ENZYME-CATALYZED HYDROLYSIS OF MONOFLUOROPHOSPHATE TO ORTHOPHOSPHATE: VISUALIZATION BY FLOW INJECTION ANALYSIS AND P-31 NMR

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ABSTRACT

Monofluorophosphate (MFP, PO_3F^{2-}), a fluorine derivative of orthophosphate (P_1, PO_4^{3-}), has been widely used as a caries-preventive additive in tooth-pastes. Selective and rapid determination of P_1 in the presence of MFP was achieved by two spectrophotometric flow injection methods that are based on classical procedures using a single Mo(VI) reagent and a mixed reagent of Mo(VI), Sn(II) and ascorbic acid. Both methods were optimized to monitor alkaline phosphatase (EC3.1.3.1)-catalyzed hydrolysis of MFP. Multi-point measurements of kinetic processes were made to visualize the zero-order aspect of MFP hydrolysis. The kinetic processes could be discussed in terms of Michaelis-Menten model. Other techniques, P31-NMR and HPLC, were also employed to confirm the conversion of MFP to orthophosphate.

INTRODUCTION

Monofluorophosphate (MFP, PO_3F^{2-}) is a fluorine derivative of orthophosphate (P_1, PO_4^{3-}). Its disodium salt has been chemically prepared and widely used as a caries-preventive additive in tooth-pastes[1,2]. MFP has also been examined as a drug for the treatment of osteoporosis[3-6]. Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, a main constituent of teeth and bones, is soluble in an acidic medium of lactic acid that may be produced by the bacterial metabolism of sugar on the surface of teeth. The dental and medical effects of MFP have been considered to be based on the conversion

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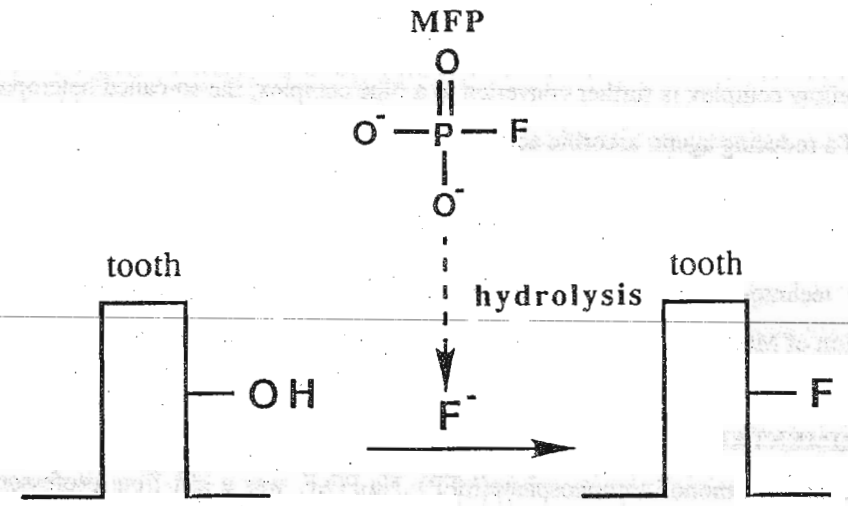
of hydroxyapatite to fluoroapatite, $Ca_{10}(PO_4)_6F_2$, by the action of fluoride ion. Fluoroapatite thus formed is less soluble and more acid-resistant than hydroxyaptite. As shown in Scheme 1 fluoride ion is supplied by the hydrolysis of MFP.



Scheme 1. Conversion of hydroxyapatite to fluoroapatite by monofluorophosphate.

The mechanistic model of fluoridation is based on the assumption that MFP might be hydrolyzed very rapidly to give fluoride ion during a short time of tooth-brushing , usually a few minutes. However, MFP is very stable in the absence of catalysts; the half-life of hydrolysis at neutral pH is as long as a few years[7]. Some catalysts are needed to provide the rapid hydrolysis of MFP.

