Note

Utilization of Bacterial Xanthine Oxidase for Inorganic Phosphorus Determination

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Inorganic phosphorus (Pi) is involved in many metabolic reactions, and plays an important role especially in glycolysis and gluconeogenesis. Therefore, the determination of Pi is one of the most frequently used techniques for many biochemical reactions.

An enzymatic assay method based on the reaction of phosphorylase *a* as the phosphoryl acceptor was proposed by Fawaz *et al.*¹⁾ and studied more completely by Schulz *et al.*²⁾ Furthermore, methods based on the reactions of glyceraldehyde phosphate dehydrogenase^{3~6)} and purine

nucleoside phosphorylase (PNPase) (EC 2.4.2.1)^{6,7)} have been reported.

The method based on the PNPase reaction was coupled with milk xanthine oxidase (XOD) (EC 1.2.3.2). The released hypoxanthine during the phosphorylase of inosine is measured from the absorbance at 293 nm.

As previously reported we found a new type of XOD in *Enterobacter cloacae* KY3074, which was different from milk XOD in the electron acceptor specificity. *Enterobacter* XOD showed the highest activity with O₂ as electron acceptor.⁸⁾ *Enterobacter* cells grown in the medium for XOD production also contained uricase.⁹⁾

We applied *Enterobacter* XOD instead of milk XOD to the assay system of Hwang and Cha,⁷⁾ and it was possible to measure the amount of Pi in the visible region. The following equations show the general principles for Pi determination:

Hwang and Cha⁷⁾ have determined Pi by measuring the

FIG. 1. Development and Disappearance of Quinoneimine Dye during the XOD Reaction.

[A] The absorbance change at 500 nm was measured for quinoneimine dye formation, after the reaction was performed with a mixture (3 ml) containing 100 μmol Tris-HCl buffer, pH 7.3, 1 μmol hypoxanthine, 5 μmol 4-aminoantipyrine and phenol, 2.5 units peroxidase, and cow milk XOD at 37°C for each time. For uric acid formation, the absorbance change at 293 nm was measured. Experimental conditions were as described for the quinoneimine dye formation, except that 4-aminoantipyrine, phenol, and peroxidase were not included. Quinoneimine dye formation: 0.16 units milk XOD, $\bullet$ — $\bullet$; 0.4 units milk XOD, $\circ$ — $\circ$; uric acid formation: 0.16 units milk XOD, $\bullet$ — $\bullet$.

[B] Quinoneimine dye formation was measured using *Enterobacter* XOD. Experimental conditions were as described in [A], except that 5 μmol hypoxanthine and 1 unit *Enterobacter* XOD were used.

FIG. 2. Standard Curve for Phosphate Determination.

The assay mixture in a total volume of 3 ml contained 100 μ mol Tris-HCl buffer (pH 7.5), 1 μ mol inosine, 2 μ mol 4-aminoantipyrine and phenol, 10 units peroxidase, 0.16 units PNPase,¹²⁾ 6 units XOD and 1.2 units uricase from *E. cloacae* KY3074^{8,9)} and the indicated amounts of KH_2PO_4 . The reaction was performed at 37°C for 15 min, and the quinoneimine dye production was measured as absorbance at 500 nm.

formation of uric acid at 293 nm. On the other hand, the measurement of H_2O_2 , which is another product of hypoxanthine oxidation, by the method of Allain *et al.*¹⁰⁾ seems to have the advantage of being free from the interference by substances showing absorption in the ultraviolet region.

We measured the formation of H_2O_2 following the development of quinoneimine dye, as shown in Fig. 1. During the reaction of milk XOD, the color developed gradually decreased, although the formation of uric acid continued. Furthermore, the addition of more milk XOD caused a rapid development of the color and a subsequent decreasing of the developed color. This observation indicates that milk XOD is not suitable for the determination of hypoxanthine by the method of Allain *et al.*

In the reaction of *Enterobacter* XOD, on the other hand,

the color developed showed no decrease in the absorbance up to 1.5. Thus the determination of quinoneimine dye became practical with the use of *Enterobacter* XOD.

XOD allows the use of various compounds such as ferricyanide, O_2 , or quinones as electron acceptors.¹¹⁾ Of these acceptors, ferricyanide is the most effective acceptor for milk XOD, but for *Enterobacter* XOD O_2 is most effective.⁸⁾ The difference in the specificity for electron acceptor, of the two XODs, probably resulted in the difference in the reduction of quinoneimine dye.

The standard curve for the assay of Pi was obtained with a method involving four enzymes (Fig. 2). The curve was linear for the amounts of Pi but with a relatively high blank value of 0.045. In other enzymatic assay methods for Pi a considerable blank value is also observed^{2,3,7)} and this is probably due to the incomplete removal of Pi from enzyme preparations.

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