Intercalation of Enzymes in γ-Titanium(IV) Phosphate

Yasushi KANZAKI*,† and Mitsuo ABE
Department of Chemistry, Faculty of Science, Tokyo Institute of Technology,
2-12-1 Ohokayama, Meguro-ku, Tokyo 152
† Laboratory of Analytical Chemistry, Showa College of Pharmaceutical Sciences,
3 Higashi-Tamagawagakuen, Machida, Tokyo 194
(Received February 16, 1991)

Synopsis. The intercalation of enzymes with molecular weights of 23000—240000 was examined for layered inorganic compounds. The intercalation of papain and catalase into γ-type titanium(IV) phosphate was achieved by coexisting propylamine in an aqueous medium. The intercalation compound of papain was enzyme active for *N*-benzoyl-L-arginine ethyl ester and a catalase intercalation compound for H_2O_2 decomposition.

A great variety of methods for the preparation of immobilized enzymes have been reported and practically applied in many quarters. Inorganic materials, such as clay minerals, have been used as supports of immobilized enzymes.[1] However, such immobilized enzymes are prepared by simple adsorption or ion-exchange reaction of enzymes on the surface of clay minerals. Intercalation occurs in layered inorganic compounds in which each lamella is combined by weakly interacting forces, such as van der Waals force. Enzymes which are intercalated between such weakly interacting lamellae are expected to be utilized as immobilized enzymes. Such an approach may develop different conceptions regarding the immobilization of enzymes.

This paper deals with the intercalation of some common enzymes in layered γ-type titanium(IV) phosphate ($Ti(HPO_4)_2 \cdot 2H_2O$: γ-TiP). The enzyme activity of the intercalation compounds was also examined.

Experimental

Layered Materials. The H^+ form γ-TiP was prepared according to the procedure reported by Kobayashi and Yamazaki[2] as follows. Amorphous titanium(IV) phosphate was heated hydrothermally in 10 M (M=mol dm^{-3}) phosphoric acid for 24 h at 280 °C. Well crystallized γ-TiP with an average diameter of 10—20 μm was prepared.[3] The crystal thus prepared was carefully washed with deionized water and air-dried.

Procedure. The direct intercalation of enzymes into inorganic layered compounds seemed to be improbable because of the tremendously large size of the enzymes. The following procedures were employed according to the intercalation reaction of α-zirconium(IV) phoshate with propylamine reported previously.[4] It was found that delamination occurred in the reaction of α-zirconium(IV) phosphate with an aqueous propylamine solution, and gave a colloidal solution of α-zirconium phosphate. Relamination, which gave a transparent film, was found to occur when the solvent was removed from the colloidal soluion. If delamination and the following relamination occurred in the presence of enzymes, the intercalation of enzymes with large moleculr weights could be achieved by coexisting enzymes in such a colloidal solution.

We have found that γ-TiP gave similar colloidal (delaminated) materials in an aqueous propylamine solution.[5] Two procedures for the intercalation were examined in this study according to the above-mentioned findings.

Table 1. Enzymes and Their Molecular Weight

Enzymes	Mol. weight
Papain (Protein decomposition)	23000
Lipase (Triglyceride decomposition)	38000
Peroxidase (H_2O_2 decomposition)	40000
α-Amylase (Starch hydrolysis)	51000
Gluco-Amylase (Starch-Glucose)	70000
Glucose-Oxidase (Glucose oxidation)	153000
Catalase (H_2O_2 decomposition)	240000

Procedure (1): Enzymes were reacted with a colloidal solution of γ-TiP which was prepared in an aqueous propylamine solution.

Procedure (2): γ-TiP was reacted with an enzyme–propylamine mixed solution (cointercalation).

Enzymes. Seven commonly available enzymes were selected for the intercalation, and are listed in Table 1. They were used without any pretreatment. Intercalation was carried out at 30 °C for 1 week unless otherwise stated. The reaction product was developed on a glass plate and the X-ray diffraction spectrum was measured using Mn-filtered Fe-$K\alpha$ radiation. The X-ray diffractometer used could measure down to 2θ=0.9° which corresponds to the *d*-value of about 12 nm.

