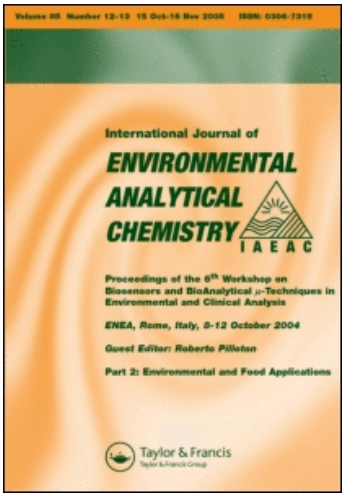

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Enzymic Phosphate Analysis using Immobilized Nucleoside Phosphorylase in an Enzyme Thermistor

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Enzymic Phosphate Analysis using Immobilized Nucleoside Phosphorylase in an Enzyme Thermistor

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Inorganic phosphate has been analyzed using immobilized purine nucleoside phosphorylase in combination with a simple flow calorimeter—an enzyme thermistor. Phosphate was analyzed in the concentration region 30 μM–10 mM. A response signal was obtained 2–3 min after introduction of phosphate into the flow stream of the analysator and the maximal capacity was 10 samples per hour.

KEY WORDS: Phosphate analysis, enzyme analysis, immobilized enzymes, enzyme thermistor, nucleoside phosphorylase.

INTRODUCTION

Purine nucleoside phosphorylase (E.C. 2.4.2.1) catalyzes the phosphorolytic cleavage of purine nucleosides to free purines and ribosyl-l-phosphate.[1] Previously, inorganic phosphate has been analyzed by an enzymic method based on the catalytic activity of this enzyme.[2]

In spectrophotometric measurements as little as 4 nmoles of orthophosphate was detected.

More sensitive enzymic procedures for phosphate analysis have been based on fluorometry and the inhibitory action of phosphate on alkaline phosphatase.[3,4] Immobilized on porous glass this enzyme has been used in a flow-system for analysis of phosphate in the concentration range 0.1–1 mM.[5] A similar sensitivity was obtained with a phosphate-sensitive electrode based on the inhibition of the hydrolysis of glucose-6-phosphate catalysed by alkaline phosphatase.[6]

The enzyme thermistor has been used to analyze a number of substances.[7] It was applied to continuously monitor constituents in a fermentation process[8] in order to develop a process control system. The application in environmental control for analysis of substances in effluents has also been described.[9]

The present report deals with the use of immobilized nucleotide phosphorylase in the enzyme thermistor to monitor orthophosphate, aiming at a continuous monitor in industrial process control.

MATERIALS AND METHODS

Purine nucleoside phosphorylase (E.C. 2.4.2.1) 13 U/mg, from calf spleen was obtained from Boehringer, Mannheim, G.F.R. Enzyme was immobilized on aminopropyl substituted porous glass with glutaraldehyde.[10] Controlled porous glass ZIR-CLAD CPG 250 and 3-aminopropyl triethoxysilane were purchased from Pierce Chem. Comp., Rockford, Ill., U.S.A.

The protein content of the glass supported enzyme was analyzed with ninhydrin[11] after alkaline hydrolysis of the peptide bonds of the enzyme.[12] A mixture of aminopropyl substituted porous glass and known amounts of soluble enzyme was used as a reference.

The enzyme activity of purine nucleoside phosphorylase was analyzed with inosine and phosphate as substrates and an excess of the auxiliary enzyme xanthine oxidase (Sigma Chem. Co., St. Louis, U.S.A.).[2]

The change in A_{293} due to production of uric acid was followed in a Gilford model 125 spectrophotometer equipped with a potentiometric recorder. The assay volume was 2.5 ml and its composition was 0.2 mM in inosine, 2.5 mM in phosphate, and 50 mM in Tris-

acetate buffer, pH 7.4. The assay mixture also contained xanthine oxidase corresponding to 0.12 units per ml. To start the reaction 0.10 ml of purine nucleoside phosphorylase ($\cong$30 mU/ml) was added. For calculation of the enzyme activity a molar absorptivity of 13,000 $M^{-1}\,cm^{-1}$ was used and one unit (U) corresponds to the production of 1 μmole of uric acid per min.[2]

Enzyme activity of the immobilized enzyme was analyzed by spectrophotometry using suspensions of glass conjugates of the enzyme at the conditions given above for activity determination for the soluble enzyme.

Enzyme thermistor assays were performed using a single column device as previously described.[7,9] The volume of glass–enzyme conjugate packed in the column was either 0.5 or 1.0 ml. Pulses of 1 ml solutions of phosphate with varied concentrations (0.001–10 mM) were introduced into the continuous flow system (flow rate 1 ml/min). The buffer composition was either 50 mM Tris-acetate or 50 mM sodium barbitate-buffer, pH 7.4, containing 1 mM inosine. The phosphate solutions were prepared in the same buffer.

RESULTS AND DISCUSSION

This paper deals with the enzymic analysis of orthophosphate. The enzyme activity of purine nucleoside phosphorylase on phosphate and inosine in the production of ribose-1-phosphate was utilized:

$$\text{o-PHOSPHATE} + \text{INOSINE} \longrightarrow \text{HYPOXANTHINE} + \text{RIBOSE-1-PHOSPHATE}$$

Purine nucleoside phosphorylase was immobilized on porous glass. Typically, 0.2–1.5 U/g wet conjugate was obtained when 0.3–1.0 mg of enzyme protein was added per g of activated glass. As the yield on protein basis was 80–90% the apparent specific activity was only 6–10% of that of the soluble enzyme.

The enzyme activity was analyzed in conjugates that had been kept in a buffered suspension at 4°C. The results are shown in Figure 1. Within the first week the activity decreased to about 50%, but for the next month it remained at 40–50% of the initial value.

