Video Article

Analysis of *N*-glycans from *Raphanus sativus* Cultivars Using PNGase H$^+$

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Abstract

In recent years, the carbohydrate moieties of plants have received considerable attention, as they are a potential source of cross-reactive, allergy-provoking immune responses. In addition, carbohydrate structures also play a critical role in plant metabolism. Here, we present a simple and rapid method for preparing and analyzing *N*-glycans from different cultivars of radish (*Raphanus sativus*) using an *N*-glycanase specific for the release of plant-derived carbohydrate structures. To achieve this, crude trichloroacetic acid precipitates of radish homogenates were treated with PNGase H$^+$, and labeled using 2-aminobenzamide as a fluorescent tag. The labeled *N*-glycan samples were subsequently analyzed by ultra performance liquid chromatography (UPLC) separation and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry for a detailed structural evaluation and to quantify relative abundancies of the radish-derived *N*-glycan structures. This protocol can also be used for the analysis of *N*-glycans from various other plant species, and may be useful for further investigation of the function and effects of *N*-glycans on human health.

Video Link

The video component of this article can be found at https://www.jove.com/video/57979/

Introduction

N-glycans in plants have drawn increased attention in recent years, as previous research has highlighted *N*-glycans as a potential source of immunological cross-reactions that may provoke allergic responses[1,2]. It has been demonstrated previously that *N*-glycans on plant glycoproteins can affect catalytic activity[3,4], thermostability and folding[5,6] or subcellular localization and secretion[7]. In order to correlate the glycan structures with their respective functions, *N*-glycans must first be released from glycoproteins either chemically or enzymatically. The classical chemical method for releasing both *N*- and *O*-glycans is β-elimination, in which alkaline sample treatment is accompanied by reduction with borohydride to yield an alditol[8]. However, this procedure precludes labeling with a fluorophore and causes significant decay of monosaccharide units from the reducing end of the glycan structure. Chemical deglycosylation based on ammonium hydroxide/carbonate treatment is also a commonly used alternative method[9]. Neither of these chemical release methods degrades intact proteins, which allows mass spectrometric analysis of unlabeled glycan pools without the interference of peptide fragments in the same mass range. However, one drawback of these methods is the increased degradation rate of α1,3-fucosylated *N*-glycans, a common carbohydrate structure found in plants[10]. Alternatively, enzymatic release methods using peptide:*N*-glycanases (PNGases, EC 3.5.1.52) are also widely applied. Recombinant PNGase F (from *Flavobacterium meningosepticum*) is the most common choice and permits the release of all types of *N*-glycans, except the structures bearing a core α1,3 fucose[11,12]. Therefore, PNGase A (isolated from almond seeds) is usually used for the analysis of plant *N*-glycans[13]. However, this enzyme deglycosylates only proteolytically-derived glycopeptides, and is unable to deglycosylate native glycoproteins[14]. Hence, a multi-step sample workup is required before further analysis, which causes extensive loss of glycans, especially those of low abundance[15].The overall goal of the method is to present an optimized workflow for *N*-glycan release and fluorescence labeling in a simple and robust manner. The underlying rationale is that PNGase H$^+$, which was recently discovered in *Terriglobus roseus* and can be recombinantly expressed in *E. coli*, can hydrolyze *N*-glycans directly from the protein scaffold in acidic conditions[16]. A key advantage of using PNGase H$^+$ over alternative methods is that fluorescent labeling reactions can be performed in the same reaction tube without changing the reaction buffers[17,18]. The simple preparation conditions and high recovery of low-abundance oligosaccharides make this method a valuable tool in the analysis of *N*-glycans. This protocol is suitable for the analysis of *N*-glycans from various plant species.

Protocol

1. Sample collection

1. Purchase different cultivars of fresh radish (*Raphanus sativus* L.).

2. Isolation of Protein from Radish

1. Homogenize approximately 100 g of fresh radish with a kitchen blender for 10 min.
2. Transfer the slurry to a 50 mL centrifuge tube and centrifuge at 14,000 × *g* at 4 °C for 20 min to remove the insoluble material.
3. Transfer the supernatant carefully into a new 50 mL centrifuge tube and add an equal volume of 2 M trichloroacetic acid (TCA) solution.
   NOTE: Adding TCA will precipitate soluble (glyco)proteins.
4. Centrifuge at 14,000 × *g* at 4 °C for 30 min and remove the supernatant from the pelleted (glyco) proteins.
5. Wash the pellet with 20 mL of deionized water and centrifuge at 14,000 × *g* at 4 °C for 5 min to remove soluble oligosaccharides and polysaccharides.
6. Repeat step 2.5. four times.
7. Re-suspend the pellets in 1 mL of deionized water and transfer to a fresh 1.5 mL centrifuge tube.

