. 10, for example, the permethylated Fmoc derivatives of the pollen glycan $Hex_3HexNAc_2Fuc_1Xyl_1$ at *m/z* 1578.42 generates a series of fragment ions during sequential MS^n disassembly, which provide plentiful structural details, such as linkages and branching of glycans. Fig. 10B and C show the MS^3 spectra at *m/z* 1372 and *m/z* 1054 from a singly-charged parent ion at *m/z* 1578, respectively. These ions produces some diagnostic fragments, such as those at *m/z* 1299 ($^{0,4}X_2$), 1093 ($^{0,4}X_2$), 547 (Y_1) and 484 ($^{0,2}X_2$). According to the diagnostic fragment ions at *m/z* 547 and 484, we deduced that the fucose residue was core-linked and xylose was 1,2-linked. Based on the diagnostic fragment ions at *m/z* 1299, 1093 and 484, the branches were deduced to be 1,3-linked and 1,6-linked to the core mannose. In addition, the permethylated Fmoc derivatives of the sialylated glycan $Hex_5HexNAc_4Neu5Ac_2$ at *m/z* 1444 ($[M + 2Na]^{2+}$) was fragmented to generate an MS^2 spectrum (Supporting information Fig. S19). According to the cross-ring fragments at *m/z* 1226 ($^{0,4}X_{5\alpha}$) and *m/z* 1044 ($B_6{}^{2,4}X_{3\alpha}$), we demonstrated that both α-2,6 and α-2,3 linkages of sialic acid are present. On this basis, we chose the MS^2 fragment at *m/z* 1256 ($[M + 2Na]^{2+}$) as a parent ion to generate a MS^3 spectrum (Supporting information Fig. S20). The observed cross-ring fragments at *m/z* 398 ($B_{3\beta}{}^{0,4}X_{5\beta}$) and *m/z* 494 ($B_{4\beta}{}^{2,4}X_{5\beta}$) indicated that the other branch also include both α-2,6 and α-2,3-linked sialic acid. Because this disialylated *N*-glycan exhibits three isomers in LC/MS analysis, we deduced that the linkage of its sialic acids were α-2,3 and α-2,3, α-2,3 and α-2,6, and α-2,6 and α-2,6. These results are consistent with those reported in the literature [6]. In conclusion, sequential MS^n fragmentation of permethylated 1-amino alditols can be utilized for glycan sequence identification, while sequential MS^n fragmentation of permethylated Fmoc derivatives provides more types of linkage-specific or diagnostic ions for linkage elucidation. Therefore, sequential MS^n analysis after

