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Analysis of sugar phosphates in plants by ion chromatography on a titanium dioxide column with pulsed amperometric detection

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Abstract

This paper describes the development of a practical method for the analysis of sugar phosphates from the model higher plant *Arabidopsis thaliana* by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD). The extraction method of sugar phosphates from higher plants was first optimized for HPAEC–PAD analysis. In order to improve the resolution in HPAEC–PAD, a column packed with titanium dioxide resin was used. The titanium dioxide column was used as a trap-column for sugar phosphates and nucleotides, for the removal of sample matrices. Sample pretreatment was achieved in-line and automatically using a six-port valve placed after the injection valve.

Keywords: *Arabidopsis thaliana*; Titanium dioxide resin; Sugar phosphates; Phosphates

1. Introduction

Phosphorus is one of the most essential elements for biological organisms. Phosphorus in not only plants but also animals, originates from phosphate molecules absorbed by plants. The inorganic phosphate which is taken up from soil by plants is incorporated into various phosphoric compounds, for example, sugar phosphates, nucleotide phosphates and phospholipids. The metabolic fate of phosphate absorbed into the cell is important for understanding the phosphate metabolism of the cell. Post-genome approaches to understanding whole metabolic patterns in both wild and genetically modified organisms (metabolomics) require comprehensive analyses of metabolites. We have focused on developing methods for use with phosphate-related metabolomic studies in plant cells. There have been numerous research reports on phosphate metabolism but most studies have dealt with individual metabolic processes. In the present study, we have examined sugar phosphates at the level of the whole plant. Sugar phosphates play a central role in phosphate metabolism and it is therefore important to have a reliable and sensitive method for their detection, separation and determination. There is a variety of techniques that have been used for analysis of sugar phosphates (e.g. LC [1–7], LC–MS [8], GC–MS [9], CE–MS [10], etc.). Each of those analytical methods relies on some form of chromatography. Among them, a combination of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is one of the most useful of the chromatographic techniques for measurements of sugar phosphates [4–7].

Although there are some reports of analysis of sugar phosphates with HPAEC–PAD, they have used simple materials such as cultured cells or yeast, and have used different extraction methods. In plant metabolomics, the most suitable material is *Arabidopsis*, whose genome has been completely sequenced, and which has many advantages such as its rapid life cycle and its well-dissected gene function [11]. However, whole plants such as *Arabidopsis* are complicated structures,

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with different thicknesses and water content in each organ, and it is therefore more difficult to apply existing extraction methods to this material. It is essential that enzymes capable of transformation or breakdown of sugar phosphate be immediately inactivated and this is more problematic in bulky tissue. Furthermore, inactivation of enzymes by high concentration of strong acids or bases solution is not suitable for HPAEC–PAD analysis, because of interference with the chromatographic separation.

In order to obtain a reliable and practical method for analysis of sugar phosphates in plant tissues, we have developed a new method for extraction that is compatible with analysis by HPAEC–PAD. A further advance involved the use of a Titansphere TiO column packed with titanium dioxide resin—which could be used with crude samples to retain only phosphate compounds [12].

2. Materials and methods

2.1. Chemical materials

Deionized water with a specific resistance of over 18 MΩ from a Milli-RO/Milli-Q system (Millipore, Bedford, MA, USA) was degassed by aspiration and used to prepare all eluent and standards. Sodium hydroxide was purchased from Fisher Scientific (Hampton, NH, USA) as a 50% solution, and sodium acetate and acetic acid were purchased from Wako (Osaka, Japan). Metabolites for standards were purchased from Sigma (St. Louis, MO, USA). Galactose 1-phosphate, glucose 1-phosphate, sucrose 6-phosphate, glucose 6-phosphate, fructose 6-phosphate, glucose 1,6-diphosphate, fructose 1,6-bisphosphate, and UDP-glucose (uridine 5′ diphosphate-glucose) were selected as standards for sugar phosphate analysis by HPAEC–PAD. The stock solutions (0.5 mM each) of the target analytes were made up in deionized water and then stored in a refrigerator for further use. Working standard solutions were prepared daily by appropriate dilution of the stock solution.

