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Reversed-phase high-performance liquid chromatography of disaccharides labeled by 4-aminobenzoic acid ethyl ester

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ABSTRACT

Disaccharides and oligosaccharides are important as the units of structure of polysaccharides and as molecules that associate with glycoproteins and glycolipids. This research addressedthe high-performance liquid chromatography (HPLC) analysis of disaccharides labeled by 4-aminobenzoic acid ethyl ester (ABEE) on a reversed-stationary-phase column. The results of this study demonstrated the successful resolution of ABEE-labeled disaccharides and their UV detection at 305nm.

Keywords: Disaccharide, RP-HPLC, ABEE, derivatization

INTRODUCTION

Monosaccharides, disaccharides, and oligosaccharides are the components of polysaccharides [1, 2]. Moreover, they associate with glycoproteins and glycolipids in living organisms [1]. High-performance liquid chromatography of these saccharides is important [3, 4] for determining the quantity and the composition of these compounds in the materialsthat make upliving organismsand foods [2, 4]. The functional groups of thecompounds are basically alcohols, aldehydes, and ketones, which yield intramolecular hemiacetals and ketals with very weak UV absorbance in thewavelength range higher than 200nm. Therefore, it is very difficult to analyze these compoundsdirectly using common UV detectors. However, superior UV detectors that work within awide range of wavelengthsbelow200nm, refraction index (RI) detectors, or mass spectrometry may allow their analysis. Moreover, ascommon reversed-phase analytical columns do not retain hydrophilic compounds such asoligosaccharides, hydrophilic stationary phases bearing amino groups [5] have been used in the direct analyses of oligosaccharides.

However, saccharide derivatives labeled withhydrophobic functional groups with UV absorbance can be analyzed using a setting of a reversedstationary phase and a common UV detector. 4-Aminobenzoic acid ethyl ester(ABEE) [6–8] is an example of derivatizing agents that can be used to introduce functional groups into monosaccharides, disaccharides, and oligosaccharides.ABEE was originally applied to the analysis of labeled saccharides using normal-phase HPLC. Although the ABEE-based labeling method has been applied and optimized for the analysis of monosaccharides [7, 8], the mode of the analysis has not been applied to disaccharides using reversed-phase HPLC.

This research describes the HPLC analysis of disaccharides by ABEE derivatization on a reversed-stationary-phase column. Complete baseline separation of four disaccharides was achieved.

MATERIALS AND METHODS

Chemicals and derivatization reagents

D-Glucose and D-maltose were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). D-Cellobiose and D-lactose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).D-Isomaltose was purchased from Hayashibara Co., Ltd. (Okayama, Japan). An ABEE labeling kit including ABEE, acetic acid, and pyridine–boron

hydride complex was purchased from J-Oil Mills (Osaka, Japan).

Workup of derivatization

An ABEEmethanol solution (810μL) and glacial acetic acid (170μL) weredissolved at 40–50°C in a polypropylene microtube. A pyridine–boron hydride complex (145μL) was added to the microtube, which was sealed with a screw cap and vortexed to give an ABEE stock solution that was sufficient for 20 analyses.An aliquot (40μL) of the ABEE stock solution and a saccharide solution (1.0mM, 10μL) were mixed by vortexing, and the resulting solution was heated at 80°C for 6 h. After cooling and centrifuging for 1 min, pure water (200mL) and chloroform (200mL) were added to the microtube, which was then vortexed. The resulting upper layer was extracted, centrifuged, and filtered using a micromembrane (0.45mm pore size), to afford a sample solution for HPLC.

Instrumentation and analysis

The HPLC system was composed of an880-PU flow pump (JASCO, Tokyo), a TSK80TManalytical column (150 × 4.6mm I.D., TOSOH, Tokyo), an875 UV detector (JASCO, Tokyo), and a Sic Chromatocorder 21 (System Instruments, Tokyo). Elution was performed using a mixed solution that was composed of 0.02% trifluoroacetic acid:acetonitrile (90:10% (v/v)) at a flow rate of 1.5 mL/min.

