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Single-Enzyme-Based Amperometric Assay for Phosphate Ion

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ABSTRACT

An amperometric assay for phosphate ion, using a single enzymatic process, has been developed. The enzyme used was pyruvate oxidase, which catalyzes the oxidation of pyruvate in the presence of oxygen and phosphate ion. The products of the enzymatic reaction are acetyl phosphate, carbon dioxide, and hydrogen peroxide. The latter was monitored by means of a hydrogen peroxide electrode and an oxidase meter. Phosphate ion in the concentration range of 50–500 μM can be measured within 4 min. Anions, such as pyrophosphate, sulfate, nitrate, nitrite, and acetate, gave only marginal responses.

Index Entries: Amperomatric assay, single-enzyme based, for phosphate ion; pyruvate oxidase, catalysis of oxidation of pyruvate with; phosphate ion.

INTRODUCTION

Several enzymatic methods for determining phosphate ion are currently available. These methods can be divided into two groups, according to the type of substrates being used (*1*,*2*). In one type, which is herein called the phosphate-utilizing system, the phosphate ion serves as a cosubstrate and is consumed in the enzymatic processes to produce a phosphorylated product for each phosphate ion used. In the other type, called the phosphate-generating system, phosphate ions are produced from phosphorylated substrates in the enzymatic process. Examples of the phosphate-utilizing systems are: (a) The method that uses muscle

phosphorylase a, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. The reaction can be measured by monitoring the production of NADH, either spectrophotometrically or fluorimetrically (*1–3*); (b) the method that uses glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, hexokinase, and glucose-6-phosphate dehydrogenase (*2–3*); and (c) the method that uses purine nucleoside phosphorylase and xanthine oxidase (*4*). Examples of the phosphate determination utilizing the phosphate-generating system include: (a) The method developed by Weetall and Jacobson (*5*), which make use of the inhibitory action of phosphate ion on phosphatase reactions; and (b) the amperometric method developed by Guilbault and Nanjo (*6*), which uses two enzymes, alkaline phosphatase and glucose oxidase.

The disadvantages of the currently available enzymatic phosphate assays based on the phosphate-utilizing approach are the complexity of the experimental procedure and the cost associated with using multiple enzymes. On the other hand, the assays based on the enzymatic phosphate-generating system generally lack a good sensitivity, and when used in conjunction with an electrochemical sensor, they require auxiliary enzymes to generate electroactive species.

In this communication, I describe a direct, single-enzyme method for phosphate determination. The method uses a phosphate-utilizing enzyme, pyruvate oxidase, and a hydrogen peroxide electrode.

MATERIALS AND METHODS

Pyruvate oxidase [EC 1.2.3.3, pyruvate:oxygen 2-oxidoreductase (phosphorylating)], sodium pyruvate, flavin adenine dinucleotide (FAD), and thiamine pyrophosphoric acid (cocarboxylase, TPP) are from Boehringer Mannheim Biochemicals. 3-(*N*-Morpholino)-propanesulfonic acid (MOPS) is from Behring Diagnostics. Sodium phosphate, sodium sulfate, sodium nitrate, sodium nitrite, and potassium chloride are from Fisher Scientific Co. The oxidase meter, YSI model 25 and hydrogen peroxide electrode probe are from Yellow Spring Instruments.

Reagent Preparation

Substrate Solution

The substrate solution is prepared in 0.05*M* MOPS buffer, pH 7.3, and consists of 25 m*M* sodium pyruvate, 0.1 m*M* FAD, 1 m*M* TPP, 10 m*M* MgCl, and varying amounts of sodium phosphate, as indicated in Fig. 1. Five milliliters of substrate solution are used in each assay run.

Enzyme Solution

The enzyme solution is prepared by dissolving the lyophilized pyruvate oxidase in 0.05*M* MOPS buffer, pH 7.3, containing 0.1 m*M*

