CHEMISTRY LETTERS, pp. 1433-1436, 1983.

MEASUREMENT OF ENZYMATIC ACTIVITY OF INORGANIC PYROPHOSPHATASE FOR PYROPHOSPHATE BY FLOW INJECTION ANALYSIS

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A flow injection system was designed for the spectrophotometric measurement of enzymatic hydrolysis of pyrophosphate by inorganic pyrophosphatase (EC 3.6.1.1). Rapid hydrolysis with a very short half-life (2 min) could be easily monitored by the selective detection of orthophosphate using a Mo(VI) reagent.

Inorganic pyrophosphatase (EC 3.6.1.1, P_2ase) is an enzyme that catalyzes the hydrolytic conversion of inorganic pyrophosphate (diphosphate, P_2) to orthophosphate.

$$P_2 \xrightarrow{P_2\text{ase}} 2\ P_1 \qquad (1)$$

Extensive investigations [1-6] by biochemists have been reported on the catalytic properties of P_2ase for P_2, because the enzyme P_2ase is known to be widely distributed in nature and may play an important role in cell metabolism.

Our major concerns are the catalytic activity of P_2ase for phosphorus compounds in environmental water and the utility of P_2ase in inorganic phosphorus chemistry. One of important tasks in these fields is the development of automated techniques to be used for the rapid measurement of enzymatic activities. This paper describes an analytical method based on flow injection analysis (FIA) [7] by which the rate of enzymatic hydrolysis of P_2 with a half-life less than a few minutes can be easily measured.

The FIA manifold in Fig. 1 was designed so that the enzymatic reaction in

Eq. 1 could be stopped instantaneously and the product P_1 could be detected selectively in the presence of the substrate P_2. Each flow rate of water and Mo(VI) reagent (0.03 M Mo(VI) + 0.3 M H_2SO_4 + 0.01 mM P_1; 1 M = 1 mol dm^{-3}) was adjusted to 1.0 ml/min by using a reciprocating pump (Kyowa KHU-W-52 or Jasco RP-4). A sample solution (S) was injected into the water stream via a loop-valve injector (Seishin VMU-6, 100 µl loop). Only P_1 reacted with the Mo(VI) reagent in the reaction coil (RC, 0.5 mm ID x 5 m PTFE, 30 °C) to form molybdophosphoric acid. The absorbance of this yellow complex was monitored with a flow-through cell attached to a spectrophotometer (D, Hitachi 200-10 or Jasco UVIDEC-320).[8] A back-pressure coil (BC, 0.25 mm ID x 2 m) was located at the exit of the cell. The addition of P_1 to the Mo(VI) reagent is recommended to stabilize the base-line level if molybdophosphoric acid tends to be adsorbed on the flow system.

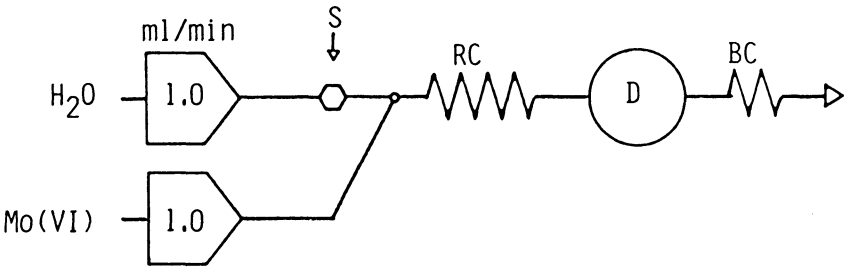

Fig. 1. FIA manifold for the selective determination of P_1.

Standard samples of P_1 (KH_2PO_4), P_2 ($Na_4P_2O_7 \cdot 10H_2O$), and P_3 (triphosphate, $Na_5P_3O_{10}$) were injected into the manifold (Fig. 1) at 1 min intervals. The residence time in the reaction coil was about 40 s. Calibration profiles are shown in Fig. 2. Orthophosphate could be determined reproducibly with a relative standard deviation of less than 1% in the dynamic range of 0.01 mM - 1 mM P_1. This working range was satisfactory for the activity measurement of P_2ase in most cases. On the other hand polymeric P_2 and P_3 did not show positive signals. The negative signals observed at 10 mM may be ascribed to a matrix effect.

