Sweet Potato Acid Phosphatase Immobilized on Glutaraldehyde-Activated Aminopropyl Controlled-Pore Glass: Activation, Repeated Use and Enzyme Fatigue

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Sweet potato acid phosphatase was covalently coupled with glutaraldehyde to aminopropyl controlled-pore glass, and used as a pre-column enzyme reactor. The immobilized enzyme reactor (IMER) was continuously operated using an automated chromatographic detection system we developed. Functional evaluation of the IMER was carried out by injecting ten samples on the same day at an injection amount of 1.25 nmol (62.5 nmol per ml) using riboflavin sodium phosphate (FMNs) as a substrate, and by prolonged use for ten months. The IMER exhibited decreased activity after repeated use for a total of 3000 samples, but about 75% of its original activity remained. The conversion rate of FMNs to riboflavin by IMER was increased from 89 to 97% by adding citrate, ethylenediaminetetraacetic acid disodium salt, etc., but especially by adding citrate. The increased conversion of FMNs to riboflavin due to the addition of citrate was probably not due to the chelation of heavy metal ions by citrate. We also investigated the complex formation of acid phosphatase with the substrate FMNs using surface plasmon resonance to determine the effect of citrate on the processes of association and dissociation between the enzyme and substrate. Enzyme fatigue was also observed during the course of prolonged and repeated use.

Key words acid phosphatase; immobilized enzyme; enzyme fatigue; riboflavin phosphate; repeated use; activation

Acid phosphatase (orthophosphoric monoester phosphohydrolase) from sweet potato is a non-specific enzyme, similar to those of other plant and animal origins.1,2) Covalent attachment of the enzyme to controlled-pore glass, a highly porous hydrophilic material, offers several advantages for use in packed reactors because of its mechanical and chemical stability, selectivity of the enzymatic reaction, economical use gained by immobilization and ability to use repeatedly without serious loss of activity.3,4)

Riboflavin monophosphate (flavin mononucleotide, FMN) is one of two flavin coenzymes derived from riboflavin, vitamin B2. Commercial FMN preparations contain seven different flavin phosphates, i.e., riboflavin 5'-phosphate (5'-FMN), riboflavin 4'-phosphate (4'-FMN), riboflavin 3'-phosphate (3'-FMN), riboflavin 4',5'-diphosphate, riboflavin 3',4'-diphosphate, riboflavin 3',5'-diphosphate and riboflavin.5) Immobilized sweet potato acid phosphatase catalyzed the hydrolysis of FMN.6)

In a previous paper, we described the development of an automated chromatographic detection system for the determination of total riboflavin phosphates using a sweet potato immobilized enzyme reactor (IMER) as a pre-column reactor, and observed some increase in the conversion rate from FMN to riboflavin by additives in sample solutions.7) However, it is unclear which kinds of additives increase the conversion rate, why they do so, and to what extent the sweet potato acid phosphatase bound to controlled-pore glass.

MATERIALS AND METHODS

Chemicals Acid phosphatase (orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from sweet potato (Type XA, suspension in 1.8 M ammonium sulfate and 10 mM magnesium chloride, pH 5.3, 18 U/mg protein) was purchased from Sigma (St. Louis, MO, U.S.A.). Aminopropyl controlled-pore glass (aminopropyl-CPG, 1400 Å pore diameter, 120—200 mesh) was obtained from CPG (Lincoln Park, NJ, U.S.A.), p-Nitrophenyl phosphate (disodium salt, hexahydrate) and p-nitrophenol were obtained from Wako (Osaka, Japan). Riboflavin sodium phosphate (FMNs) were purchased from E. Merck (Darmstadt, Germany). Riboflavin standard, the Japanese Pharmacopoeia standard, was a product of the Japanese National Institute of Health (Tokyo, Japan). HPLC-grade methanol was obtained from Kanto (Tokyo). A CM5 sensor chip, HBS-EP buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20) and a 10% solution of Tween 20 (Surfactant P20) were purchased from Biacore AB (Uppsala, Sweden). N-Ethyl-N'-dimethylaminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of analytical-reagent grade.

Preparation of Immobilized Acid Phosphatase and Enzyme Reactor The procedure for the preparation of immobilized acid phosphatase and IMER was described in our previous papers.6,7) In brief, sweet potato acid phosphatase was immobilized by covalent coupling with glutaraldehyde to aminopropyl controlled-pore glass (aminopropyl-CPG). The Schiff base double bond and the residual aldehyde groups were reduced with sodium borohydride. The immobilized acid phosphatase thus obtained was packed into a stainless
steel column of 50×4 mm ID, and used on-line as a pre-column IMER at 30 °C.