RESULTS AND DISCUSSION

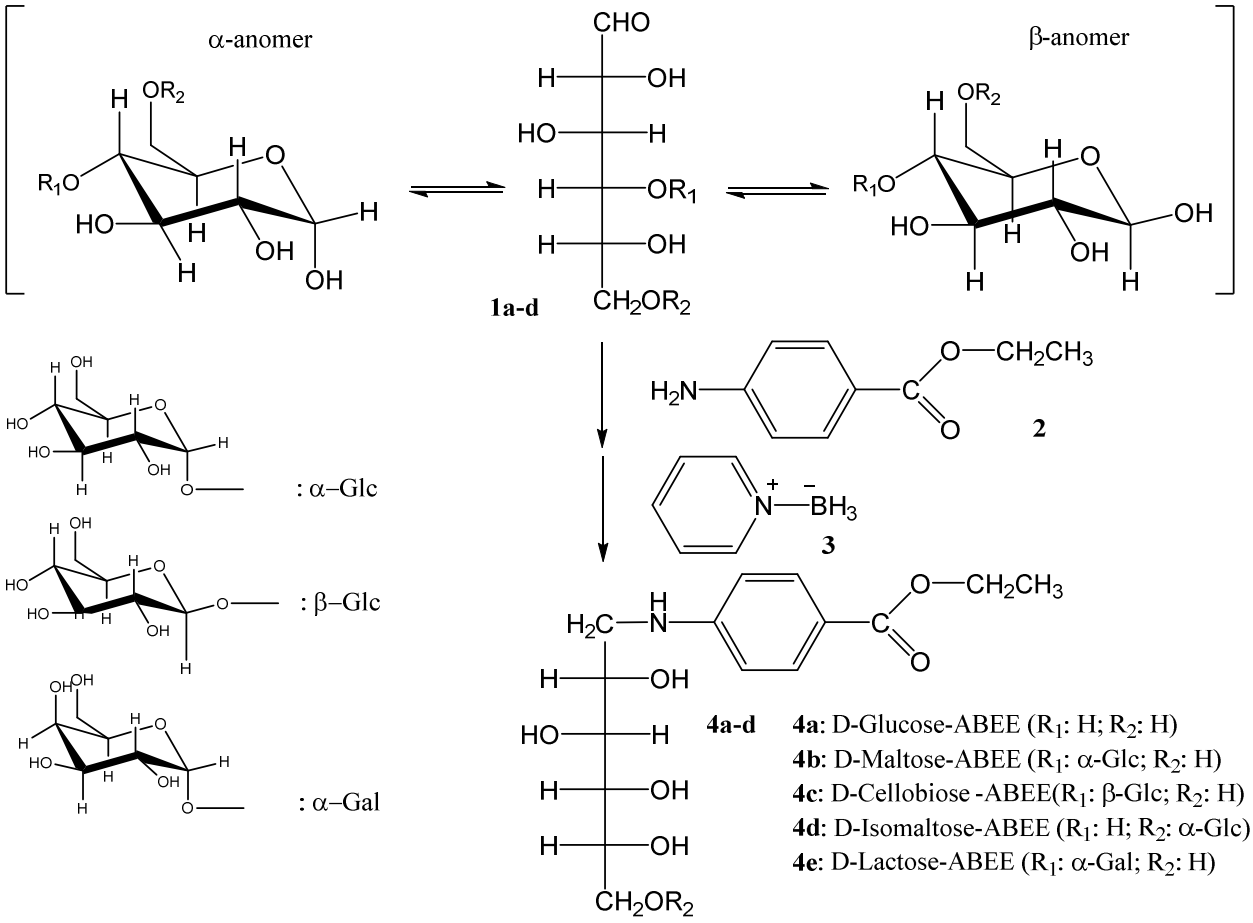


Figure1. Procedure used for the labeling of glucose (1a) and disaccharides (1b–e) with ABEE (2)

Glucose (**1a**) and disaccharides (**1b-e**) reacted with 4-aminobenzyic acid ethyl ester to yield imines, which underwent the reduction by pyridine boron hydride in situ to amine derivatives (**4a-e**). The imine formation proceedsin case of aldehydes.. Therefore, the reaction is a selective labeling for aldose, not for ketoses and sucrose. Figure 2 shows a typical chromatogram of the four disaccharides derived based on ABEE.