This work was undertaken to examine the catalytic ability of alkaline phosphatase (APase, EC3.1.3.1) to accelerate the hydrolysis of MFP to orthophosphate. Alkaline phosphatase is widely distributed in nature and is known as an active enzyme toward the P-O-C bond hydrolysis of phosphate monoesters[8,9].

$$\text{MFP} \xrightarrow{\text{APase}} P_1 + F^- \qquad (1)$$

Two spectrophotometric methods were optimized to monitor the rapid kinetic process of MFP hydrolysis. Both methods are based on the selective detection of the product, orthophosphate, in the

presence of the substrate, MFP. Orthophosphate reacts with a Mo(VI) reagent to form a yellow complex, molybdophosphate.

$$P_1 + Mo(VI) \longrightarrow P_1 - Mo(VI) \quad (2)$$

The yellow complex is further converted to a blue complex, the so-called heteropoly blue, by the action of a reducing agent, ascorbic acid.

$$P_1 - Mo(VI) \xrightarrow{Asc} P_1 - Mo(V) - Mo(VI) \quad (3)$$

Other techniques, P-31 NMR and HPLC[7-13], were employed to confirm the enzymatic conversion of MFP to P_1 by measuring both the substrate and the product.

EXPERIMENTAL

Disodium monofluorophosphate(MFP) ,Na_2PO_3F, was a gift from professor T. Nakamura. Alkaline phosphatase (APase, E.C.3.1.3.1, Sigma P-5521) was used for enzymatic hydrolysis of MFP[8,9].

Main components of an FIA system were indicated in Fig.1. The Mo(VI) reagent was prepared by dissolving 5.3 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, in 1 L of 0.3 M H_2SO_4. The reagent was stable at room temperature for at least one month. The Sn(II) - ascorbic acid solution used as a reductant was freshly (daily) prepared. It consisted of $8x10^{-4}$M $SnCl_2$, $1x10^{-2}$ M ascorbic acid($C_6H_8O_6$) and 0.3 M H_2SO_4.

RESULTS AND DISCUSSION

Design of FIA systems

The three-channel FIA system in Fig.1 was designed so that the enzymatic reaction in equation 1 could be stopped instantaneously and the product P_1 could be detected selectively in the presence of the substrate MFP. A sample solution (S) was injected into the water stream(1ml/min) via a loop valve injector . The molybdenum(VI) reagent from the second channel was acidic enough to stop the enzymatic reaction by acidifying the sample zone at the confluence point.

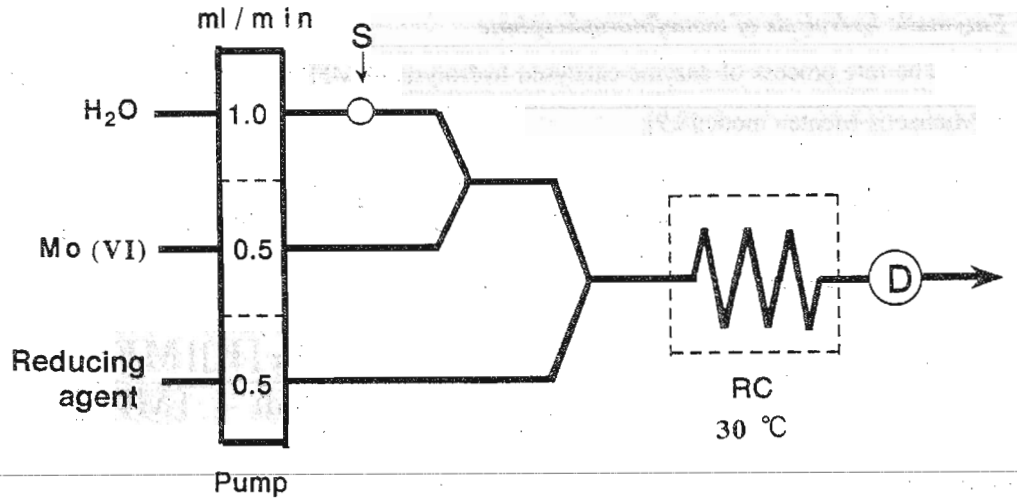


Fig. 1. FIA system for the selective determination of orthophosphate in the presence of monofluorophosphate.

S, sample injector(0.1 mL); RC, reaction coil (0.5 mm I.D. x 5 m, PTFE); D, spectrophotometric detector (400 nm and 690 nm).