Assay. An enzme assay for the intercalation compounds was examined by contacting the substrate solutins (*N*-benzoyl-L-arginine ethyl ester (BAEE) for papain and H_2O_2 for catalase) with those corresponding intercalation compounds which were developed on glass plates and were fixed by air-drying.

Results and Discussion

Procedure (1): Few systematic results were obtained although a variety of reaction products were observed in the reaction of enzymes with colloidal solution of γ-TiP. The only result that implied the success of enzyme intercalation was observed in a reaction with papain. Figure 1a shows the X-ray diffraction pattern of the reaction products with respect to the added amount of papain. The peak for the propylamine intercalation compound[6] (2θ=5.9°; *d*-value=1.9 nm,) which was observed in a dilute papain solution, completely disappeared at a papain content of 30 mg, suggesting the perfect conversion of γ-TiP into the papain intercalation compound. The first two sequences of the baseal plane reflection ($n\lambda$: n=1,2,3...) were observed. Such sequential reflections generally imply the formation of an intercalation compound. The average interlayer spacing of the product calculated from the peak angle was 5.6 nm, which was compared with 1.2 nm for the host γ-TiP or 1.9 nm for propylamine intercalated γ-TiP. The line width of the peak was 1.7°—2.3°, corresponding to the interlayer spacing of 6.5—4.8 nm. The large line

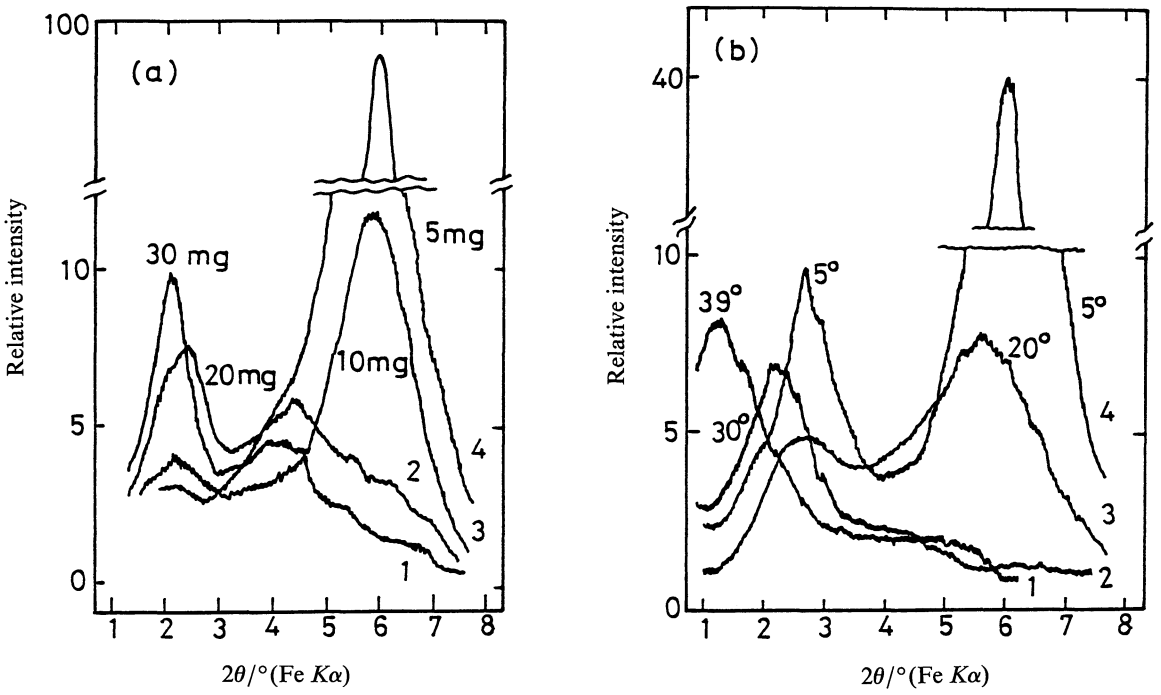

Fig. 1a. X-Ray diffraction patterns of papain intercalation compound of γ-TiP. Solution: 10 ml-aq, propylamine: 25 μL (0.04 mol dm^{-3}), γ-TiP: 0.1 g, Temp 30°C. Papain: (1): 30 mg, (2): 20 mg, (3): 10 mg, (4): 5 mg.