P_2ase was allowed to react with P_2 at 30 °C in a buffered medium (pH 7.2, 5 mM Tris-HCl buffer) containing 1 mM $MgCl_2$ as an activator. Aliquots of the reaction mixture (each 100 µl) were successively injected at 1 min intervals into the manifold (Fig. 1) to monitor the reaction product P_1. Figure 3 shows kinetic FIA profiles obtained by keeping the substrate concentration constant, $[P_2]$ = 0.5 mM, and varying the enzyme concentrations. The concentrations of P_2ase from yeast (Sigma) were estimated on the basis of the protein content specified and the

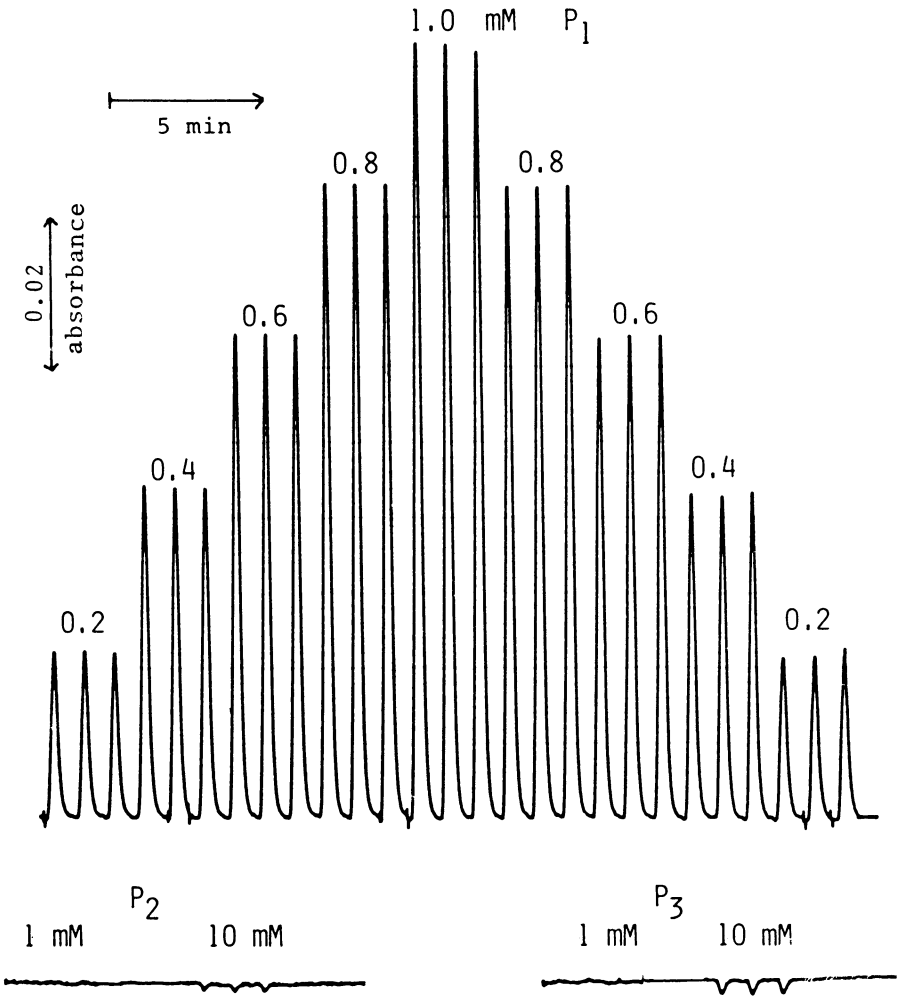

Fig. 2. FIA calibration profiles for P_1, P_2, and P_3.

molecular weight (64000) in the literature.[4] The peak height or the amount of P_1 in each FIA profile increased linearly with time and then tended to become constant toward the end of the enzymatic reaction. The slopes in FIA profiles or the reaction rates were proportional to the enzyme concentrations, but independent of the substrate concentrations (0.2 mM, 0.5 mM, and 1 mM P_2). The observed reaction rate, V, is considered to be the maximum reaction rate, V_{max}, in the well-known Michaelis-Menten expression[9]; $V = V_{max}[P_2]_o / ([P_2]_o + K_m)$. The Michaelis constant K_m for P_2ase has been shown to be as low as 0.008 mM.[2]