It was preliminarily confirmed, by use of the channels of H_2O and Mo(VI), that only P_1 reacted rapidly with the Mo(VI) reagent in a reactor at 25 ~ 40℃ to form molybdophosphoric acid. Selective and quantitative determination of P_1 could be achieved at 30℃, in the presence of MFP, by measuring the absorbance at 400 nm of the yellow complex. At temperature above 50℃ MFP gave an FIA signal due to its conversion to orthophosphate in the reactor. Both MFP and P_1 indicated FIA signals of similar sensitivity at temperature above 130 ℃.

The third channel of a reductant in Fig.1 was installed to achieve 20-fold enhancement in sensitivity by converting the yellow complex (absorption maximum; 336 nm) to a blue complex (maximum; 709 nm). The reductant solution is composed of ascorbic acid and Sn(Ⅱ). The combined use of the two reductants was synergistically effective to accelerate the color development. It is noted that the yellow method is favorable in the higher concentration range of P_1 above 10^{-5} M and the blue method is useful in the lower working range, 2×10^{-4}M ~ 2×10^{-7} M P_1.

Enzymatic hydrolysis of monofluorophosphate

The rate process of enzyme-catalysed hydrolysis of MFP in equation 1 is discussed in terms of Michaelis-Menten model[8,9];

$$MFP + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MFP\text{-}E \xrightarrow{k_3} P_1 + E + F^- \quad (4)$$

$$v = \frac{k_3 [E][MFP]}{Km + [MFP]} \quad (5)$$

$$k_3 [E] = Vmax \quad (6)$$

v is rate or velocity of hydrolysis. k_1, k_2 and k_3 are rate constants. Km is Michaelis constant. The second forward step with k_3 is assumed to be a rate-determining process.

FIA methodology with the Mo(VI) reagent in Fig.1 was employed to analyze the rapid hydrolytic process of MFP to P_1. As mentioned above only P_1 could be detected selectively. The enzymatic hydrolysis was initiated by the addition of APase to the pH7.2-buffered MFP-Mg^{2+} mixture at 30℃ and the reaction mixture was analyzed at appropriate interval. In the case where no enzyme was added very small signals due to the contaminant in the original MFP sample were observed throughout multiple injections at 1.5 min interval (Fig.2 a). On addition of the enzyme, however, the P_1 signal increased linearly with incubation time, in accordance with the zero-order reaction rate. Toward the completion of MFP hydrolysis the peak height tended to become constant. The linear slopes in Figs.2b and 2c are dependent on the enzyme concentrations and are related to the maximum velocity, k_3[APase], in Michaelis-Menten equation. The catalytic rate constant, k_3, can be calculated when the enzyme concentration, [APase], is given. The molecular weight of the enzyme has been reported to be in the range, 80.000 ~ 140,000. The lower value, 80,000, was adopted in this paper to calculate the k_3 value, 4×10^3 min^{-1}, at 30℃ and pH7.2.