Fig. 1b. X-Ray diffraction patterns of catalase intercalation compound of γ-TiP. Solution: 10 ml-aq, γ-TiP: 0.1 mg, propylamine: 25 μl. Intercalation was carried out at (1): 39°C, (2): 30°C, (3): 20°C, (4): 5°C.

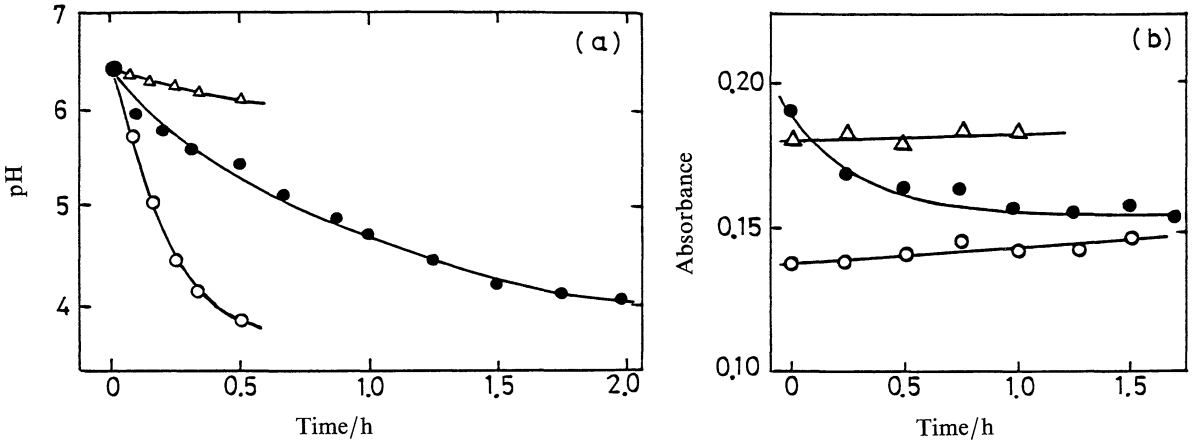

Fig. 2a Activity measurement for papain intercalation compound of γ-TiP (on decomposition of BAEE). Absorbance at 270 nm for buffer solutions containing; (○): aqueous papain solution, (●): papain intercalated γ-TiP, (△): supernatant solution of Fig. 1a-1.

Fig. 2b. Activity measurement for catalase intercalation compound of γ-TiP (on decomposition of H_2O_2). Absorbance at 240 nm for buffer solutions containing, (○): H_2O_2, (△): Catalase intercalated γ-TiP, (●): H_2O_2+catalase intercalated γ-TiP.

width might reflect the irregular arrangement of enzymes in the layered material.

An enzyme assay for the intercalation compound was carried out through the time dependence of the absorbance at 270 nm for BAEE according to the standard procedure.[7] The results are shown in Fig. 2b. A decay in absorbance was observed (closed circles), and indicating that the intercalated papain retained enzyme activity against BAEE. The decay was slow in comparison with that observed in a papain solution. Such slowness may be ascribable to the difficulty in contacting BAEE with papain in layered lattices. In addition, the

reaction was heterogeneous while the reaction of papain solution was homogeneous. The activity of papain in the supernatant solution was rather low since the intercalation reaction was carried out in a basic medium (propylamine solution). The activity of papain decreased rapidly in such a basic solution. (Papain must be kept in a buffer solution at pH=6.2.)