**Preparation of Sample Solution and Standard Solution**

The preparation of both FMNs and riboflavin standard solution has also been described in detail in a previous paper. The solutions were carefully preserved in light-resistant containers.

**Assay of Enzyme Activity**

The effect of a heavy metal ion (cupric ion, Cu²⁺) on the soluble acid phosphatase activity was measured as follows: 50-µl of a known concentration of cupric chloride and 20-µl of the enzyme solution were added to 0.9 ml of 0.5 M acetate buffer, pH 5.6, and then incubated for 30 min at 40 °C. After incubation, the enzyme reaction was started by adding 30 µl of 0.6 M disodium p-nitrophenyl phosphate solution as a substrate. The reaction was allowed to continue for 10 min at 40 °C, when 2 ml of 0.5 M NaOH was added, and then the absorbance of the liberated p-nitrophenol was measured at 405 nm by a spectrophotometer using p-nitrophenol as a standard. For measurement of the recovery effects of the additives on the enzyme inhibition by Cu²⁺, known concentrations of additives (citrate or ethylene-diaminetetraacetic acid disodium salt, EDTA) were co-incubated with cupric chloride, and assayed as described above. Enzyme activity was expressed as a percentage of that without metal ion and/or additives.

**On-Line Automated Analytical System for Continuous Use IMER**

A schematic diagram of the pre-column IMER/reversed-phase high-performance liquid chromatographic (HPLC) system is shown in Fig. 1. The analytical system used throughout this study was the same as that described in a previous paper. Three Shimadzu LC-6A pumps (Kyoto) and two “six-port” switching valves (FCV-2AH, Shimadzu) were controlled by a system controller (SCL-6B, Shimadzu). Three different solutions, specifically two carrier streams, i.e., a weak solution of acetic acid (5 mM) and 0.5 M acetate buffer, pH 5.0, and a mobile phase, were pumped through the individual pumps. Sample solutions containing 1.25 nmol amounts of FMNs were injected through an automated sample injector (SIL-6B, Shimadzu) at a sample volume of 20 µl. The IMER was thermostated at 30 °C. The sample solution was passed through the IMER via a carrier stream containing 0.5 M acetate buffer, pH 5.0, at a flow-rate of 0.5 ml/min, and the flow was then passed through a trap column (TSK guardgel ODS-80Ts, 15×3.2 mm ID, Tosoh, Tokyo). After 10 min, the switching valve (SV) on this line, SV-2, was switched, and then the unaltered FMNs and enzymatically hydrolyzed riboflavin were back-eluted from the trap column using a mobile phase containing methanol. The mobile phase consisted of 0.1 M ammonium formate buffer, pH 3.7–methanol (73 : 27, v/v) and was pumped at a flow-rate of 1.0 ml/min. FMNs and riboflavin were subsequently chromatographed on an analytical column (TSK gel ODS-80Ts, 150×4.6 mm ID, 5-µm particle, Tosoh), and detected by UV absorption at 280 nm with a spectrophotometer (SPD-6A) equipped with an 8-µl flow cell. The trap column and analytical column were thermostated at 50 °C in a column oven (CTO-6A, Shimadzu). Chromatographic data were recorded on a Chromatopac CR-3A integrator (Shimadzu). During the period of separation and detection, the trap column was programmed to wash with 5 mM acetic acid via an opposite IMER line, i.e., a column-free line, and then buffered with 0.5 M acetate buffer, pH 5.0, for the next analysis. The time program of the on-line pre-column IMER/HPLC analytical system for the determination of total FMNs was described in detail in a previous paper.