2.2. Plant material

Seeds of *Arabidopsis thaliana* (L.) Heynh. cv. Columbia wild-type were sterilized by a 5 min treatment in 70% ethanol and 10 min in a solution of 1% Triton-X and 0.4% sodium hypochlorite. The seeds were washed four times with autoclaved deionized water, and then spread on 0.2% gellum gum plates containing Murashige and Skoog nutrient solution. Plates were placed at 4 °C for 2 days, and then they were grown in a growth cabinet continuously illuminated (light level 50 μmol m^{-2} s^{-1}) at 22 ± 1 °C for 8 weeks until required.

2.3. Plant extraction

A single whole Arabidopsis plant was frozen in liquid nitrogen and lyophilized for 4 h in a freeze dryer (Freezone 1L, Labconco, Kansas City, MO, USA), and stored at −80 °C. The sample was crushed into small pieces and placed in a 2.0 ml eppendorf tube. Boiling water (0.5 ml) was added and the extract was then immediately irradiated with 600 W microwave irradiation for 15 s. After cooling on ice, the extract was centrifuged at 15 000 rpm at 4 °C for 10 min. The supernatant was filtered through a 0.45 μm

Table 1
HPAEC–PAD analytical conditions

Columns	CarboPac PA1 (250 mm i.d. × 4.0 mm), CarboPac PA1 guard (50 mm i.d. × 4.0 mm)					
Trap column	Titansphere TiO (50 mm i.d. × 4.6 mm)					
Eluents	E1: 75 mM NaOH, E2: 75 mM NaOH/500 mM sodium acetate					
	Gradient program					
	Time (min)	E1 (%)	E2 (%)	Valve A	Valve B	Pump I
	Initial	100	0	Dotted path	Dotted path	
	−15	100	0	Solid path		On
	0.0	100	0	Dotted path	Solid path	
	5.0	100	0		Dotted path	
	18.0	56	44			Off
	22.0	60	40			
	32.0	0	100			
	38.0	0	100			
	38.1	100	0			
	45.0	100	0			
Eluent flow rate	1.0 ml min^{-1}					
Rinse reagent	Deionized water					
Reagent flow rate;	1.5 ml min^{-1}					
Detector	Amperometry detector (AU/AgCl electrode)					
Waveform	Time = 0.00, potential = 0.05; time = 0.20, potential = 0.05, integration = begin; time = 0.40, potential = 0.05, integration = end; time = 0.41, potential = 0.75; time = 0.60, potential = 0.75; time = 0.61, potential = −0.15; time = 1.00, potential = −0.15					

Dotted path and solid path were shown the path of valves in Fig. 1.

filter (Ekicrodisc AcroLC, Gelman Sciences Japan) and applied to the HPAEC–PAD.

2.4. HPAEC–PAD analysis

A DX-500 ion chromatography system (Dionex, Sunnyvale, CA, USA) consisting of a GP50 gradient pump, LC30 chromatography oven and ED50A electrochemical detector was used in this study. Dionex Chromeleon software version 6.5 was used for data processing. Dionex CarboPac PA1 (250 mm × 4 mm) and PA1 guard (50 mm × 4 mm) columns packed with anion-exchange resin were used as the separation columns, and a 50 mm × 4.6 mm Titansphere TiO column (GL Sciences, Tokyo, Japan) packed with titanium dioxide resin, was used as the inline sample pretreatment column. The mechanistic principle of the TiO column separation seems to be the ligand exchange between phosphate and titanium dioxide [13]; this should be a future subject of research. In all analyses, 25 μl was injected with a Dionex AS50 autosampler. The AS50 sample tray was cooled to 4 °C by a tray cooler during analysis. The analysis was performed at 35 °C with the flow rate set at 1.0 ml min^{1}. The gradient and valve switching program is shown in Table 1. The detection was carried out by a pulsed amperometry cell equipped with a working gold electrode and a combined pH–Ag/AgCl reference electrode. The potential–time sequence is also shown in Table 1.

