Highly Sensitive Densitometry for Sugarphosphoesters and Nucleotides Using Enzymatic Hydrolysis on a TLC Plate

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Sensitive TLC for the determination of sugarphosphoesters and nucleotides was established. The individual separation of four sugarphosphoesters and three nucleotides on a silica gel plate was carried out in 1-butanol–acetic acid–water (1 : 1 : 1, v/v). Following the development of phosphate esters, alkaline phosphatase solution was sprayed on the plate at 45 °C to liberate phosphate from each phosphate ester. After spraying a mixture of molybdic acid and Malachite Green on the plate, the liberated phosphate appeared as blue-green spots of molybdophosphate–Malachite Green aggregates on a yellow-brown back ground. The absorbance of each spot on the plate was measured at 620 nm with a densitometer to obtain a chromatogram. Fructose 1-phosphate, fructose 1,6-phosphate, glucose 6-phosphate, mannose 6-phosphate, adenosine 5′-triphosphate, adenosine 5′-diphosphate and adenosine 5′-monophosphate could be detected separately at a sensitivity of as little as 0.5 pmol/spot. The values of relative standard deviation for the esters was less than 3.9% ($n=7$) at 20 pmol/spot.

Key words sugarphosphoester; molybdophosphate–Malachite Green aggregate; TLC; alkaline phosphatase; densitometry; nucleotide

Phosphate esters such as sugarphosphoesters and nucleotides are essential to living organisms. Several methods are presently available for determining the phosphate esters in biological substances. Due to their low molar absorptivity, it is difficult to detect phosphate esters directly with high sensitivity. A colorimetric method, in which molybdenum blue is formed, is applied to the determination of these esters through the hydrolysis of nucleotides in sulfuric acid.[1] An enzyme electrode method based on competition with fructose and glucose for hexokinase[2] and ion chromatographic determination[3] are examples of reported methods. However, the sensitive determination of phosphate esters on a biological scale is rarely if at all possible using any of these methods.

In the present study, the authors aimed at establishing a simple and highly sensitive method for determining phosphate esters. The procedure involving the hydrolysis of phosphate esters with an enzymatic reaction and color reaction with molybdophosphate–Malachite Green aggregate formation, followed by densitometry, can be conducted entirely on a silica gel plate for TLC.

Materials and Methods

Chemicals and Reagents Reagents and solvents were of a guaranteed grade. Water was purified by a Milli-Q water system (Millipore, Bedford, MA). The sugarphosphoesters used were fructose 1-phosphate (F1-P), fructose 1,6-phosphate (F1,6-P), glucose 6-phosphate (G6-P), mannose 6-phosphate (M6-P), and the nucleotides; adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Standard solutions of the phosphate esters were prepared by dissolving each individually in an adequate amount in water. Malachite Green oxalate was purchased from Tokyo Chem. Ind. (Tokyo, Japan).

The molybdate–Malachite Green (Mo–MG) reagent was obtained by successively mixing 200 ml 38 mM ammonium molybdate, 100 ml 8.7 M sulfuric acid and 200 ml 3.2 mM Malachite Green, and 500 ml water. The mixture was allowed to stand for 30 min and filtered through a 0.45 μm pore size membrane filter. This solution was stable for use for three months with refrigeration.

Enzyme Alkaline phosphatase (Al-P, from Human Placenta) was purchased from Sigma. An Al-P solution was prepared by being dissolved at 2.0 U/ml in 175 mM diethanolamine–hydrochloride (DEA) buffer (pH 9.6) containing 3.5 mM $MgCl_2$.

Pretreatment of Silica Gel Plate A silica gel plate, 0.25 mm in thickness (Silica gel 60, E. Merck, Darmstadt, Germany), was pretreated by being developed in 1-butanol–acetic acid–water (1 : 1 : 1, v/v) for impurity elimination.

