Enzymatic Assay of Inorganic Phosphate with Use of Sucrose Phosphorylase and Phosphoglucomutase

Minoru Tedokon, Kenji Suzuki, Yuzo Kayamori, Seichi Fujita, and Yoshiaki Katayama

We developed a new enzymatic method for the assay of inorganic phosphate (Pi) by using sucrose phosphorylase (SP; EC 2.4.1.7) and phosphoglucomutase (PGM; EC 5.4.2.2). Pi is transferred to sucrose by SP, producing α-D-glucose 1-phosphate (G1P) and α-D-fructose. G1P is transphosphorylated by PGM in the presence of α-D-glucose 1,6-bisphosphate to form α-D-glucose 6-phosphate, which is oxidized by NAD⁺ and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) to form 6-phosphogluconolactone (6PG) and NADH. Finally, the oxidation of 6PG by NAD⁺, catalyzed by 6-phosphogluconic dehydrogenase (EC 1.1.1.44), yields D-ribulose 5-phosphate and NADH. Thus two molecules of NADH are formed for each molecule of Pi, and the reaction is monitored at 340 nm. The Kᵐ values for SP and sucrose were 4.44 and 5.31 mmol/L, respectively. The best buffer was 1.4-piperazinediethanesulfonic acid (Pipes) at 50 mmol/L and pH 6–7. Implementing this method with a Cobas-Bio centrifugal analyzer allowed us to measure Pi accurately and precisely.

Additional Keyphrase: centrifugal analyzer

The molybdenum blue method of Fiske and Subbarow (1) and its modifications are widely used for assay of inorganic phosphate (Pi). Some molybdenum blue methods require deproteinization; those without a deproteinization step are affected by the protein concentration in the sample. Among the enzymatic methods described for Pi, Guynn et al. (2) developed a spectrometric determination with glyceraldehyde-3-phosphate dehydrogenase; Schulz et al. (3) used phosphorylase, phosphoglucomutase, and glycogen; and Pierre et al. (4) used maltose phosphorylase and β-phosphoglucomutase. All of these methods, however, have low sensitivity, and the Pi present as a contaminant in the reagents precludes their routine use. In one enzymatic method (5), purine-nucleoside phosphorylase (PNP; EC 2.4.2.1), xanthine oxidase (XOD; EC 1.1.3.22), and peroxidase (POD; EC 1.11.1.7) are used in a colorimetric assay. A kit based on this PNP–XOD–POD scheme is commercially available from Kyowa Medex Co., Ltd. (Tokyo, Japan), but is susceptible to interference from reducing substances such as bilirubin, uric acid, and ascorbic acid.

Our goal was to develop a new enzymatic assay of Pi that would be specific, rapid, easy to use, and applicable to an automated analyzer such as a Cobas-Bio analyzer. We wanted to assess the Kᵐ values of sucrose phosphorylase (SP; EC 2.4.1.7) for Pi and sucrose and to find an appropriate buffer and determine its optimum concentration and pH. Furthermore, we wanted to assess the method's linearity, accuracy, and imprecision; the effect of potential interferences; and the agreement of our results with those from other widely used methods.

Materials and Methods

Materials

Reagents. SP (from Leuconostoc mesenteroides; sucrose:orthophosphate α-D-glucosyltransferase; EC 2.4.1.7) was purchased from Sigma Chemical Co. (St. Louis, MO 63178); α-D-glucose 1,6-bisphosphate (G1,6P), phosphoglucomutase (PGM, from rabbit muscle; α-D-glucose 1,6-phosphomutase; EC 5.4.2.2), and glucose-6-phosphate dehydrogenase (G6PDH, from L. mesenteroides; EC 1.1.1.49) were obtained from Boehringer Mannheim Yamanouchi K. K. (Tokyo, Japan). NAD⁺, 6-phosphogluconic dehydrogenase (6PGDH, from L. mesenteroides; 6-phospho-D-gluconate:NAD⁺ 2-oxidoreductase (decarboxylating); EC 1.1.1.44), and bovine serum albumin (BSA) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). KH₂PO₄, sucrose, bilirubin, and MgCl₂·6H₂O were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1,4-Piperazinediethanesulfonic acid (Pipes) was purchased from Dojindo Labs. (Kumamoto, Japan). G6PDH and 6PGDH were used after dialysis against distilled water to remove any contaminating Pi. The complete reagent mixture is shown in Table 1.

<table>
<thead>
<tr>
<th>Pi stock standard solution</th>
<th>Dissolve 4.39 g of dried KH₂PO₄ in 1 L of distilled water to make a 32.3 mmol/L (1000 mg/L) Pi stock standard solution.</th>
</tr>
</thead>
</table>

Apparatus. We used an automated spectrophotometer (Model UV-240; Shimazu Co., Kyoto, Japan) and a Cobas-Bio analyzer (Roche Analytical Instruments Inc., Nutley, NJ 07110) for the assays.

