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Assay of Purine Nucleoside Phosphorylase in Erythrocytes by Flow-Injection Analysis with Fluorescence Detection

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A sensitive assay for purine nucleoside phosphorylase (PNP) in human erythrocytes is described. Erythrocyte lysate is incubated with the substrate inosine in the presence of phosphate for the enzyme reaction, and the resulting mixture is deproteinized with perchloric acid and neutralized. The supernatant containing the reaction product, hypoxanthine, is applied to a flow-injection system in which immobilized enzyme columns of xanthine oxidase, urate oxidase and horseradish peroxidase are connected in series in that order in the flow line. Hydrogen peroxide formed in the enzymatic conversion of hypoxanthine is measured fluorimetrically by reaction with 3-(*p*-hydroxyphenyl)propionic acid in the system. The lower limit of determination (signal-to-noise ratio = 5) for hypoxanthine is 0.3 pmol in a 20-μl injection volume, which corresponds to a PNP activity of 0.117 μmol/min/ml erythrocytes. This method permits the assay of PNP in only 10 nl of human erythrocytes in 50 μl of erythrocyte lysate.

Keywords——purine nucleoside phosphorylase activity; flow-injection analysis; hypoxanthine determination; immobilized enzyme; xanthine oxidase; urate oxidase; horseradish peroxidase; fluorescence detection; hydrogen peroxide; 3-(*p*-hydroxyphenyl)propionic acid

Purine nucleoside phosphorylase (PNP; EC 2.4.2.1) catalyzes the conversion of purine nucleosides such as inosine, guanosine and their 2′-deoxyribonucleosides to purine bases and ribose 1-phosphate or 2-deoxyribose 1-phosphate. Deficiency of PNP is closely associated with immunodeficiency characterized by severely defective T-cell function with normal B-cell immunity.[1,2] Therefore, the assay of this enzyme activity in erythrocytes[3-7] or lymphocytes[8] is useful in the diagnosis of this disease.

PNP in erythrocytes and lymphocytes has been assayed by spectrophotometric[5-11] and radiochemical[3,4,12,13] methods. We have reported a sensitive fluorimetric flow-injection analysis (FIA) of hydrogen peroxide using an immobilized horseradish peroxidase (HRP) column,[14] and it was successfully applied to the flow-injection determination of adenosine and inosine in human plasma[15] and serum guanase activity using immobilized enzyme

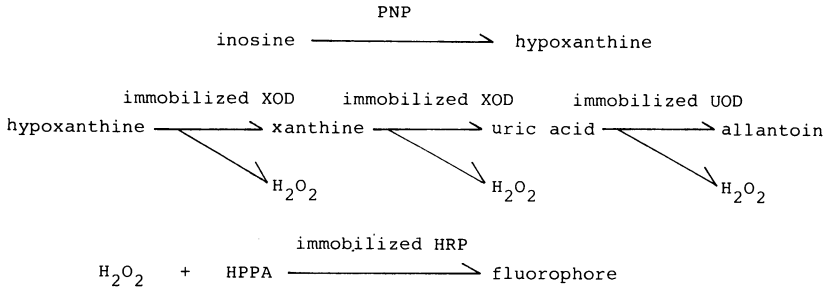

Chart 1

columns connected in series.[16] Based on the enzyme column technique, a highly sensitive FIA method for the assay of PNP in human erythrocytes has been developed. Hypoxanthine formed enzymatically from the substrate inosine is degraded to hydrogen peroxide by using immobilized xanthine oxidase (XOD) and urate oxidase (UOD, uricase) columns (Chart 1). The hydrogen peroxide is quantified by an immobilized HRP-mediated reaction using 3-(*p*-hydroxyphenyl)propionic acid (HPPA), an efficient fluorogenic substrate for HRP[17] (Chart 1).

Experimental

Chemicals and Reagents——Double-deionized water was filtered through a Milli-QII system (Japan Millipore Ltd.; Tokyo, Japan) just before use. HPPA was obtained from Dojindo Laboratories (Kumamoto, Japan) and Otsuka Chemical Co. (Osaka, Japan). HRP (285 purpurogallin unit/mg), UOD (grade II, 4.0 U/mg, from *Candida* sp.) and XOD (0.4 U/mg, from cow's milk, in 3.2 M $(NH_4)_2SO_4$ suspension, 10 mM in ethylenediaminetetra acetic acid disodium (EDTA·2Na)) were from Sigma (St. Louis, U.S.A.), Toyobo Biochemicals (Osaka, Japan) and Boehringer Mannheim Yamanouchi (Tokyo, Japan), respectively. Inosine and hypoxanthine were purchased from Nakarai Chemicals (Kyoto, Japan). Unless otherwise noted, all other chemicals were of reagent grade.

Preparation of Erythrocyte Lysate——Venous blood samples (1 ml) obtained from healthy volunteers were collected in glass tubes containing 10 μl of 200 mM EDTA·2Na. Each blood sample was centrifuged for 10 min at 1000 *g*. Then, the plasma and the buffy coat were removed by centrifugation and the remaining erythrocytes (packed cells) were washed twice with 5 ml of ice-cold 150 mM NaCl. The erythrocytes were lysed with cold water to obtain a 5000-fold dilution.