The Titansphere TiO column was connected to the six-port column switching valve which was placed after the injection valve in the autosampler (Fig. 1). After sample loading of the injection loop, the injection valve was switched to inject position (dotted line path of valve A in Fig. 1) and then the sample in the injection loop was passed into the TiO column by pump I with deionized water. The sample matrix was rinsed away and phosphate compounds were retained in the TiO column. Valve B was then switched to the path through the TiO column, in order to desorb phosphate compounds from it.

2.4.1. Elution

In order to confirm the elution of analytes retained in the TiO column, the recoveries of standards were compared in the presence or absence of the TiO column. In this procedure, 75 mM sodium hydroxide was used as the sample carrier supplied by pump I in Fig. 1, and the guard column, analytical column and amperometry cell were connected to waste line of valve B.

2.4.2. Optimization of a procedure for the removal sample matrix

The sample loaded into the injection loop should normally be transferred to the TiO column by the rinsing reagent. Three different pH reagents were examined as eluting reagents, to try to find an eluent that would allow retention of phosphate compounds on the TiO column but would elute non-analytes. Deionized water, 0.1 M acetic acid and 0.1 M sodium acetate were examined rinsing reagents. These reagents do not impair the chromatography resolution.

3. Results and discussion

3.1. Extraction of phosphate compounds from a whole plant

Conventional HPAEC–PAD measurement was first applied to the analysis of sugar phosphate in whole Arabidopsis plants. The recoveries of spiked sugar phosphates standards were examined, in order to confirm the extraction efficiency. This extraction procedure was compared to the well-discussed report by Gonzalez et al. [14], which used boiling buffered ethanol extraction. The recoveries of sugar phosphate standards in each extraction method are shown in Table 2. The recoveries were almost 100% for the microwave extraction procedure except fructose 1,6-diphosphate and UDP-glucose. We have also confirmed that none of the standards was degraded during microwave treatment (data not shown). These assays demonstrated that the extraction procedure did not alter the sugar phosphates. The reason why recoveries of some sugar phosphate standards exceeded 100% cannot be explained only by decrease of volume of the extraction solvent by heating during microwave extraction, but it may be partly caused by the deviated disturbance of unknown peaks in chromatogram. Since

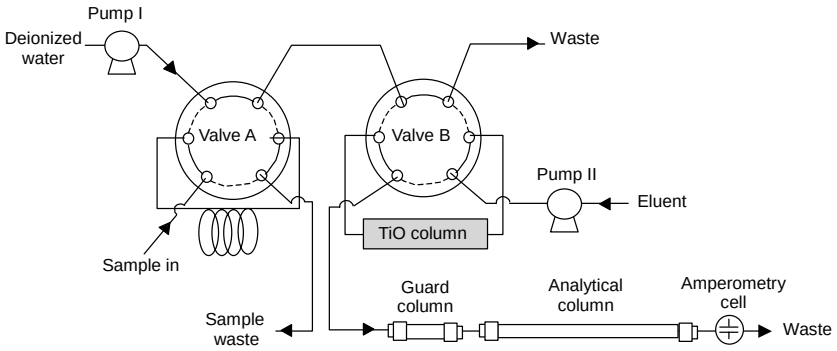

Fig. 1. Schematic of flow diagram of the IC system. For experimental details, see Table 1.

Table 2
Comparison of recoveries of standards spiked into extracts from Arabidopsis in two extraction methods

	Recovery (%)	
	Microwave	Boiling buffered ethanol
Galactose 1-phosphate	109	93
Glucose 1-phosphate	106	111
Sucrose 6-phosphate	106	85
Glucose 6-phosphate	107	90
Fructose 6-phosphate	104	107
Fructose 1,6-diphosphate	33	71
UDP-glucose	84	50

50 μM standards were added to extracts prepared either by microwave heating or by boiling buffered ethanol. Details of the extraction procedures are outlined in Section 2.

fructose 1,6-diphosphate and UDP-glucose were sometimes strongly disturbed to be detected by unknown peaks in chromatogram, their recoveries apparently were low when their concentrations in sample are not high.

