Organic Phosphates Analyzer Using High-Performance Anion-Exchange Chromatography and a Postcolumn Phosphomolybdic Acid Reaction

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An organic phosphates analyzer was developed based on high-performance anion-exchange chromatography and a postcolumn phosphomolybdic acid reaction. The organic phosphates were separated on an Asahipak NH2P-50 column and then introduced into a postcolumn reaction detector in which organic phosphates were heated at 170°C in 2.0 M sulfuric acid for 5.8 min. The resultant orthophosphate was monitored at 830 nm after a selective reaction with a molybdenum reagent. When 1.0 nmol of each sugar phosphate solution was employed (injection volume, 10 μ l) the relative standard deviations were 1.9–2.6% ($n=8$). The detection limits ($S/N=3$) of the organic phosphates were ca. 100 pmol as the injected amount.

Keywords Organic phosphate, phospho-amino acid, sugar phosphate, high-performance anion-exchange chromatography, on-line acid hydrolysis, phosphomolybdic acid reaction, heteropoly blue complex

Several methods have been reported for the detection and determination of organic phosphates. Baba *et al.*¹ have reported on a flow injection analysis (FIA) method for nucleotides involving the hydrolysis of nucleotides in 0.53 M sulfuric acid and successive conversion of the resultant orthophosphate to the so-called heteropoly blue complex with a molybdenum reagent. Baba *et al.*² also developed a coupled high-performance liquid chromatography (HPLC) and FIA system for the separation and detection of nucleoside H-phosphonates. Recently, we reported on the FIA method³, which enables the determination of various organic phosphates by a phosphomolybdic acid reaction following quantitative hydrolysis. On the other hand, ³²P-labeled phospholylated peptides, which are the final products in protein kinase assays, have been measured based on their radioactivity counts.^{4–7} Although a selective separation of nucleosides⁸ and organic phosphates⁸ was achieved on a titania column, no analytical method that is selective to organic phosphates is available, despite their importance in bioscience. In this study, therefore, the development of an organic phosphates analyzer was attempted by combining the FIA method³ as the postcolumn detection system with the anion-exchange HPLC of organic phosphates.

Experimental

Materials and reagent

Adenosine 5'-monophosphate sodium salt (AMP), adenosine 5'-triphosphate disodium salt (ATP), *O*-phospho-DL-serine (P-Ser), *O*-phospho-L-threonine (P-Thr), *O*-phospho-DL-tyrosine (P-Tyr) and D-fructose 6-phosphate disodium salt (Fru-6-P) were purchased from Sigma (St. Louis, MO, USA). Glucose 1-phosphate dipotassium salt (Glu-1-P), D-glucose 6-phosphate dipotassium salt (Glu-6-P) and fructose 1,6-diphosphate barium salt (Fru-1,6-PP) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Adenosine 5'-diphosphate disodium salt (ADP) was purchased from Kohjin (Tokyo, Japan). Boric acid (H₃BO₃), L(+)-ascorbic acid and ammonium molybdate ((NH₄)₆Mo₇O₂₄·4H₂O) were purchased from Wako (Osaka, Japan). Sodium chloride (NaCl), sodium sulfate (Na₂SO₄), potassium phosphate, dibasic (K₂HPO₄), sodium hydroxide (NaOH) and sulfuric acid (H₂SO₄) were purchased from Kanto (Tokyo, Japan). They were all guaranteed reagents and were used without further purification. Organic phosphates were dissolved in a Milli-Q water (Nihon Millipore Kogyo, Yonezawa, Japan) to make 100 μ M sample solutions. The molybdenum reagent described by Baba *et al.*¹ was modified to contain 5.0 M NaOH, 0.65% (w/v) ammonium molybdate and 1.65% (w/v) L(+)-ascorbic acid.

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Established organic phosphates analyzer system

A schematic diagram of the organic phosphates analyzer is shown in Fig. 1. A borate buffer (A) was delivered as an eluent at a flow rate of 0.5 ml min^{-1} with a JASCO TRIROTAR-VI pump (Tokyo, Japan). Ten microliters of the sample solution were introduced via a Rheodyne 7125 injector (Cotati, CA, USA) with a $10 \mu\text{l}$ -microsyringe. Separation of the organic phosphates was performed using an Asahipak NH2P-50 column (4.6 mm i.d. $\times$ 250 mm) (5) at either 40 or 50°C . The absorbance of the effluent was measured at 220 nm (JASCO UVIDEK-100-VI) (6). The effluent was added with a 4.0 M H_2SO_4 solution (B) delivered through an SNK FI-3000 double-plunger pump (Sanuki Industry, Tokyo, Japan) at 0.5 ml min^{-1} , introduced into a hydrolysis coil (PTFE, 0.5 mm i.d. $\times$ 30 m) in a KWD-2R postcolumn reactor (Kyowa Seimitsu, Tokyo, Japan) (8) and heated at 170°C in order to liberate orthophosphate. The resultant orthophosphate was reacted at 120°C with a molybdenum reagent (C), which was delivered through the SNK FI-3000 double-plunger pump at 0.5 ml min^{-1} , to form the so-called heteropoly blue complex, and cooled in a 2 m cooling coil (13). Finally, the absorbance was measured at 830 nm with a Soma S-3250 spectrophotometer (Soma Optics, Tokyo, Japan) (14). A back-pressure coil (0.25 mm i.d. $\times$ 3.5 m) (17) was set so as to suppress any air bubbling at high temperature,