**Surface Plasmon Resonance Analysis**

All measurements were performed on a Biacore X instrument from Biacore AB (Uppsala, Sweden). The principle and application of the system for surface plasmon resonance (SPR) detection were described by Karlsson et al. Acid phosphatase was immobilized to a CM5 sensor chip via primary amine groups as described in the literature. The carboxylated dextran matrix on the sensor surface was washed with HBS-EP buffer at a flow rate of 5 µl/min at 30 °C, and then activated by injecting a mixture (75 µl) of 100 mM NHS and 400 mM EDC in equal volumes. In order to immobilize acid phosphatase to the activated dextran matrix, 300 µl of acid phosphatase solution at a concentration of 560 µg protein/ml (20.9 unit/mg protein) in 10 mM sodium acetate buffer, pH 4.0, was successively injected using a Biacore control program. The remaining activated NHS-ester groups were blocked by injection of 75 µl of 1 M ethanolamine hydrochloride, pH 8.5. The SPR measurements for riboflavin binding to acid phosphatase with or without citrate were performed in 10 mM sodium acetate buffer, pH 5.0, 0.15 M NaCl and 0.005% Tween 20, at a flow rate of 20 µl/min for 3 min. The sensor surface was regenerated with a 60-µl injection of 10 mM glycine–HCl buffer, pH 2.5. The rates of association (Kₐ) and dissociation (Kₐ) were determined using the BIAevaluation 3.0 software program, and the equilibrium dissociation constant (Kₐ) was estimated using the formula Kₐ = Kₐ / Kₐ.

**RESULTS AND DISCUSSION**

In a previous paper, we partially observed that the enzymatic conversion of FMNs to riboflavin by IMER was enhanced by the addition of certain additives. When a sample solution without additives was chromatographed, a small 4′-FMN peak was observed on the chromatogram, but when sample solutions containing additives were analyzed, the 4′-FMN peak disappeared and the conversion rate increased. The conversion rate of FMNs to riboflavin by the IMER without additives was about 89%. The addition of KCl, ethanol or methanol at a final concentration in the sample solution of 150 mM and 50% for KCl and alcohols, respectively, brought the conversion rate up to 91—93%.
crease the conversion rate by adding malonate, succinate, glutarate, tartarate, lactate, malate, citrate and EDTA. Figure 2 shows the results of an experiment to measure the conversion ratio as a function of the final concentration of additives in the sample solution. Citrate, malate, EDTA and tartarate were all effective, with the addition of citrate to the sample solution being extremely effective, and a final concentration of 50 mM was sufficient. The conversion ratio increased about 1.09-fold, and the conversion rate of FMNs to riboflavin was approximately 97%. Certain phosphatases had associated phosphotransferases, which transfer the phosphate group approximately 1.09-fold, and the conversion rate of FMNs to riboflavin was lower than that of EDTA, whereas the concentration of citrate for complete recovery of inhibition of the enzyme by Cu2+ required about a 10-fold excess compared with EDTA. It was thus found that the effective addition of citrate did not result from the chelation of heavy metals in the sample solution. SPR detection allows direct observation of the molecular interaction between an analyte in solution and its immobilized binding partner, and this observation includes the monitoring of low-molecular-weight analytes and that of low affinity interactions. We investigated the complex formation of acid phosphatase with the substrate FMNs using SPR in order to clarify the effect of citrate on the processes of association and/or dissociation between the enzyme and substrate. The SPR signal for immobilized acid phosphatase was found to be about 13500 resonance units (RU). The calculated maximum binding capacity, Rmax, was 64.7 RU. All independent experiments were performed over the concentration range of 0.5 mM to 2.5 mM FMNs in triplicate. The citrate/FMNs ratio was kept constant at 4. Figure 4 depicts representative BIAcore sensorgrams of FMNs with or without the addition of citrate. Kinetic analyses of individual sensorgrams gave $K_a = 7.39 \times 10^2$ M$^{-1}$ s$^{-1}$ (mean value), $K_d = 1.15$ s$^{-1}$ (mean value) and $K_D = 1.56 \times 10^{-3}$ M with a standard deviation of $1.4 \times 10^{-4}$ M without citrate, and $K_a = 5.79 \times 10^2$ M$^{-1}$ s$^{-1}$, $K_d = 1.05$ s$^{-1}$ and $K_D = 1.81 \times 10^{-3}$ M with a standard deviation of $2.6 \times 10^{-3}$ M with citrate. This indicates that the $K_p$ for the acid phosphatase riboflavin phosphate complex was slightly increased by the addition of citrate. The addition of citrate to FMNs may induce a conformational change in the enzyme and make the catalytic site more easily accessible to FMNs, thereby increasing the enzymatic turnover reaction. Interestingly, the addition of citrate increased the conversion rate, although the increase was slightly different after repeated use of the IMER. The immobilized acid phosphatase was freshly packed and used in the IMER in order to determine the stability behavior of the IMER and to investigate the activation by citrate during the course of prolonged, repeated use. IMER function was evaluated using the on-line automated pre-column IMER/reversed-phase HPLC system we constructed. The IMER was thermostated at 30°C. Five samples without citrate, followed...
by five samples with citrate, were each continuously injected on the same day in amounts of 1.25 nmol (62.5 nmol per ml) using FMNs as a substrate, and the IMER was repeatedly used over an extremely long period of time. Therefore, sample injection was performed at ten samples per day, with 3000 samples over ten months. The change in conversion rate from FMNs to riboflavin is shown in Fig. 5. The IMER did not require the addition of so much citrate at the beginning of use, but the effectiveness of citrate addition was gradually observed. This result also indicates that IMER activity decreased during the course of prolonged, repeated use. However, even after 1500 continuous injections of individual samples with and without citrate had been made, i.e., a sum total of 3000 samples, about 75% of its original activity remained. Chromatograms obtained during the course of repeated use are shown in Fig. 6, in which those for the beginning, middle and end of experiments are represented. The FMN preparations used throughout this study contained 3'-FMN, 4'-FMN, 5'-FMN and riboflavin, in proportions of approximately 8:11:69:12. 4'-FMN was somewhat resistant to enzymatic hydrolysis (Fig. 6C), but underwent hydrolysis after the addition of citrate (Fig. 6D). The peak of 4'-FMN increased following continuous injection of the samples (Figs. 6E, G). The peak of 4'-FMN did not disappear even after the addition of citrate (Figs. 6F, H). The appearance of individual non-hydrolyzed components is illustrated clearly in Fig. 7. As shown in Fig. 7A, 4'-FMN was first detected, and 3'-FMN subsequently appeared. 5'-FMN was the most susceptible FMN to the enzyme, but 5'-FMN appeared slowly after prolonged, repeated use. Concurrently, Fig. 7B shows that the addition of citrate suppressed the appearance of non-hydrolyzed 3', 4'- and 5'-FMNs. For example, non-hydrolyzed 3'-FMN appeared after 700 continuous injections of each individual sample without citrate had been made, i.e., a sum total of 1400 samples (Fig. 7A). On the other hand, non-hydrolyzed 3'-FMN appeared after 900 continuous in-
jections of each individual sample with citrate had been made, i.e., a sum total of 1800 samples (Fig. 7B). This finding also suggests that the addition of citrate can retard the appearance of non-hydrolyzed FMNs. Nevertheless, enzyme fatigue due to prolonged, repeated work was observed by the end of the experiments. Even 5′-FMN, i.e., the most abundant and susceptible phosphate ester among FMNs, was then not completely hydrolyzed. We interpret this phenomenon to indicate enzyme fatigue.