3. Preparation of *N*-Glycans

1. Mix 50 µL of protein solution from step 2.7. (equal to 5 g of fresh radish) with 0.2 mU of recombinant PNGase H^+ in 10 mM acetic acid.
2. Incubate the reaction mixture at 37 °C for 12 h. After the incubation, centrifuge at 14,000 × *g* for 5 min to remove the extra protein and enzyme.
3. Transfer the supernatant to a fresh 1.5 mL centrifuge tube.

4. Purification of *N*-Glycans

1. Prepare the solid-phase extraction (SPE) column to enrich the *N*-glycans and remove salts and other impurities from the reaction mixture, increasing the selectivity of the fluorogenic 2-aminobenzamide (2-AB) labeling agent for *N*-glycan derivatization.
   1. Add 3 mL of deionized water to wash the column.
   2. Add 3 mL of 80% acetonitrile solution containing trifluoroacetic acid (TFA, 0.1% v/v) to active the SPE-column.
   3. Add 3 mL of deionized water to equilibrate the SPE-column.
2. Transfer the sample from step 3.3 onto the column and discard the flow-through.
3. Add 1.5 mL of deionized water to wash the column and discard the flow-through.
4. Elute the released *N*-glycans from the column using 1.5 mL of aqueous 20% acetonitrile solution, containing 0.1% TFA (v/v) into a 2 mL centrifuge tube.
5. Remove the solvent by centrifugal evaporation at room temperature. Evaporate until the sample is completely dry.

5. Fluorescence Derivatization of *N*-Glycans

1. Prepare 1 mL of 2-AB solution, consisting of 35 mM 2-AB and 0.1 M sodium cyanoborohydride in dimethyl sulfoxide/acetic acid solution (7:3, v/v).
2. Add 5 µL of 2-AB solution to the dried samples (step 4.5.) derived from six different radishes. Vortex each sample until completely dissolved.
3. Incubate the mixture for 2 h at 65 °C.
4. Let the sample cool down to room temperature for 5 min, and add 5 µL of deionized water and 40 µL of acetonitrile. Centrifuge the tubes at 14,000 x g for 3 min.
5. Transfer 48 µL from the supernatant into a 300 µL high-recovery HPLC vial. Store the derivatized *N*-glycan samples at -20 °C for up to one month.

6. HILIC-UPLC profiling of *N*-glycans

1. Analyze the samples using a standard UPLC system connected to an online fluorescence detectorset at the excitation/emission wavelengths of 330 nm/420 nm, respectively.
2. Use a hydrophobic interaction liquid chromatography (HILIC)-UPLC glycan column at a column temperature of 60 °C for the analysis.
3. Prepare solvent A by diluting 50 mL of stock solution (1 M ammonium formate solution, pH 4.5) with 950 mL of liquid chromatography-mass spectrometry (LCMS)-grade water. Prepare the stock solution as follows:
   1. Add 43 mL of formic acid to 700 mL of LCMS-grade H_2O.
   2. Adjust the pH to 4.5 by dropwise addition of aqueous ammonia solution (25% w/w).
   3. Transfer the solvent to a measuring cylinder and fill up to 1000 mL with LCMS-grade H_2O. Store at 4-6 °C for up to 3 months.
4. Use LCMS-grade acetonitrile as solvent B.
5. Separate *N*-glycans with the following gradient elution:
   1. Start by adding 95% of solvent B into solvent A. Set the flow rate at 0.5 mL/min from 0 to 44.5 min.
   2. From 0 min to 6 min, gradually decrease the proportion of solvent B with a linear gradient to78%.
   3. From 6 min to 44.5 min, gradually decrease the proportion of solvent B with a linear gradient to 55.9%.
   4. From 44.5 min to 46.5 min, rapidly decrease the proportion of solvent B with a linear gradient from 55.9% to 0% and hold at 0% for 2 min, and initiate washing of the column. Reduce the flow rate to 0.25 mL/min from 44.5 to 55 min.
   5. Wash the column further by increasing solvent B to 95% from 48.5 min to 50.5 min and hold at 95% for 4.5 min.
   6. Re-equilibrate the column to the starting conditions of 95% solvent B at 0.5 mL/min from 50.5 to 57 min.
6. Inject 45 µL of the sample into the UPLC system.
7. Elute the *N*-glycans by UPLC with retention times between 15 and 40 min.