A treatment of samples with alkaline phosphatase diminished peaks of sugar phosphates. Measurements of in vivo sugar phosphates under different phosphate nutrient conditions also suggested that peaks measured here were really derived from sugar phosphates (data not shown).

However, using this procedure, it was found that various unknown compounds interfered with the determination of sugar phosphates at low levels. In order to try to reduce the matrix effects and to reduce interference, we investigated the use of a TiO column. It was found to be suitable for the measurement of phosphate compounds such as nucleotides [12] and phosphorylated amino acids (data not published).

3.2. Selection of eluting reagent for TiO column

First, we have examined an eluting reagent for TiO column. In a previous study, utilizing a TiO column, 90% acetonitrile was used as the rinsing reagent for non-phosphate compounds, and sodium hydrogen phosphate was used as the eluting reagent for phosphate compounds [12]. Since these reagents are not suitable for sugar-specific HPAEC–PAD analysis, the following rinsing and eluting reagents were examined. Based on the recommendation of Kuroda [13] to use a high pH eluting reagent, sodium hydroxide (75 mM) was chosen. The initial eluent for the CarboPac PA1 column was also 75 mM sodium hydroxide, so there should be no interference with the separation of sugar phosphates. Table 3 shows the comparison of standards, which were determined with HPAEC–PAD in the presence of TiO column to the absence of TiO column. Standards were eluted by 75 mM sodium hydroxide. The recoveries in the presence of TiO column were almost 100% except for fructose 6-phosphate. Signal of fructose 6-phosphate fluctuated very much and sometimes

Table 3
Comparison of standards with/without TiO column as a trap column

Sugar phosphates	Ratio of peak area (%)
Galactose 1-phosphate	97
Glucose 1-phosphate	98
Sucrose 6-phosphate	102
Glucose 6-phosphate	101
Fructose 6-phosphate	0–70
Glucose 1,6-diphosphate	106
UDP-glucose	107

The peak areas of standards which were analyzed with TiO column, were compared as the ratio to those which were analyzed without TiO column.

was not detected by using the TiO column. It may be possible for fructose structure to partly interact with titanium dioxide, since fructose 1,6-diphosphate and fructose 2,6-diphosphate showed the same tendencies. This is the future subject.

3.3. Selection of rinsing reagent and optimization of rinse time for TiO column

In the procedure adopted, plant extracts loaded into the injection loop are transferred to the TiO column by the rinsing reagent. It is important to select a rinsing reagent that does not itself elute sugar phosphates from the TiO column.

Table 4 shows the recoveries of spiked sugar phosphates standards in whole plant extracts using three different reagents; acetic acid, deionized water and sodium acetate. The recoveries of sugar phosphates were between 93 and 114% using deionized water, which was the best among three rinsing reagents. Although some recoveries exceeded 110%, they were caused by the disturbance of unknown peaks which may be removed by optimization of rinsing time. Table 5 shows the recoveries of spiked sugar phosphates standards in plant extracts for rinsing times of 5, 10, 15 and 20 min. Since the recoveries of standards were better in 15 and 20 min rinsing time than others, 15 min rinse was selected for rinsing time.

Fig. 2 shows the chromatogram of standards, which was obtained by optimized analytical condition.

Table 4
Recovery of standards spiked into plant extracts, for different rinsing reagents that were used to load the sugar phosphates onto the TiO column

Sugar phosphates	Recovery (%)		
	0.1 M Acetic acid	Deionized water	0.1 M Sodium acetate
Galactose 1-phosphate	85	101	38
Glucose 1-phosphate	74	101	27
Sucrose 6-phosphate	70	102	40
Glucose 6-phosphate	87	93	38
Glucose 1,6-diphosphate	71	112	231
UDP-glucose	71	114	89

Table 5
Recovery of sugar phosphate standards spiked into plant extracts with increasing rinse times

Sugar phosphates	Recovery (%)			
	5 min	10 min	15 min	20 min
Galactose 1-phosphate	82	89	94	93
Glucose 1-phosphate	82	89	94	97
Sucrose 6-phosphate	90	95	106	105
Glucose 6-phosphate	92	93	93	93
Glucose 1,6-diphosphate	97	99	103	110
UDP-glucose	124	113	110	109

Deionized water was used as the rinsing reagent, and 75 mM sodium hydroxide was used as the eluting reagent.

