Purification of Pyridylaminated Oligosaccharides Using 1,2-Dichloroethane Extraction

Yusuke Suzuki,*† Aya Okano,* Kazuya Kabayama,** Atsuyoshi Nishina,* Minoru Tanigawa,* Katsushi Nishimura,* and Yasunori Kushi†

*College of Science and Technology, Nihon University, 228A College of Sci. and Tech. Bldg. No. 2, 1-5-1 Kanda Surugadai, Chiyoda, Tokyo 101-0062, Japan
**Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

Fluorescence derivatization of the oligosaccharides released from glycoconjugates is widely used for precise structural characterization. To ensure labeling of the oligosaccharides, a large excess of fluorescence reagents is usually added to the reaction tube. Therefore, any excess reagents and by-products of the labeling reaction should be removed by several column chromatographies, including using a cellulose cartridge or spin columns. However, these purification steps are often time-consuming, expensive, and laborious. In this study, we found that 1,2-dichloroethane extraction could effectively and easily purify pyridylaminated oligosaccharides with a high recovery rate.

Keywords Pyridylaminated oligosaccharides, HPLC, cellulose cartridge, 1,2-dichloroethane extraction

(Received April 5, 2016; Accepted April 14, 2016; Published May 10, 2016)

Introduction

A plasma membrane is covered with oligosaccharides in the form of glycoconjugates, glycoproteins, and glycolipids. It is now well known that the composition of glycoconjugates regulates various biological phenomena and diseases.1–3 The above-mentioned highly divergent functions are generated by complicated oligosaccharide moieties of glycoconjugates. To resolve their functions, a detailed structural characterization of glycoconjugates is indispensable, especially that of oligosaccharide moieties. Pyridylamination on the reducing end of oligosaccharides released from glycoconjugates is one of the most powerful tools for sensitively analyzing them, using high-performance liquid chromatography (HPLC), various types of mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy.4–7 Numerous reports of concerning HPLC analyses of pyridylamino (PA)-oligosaccharides have been published, and it is easy to compare the obtained HPLC data, and thus enabling the oligosaccharide structure to be deduced by two- or three-dimensional (2D/3D) HPLC mapping techniques that combine ion-exchange, normal, and reversed-phase HPLC.8–11 Furthermore, fragment ions of the PA-oligosaccharides in MS/MS spectra can be easily characterized because there are differences in the mass number between a reducing-PA tagged end and a non-reducing free end; some databases that can automatically deduce the oligosaccharide structure from the fragmentation pattern in MS/MS spectra have been established.12–14

However, an excess of 2-aminopyrididine (2-AP) of more than a few thousand-fold is required in the reaction tube to reduce non-labeled oligosaccharides. These excess PA reagents generate by-products, which interfere with further analyses using various analytical instruments; therefore, steps for the purification of PA-oligosaccharides are indispensable. Although there have been many reports on different purification methods, such as gel filtration, organic solvent extraction, and the use of a cellulose cartridge, there is still no simple and convenient procedure to effectively remove 2-AP, especially small oligosaccharides.15,16 Furthermore, the available procedures are time-consuming, suffer from sample losses, and the employed resin is expensive. Against this background, we recently found that 1,2-dichloroethane (DCE) extraction could rapidly and simply remove various detergents in glycolipid samples or backgrounds after glycolipid extraction from a thin-layer chromatography plate.17,18 The principle of this established method is believed to depend on hydrogen-bond interactions between the oligosaccharide moiety of glycolipids and the hydroxyl group on a glass surface. Because almost all by-products and excess 2-AP reagents would lack hydroxyl groups after the PA reaction, we therefore applied the above method to purify PA-oligosaccharides in this study.