8. Collect each observed UPLC peak fraction using 2 mL centrifuge tubes and dry the samples by centrifugal evaporation.
9. Inject approximately 1 pmol of 2-AB labeled dextran standard (2−20 glucose units) to calibrate the plant-*N*-glycan profiling, and to assign their elution times into standardized glucose units.

7. MALDI-TOF MS Analysis

1. Dissolve the samples from step 6.8 in 5 µL of LCMS -grade water. Mix 1 µL of the sample solution and 1 µL of 6-aza-2-thiothymine (ATT) solution (0.3% w/v in 70% v/v aqueous acetonitrile), and pipette the mixture onto the MALDI-TOF sample carrier.
2. Analyze the samples using a MALDI-TOF mass spectrometer instrument in positive ion mode by pressing the **Start** button.
3. Analyze the mass spectra by using the accompanying MALDI-TOF-MS analysis software.
4. Interpret the spectra using an open source glycan interpretation software[19]. Set the search parameters for 2-AB labeling, $[M+Na]^+$, and set the accuracy parameter to 2.0 Da.

Representative Results

Figure 1 shows a schematic overview of the described protocol, including the isolation of (glyco-)proteins from radish, the preparation of *N*-glycans, the UPLC analysis, and the MALDI-TOF-MS analysis of these components. **Figure 2** shows representative UPLC chromatograms of derivatized *N*-glycans of the analyzed radish cultivars. **Figure 3** shows the obtained results of the 2AB-derivatized *N*-glycan structures using MALDI-TOF-mass spectrometry. **Figure 4** shows the quantitative composition of *N*-glycans from each radish cultivar.

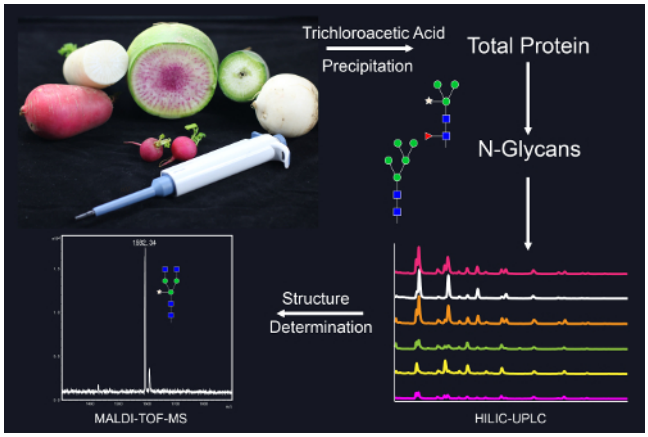

Figure 1: Schematic overview of the described analysis method for *N*-glycans released from radish glycoproteins. The method includes the preparation of (glyco-)proteins and *N*-glycans, UPLC profiling and MALDI-TOF-MS analysis. Please click here to view a larger version of this figure.

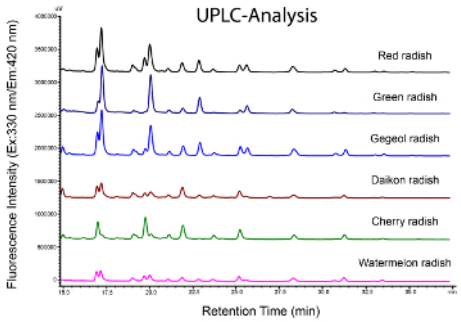

Figure 2: *N*-glycan profiles of 2-AB labeled *N*-glycans. *N*-glycans are isolated from red radish, green radish, gegeol radish, daikon, cherry radish and watermelon radish. Please click here to view a larger version of this figure.