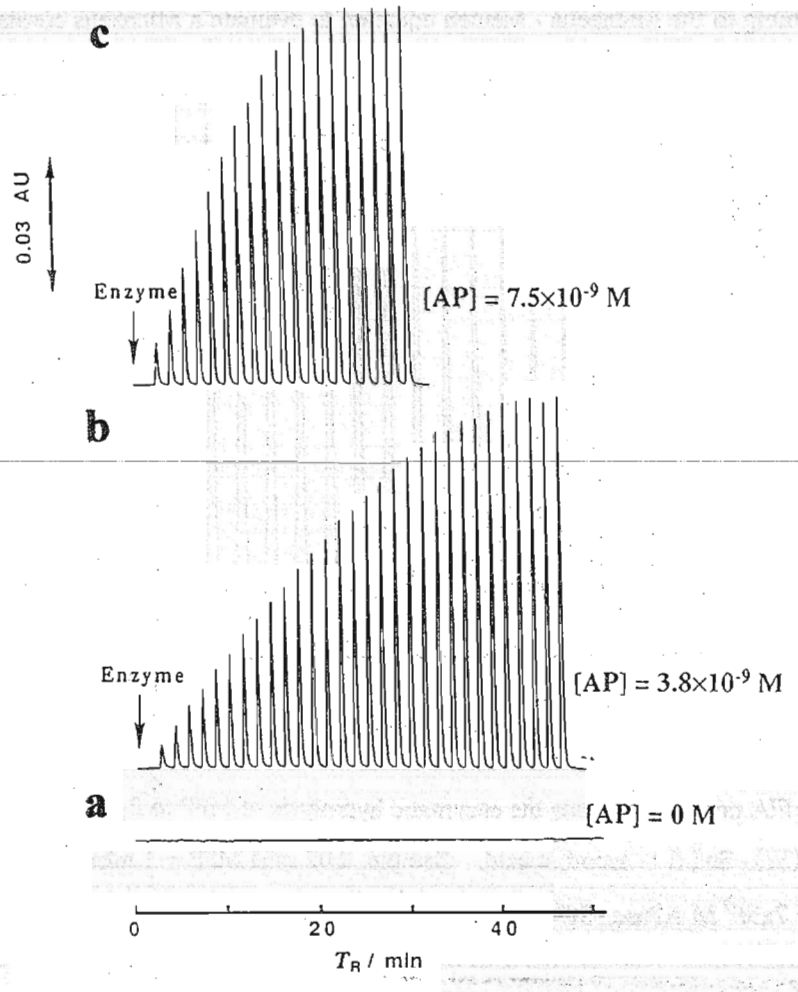


Fig.2. Kinetic FIA profile indicating the enzymatic hydrolysis of MFP to P_1.
Reagent; Mo (VI). Sample; 0.5 mM MFP + 1 mM $MgCl_2$ + 10 mM Tris-HCl(pH7.2) + APase as indicated.

The FIA method using the Mo(VI) reagent was not sensitive enough to monitor the initial kinetic process of MFP hydrolysis when the initial MFP concentration was less than 10^{-4} M. The more sensitive regent, Mo(VI)-Sn(II)-Asc, was favorable to obtain the kinetic profile at lower MFP concentration, 2 x 10^{-5} M. The FIA signals in Fig.3 increased non-linearly with incubation time, in contrast to the linear slope in Fig.2 at higher MFP concentration. The progress curve in Fig,3 was

analyzed according to the Michaelis - Menten equation to evaluate a Michaelis constant; *Km* = 3 x 10^{-5} M.

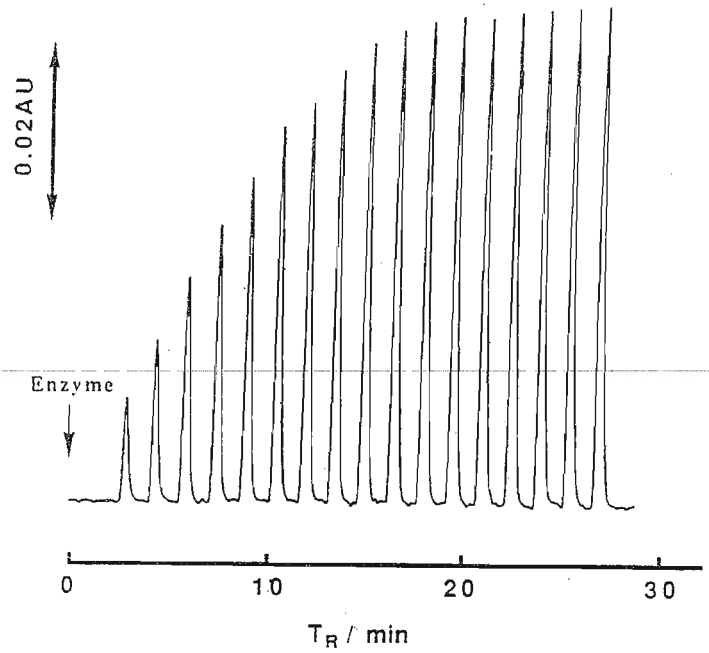


Fig.3. Kinetic FIA profile indicating the enzymatic hydrolysis of MFP to P_1.
Reagent; Mo(VI) - Sn(II) - ascorbic acid. Sample; 0.02 mM MFP + 1 mM $MgCl_2$ + 10 mM Trs-HCl(pH7.2) + 7x!0^{-9} M APase., 30℃.