Specimens. Pi in 126 serum samples was assayed in our center as part of a routine screen of inpatients and outpatients.

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Table 1. Composition of the Enzyme Reagent

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>Sucrose, mmol/L</td>
</tr>
<tr>
<td>0.01</td>
<td>α-D-Glucose 1,6-bisphosphate, mmol/L</td>
</tr>
<tr>
<td>3</td>
<td>NAD⁺, mmol/L</td>
</tr>
<tr>
<td>400</td>
<td>Sucrose phosphorylase, U/L</td>
</tr>
<tr>
<td>2000</td>
<td>Phosphoglucomutase, U/L</td>
</tr>
<tr>
<td>5000</td>
<td>Glucose-6-phosphate dehydrogenase, U/L*</td>
</tr>
<tr>
<td>200</td>
<td>6-Phosphogluconic dehydrogenase, U/L*</td>
</tr>
<tr>
<td>0.84</td>
<td>Bovine serum albumin, g/L</td>
</tr>
<tr>
<td>3</td>
<td>MgCl₂·6H₂O, mmol/L</td>
</tr>
<tr>
<td>50</td>
<td>PIPES buffer (pH 7.0), mmol/L</td>
</tr>
</tbody>
</table>

* Dialyzed before use to remove endogenous Pi.

Procedures

**Principle of the method.** The reaction sequence is as follows:

\[ \text{Sucrose} + \text{Pi} \xrightarrow{\text{SP}} \alpha\text{-D-glucose 1-phosphate} + \text{D-fructose} \]

\[ \alpha\text{-D-Glucose 1-phosphate} \xrightarrow{\text{PGM}} \alpha\text{-D-glucose 6-phosphate} \]

\[ \alpha\text{-D-Glucose 6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} 6\text{-phospho-D-gluconate} + \text{NADH} \]

\[ 6\text{-Phospho-D-gluconate} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} \text{D-ribulose 5-phosphate} + \text{NADH} \]

Pi is transferred to sucrose by the action of SP; the subsequent reaction in the presence of PGM forms G6P. Maximum activity of PGM is obtained only in the presence of G1,6P. The rate of NADH generation is proportional to the serum Pi concentration, two molecules of NADH being generated for each molecule of Pi. The reaction is monitored by the change in absorbance at 340 nm. All experiments were performed at 37°C.

**Kₘ values of SP for Pi and sucrose.** Kₘ values were estimated from Lineweaver–Burk plots. We kept the concentration of SP at 500 U/L and varied the concentrations of Pi or sucrose as shown in Figure 1.

**Selection of buffer.** We prepared the reagent mixture (Table 1) in three different buffers at 50 mmol/L and pH 7.0: triethanolamine (TEA), PIPES, or Tris·HCl. We added 15 µL of 4.64 mmol/L Pi standard or distilled water zero blank to 1 mL of the reagent mixture and then performed the assay. The change in absorbance at 340 nm was measured for 300 s, at intervals of 30 s.

**Optimum SP concentration.** We varied the SP concentrations from 200 to 1200 U/L in 200 U/L steps and followed the reactions with time. A 3.23 mmol/L Pi standard was used as the specimen.

**Linearity.** We prepared working Pi standards of 0, 2.84, 5.68, 8.52, 11.36, and 14.20 mmol/L by diluting the stock standard; we assayed the standards by adding 5 µL of standard to 370 µL of the reagent mixture.

**Recovery studies.** We added portions of the stock Pi standard to pooled sera and performed the assay without delay.

**Imprecision.** We estimated within-run imprecision from 10 replicate assays of three concentrations of Pi (0.41, 1.03, and 1.96 mmol/L) in pooled sera; we estimated day-to-day imprecision by assaying these sera several times over 10 days.

**Examination of potential interferents.** We added solutions of glucose at 100 g/L, ascorbic acid at 2 g/L, bilirubin at 2 g/L, hemoglobin (from lysed erythrocytes) at 60 g/L, or 10% Intralipid (KabiVitrum AB, Stockholm, Sweden) to two pools of sera with endogenous Pi of 0.84 and 1.68 mmol/L to give solutions of 0–10 g/L glucose, 0–200 mg/L ascorbic acid, 0–200 mg/L bilirubin, 0–6 g/L hemoglobin, or 0–5 g/L Intralipid. We dissolved bilirubin in a small volume of 1 mol/L NaOH, diluted this solution with distilled water, and adjusted the pH to 7.0.

**Comparison of methods.** We analyzed serum samples from patients for Pi by three different assay methods with the analyzer: the method described here, the PNP–XOD–POD method (5), and a molybdenum blue method (6).