Development of Phosphate Esters and Color Reaction The plate spotted by 1 μl sample solution at 0.5—50 pmol/spot was developed in 1-butanol–acetic acid–water (1 : 1 : 1, v/v). After being dried, the plate was sprayed with Al-P solution to liberate phosphate from the phosphate esters. The volume of enzyme solution was 2 ml per 10^2 cm^2 area of the plate. Enzymatic reaction on the plate was carried out for 20 min at 45 °C. After spraying 2 ml of Mo–MG reagent per 10^2 cm^2 area, the phosphate thus produced reacted with the reagent to form molybdophosphate–Malachite Green (P-Mo–MG) aggregates of blue green spots at 45 °C. After 5 min, the colored spots were scanned at 620 nm with a flying spot scanner CS-9000 densitometer (Shimadzu Co., Kyoto, Japan). The extinction profiles were recorded and amounts of phosphate esters determined based on peak areas in the obtained chromatograms.

Results and Discussion

Separation of Phosphate Esters The solvent system for phosphate ester development was a slight modification of the 1-butanol–acetic acid–water (1 : 0.3 : 0.3, v/v) system originally proposed by Okumura[4] for a similar purpose. To determine the optimal conditions for phosphate ester separation, the effects of varying the ratio of acetic acid and water concentrations on *Rf* were examined using G6-P, F1-P, F1,6-P and M1-P. An acetic acid to 1-butanol ratio in the range 0.16—1.6 was found best for this separation. The proportion of acetic acid to 1-butanol was thus adjusted to 1. Although the distribution of *Rf* increased in the proportion with water to 1-butanol to more than 1.3, a phase separation of the developing solvent occurred. The proportion of water was thus adjusted to 1. The *Rf* obtained by the solvent system ranged from 0.30—0.73. The solvent system for phosphate ester separation was thus established as 1-butanol–acetic acid–water (1 : 1 : 1, v/v) based on the above. For F1,6-P

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and ADP, the range of *Rf* was essentially the same as that of this solvent system, thus making clear separation difficult. *Rf*s of other phosphoester separations were found to be satisfactory.

Enzymatic Reaction Sugarphosphoesters and nucleotides liberate constituent phosphate through hydrolysis with Al-P. For instance, the hydrolysis of G6-P occurs as follows:

$$\text{glucose 6-phosphate} \xrightarrow[Mg^{2+}]{\text{Al-P}} \text{glucose} + PO_4^{3-}$$

To determine the optimal conditions for enzymatic reaction on the plate, the effects of Al-P and Mg^{2+} ion concentrations and hydrolysis time on the peak area of each chromatogram were examined using G6-P (25 pmol/spot) as the substrate.

Figure 1 shows the effect of Al-P concentration on the peak area. This area was constant and maximal at 2.0—4.0 U/ml Al-P. Al-P was thus adjusted to 2.5 U/ml. The activity of Al-P is enhanced in the presence of Mg^{2+} ions. The concentration of the latter was thus adjusted to 3.5 mM, with a consequent maximal peak area (Fig. 2). As shown in Fig. 3, a constant and maximal peak area was attained by heating at 45 °C for 15—30 min. An Al-P reaction was thus carried out at 45 °C for 20 min. It is evident from the present results that the optimal conditions for the liberation of phosphate from sugarphosphoesters and nucleotides through enzymatic reaction on the plate are those indicated in "Materials and Methods."

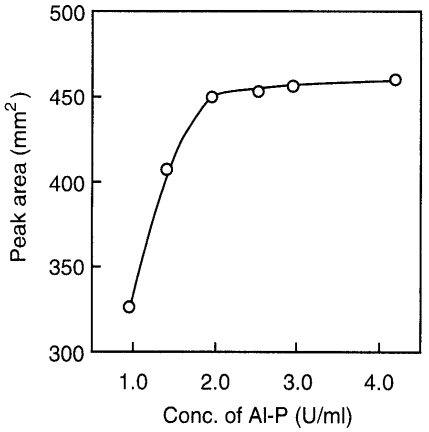

Fig. 1. Effect of Al-P Concentration on Peak Area

G6-P (25 pmol/spot) was used as the substrate. Al-P was dissolved in 175 mM DEA–HCl buffer (pH 9.6) containing 3.5 mM $MgCl_2$. The reaction was carried out at 45 °C for 20 min.