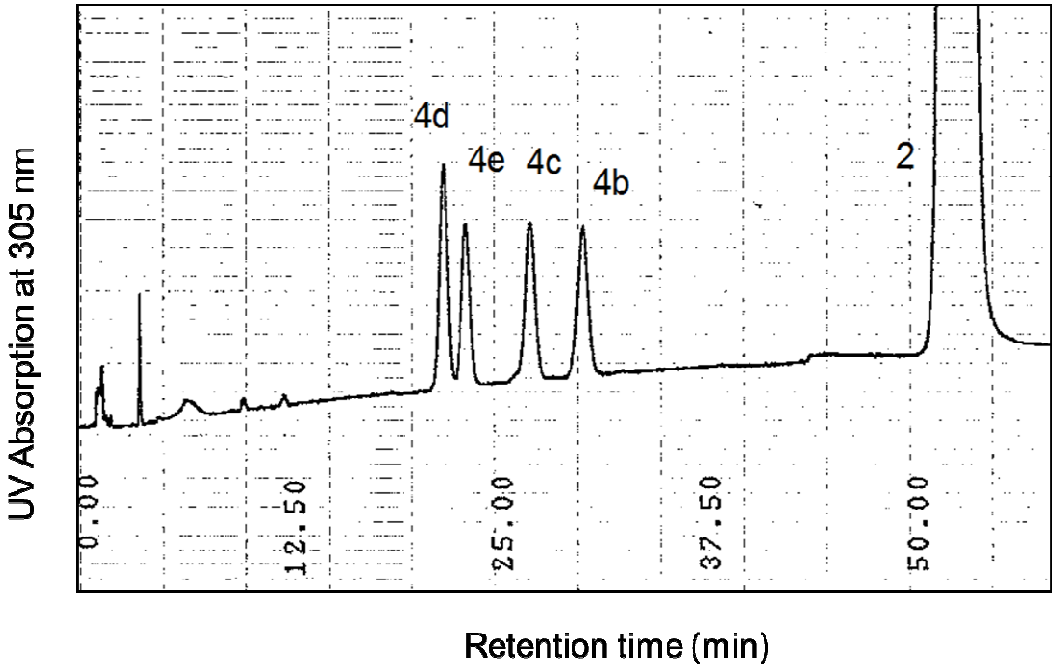
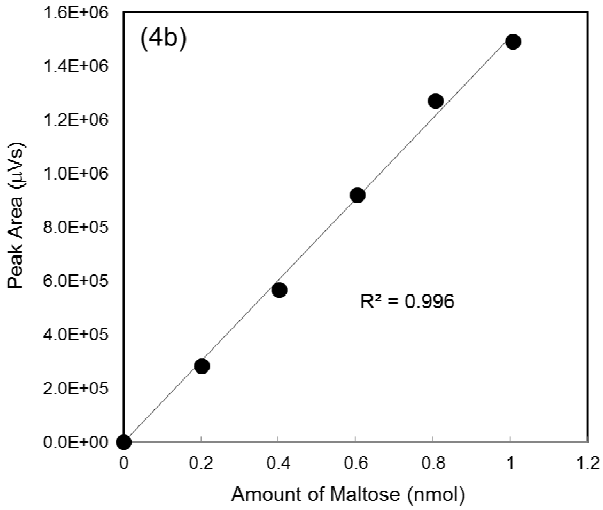
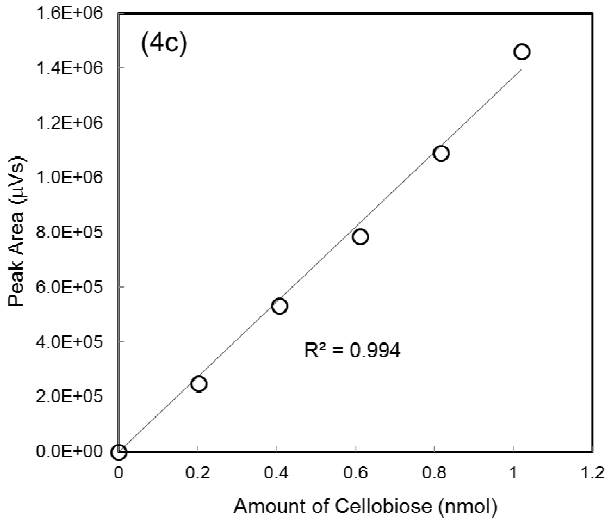