P-31 NMR evidence for enzymatic MFP hydrolysis

As can be seen in Figs. 2 and 3 only the progressive formation of P_1 from MFP is monitored by the FIA technique, without the direct evidence for the degradation of MFP. A simple P-31 NMR experiment was made to ascertain the simultaneous variation of both MFP and P_1. The result in Fig.4 with the MFP-enzyme incubate indicates that P-31 NMR signal intensity of P_1 (singlet) increases with the decrease in signal intensity of MFP(doublet). The fact rationalizes that the time course of MFP hydrolysis can be evaluated from the variation in P_1 concentration, as can be easily and rapidly achieved by FIA experiments.

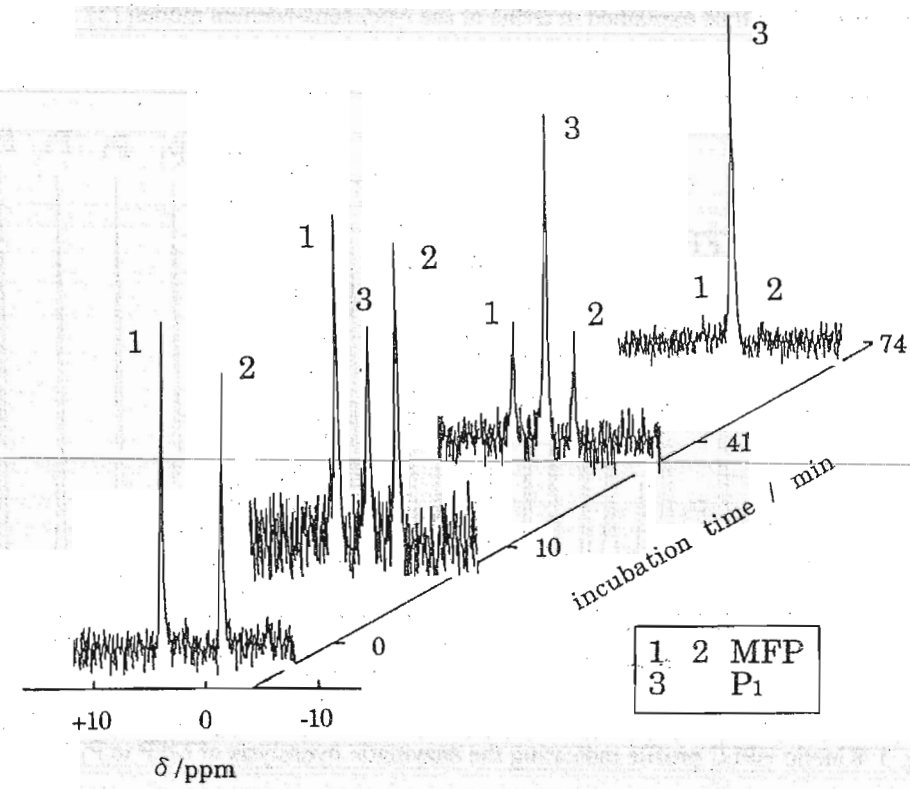


Fig.4. P-31 NMR spectra indicating the kinetic process of enzymatic conversion of monofluorophosphate to orthophosphate.

A mixture of 10 mM MFP, 1mM $MgCl_2$, 5 mM Tris-HCl buffer (pH7.2) and $1x10^{-7}$M APase was incubated at room temperature (ca.25℃) and was subjected to P-31 NMR measurement at time intervals indicated. The doublet signals (1 and 2) are for MFP and the singlet (3) is for P_1.

HPLC evidence for enzymatic MFP hydrolysis

HPLC was also able to detect both MFP and P_1 in hydrolytic reaction. An HPLC system was employed which consisted of an anion-exchange column and a high-temperature(high-pressure) FIA reactor[11,12]. An incubated mixture of MFP and enzyme was repeatedly analyzed at 6 min interval to obtain a kinetic HPLC profile in Fig.5. The first pair of MFP and P_1 is for a reference aliquot that contains no APase. The subsequent pairs were obtained by injecting the MFP-APase incubate. The HPLC signal of MFP decreased with incubation time, with consequent increase of P_1

signal, which could be explained in terms of the Michaelis-Menten model[13]..