Enzyme Reaction and Sample Solutions for FIA——The substrate solution was 1.0 mM inosine in 50 mM phosphate buffer (pH 6.0). The solution (450 μl) was preincubated at 37 °C for *ca.* 5 min and again incubated for exactly 10 min after adding 50 μl of the erythrocyte lysate. The reaction was stopped by addition of 100 μl of 4.0 M $HClO_4$. The resulting solution was mixed with 180 μl of 2 M K_2CO_3 to remove $KClO_4$ and centrifuged for 10 min at 1000 *g*. An aliquot (20 μl) of the supernatant (sample solution for testing) was applied to the flow-injection system. For the blank (reflecting endogenous uric acid, xanthine and hypoxanthine), the same procedure was carried out except that the erythrocyte lysate and the substrate solution were added to the $HClO_4$ solution, incubation being omitted.

Flow-Injection System and Assay Procedure for Hypoxanthine——A schematic flow diagram of the flow-injection system is shown in Fig. 1. The immobilized XOD, UOD and HRP columns were prepared as previously described[15] and connected in series in that order in the flow line using Teflon tubing (0.25 mm i.d.; Gasukuro Kogyo, Tokyo, Japan). The reagent solution, which was 5 mM HPPA in 0.1 M Tris–HCl buffer (pH 8.0), 0.15 M in NaCl, and 10 mM in EDTA·2Na and the carrier solution, which was the same as the reagent solution but without HPPA, were pumped separately with a Sanuki DM2M-1026 pump; the flow rates were both 0.25 ml/min. The sample solution for the test or blank was injected through a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) into the carrier stream. A mixing coil (1 m) and the three enzyme columns were immersed in a water bath at 37 °C. The fluorescence intensity was measured at an excitation wavelength of 305 nm and an emission wavelength of 405 nm with a Shimadzu RF 530 fluorescence spectromonitor equipped with a 12-μl flow cell and a Hitachi 056 chart recorder.

To prepare a calibration curve, hypoxanthine standard solutions (0.25—25 μM) were taken through the procedure. The concentration of hypoxanthine formed enzymatically was read on the calibration curve in terms of net peak height. PNP activity was expressed as micromoles of hypoxanthine formed per minute per milliliter of erythrocytes at 37 °C.

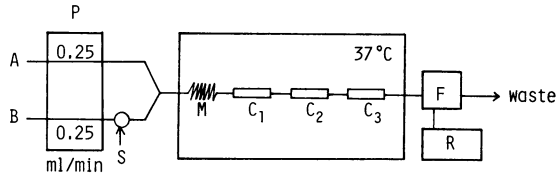

Fig. 1. Schematic Diagram of the Flow-Injection System

A, reagent solution; B, carrier solution; C, immobilized enzyme columns (1, XOD; 2, UOD; 3, HRP); F, fluorescence detector; P, pump; S, sample injector; M, mixing coil; R, recorder.

Results and Discussion

The conditions of the FIA were optimum and essentially the same as described previously.[16] The PNP-catalyzed reaction requires inorganic phosphate, and thus phosphate buffer was used for the enzyme reaction. Although the relationship between pH and PNP activity was biphasic for an unknown reason, the pH at which the activity was highest was 6.0 (Fig. 2). Phosphate buffer gave a maximum and constant activity at concentrations of 25—75 mM. Therefore, 50 mM phosphate buffer of pH 6.0 was selected for the enzyme reaction. A maximum and constant activity was obtained with 0.75—2.0 mM inosine in the substrate solution, with an observed K_m value of 95 μM; 1 mM in the substrate solution was employed as a saturating concentration for the enzyme reaction.

The amount of hypoxanthine formed enzymatically was strongly influenced by the incubation temperature in the reaction and higher temperatures resulted in faster formation of hypoxanthine up to at least 50 °C for the incubation time of 10 min (Fig. 3), though deactivation of the enzyme occurred on prolonged incubation; 37 °C was chosen for convenience. The enzyme reaction rate was linear with time up to at least 60 min at 37 °C. The amount of hypoxanthine formed during the incubation time of 10 min was proportional to the amount of erythrocytes up to at least 100 nl; 10 nl of erythrocytes (in 50 μl of erythrocyte lysate) was chosen for the standard procedure.

Components of the erythrocyte lysate had no effect on the determination of hypoxanthine; the recoveries of hypoxanthine added to the erythrocyte lysate in the concentration range of 1—400 μM were approximately 100%. Thus, a calibration curve was prepared by subjecting hypoxanthine standard solutions directly to the FIA. The calibration curve was linear up to 500 pmol per 20-μl injection volume and passed through the origin. The lower limit of determination (signal-to-noise ratio = 5) for hypoxanthine was 0.3 pmol in a 20-μl injection volume, which corresponds to a PNP activity of 0.117 unit.