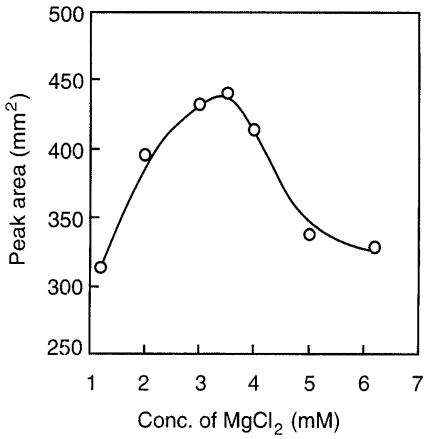

Fig. 2. Effect of $MgCl_2$ Concentration in Al-P Solution on Peak Area

G6-P (25 pmol/spot) was used as the substrate. The Al-P solution used contained 2.5 U/ml Al-P in 175 mM DEA–HCl buffer (pH 9.6). The reaction was carried out at 45 °C for 20 min.

The hydrolysis of phosphate esters by the present method using an enzyme required heating at 45 °C. For hydrolysis using sulfuric acid, a temperature of 140 °C was required.[5]

The results of the enzymatic reaction by Al-P indicated F1-P, F1,6-P, G6-P, M6-P, ATP, ADP and AMP to be hydrolyzed to liberate phosphate in yields sufficient to make possible the accurate quantification of phosphate esters. Yields are shown in Table 1.

Color Reaction By spraying Mo–MG reagent on the plate, liberated phosphates appeared as blue-green spots of P-Mo–MG aggregates on a yellow-brown back ground. The absorption spectrum of each spot was essentially the same as that of P-Mo–MG in methyl cellosolve solution. A sharp absorption peak with a maximum absorbance at 620 nm indicated $(MG)_3(PMo_{12}O_{40})$ to possibly be present as an ion association complex. An increace in sensitivity has been shown on using the Mo–MG reagent instead of ammonium molybdate reagent to stain the phosphate; this reagent reacts with phosphate to form P-Mo–MG aggregates whose molar absorptivity in methyl cellosolve is $2.7 \times 10^5\ M^{-1}\,cm^{-1}$ at λ_{max} 627 nm.[6] The optimal conditions for using the Mo–MG reagent for staining phosphate on the plate are indicated in our previous paper.[7]

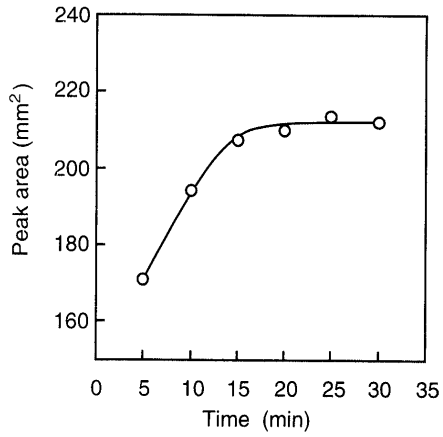

Fig. 3. Effect of Reaction Time of Al-P on Peak Area

G6-P (25 pmol/spot) was used as the substrate. The Al-P solution used contained 2.5 U/ml Al-P and 3.5 mM $MgCl_2$ in 175 mM DEA–HCl buffer (pH 9.6). The reaction was carried out at 45 °C.

Table 1. Yields of Hydrolyzed Phosphate Esters

Phosphate ester	Yield (%)[a]
G6-P	73
F1,6-P	42
F1-P	38
M1-P	87
ATP	21
ADP	47
AMP	90

a) Peak area for orthophosphate at same content was set at 100%.