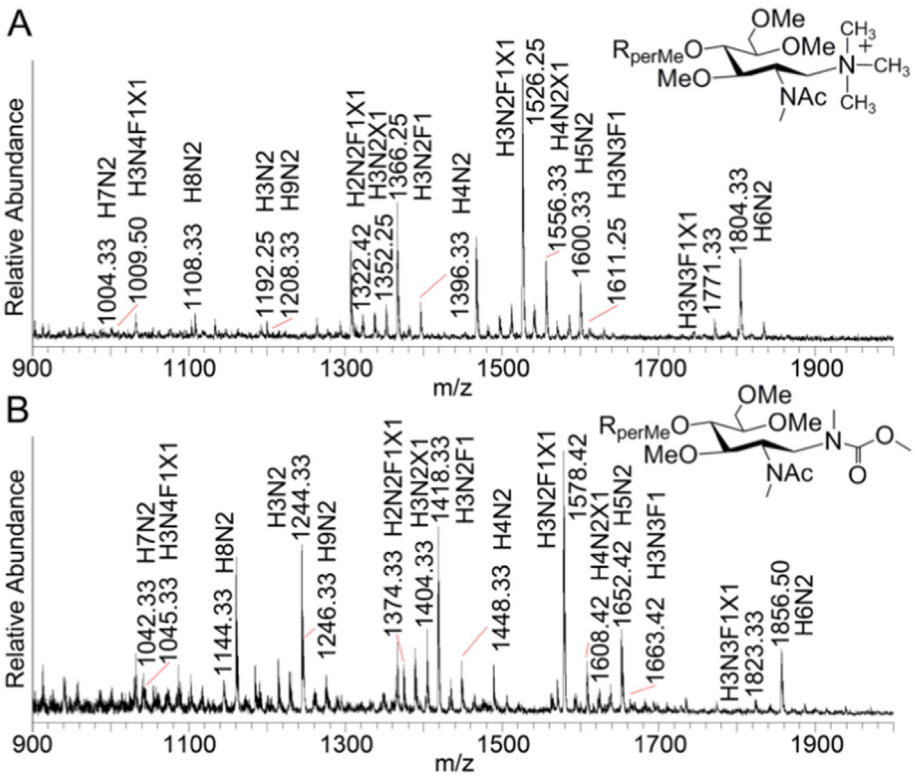

Fig. 8. Positive-ion mode ESI-MS profiles of permethylation products of 1-amino alditols (A) and corresponding Fmoc derivatives (B) derived from *Fagopyrum esculentum* Moench pollen. All of the MS signals shown in (A) are assigned to $[M]^+$ type ions, while all of the MS signals shown in (B) are assigned to $[M + Na]^+$ or $[M + 2Na]^{2+}$ type ions.

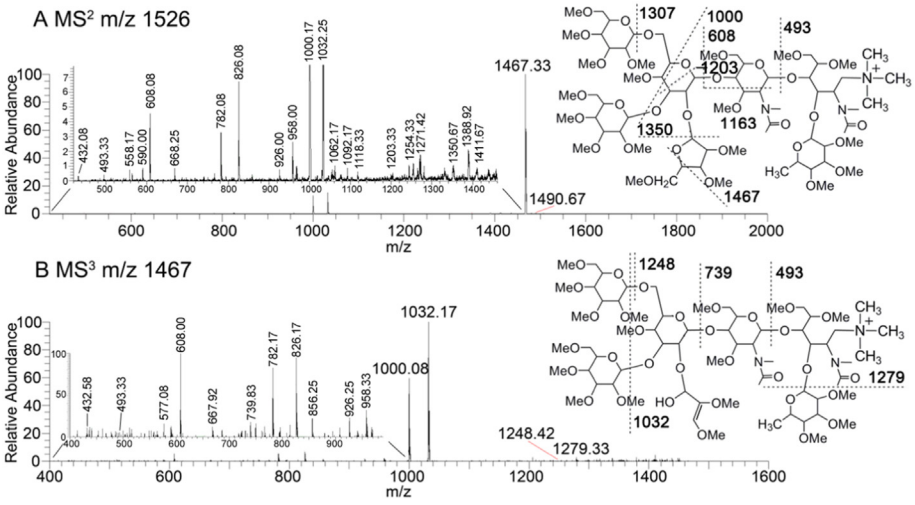

Fig. 9. MS^n data of the permethylated 1-amino alditol-form *N*-glycan $Hex_3HexNAc_2Fuc_1Xyl_1$ derived from *Fagopyrum esculentum* Moench pollen. Panel A shows the MS^2 spectrum of the parent ion at *m/z* 1526 ($[M]^+$). Panel B shows the MS^3 spectrum of the MS^2 fragment ion at *m/z* 1467 ($[M]^+$). The MS^n fragment ions were assigned using GlycoWorkbench [5].

permethylation is an efficient method for the structural characterization of *N*-glycan 1-amino alditols and their Fmoc derivatives after permethylation.

4. Conclusions

We developed a novel method for the reductive chemical release of *N*-glycans as 1-amino alditols from glycoproteins in aqueous ammonia solution containing reducing agents. Using this method, we successfully released typical neutral *N*-glycans and acidic *N*-glycans from glycoproteins, without peeling degradation of core α-1,3-fucosylated *N*-glycans or detectable deacetylation reaction. *N*-Glycans obtained from 1 μg of RNase B could be clearly observed in MS spectra, demonstrating high sensitivity of this method. The released *N*-glycans, such as those from chicken ovalbumin, *Fagopyru mesculentum* Moench pollen and FBS, can be efficiently derivatized with Fmoc, allowing for glycan isomer separation and identification by detailed online LC/MS analysis. In addition, permethylation of the 1-amino alditol-form *N*-glycans generates tertiary ammonium cations at the reducing end, which enhance the sensitivity of MS detection and permit glycan sequencing by MS^n. Permethylation of Fmoc derivatives of *N*-glycans produce methylated carbamates, which give rich cross-ring fragments during MS^n fragmentation and thus allow detailed characterization of glycan linkages and branching. This strategy has shown satisfactory applications in high-throughput preparation and analysis of various *N*-glycans. At the same time, the authors' group is devoted to separation of various Fmoc-labeled *N*-glycans by 2D-HPLC, which are suitable for further functional glycomics studies such as microarray analysis after simple removal of Fmoc group.