Experimental

Materials

Maltose was purchased from Wako Chemicals (Osaka). Maltolheptaose was purchased from Tokyo Chemical Industry (Tokyo). A PALSTATION kit and a cellulose cartridge were purchased from TAKARA Bio (Shiga), and used for the pyridylamination of oligosaccharide and purification. A MonoSpin NH2 column was purchased from GL Science (Tokyo).
After maltose and maltoheptaose (each 50 nmol) were transferred into glass tubes and dried completely by air flow, 20 μL of a 2-AP solution in acetic acid (12.76 mmol/mL) was added and mixed well. The reaction tubes were heated at 80°C for 60 min in a heating block. Next, 20 μL of a borane-dimethylamine solution in acetic acid (200 mg/mL) was added into the above-mentioned tubes and heated at 80°C for 60 min, respectively. After heating, 20 μL of a trimethylamine–ethanol solution and 40 μL of toluene were mixed well, and then dried at 60°C for 10 min under air flow. The samples were subsequently diluted with 20 μL of methanol and 40 μL of toluene, and then dried at 60°C for 10 min under air flow again. Finally, the samples were dissolved with 50 μL of toluene and dried completely at 60°C for 10 min under an air flow.

Organic solvents extraction for removal of excess PA-reagents
To confirm the removal ability of excess PA-reagents from PA-oligosaccharides with various organic solvents, 50 pmol of PA-oligosaccharides was transferred in each Pyrex glass tube, and dried. The PA-oligosaccharides were washed three times with 2 mL of various organic solvents. In this study, we used 11 organic solvents: methanol, acetone, 1-butanol, pyridine, acetonitrile, diethyl ether, chloroform, hexane, heptane, dichloromethane, and DCE. The residues of PA-oligosaccharides were dissolved with a mobile-phase buffer, and one-third of PA-maltose and the whole quantity of PA-maltoheptaose were analyzed by high-performance liquid chromatography (HPLC), respectively.

HPLC analysis
The PA-oligosaccharides were separated by reversed-phase HPLC with a PALPAK-type R column (4.6 mm i.d. × 250 mm, Takara Bio). The PA-oligosaccharides were detected at an excitation wavelength of 310 nm and an emission wavelength of 400 nm. PA-maltose was separated at a flow rate of 0.75 mL/min at 40°C with 100 mM acetic-acid-triethylamine (pH 4.0) for 15 min. PA-maltoheptaose was separated at a flow rate of 0.75 mL/min at 40°C using a gradient elution system with solvent A (100 mM acetic-acid-triethylamine, pH 4.0), solvent B (solvent A containing 0.5% 1-butanol), and a linear gradient: from 5 to 55% B for 60 min. Each peak was fractionated and evaporated completely in a speed Vac apparatus; the residues were resuspended in 4 μL of water.
MALDI-TOF MS and MALDI-TOF/TOF MS analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS or MALDI-TOF/TOF MS) was performed on a Voyager RP-Pro (Applied Biosystems, California) equipped with a 337-nm nitrogen laser for PA-maltose or a JMS-S3000 mass spectrometer (JEOL, Tokyo) equipped with a 349-nm nitrogen laser for PA-maltoheptaose. MS spectra were calibrated externally with peaks from the peptide calibration standard bradykinin ([M+H]+, 757.40), angiotensin II ([M+H]+, 1046.54), and adrenocorticotropic hormone, fragment 18 - 39 ([M+H]+, 2465.20). The matrix was 2,5-dihydroxybenzoic acid (DHB) at a concentration of 10 mg/mL in water. The PA-oligosaccharides were dissolved in 4 μL of water, and mixed with 1 μL of matrix solutions, then placed on a target plate for crystallization. Crystallization was accelerated by a gentle stream of cold air.

Results and Discussion

PA-maltose and PA-maltoheptaose were prepared as described in Experimental. To confirm the peak derived from PA-maltose, we purified PA-maltose using a cellulose cartridge, which is commonly used for oligosaccharide purification, and analyzed the resulting sample using HPLC for use as a positive control (Fig. 1a). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis (data not shown) showed that the background peak derived from the PA-derivative was detected at 5.8 min (Fig. 1a, asterisk), and PA-maltose was detected at 6.8 min (Fig. 1a, arrow head).