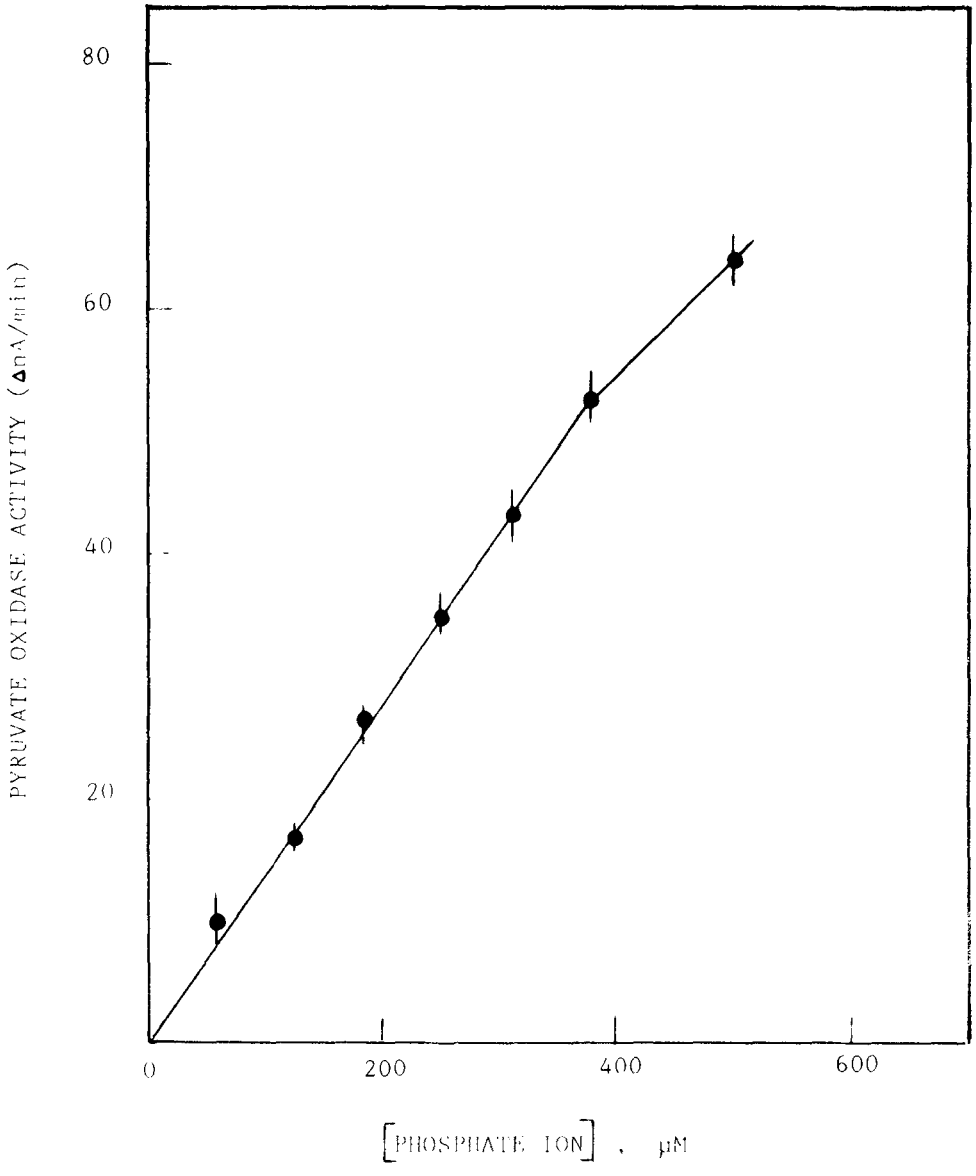

Fig. 1. Standard curve for single enzyme-based amperometric determination of phosphate ion concentration, using pyruvate oxidase and hydrogen peroxide sensors. All measurements were made at 25°C and in 0.05*M* MOPS buffer, pH 7.3. (*See* text for the composition of the substrate and enzyme solutions.)

FAD and 1 m*M* TPP. Routinely, the enzyme solution is prepared at a concentration of 3.3 mg/mL. In each assay, 0.1 mg of enzyme is used.

Amperometric Assay for Pyruvate Oxidase and Phosphate Ion

Pyruvate oxidase catalyzes the oxidation of pyruvate in the presence of phosphate and oxygen, with the formation of acetylphosphate, carbon

dioxide, and hydrogen peroxide. The hydrogen peroxide formed in the enzymatic reaction can be measured amperometrically by using a YSI-Clark 2510 hydrogen peroxide probe and a YSI model 25 oxidase meter, which supplies a polarizing voltage of 700 mV. The amperometric response is displayed on a Fisher chart recorder. Typically, the enzymatic reaction is performed with a well-stirred 5-mL substrate solution at 25°C. After the tip of the electrode is immersed in the stirred substrate solution and a stable base line reading is obtained, the enzymatic reaction is initiated by addition of 30 μL of pyruvate oxidase, containing 3 U of enzyme. The reaction is continuously monitored for about 4 min at the most. At the completion of each assay, the reaction vessel and the electrode probe are thoroughly washed with distilled water, followed by 0.05*M* MOPS buffer, pH 7.3.

RESULTS AND DISCUSSION

Several attempts have been made to develop a reliable, ion-selective electrode for measuring phosphate ion by using inorganic phosphate compounds as the active elements of the sensing membrane (*7,8*). These attempts, however, have not been too successful. Alternative approaches using enzymes as the key sensing elements have been described by Guilbault and coworkers (*3,6*). As previously mentioned, all enzyme-based electrochemical assays that have been developed for phosphate utilized multiple enzyme systems. The method described in this communication is the first enzymatic phosphate determination that uses a single enzyme, pyruvate oxidase, to generate an electroactive species, hydrogen peroxide. Pyruvate oxidase is a flavoprotein and requires thiamine pyrophosphate for its function. In the presence of phosphate ion and oxygen, it catalyzes the oxidation pyruvate to form acetyl phosphate, carbon dioxide, and hydrogen peroxide. Figure 1 shows the standard curve for phosphate determination, using pyruvate oxidase and an

TABLE 1
Effects of Phosphate and other Anions on Enzyme (Pyruvate Oxidase)-Generated Amperometric Readings

Anion	Amperometric readings, % of phosphate response
Phosphate	100
Pyrophosphate	9
Sulfate	8
Nitrate	8
Nitrite	2
Acetate	5

amperometric hydrogen peroxide sensor system. The vertical bars in Fig. 1 indicate the standard deviation. Phosphate ion in the concentration range of 50–500 μM can be determined within 4 min. Phosphate ion concentrations up to 400 μM give a linear response. Furthermore, the enzyme-generated amperometric response appears to be specific for phosphate ion only (Table 1). Anions, such as pyrophosphate, sulfate, nitrate, nitrite, and acetate, did not seem to serve as a substrate for the enzyme. The error caused by the presence of these anions is in all cases less than 10%.

CONCLUSION

In conclusion, a single-enzyme-based amperometric assay for phosphate ion, which is rapid and free of interference from anions, has been described. The enzyme used is pyruvate oxidase and the electroactive species is hydrogen peroxide.

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