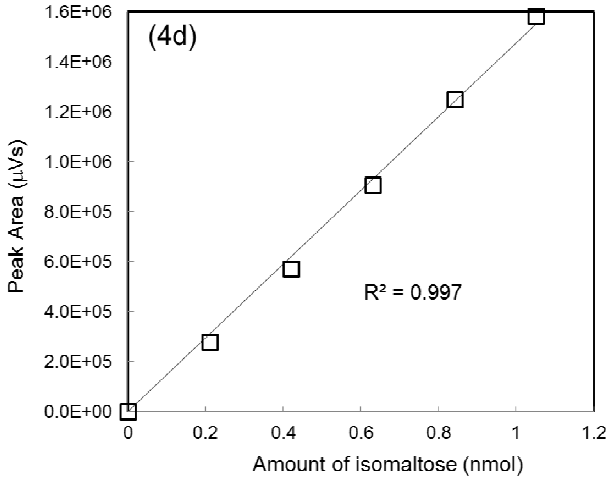
Figure 2.Typical chromatogram of the four ABEE-labeled disaccharides

Analytical column: TSK gel 80TM (150×4.6 mm I.D.); eluate: 0.02% trifluoroacetic acid:acetonitrile (90-10% (v/v)) at a flow rate of 1.5 mL/min; detection: 305nm. **1**: 4-aminobenzoic acid ethyl ester;**4b**: D-maltose–ABEE (retention time: 30.07min); **4c**: D-cellobiose–ABEE (27.17min); **4d**: D-isomaltose–ABEE (22.01min); **4e**: D-lactose–ABEE (23.21min).

Four disaccharideswere almost completely resolved down to the baseline. The first eluted peak, D-isomaltose–ABEE (**4d**),was linked byan α (1→6) bond between two glucose molecules, whereas the other disaccharide derivatives(**4b**, **4c**, and**4e**) were linked by an α (1→4) bond between two D-glucose molecules or between a D-glucose and a D-galactose molecule. The difference in structure between these compounds may render D-isomaltose–ABEE(**4d**) more hydrophilic than the others. Although the other disaccharides were very similar in structure, the ABEE derivatives of these compounds separated to each other. Conversely, D-glucose–ABEE was analyzed using the same HPLC mode. However, its peak emerged at almost the same position as that corresponding toD-maltose–ABEE (**4b)**. The complete resolution of **4a** and **4b**was not achieved. Figure 3 shows the calibration curves of the disaccharide derivatives (**4b**–**e**).







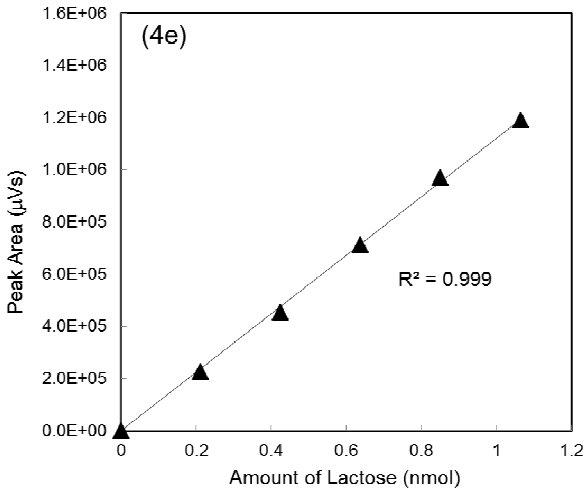


Figure 3.Calibration lines of ABEE-labeled disaccharides (4b–e)

All lines showed excellent linearity between the amount of compound and the peak area. These results suggest that the quantitative analysis of these compounds is possible in the range of 0–1.2 nmol. This research demonstrated the clear resolution of the four disaccharides labeled with ABEE. This example of this analytical mode may be useful for determining the composition of oligosaccharides [8] and monitoring glycosylation [9, 10] ofnatural products.

CONCLUSION

This research demonstrated that the four ABEE-labeled disaccharides (**4b-e**) were successfully separated by RP-HPLC. The linearity of peaks of ABEE-labeled disaccharides was performed. This method may be used for the quantification of disaccharides, the determination of glycosylationrate of natural products.

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