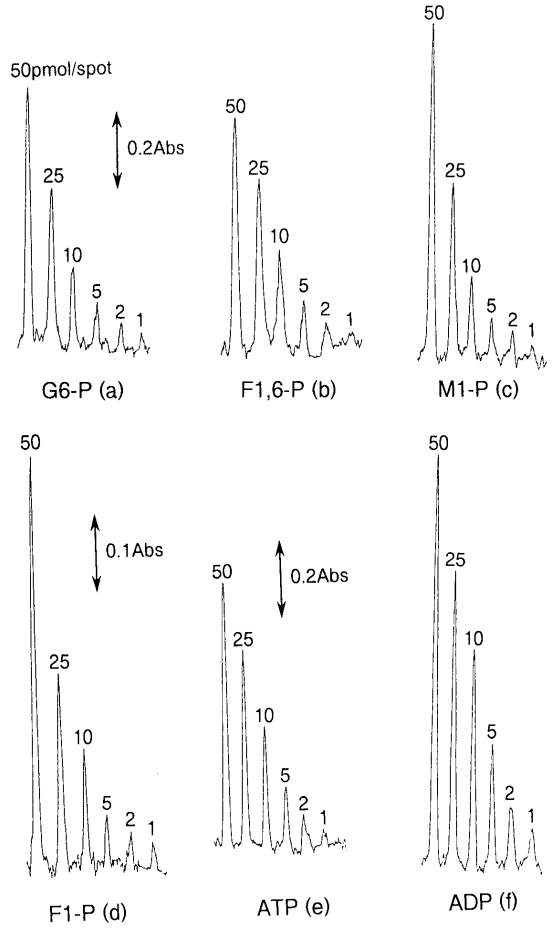

Fig. 4. Typical Chromatograms of Sugarphosphoesters (a, b, c and d) and Nucleotides (e and f)

Sensitivity scales of absorbance for all compounds were the same, except for (d).

Calibration Curve Figure 4 shows chromatograms obtained using standard solutions of sugarphosphoesters and nucleotides. The seven phosphate esters each displayed a calibration curve, since the yields of phosphate by Al-P hydrolysis were due to differences in the substrate (Fig. 5). F1-P and F1,6-P, whose sugars are identical, showed essentially the same hydrolysis rate of about 40%. The slopes of their calibration curves were proportional to the number of phosphates comprising them. Though their nucleosides are identical, the slopes of the calibration curves of ATP, ADP and AMP did not depend on the number of phosphates, this possibly being due to

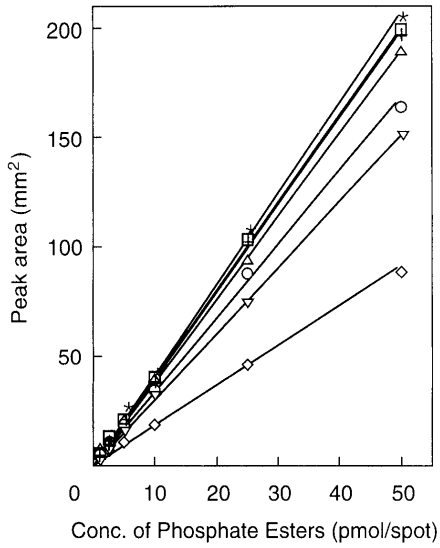

Fig. 5. Calibration Curves of Sugarphosphoesters and Nucleotides
○, G6-P; △, F1, 6-P; ◇, F1-P; □, M1-P; ▽, AMP; *, ADP; +, ATP.

differences in the reactivity of Al-P to each nucleotide. The seven esters had essentially the same correlation coefficients ($r=0.999$, at 1—50 pmol/spot), and relative standard deviation was less than 3.9% ($n=7$, at 20 pmol/spot). The detection limit of phosphate esters by the present method were 0.5 pmol/spot on the silica plate, which is one-hundredth that of flow injection analysis using the molybdenum blue method.[1] In the present study, four sugarphosphoesters and three nucleotides, which play important roles in metabolism and biosynthesis related to phosphates, were determined to be a model for phosphate esters. The method is characterized by high sensitivity and simplicity.

References

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