Fig. 1 Schematic diagram of the organic phosphates analyzer. A, eluents; B, 4.0 M H_2SO_4 solution; C, molybdenum reagent. 1, pump (JASCO TRIROTAR-VI; 0.5 ml min^{-1}); 2, pump (SNK FI-3000; 0.5 ml min^{-1} , respectively); 3, injector (Rheodyne 7125); 4, column oven (JASCO TU-300; 40 or 50°C); 5, anion-exchange column (Asahipak NH2P-50; 4.6 mm i.d. $\times$ 250 mm); 6, spectrophotometer (JASCO UVIDEK-100-VI; 220 nm); 7, integrator (JASCO 807-IT); 8, postcolumn reactor (Kyowa Seimitsu KWD-2R; 170°C); 9, hydrolysis coil (PTFE; inner diameter 0.5 mm, outer diameter 1.5 mm $\times$ 30 m); 10, aluminum block bath (120°C); 11, coloration reaction coil (0.5 mm i.d. $\times$ 10 m); 12, cooling bath (ice/water); 13, cooling coil (0.5 mm i.d. $\times$ 2 m); 14, spectrophotometer (Soma S-3250; 830 nm); 15, signal cleaner (Tomsic SC77); 16, integrator (JASCO 807-IT); 17, back-pressure coil (0.25 mm i.d. $\times$ 3.5 m); 18, waste.

giving an appropriate pressure (ca. 10 kg/cm^2).

Results and Discussion

Optimization of the separation condition

The elution conditions for the nucleotides, phospho-amino acids and sugar phosphates chosen as representatives of the organic phosphates were separately examined. Although phosphate buffers have previously been commonly used as the eluent for the anion-exchange chromatography of organic phosphates, they could not be used since they reacted with the postcolumn molybdenum reagent. In this experiment we therefore aimed to find elution conditions for organic phosphates by using borate buffers which showed no reactivity toward the molybdenum reagent.

First, the separation condition of nucleotides (AMP, ADP and ATP) was examined. By using a 50 mM borate buffer containing 300 mM Na_2SO_4 , the effect of the pH on the retention of the solutes was examined at pH 9–10. Judging from the resolution and shapes of the peaks, pH 9.5 was selected. Next, the concentration of Na_2SO_4 to be added to a 50 mM borate buffer (pH 9.5) was examined over the range 50–300 mM; 120 mM Na_2SO_4 gave the best separation for the nucleotides. Figure 2 shows the HPLC profile of the nucleotides as well as the inorganic phosphate obtained under the optimized condition.

Secondly, the separation condition of the phospho-amino acids (P-Ser, P-Thr and P-Tyr) was examined as in the case of the nucleotides. When 50 mM Na_2SO_4 in 50 mM borate buffer (pH 8.0) was used as the eluent, a satisfactory resolution was obtained (Fig. 3).

Thirdly, the elution condition was optimized for the resolution of sugar phosphates, such as Glu-1-P, Glu-6-

Fig. 2 HPLC profile for nucleotides. Eluent, 120 mM Na_2SO_4 in 50 mM borate buffer (pH 9.5); column temperature, 40°C ; injected amount, 1.0 nmol each.

Fig. 3 HPLC profile for phospho-amino acids. Eluent, 50 mM Na_2SO_4 in 50 mM borate buffer (pH 8.0); column temperature, 40°C; injected amount, 1.0 nmol each.

Fig. 4 HPLC profile for sugar phosphates. Column temperature, 50°C; injected amount, 1.0 nmol each.

P, Fru-6-P and Fru-1,6-PP, which are the main metabolic intermediates in the glycolytic pathway. In this case, all of the isocratic conditions tested gave unsatisfactory results. Therefore, gradient elution was incorporated in the separation of sugar phosphates. The optimized program was isocratic elution with 100 mM NaCl in 100 mM borate buffer (pH 8.5) for 5 min, linear gradient elution to 500 mM NaCl in 100 mM borate buffer (pH 8.5) in 20 min, and then isocratic elution with 500 mM NaCl in 100 mM borate buffer (pH 8.5) for 15 min. Figure 4 shows the HPLC profile of the sugar phosphates obtained under the

Table 1 Analytical results for organic phosphates

Compound	Correlation coefficient ^a	R.S.D. ^b , %
AMP ^c	0.9941	
ADP ^c	0.9976	
ATP ^c	0.9955	
O-Phospho-DL-serine ^d	0.9938	
O-Phospho-L-threonine ^d	0.9940	
O-Phospho-DL-tyrosine ^d	0.9972	
Glucose 1-phosphate ^e	0.9927	2.10
D-Glucose 6-phosphate ^e	0.9953	2.05
D-Fructose 6-phosphate ^e	0.9968	1.97
Fructose 1,6-diphosphate ^e	0.9920	2.56

a. $n=2$. b. $n=8$. c. HPLC condition was the same as in Fig. 2. d. HPLC condition was the same as in Fig. 3. e. HPLC condition was the same as in Fig. 4.

optimized elution condition.

Reproducibility and calibration curve

As shown in Table 1, when 100 μM each of sugar phosphate was repeatedly determined, the relative standard deviation was between 1.97 and 2.56% ($n=8$). The calibration curves for the organic phosphates showed good linearity (correlation coefficient ≥ 0.992). This method permitted the determination of organic phosphates in the 20 – 500 μM range (injection volume, 10 μM).

The organic phosphates analyzer presented here seems to be the first tool which enables a sensitive and selective determination of various types of organic phosphates. The analyzer is expected to be widely applied to various biomedical purposes, including the determination of organic phosphates and assays of phosphatases and kinases.

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