Next, the dispensed PA-oligosaccharides were extracted using various organic solvents and analyzed using HPLC as follows. PA-maltose and the backgrounds were removed together from the glass surface by extraction with methanol (Fig. 1b). Almost all background was removed and PA-maltose was recovered to some extent after extraction with acetone, 1-butanol, and pyridine (Figs. 1c – 1e). Almost none of the background was removed by extraction with acetonitrile, diethylether, chloroform, hexane, heptane, or dichloromethane; therefore, the peaks derived from PA-maltose were not confirmed (Figs. 1f - 1k). Although the peaks derived from the residual backgrounds after extraction with DCE were higher than those after purification using a cellulose cartridge in HPLC (Fig. 1l, asterisk), a PA-maltose derived peak was detected with a low-intensity background (Fig. 1l, arrow head). The peaks detected in HPLC after extraction with various organic solvents were isolated and analyzed using MALDI-TOF MS, and PA-maltose-derived peaks were assigned. However, the detected peaks derived from PA-maltose in HPLC chromatogram would be partially overlapped with background, and the MS spectra were complex because the molecular ions of PA-maltose were detected as a proton adduct at m/z 421.5 or as sodium adduct at m/z 443.5; other contaminating factors or DHB cluster ions were also detected close to the PA-maltose peak (data not shown). To obtain a simpler and more easily interpretable HPLC chromatogram and MS spectrum, we used maltoheptaose, which has a greater molecular weight than maltose, labeled it with 2-AP, performed extraction with DCE, and analyzed the residue on a glass tube using HPLC. Almost the entire background was removed by DCE extraction, and PA-maltoheptaose was detected at 6.9 min (Fig. 2a). The isolated peaks were analyzed using MALDI-TOF/TOF MS, and the presence of the sodium adduct molecular ion [M+Na]+ at m/z 1270.12 was confirmed in the MS spectrum (Fig. 2b). We also confirmed the peak derived from PA-maltoheptaose by HPLC after purification using a MonoSpin NH₄ desalting column, which was recently developed for the purification of hydrophilic compounds (Fig. 2c). The background peaks derived from the PA-derivatives were almost removed (Fig. 2c, asterisk) and PA-maltoheptaose was detected at 6.8 min after purification using a MonoSpin NH₄ desalting column (Fig. 2c, arrow head). To evaluate the established method appropriately, we compared the relative recovery ratio of PA-maltoheptaose after purification using DCE extraction and MonoSpin NH₄ desalting column (Fig. 2d). Compared to the MonoSpin NH₄ desalting column, the relative rate of the recovered PA-maltoheptaose after DCE extraction was 1.04 ± 0.05 (mean ± S.D., n = 3). These results indicated that DCE extraction is available as a simple and rapid method for PA-oligosaccharide purification.

Previous studies employed several partition methods using ethyl acetate or dichloromethane for the purification of glycolipids, or using benzene for the purification of PA-oligosaccharides. These partition methods are generally associated with such limitations as the potential for sample loss, being time-consuming because of the need to solidly separate, and the contamination of background factors into glycoconjugate fractions, especially when small amount of samples are used. In addition, there is often a need to add salts, such as sodium bicarbonate, to the sample solution. In contrast, the method established in this study just requires washing of the sample dried in a glass tube with DCE; therefore, the procedure is simple and could be applied for PA-oligosaccharide purification of multiple-samples. We confirmed that the recovery rate of PA-oligosaccharides using the established method is almost equal to those of common purification methods, including the...
use of a MonoSpin NH2 desalting column (Figs. 2c and 2d). Furthermore, the established method enables us to purify a small amount of PA-oligosaccharides of at least 50 pmol.

Conclusion

Collectively, the results obtained in this study demonstrate that organic solvent extraction is useful for removing of excess PA-reagents from PA-oligosaccharides, as well as removing detergents from glycolipids. Non-polar organic solvents with low permittivity and low solubility to water, including hexane, heptane, chloroform, and dichloromethane, showed incomplete removals of excess PA-reagents (Fig. 1). Although almost all excess reagents were removed by extraction with polar organic solvents, including methanol and acetone, pyridine, and 1-butanol, PA-oligosaccharides tended to be lost from the glass tubes. DCE was shown to be the most suitable solvent, which removed almost all excess reagents (Figs. 1 and 2). The established method is convenient and cheap compared with previously reported methods. In the future, this method should be refined, and the optimal condition of organic solvents for the removal of excess PA-reagents should be determined.

Acknowledgements

This work was supported by Nihon University College of Science and Technology Grant-in Aid for Fundamental Science Research (Y. S.), and partly supported by Grant-in-Aid for Young Scientists (B) No. 24750164 from MEXT Japan (Y. S.). The authors declare no conflicts of interest.

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