Immobilized enzymes have been employed as model systems in studying microenvironmental effects on enzyme action, as described in a prior report by Katchalsky et al. In the present study, we tested a unique immobilized enzyme application as a model system to investigate enzyme fatigue. In this manner, immobilized enzyme technology will be available in a broad range of applications in the future.

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Fig. 5. Change in Conversion Rate with Continuous Injection of FMNs
Symbols: ● = with citrate; ○ = without citrate.

Fig. 6. Chromatograms of FMNs Preparations and Riboflavin Standard Obtained Using the On-Line Automated Pre-column IMER/HPLC System
Sample solutions containing 1.25 nmol amounts of FMNs were injected through an automated sample injector at a sample volume of 20 μl. HPLC conditions were described in Materials and Methods. FMNs preparation (A) and riboflavin standard (B) were measured without IMER. (C) through (H) were measured with IMER. (C), (E) and (G) were measured in the absence of citrate. (D), (F) and (H) were measured in the presence of citrate. Chromatograms at the beginning (number of sample injections near 100), the middle (near 600) and the end (near 1200) of the experiments are represented in (C) (D), (E) through (H), respectively. Peaks: 1 = 3′-FMN; 2 = 4′-FMN; 3 = 5′-FMN; 4 = riboflavin.
REFERENCES


Fig. 7. Appearance of Non-hydrolyzed FMNs with Continuous Injection of FMNs and the Effect of Citrate on Hydrolysis of FMNs

(A){eq}^5\text{FMN, \textit{m}}^3\text{FMN, \textit{d}}^4\text{FMN, \textit{e}}^5\text{FMN.} \text{(B) without citrate; (B) with citrate. Symbols: \text{\textbullet}^3\text{FMN, \textbullet}^4\text{FMN, \textcircled{\textbullet}^5\text{FMN.}