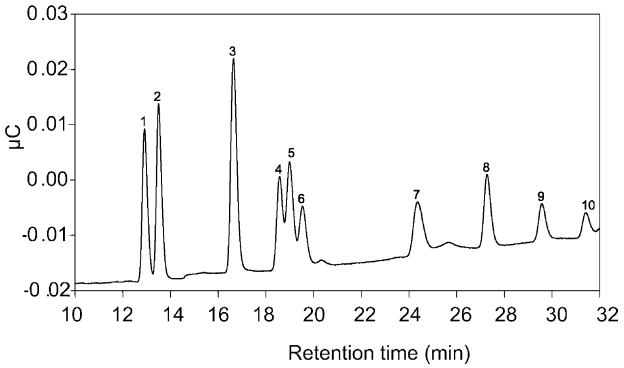

Fig. 2. Standard chromatogram analyzed by HPAEC–PAD with a TiO column. Analytical conditions as described in Section 2. Standards loaded to a TiO column were rinsed with deionized water for 15 min and then eluted with 75 mM sodium hydroxide solution. Peaks: (1) galactose 1-phosphate; (2) glucose 1-phosphate; (3) sucrose 6-phosphate; (4) glucose 6-phosphate; (5) mannose 6-phosphate; (6) fructose 6-phosphate; (7) adenosine 5′-monophosphate; (8) glucose 1,6-diphosphate; (9) uridine 5′-diphosphate-glucose; (10) adenine 5′-diphosphate. Each standard concentration was 25 μM.

3.4. Repeatability and linearity of standards

Table 6 shows the repeatability of 50 μM sugar phosphate standards at 25 μl injection. The relative standard deviation (R.S.D.) was less than 4.4% for all of the sugar phosphates analyzed. Fig. 3 shows the linearity of sugar phosphates from 0 to 150 μM, which were added to the plant extracts. Each correlation coefficient was more than 0.99.

Table 6
Peak area R.S.D.s of sugar phosphate standards spiked into plant extracts

Sugar phosphates	Area R.S.D. (%), $n = 3$
Galactose 1-phosphate	0.05
Glucose 1-phosphate	0.99
Sucrose 6-phosphate	1.26
Glucose 6-phosphate	0.98
Glucose 1,6-diphosphate	2.70
UDP-glucose	4.00

Samples were rinsed onto the column with deionized water and eluted using 75 mM sodium hydroxide. Injection volume was 25 μl.

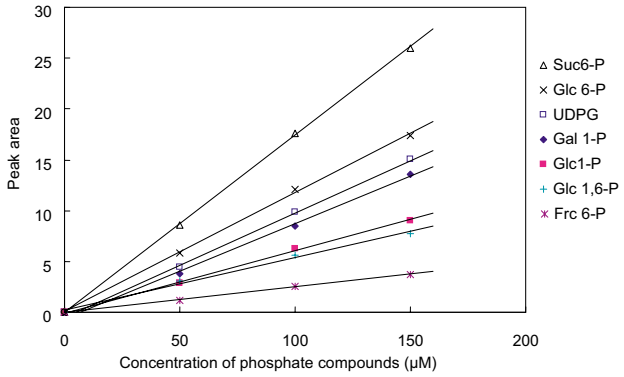

Fig. 3. Linearity of phosphate compound standards analyzed with using a TiO column. Deionized water was used as the rinsing reagent for 15 min, and 75 mM sodium hydroxide was used as the eluting reagent. Frc 6-P: fructose 6-phosphate; Gal 1-P: galactose 1-phosphate; Glc 1-P: glucose 1-phosphate; Glc 6-P: glucose 6-phosphate; Glc 1,6-P: glucose 1,6-diphosphate; Suc 6-P: sucrose 6-phosphate; UDPG: uridine 5′-diphosphate-glucose.