It was found that the enzymatic hydrolysis of P_2 of a very short half-life (2.2 min) can be measured by FIA as shown in Fig. 3 (c). The specific activity of P_2ase was estimated from the kinetic FIA profiles to be 730 U./mg, i.e., 1 mg protein liberated 730 μmol of P_1 per minute. This value is somewhat higher than

500-600 U/mg specified by Sigma, but is in agreement with 700 U/mg given by Mel'nik et al.[2)]

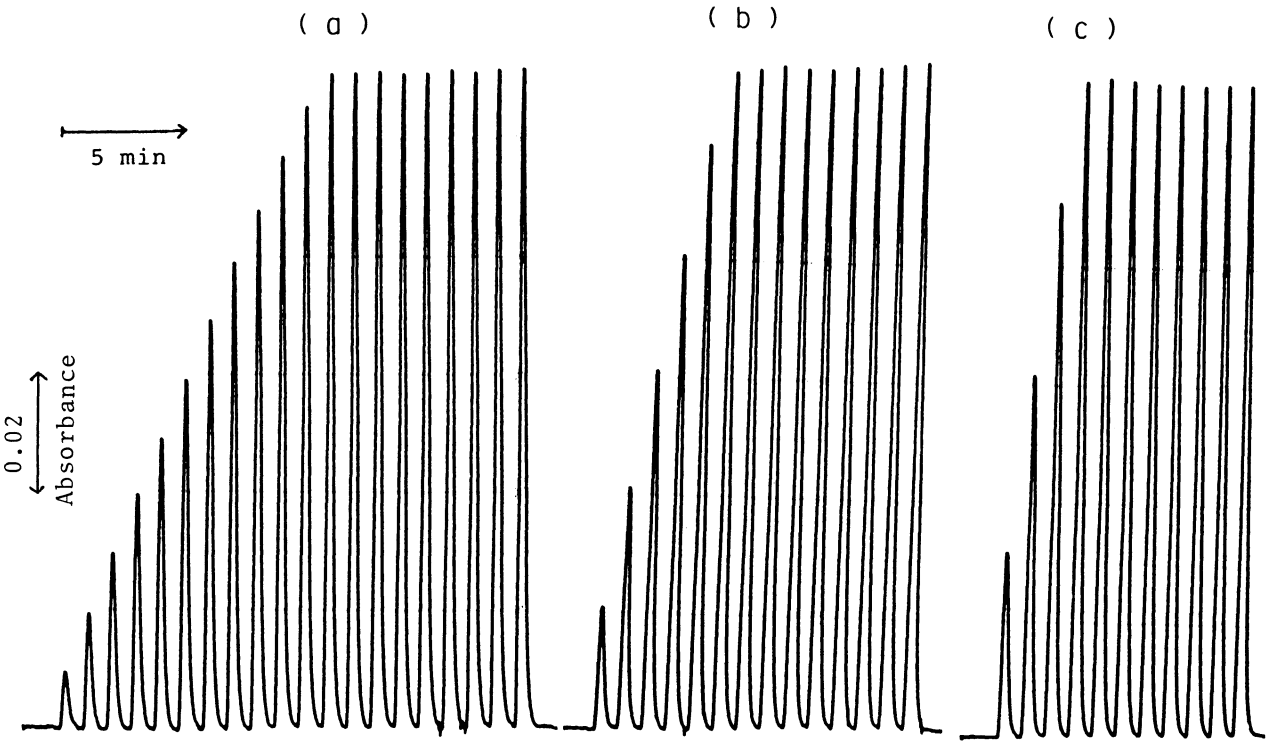

Fig. 3. Kinetic FIA profiles for enzymatic hydrolysis of pyrophosphate. P_2ase concentrations; (a) 1.5×10^{-9} M, (b) 3.0×10^{-9} M and (c) 4.5×10^{-9} M.

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(Received June 29, 1983)