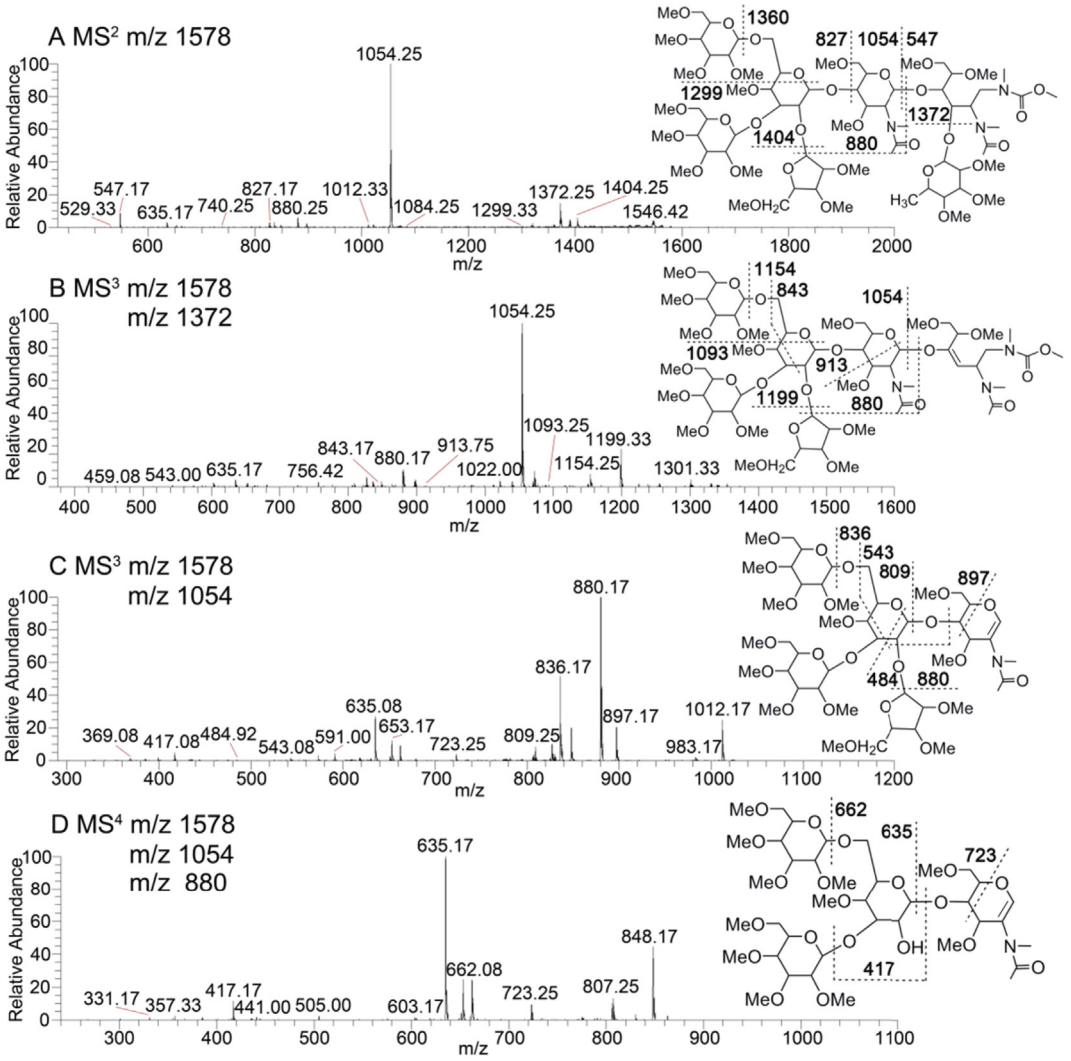

Fig. 10. MS^n data of the permethylated Fmoc derivatives of the *N*-glycan $Hex_3HexNAc_2Fuc_1Xyl_1$ derived from *Fagopyrum esculentum* Moench pollen. Panel A shows the MS^2 spectrum of the parent ion at *m/z* 1578 ($[M + Na]^+$). Panel B and C shows MS^3 spectra of the MS^2 fragment ions at *m/z* 1372 ($[M + Na]^+$) and *m/z* 1054 ($[M + Na]^+$), respectively. Panel D shows MS^4 spectrum of the MS^3 fragment ion at *m/z* 880 ($[M + Na]^+$). The MS^n fragment ions were assigned using GlycoWorkbench [5].

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by the National Natural Scince Foundation of China (31670808, 31600647, 31370804, 31300678), the Natural Science Special Fund of Shaanxi Provincial Education Department (16JK1782), the Scientific Research Program Fund for Shaanxi Province Key Laboratory (16JS109) and the Scientific Research Foundation of Northwest University, China (15NW18).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.06.002.

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