Procedure (2) Cointercalation of enzymes with propylamine was examined according to the success concerning the intercalation of phosphatidyl-ethanolamine.[8] No systematic results was obtained similar to that for Procedure (1). However, intercalation was found to occur in a catalase solution. The X-ray diffraction pattern strongly depended on the temperature in this reaction. At 5 °C, little evidence for the intercalation was observed, and most of the products were propylamine intercalation compound.[6] A product with a large interlayer spacing appeared at higher temperatures. The peak for the propylamine intercalation compound completely disappeared at around 39 °C (Fig. 1b) and a single-phase product with an average interlayer spaing of 8.5 nm (2θ=1.3°) was obtained. The line width was estimated to be 12 nm (0.9°)—5.5 nm (2.0°). The sequence of the basal plane reflection was not observed because of the large width of the spectrum.

It seems improbable that the γ-TiP formed a colloidal solution of single lamellae, which was cleaved from γ-TiP crystals in a propylamine solution. Thus the catalase intercalation compound with an interlayer spacing of around 8.5 nm must be formed through the delamination–relamination reaction of colloidal thin lamellae[4] of γ-TiP. A similar mechanism has been proposed in the intercalation of *t*-alkylamines into clay minerals.[9]

An enzyme assay of the catalase intercalation compound was carried out according to the standard procedure[10] (oxidation of H_2O_2). The enzyme activity was examine through the decrease in the absorbance at 240 nm due to H_2O_2 decomposition. Figure 2b shows the result. Catalase as well as its decomposition products show a strong absorbance at this wavelength, which often disturbs the measurements. A blank solution in the absence of H_2O_2 containing a catalase intercalation compound showed a certain residual absorbance due to catalase which might be released from the glass plates on which intercalation product was fixed. The solution of (H_2O_2+catalase intercalation compound) showed a rapid decay at this wavelength (closed circles). The infinite value did not reach zero because of the absorbance due to either catalase or its decomposition products which were released from the glass plate.

The supernatant solution which was used for the intercalation changed its original color (from brown to yellow) and did not show any enzyme activity against H_2O_2. On the other hand, the catalase intercalation compound kept its original color, suggesting that catalase was still alive. It can be concluded from the present results that the catalase intercalated in γ-TiP retained activity against H_2O_2 decomposition.

The intercalation of large-size enzymes was achieved in this study; the intercalation products exhibited enzyme activity even in an alkaline medium. Althogh the intercalation procedures adopted here seem to be somewhat tricky and no systematic results were obtained, the success of our methods offer some guiding priniciples regarding the intercalation of enzymes or new immobilizing methods for enzymes.

The authors wish to thank Professor M. Aizawa and Dr. H. Shinohara, Tokyo Institute of Technology, for their helpful suggestions and discussion concerning enzymes.

References

1) A. D. McLaren and E. F. Esterman, *Arch. Biochem. Biophys.*, **61**, 158 (1958), C. Schwabe, *Biochemistry*, **8**, 795 (1969), etc.
2) E. Kobayashi and S. Yamazaki, *Bull. Chem. Soc. Jpn.*, **56**, 1632 (1983).
3) Y. Kanzaki and M. Abe, *Bull. Chem. Soc. Jpn.*, **64**, 1846 (1991).
4) G. Alberti, M. Casciola, and U. Costantino, *J. Colloid Interface Sci.*, **107**, 256 (1985).
5) Y. Kanzaki and M. Abe, to be submitted.
6) U. Costantino, *J. Inorg. Nucl. Chem.*, **43**, 1985 (1981).
7) R. Arnon, "Methods in Enzymology," ed by G. E. Perlamnn and L. Lorand, Academic Press, New York (1970), Vol. XIX, p. 226.
8) Y. Kanzaki and M. Abe, *Bull. Chem. Soc. Jpn.*, in press.
9) Y. Okahata and A. Shimizu, *Langmuir*, **5**, 954 (1989).
10) B. Chance and A. C. Maehley, "Methods in Enzymology," ed by S. P. Colowick and N. O. Kaplan, Academic Press, New York (1955), Vol. II, p. 764.