The glass-supported enzyme was packed in a column and placed in the thermistor unit, at the outlet of which was placed a thermis-

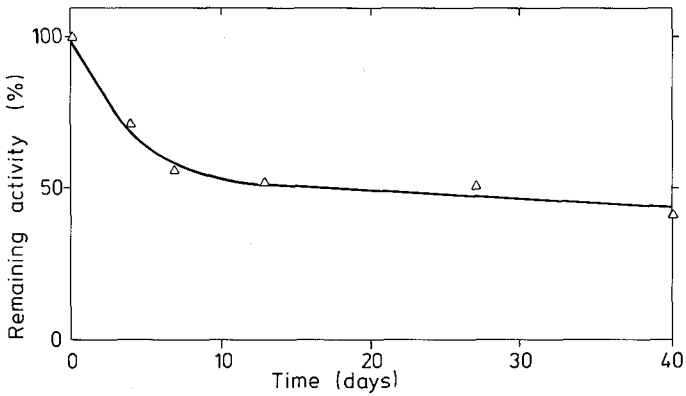

FIGURE 1 Spectrophotometric analysis of nucleoside phosphorylase activity in a preparation of enzyme immbolized on porous glass after storage at 4°C in 50 mM sodium borate, 0.02% sodium azide, pH 7.4.

tor.[7] Samples containing phosphate dissolved in the perfusion buffer, 50 mM Tris-acetate, containing 1 mM inosine, pH 7.4, were introduced as 1 min pulses into the continuous flow. The heat evolved during passage of the enzyme column was registered by the thermistor. A plot of the registered temperature change against the phosphate concentration in the sample introduced is shown in Figure 2. The diagram indicates that there is a linear correlation between temperature change and phosphate concentration. If extrapolated to zero-concentration, the curve was not crossing origo, which could indicate that there is a slight unspecific reaction going on between the support material in the column and phosphate in the samples introduced. The activity of the immobilized phosphorylase as a function of time was investigated using the enzyme preparation in the enzyme thermistor. The enzyme-bed was perfused with 1 mM inosine, dissolved in 50 mM Tris-acetate, pH 7.4, and the temperature was 27°C. The medium also contained 0.02% sodium azide to prevent bacterial growth. The heat signals decreased with time to about 50% as was observed in the activity after storage of the enzyme conjugate at 4°C.

The fact that phosphate acts as a substrate in the catalytic step should guarantee a high specificity with a low degree of interference from other compounds. However, inhibitory effects on the soluble enzyme

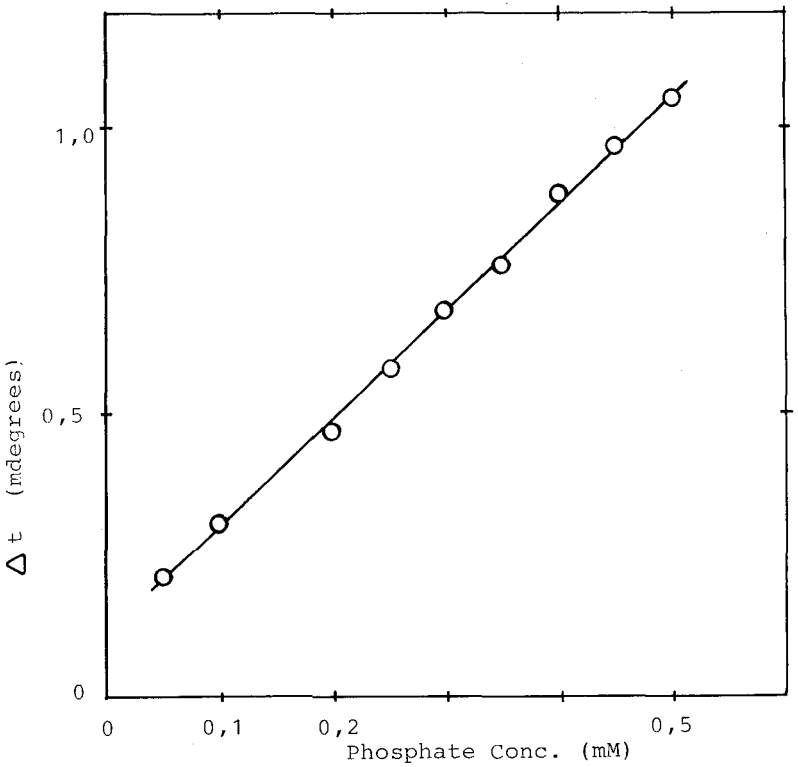

FIGURE 2 Heat response signals of a nucleoside phosphorylase enzyme thermistor towards inorganic phosphate. The enzyme activity of the conjugate was 0.40 U. The medium contained 50 mM sodium barbitate, 1 mM inosine and 0.02% sodium azide, pH 7.4.

have been reported of some organic phosphates and arsenate.[13] Effects of these substances and others on the immobilized nucleoside phosphorylase remain to be quantified.

Using the catalytic property of purine nucleoside phosphorylase and inosine, it was possible to analyse phosphate at concentrations from 30 μM to 10 mM. Within 2–3 min a heat response signal was obtained and after another 5–6 min a new sample could be analysed. We performed the pulse-wise introduction of phosphate manually, but the operation may well be conducted automatically. Furthermore, in other systems it has been shown possible to run

enzyme thermistor analysis continuously, and, if desirable, the phosphate concentration could also be followed in such a manner.[14]

In environmental analysis much debate during the last years has dealt with whether each substance shall be analyzed individually, or if the integrated effect of all the pollutants present shall be registered. For acute toxic substances this latter approach may be preferable, but for a substance like phosphate, that will contribute to the eutrophication of the environment, a specific assay may be a better choice.

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