Typical flow-injection peaks obtained with 10—80 pmol of hypoxanthine in a 20-μl injection volume and with the sample solutions for the test and blank are shown in Fig. 4. A rapid sampling rate (40 injections/h) could be attained.

Parallel tests with a spectrophotometric method[18] (enzyme reaction temperature 25 °C and pH 7.4) and the present method were performed on 15 different erythrocyte samples from

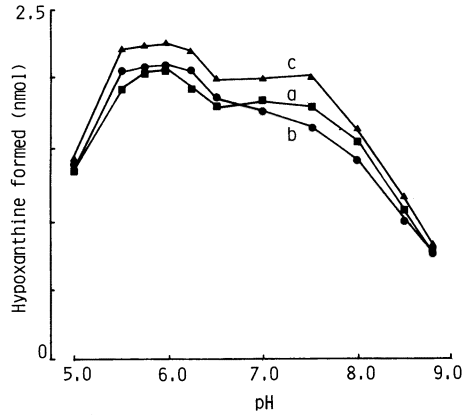

Fig. 2. Effect of pH of the Phosphate Buffer in the Enzyme Reaction on the Amount of Hypoxanthine Formed

PNP activities (μmol/min/ml): a, 20.9; b, 21.1; c, 22.6.

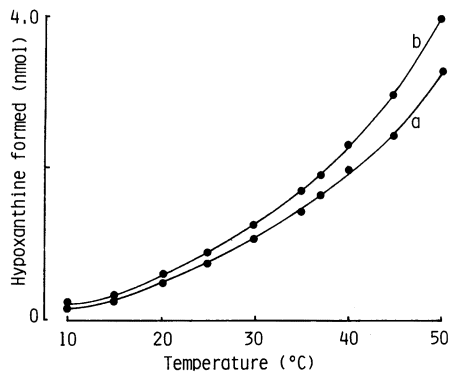

Fig. 3. Effect of Temperature of the Enzyme Reaction on the Amount of Hypoxanthine Formed

PNP activities (μmol/min/ml): a, 17.5; b, 20.9.

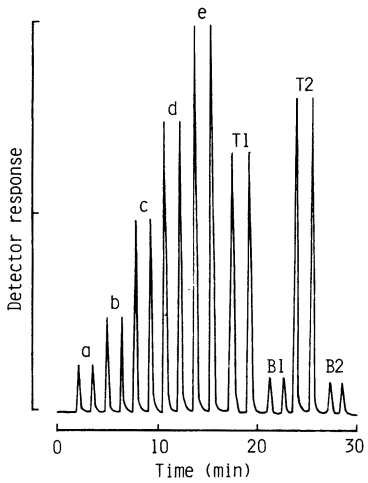

Fig. 4. Flow-Injection Peaks for Standard and Erythrocyte Samples

Amounts of hypoxanthine (pmol/20 μl): a, 10; b, 20; c, 40; d, 60; e, 80. T and B = test and blank, respectively. PNP activities (μmol/min/ml): 17.5 (obtained from T1 and B1), 22.6 (obtained from T2 and B2).

normal subjects (age, 21—56; male, 8; female 7). The linear regression equation and correlation coefficient for the two methods were y (spectrophotometric method) $= 0.34\ x$ (present method) $- 0.7$ and 0.856, respectively. Mean PNP activities obtained by the spectrophotometric method and the present method were 6.29 ± 0.87 and 20.6 ± 2.2 (mean $\pm$ S.D.), respectively. The value obtained by the present method was about three times higher than that obtained by the spectrophotometric method. The discrepancy was considered to be due to the difference in the enzyme reaction temperatures and pHs (Figs. 2 and 3). The within-day precision of the present method was examined using erythrocytes with mean PNP activities of 17.5, 21.1 and 24.6 μmol/min/ml. The relative standard deviations were 0.85, 0.75 and 0.93% ($n = 10$ each), respectively.

Uric acid (10 μM), ascorbic acid (100 μM) and glucose (10 mM) added to erythrocyte lysate at the abnormally high concentrations shown in parentheses had no effect on the hypoxanthine recovery.

To check the stability of the immobilized enzyme columns, hypoxanthine solutions (2.5 and 5 μM) were applied 50 times a week to the flow system. When not required for use, the columns were stored at 4°C after being filled with the following solutions: 3.2 M ammonium sulfate (pH 8.0) 10 mM in disodium EDTA for XOD; 50 (v/v)% glycerol (pH 10.2) 50 mM in glycine and 130 mM in sodium carbonate for UOD; and 0.1 M Tris–hydrochloric acid buffer (pH 8.5) 0.15 M in sodium chloride, 1 (w/v)% in bovine serum albumin and 25 mM in sodium merthiolate for HRP. The immobilized UOD and HRP columns were stable for at least one year; the XOD column was stable for 3 months, but a 10% loss of activity was detected after 4 months.

This study provides the first fluorimetric method for the assay of PNP. The method is highly sensitive and precise, and should be applicable to diagnosis and to biological investigations of PNP deficiency.

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