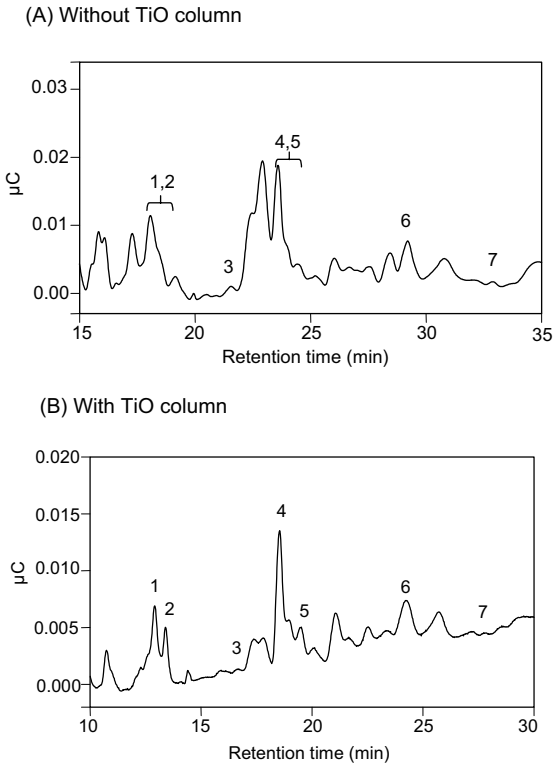

Fig. 4. Comparison of chromatograms of Arabidopsis extracts analyzed without (A) or with (B) the Titansphere TiO column. Sample was extracted with boiling water and microwave irradiation. Analytical conditions of HPAEC–PAD as described in Section 2. (A) Sample was loaded to analytical column directly. (B) Sample was loaded to column after treated in a TiO column. Peaks and concentration (μM g^{-1} fresh mass): (1) galactose 1-phosphate, 0.041; (2) glucose 1-phosphate, 0.028; (3) sucrose 6-phosphate, 0.002; (4) glucose 6-phosphate, 0.277; (5) fructose 6-phosphate, 0.083; (6) adenosine 5′-monophosphate, 0.140; (7) glucose 1,6-diphosphate, 0.010.

3.5. Comparison of chromatograms with and without TiO column

A comparison was made between plant extracts analyzed by conventional HPAEC–PAD (i.e. without TiO column) and using the new optimized method with the TiO column. Fig. 4A shows that without the TiO column, galactose 2-phosphate was not satisfactorily separated from glucose 1-phosphate, and there was an unknown peak that interfered with glucose 6-phospate. Fig. 4B shows the comparative HPAEC–PAD analysis with TiO as a trap column. Unknown peaks could be greatly diminished with this system. The concentrations of fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate obtained with this system were 0.08, 0.28 and 0.03 $\mu M\,g^{-1}$ fresh mass. They were similar to the value of 0.40 $\mu M\,g^{-1}$ fresh mass shown as the sum of fructose 6-phosphate and glucose 6-phosphate in *Arabidopsis* leaves reported by Hurry et al. [15], and the values reported by Kubota et al. [16] in *Catharanthus roseus*.

4. Conclusion

We have developed a simple method for the extraction of sugar phosphates from Arabidopsis, involving rapid freezing and microwave heating. This method gives effective inactivation of enzymes that degrade sugar phosphates and gives quantitative recovery of added sugar phosphates. The extraction reagents are compatible with analysis by HPAEC–PAD.

The use of a Titansphere TiO column to selectively retain phosphate compounds greatly improved the resolution of sugar phosphates by HPAEC–PAD and reduced interference from unknown compounds in plant extracts. These combined extraction and analysis methods provide a convenient and practical protocol for determining sugar phosphates in complex matrices such as whole plant tissue.

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