**Results**

Kₘ values. Kₘ values of SP for Pi and sucrose estimated by Lineweaver–Burk plots were 4.44 and 5.31...
mmol/L, respectively (Figure 1).

**Appropriate buffer and its optimum conditions.** We observed the changes in absorbance with time by using a Pi standard reagent mixture in a distilled water blank in the enzyme reagent mixture (Table 1) dissolved in TEA, PIPES, or Tris·HCl buffer. The reaction proceeded in TEA and PIPES but was inhibited by Tris·HCl. The blank with the PIPES buffer was the lowest; therefore, we selected PIPES buffer. Studies of the optimum PIPES concentration and pH were carried out by varying the buffer concentrations from 25 to 125 mmol/L in 25 mmol/L steps and the pH from 5.5 to 8.0 in 0.5 pH unit steps. The fastest reaction rate was found with the PIPES buffer at 25–50 mmol/L and a pH of 6.0–7.0. We chose 50 mmol/L PIPES at pH 7.0 as the optimum condition.

**Optimum SP concentration.** The reaction time course observed with several concentrations of SP are shown in Figure 2. As concentrations of SP were increased, the reaction was completed in less time; however, a decrease in absorbance was observed after the peak absorbance with the highest concentration (1200 U/L) of SP. The latter effect is caused by contaminating enzymes that oxidize NADH. With lower concentrations of SP, the effect of the contaminants was less, but then >20 min was required for the reaction to be completed. With 400 U/L SP, the analytical sensitivity of our Pi assay is 2.2 mA per 3 min (determined between 60 and 240 s) for a Pi concentration of 0.032 mmol/L, which was satisfactory. We therefore adopted a fixed-time assay with the SP concentration at 400 U/L.

**Reaction time course with the analyzer.** The reaction curves for various aqueous Pi solutions are shown in Figure 3. Because the reaction rate was linear between 90 and 300 s after the reaction began, the change in absorbance between these times was chosen as the assay condition. The assay conditions for the analyzer are shown in Table 2.

**Linearity studies.** We found that our method gives a linear response with concentrations of serum Pi from 0 to 9.6 mmol/L.

**Recovery studies.** The analytical recoveries ranged from 98.3% to 101.2% (mean 100.0%; n = 6) in the experiments where aqueous Pi solutions (0.81 and 1.62 mmol/L) were added to three different serum pools with endogenous Pi concentrations of 0.32, 0.89, and 1.74 mmol/L.

**Imprecision.** Within-run and day-to-day imprecision (CVs) were 1.55–3.17% and 2.00–4.10%, respectively. The method has acceptable precision.

**Interference studies.** No interference by glucose, ascor-
bic acid, bilirubin, or Intralipid was observed. The increase of 0.03 mol/L Pi per 1 g/L hemoglobin was attributed to Pi from the erythrocytes and could be caused by hydrolysis of the phosphoesters.

Method-comparison data. Data for correlations between this method (γ) and the PNP-XOD-POD method (α) or the molybdenum blue method (α′) are as follows: y = 0.965x + 0.002 mmol/L (n = 126, r = 0.997, S_\text{y|x} = 0.029 mmol/L) and y = 1.016x′ - 0.096 mmol/L (n = 76, r = 0.986, S_\text{y|x′} = 0.061 mmol/L).

Discussion

The assay of Pi with our method is based on the sequence of coupled reactions and provides both sensitivity and specificity. The SP-catalyzed reaction is the rate-determining step, and the rate of this reaction depends directly on Pi concentration.

The concentration of sucrose in the reagent is 200 mmol/L, which is 38-fold the K_m value. The final Pi concentration in the reaction mixture is <12.5 μmol/L, which is much smaller than the K_m value. The sample was diluted ~80-fold in the reaction mixture.

From the Michaelis–Menten theory, enzymatic reactions obey pseudo-first-order kinetics if [S] ≪ [K_m]; therefore, the kinetic determination of Pi is possible by our method. Usually, kinetic assays have a weak point in that the sensitivity is inferior to that of end-point assays. This is not true for our method; two molecules of NADH are formed for each molecule of Pi between 90 and 300 s after the reaction begins. With our method we found a linear response to Pi concentrations as great as 9.2 mmol/L and excellent recovery and precision.

Others (7) reported that glucose is a strong competitive inhibitor of the SP-catalyzed reaction. We avoided this effect by increasing the concentration of sucrose, and no inhibition was observed for as much as 10 g of glucose per liter in the specimen. The commercially available kits based on the PNP-XOD-POD method are subject to bilirubin interferences (8), something we did not find with our method.

Good correlation data between our method and both the PNP-XOD-POD and molybdenum blue methods were obtained. Our method produced less scatter of the data than did the PNP-XOD-POD procedure.

References