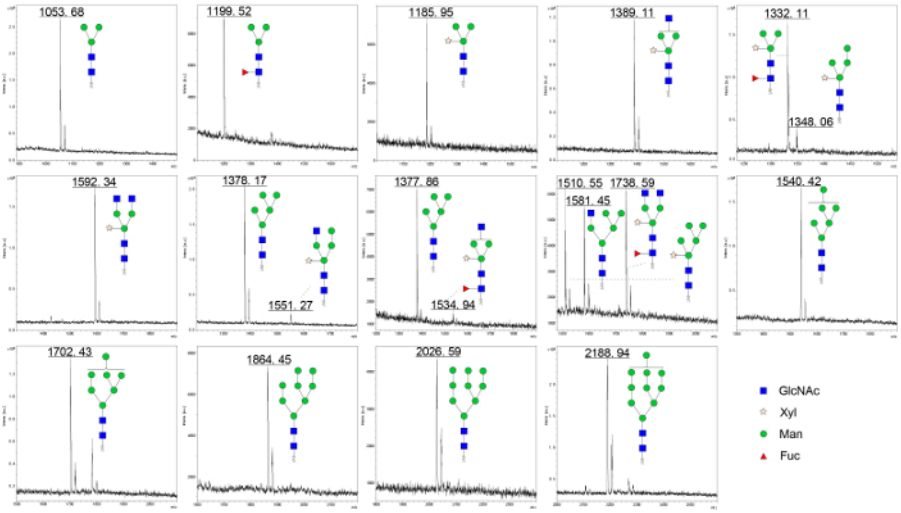

Figure 3: Mass spectrometric analysis of *N*-glycan samples isolated by HILIC-UPLC. *N*-glycans in each spectrogram are collected by the same peak. Please click here to view a larger version of this figure.

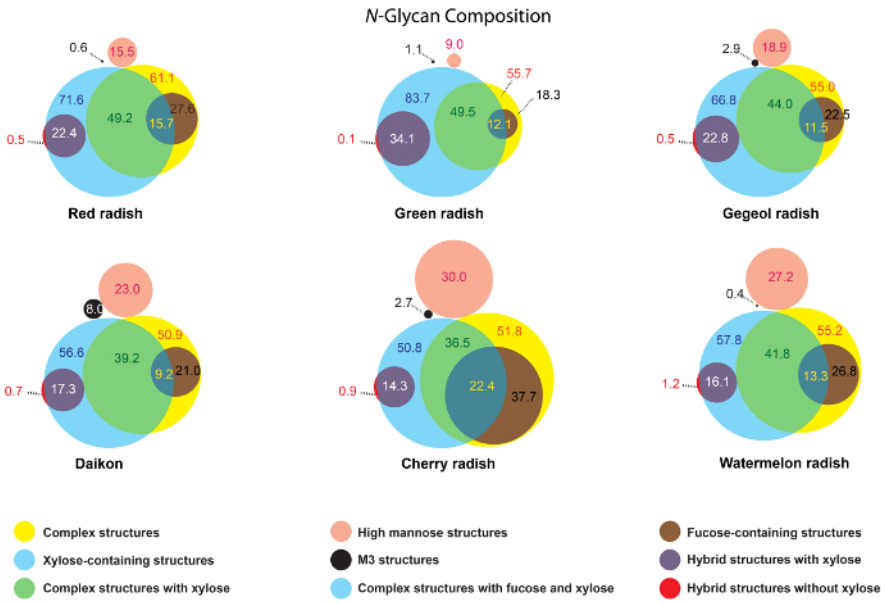

Figure 4: Quantitative composition of *N*-glycans from each radish cultivar. The sizes of circles and crescents represent the relative abundance of the 2AB-labeled *N*-glycans from radish glycoproteins. Please click here to view a larger version of this figure.

Discussion

The protocol we have presented here allows the comparison of the *N*-glycan profiles of various cultivars of radish. A significant advantage of this method compared to existing protocols is that no buffer changes between the enzymatic release of *N*-glycans and the derivatization reaction with 2-AB are required. The most critical step of this procedure is the purification of *N*-glycans using the SPE column, as failure to remove salts or other impurities in the reaction mixture may negatively impact fluorescence derivatization efficiency. The method as presented here only permits the assessment of the relative abundances of different *N*-glycans; absolute quantification would require the addition of an internal standard (such as maltopentose) in step 2.1.

Alternatively, the derivatization agent used here (2-AB) can be easily modified by using 2-aminopyridine (2-AP) or other derivatization agents. The obtained *N*-glycan derivatives from step 5.3 can be also separated by size exclusion chromatography (SEC), strong or weak anion exchange chromatography (SAX or WAX), or reverse-phase HPLC methods (although likely with inferior resolution). To further verify the structures of *N*-glycans, sequential exoglycosidase treatments can be used after step 5.3 to characterize the structure of the *N*-glycans with more details. Finally, the samples from step 6.8 could be analyzed further by using MALDI-TOF-MS^n-based fragmentation methods. Possible future applications of our protocol include investigating the function and health effects of *N*-glycans from radish or other plant species.

Disclosures

The authors have nothing to disclose.

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