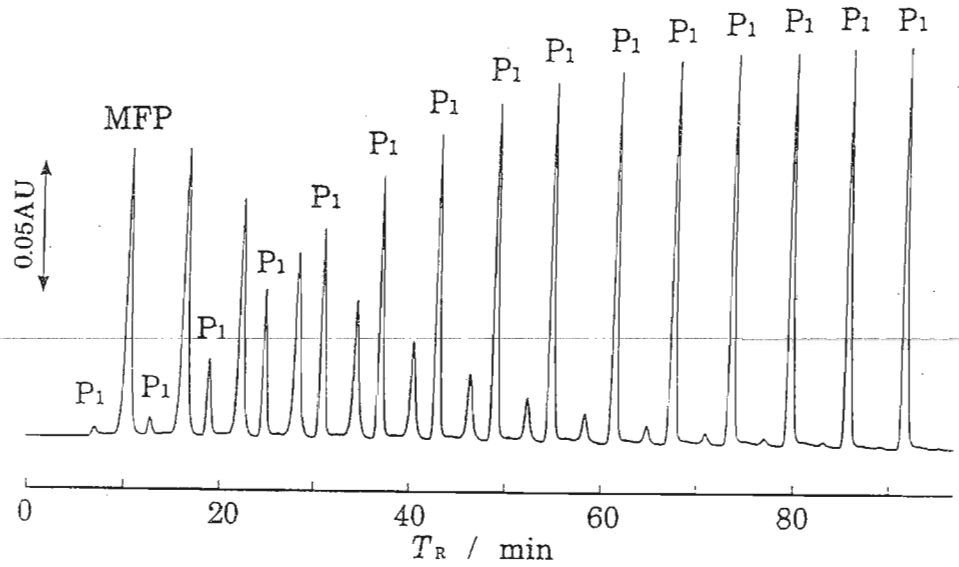


Fig. 5. Kinetic HPLC profile indicating the enzymatic hydrolysis of MFP to P_1.

Sample; 0.1 mM MFP + 1 mM $MgCl_2$ + 5 mM Tris-HCl (pH8.0) + 5 x 10^{-10} M APase, 30℃.

REFERENCES

1. W. H. Bowen(Ed), " Relative Efficacy of Sodium Fluoride and Sodium Monofluorophosphate as Anticaries Agents in Dentifrices", Royal Society Medicine Press, London(1995).
2. S. Nakashima, Thesis (M. S.), Kyushu University(1994).
3. S. Patel, J. K.-M. Chan, and D. J. Hosking, Bone, 19, 651(1997).
4. A. Rigalli, L. Estaban, L. Pera and R. C. Puche, Calcif. Tissue Int., 60, 86(1997).
5. J. Klimek, M. Jung and S. Jung, Archs. Oral Biol., 42, 181(1997).
6. A. Battmann, H. Resch, C. R. Libanati, D. Ludy, M. Fischer, S. Farley and D. J. Baylink, Osteoporosis Int., 7, 48(1997).
7. N. Ueda, Dissertation (M.S.), Kyushu University(1994).
8. N. Yoza, S. Nakashima and T. Nakazato, Chem. Lett., 53(1997).
9. N. Yoza, S. Onoue and Y. Kuwahara, Chem. Lett., 491(1997).

10. N. Yoza, N. Ueda and S. Nakashima, Fresenius J. Anal. Chem., 348, 633(1994).
11. N. Yoza, S. Nakashima, N. Ueda, T. Miyajima, T. Nakamura and P. Vast, J. Chromatogr., 664, 111(1994).
12. N. Yoza, I. Akazaki, T. Nakazato, N. Ueda, H. Kodama and A. Tateda, Anal. Biochem., 199, 279(1991).
13. H. Hirano, Y. Baba, N. Yoza and S. Ohashi, Anal. Chim. Acta, 179, 209(1986).

(Received November 27, 1997)
(Accepted December 15, 1997)