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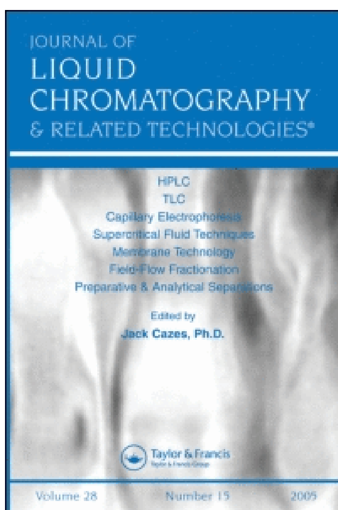

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Michaelis-Menten Analysis of Alkaline Phosphatase by Capillary Electrophoresis Using Plug-Plug Reaction

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MICHAELIS-MENTEN ANALYSIS OF ALKALINE PHOSPHATASE BY CAPILLARY ELECTROPHORESIS USING PLUG-PLUG REACTION

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ABSTRACT

This work evaluated the use of an alkaline phosphatase plug, slowly migrating in a polyacrylamide-coated capillary filled with buffer/soluble polymer, to convert *p*-nitrophenylphosphate (which was injected into the capillary as a separate plug, and migrated faster than the enzyme) to *p*-nitrophenol under a constant applied potential. The elution of assay components was monitored on-capillary at 230 nm. The initial reaction velocity of the enzyme-catalyzed reaction was estimated from the peak area ratio of the enzyme product to the internal standard. Using the Lineweaver-Burk plots, an average Michaelis constant (K_M) for alkaline phosphatase was calculated to be 4.8 ± 0.3 mM (n = 4, CV = 6%). With the constant potential electrophoresis (8 kV/57 cm), the method had a detection limit of 4.4 x 10^{-5} IU for alkaline phosphatase and a linear range up to 2.1 x 10^{-3} IU.

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INTRODUCTION

Enzymes are biological catalysts that play a vital role in biochemical reactions necessary for normal human growth, maturation, and reproduction. Measurement of enzymes in body fluids (such as blood, urine, cerebrospinal, and extracellular fluid) is routinely performed for diagnosis of disease. Due to their low concentrations in biological fluids, sometime it is difficult to measure enzymes directly by masses. However, enzymes can be measured more easily by their catalytic activities, which are the most relevant properties of enzymes in the biochemical context.

Measurements of enzyme activity by capillary electrophoresis (CE) which have been under active investigation in recent years, include (a) enzyme reaction prior to CE analysis,[1-11] (b) enzyme reaction mediated by CE (also known as electrophoretically mediated microanalysis, EMMA),[12-24] (c) reaction of immobilized enzyme on electrophoretic capillary,[25,26] and (d) enzyme reaction post CE separation.[27,28] Among them, enzyme activity assay by EMMA has shown a high degree of versatility and attracted most research attentions. EMMA utilizes differential electrophoretic mobilities of enzyme, substrate, and product to initiate enzyme-substrate reaction during capillary electrophoresis and to separate assay components from each other for final on-capillary quantitation. Up to now, three approaches have been proposed including (a) zonal injection EMMA,[12-20] the more frequently used format, (b) moving boundary EMMA,[21,22] and (c) plug-plug reaction,[23,24] the least tested format.

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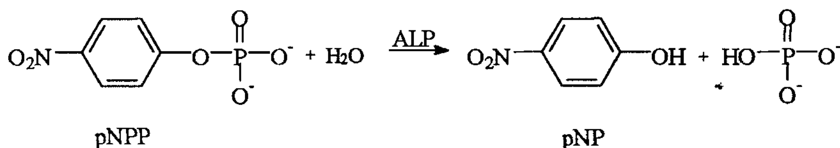
We have chosen enzyme alkaline phosphatase (ALP, EC 3.1.3.1) and substrate *p*-nitrophenylphosphate (pNPP) as a model system to illustrate the feasibility of plug-plug reaction in CE for Michaelis-Menten analysis. ALP is a membrane-bound enzyme that has engaged the interest of clinical chemists for more than 70 years.[29] The origins of ALP isoforms include tissue nonspecific ALP (e.g., liver/bone type), germ cell ALP, placental ALP, and intestinal ALP. The measurement of total serum ALP and its isoforms plays a significant role in the diagnoses of hepatocellular and cholestatic diseases,[30] colorectal cancer,[31] and bone disorders.[32] ALP catalyzes the following reaction:



Under alkaline conditions, ALP transfers the phosphate group from pNPP to water, forming pNP and inorganic orthophosphate. In the present study, a polyacrylamide-coated capillary filled with buffer/soluble polymer was used as

the reaction vessel and separation column. Quantitation of enzyme activity was achieved by internal calibration, and Michaelis constant (K_M) was calculated from the Lineweaver-Burk plots.

EXPERIMENTAL

Apparatus

A Beckman P/ACE 2050 capillary electrophoresis system (Beckman Instruments, Fullerton, CA) was used in this study with the cathode on the injection side and the anode on the detection side. The aforementioned capillary electrophoresis system contains built-in 200, 214, 230, 254, 260, 280, and 300 nm narrow-band filters for on-line detection and quantitation. The capillary tubing (Polymicro Technologies, Phoenix, AZ) was of polyacrylamide-coated fused silica (the coating procedure had been documented elsewhere[33] with an internal diameter of 100 μm before the coating and a total length of 57 cm (50 cm from the injection side to the detector). The temperature of the column was maintained at 25°C and the elution was monitored on-column at 230 nm or otherwise specified. The electropherograms were collected and analyzed using an IBM personal computer with System Gold™ software (Beckman Instruments).

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Chemicals

ALP from calf intestine (cat. no. 405612) and pNPP were purchased from Boehringer Mannheim GmbH (Germany). *p*-Nitrophenol (pNP) and glycine were obtained from Sigma Chemical (St. Louis, MO). Tris (hydroxymethyl)aminomethane (Tris) was from Bio-Rad (Richmond, CA). Magnesium chloride was from Fisher Scientific (Fair Lawn, NJ). (Hydroxypropyl)methylcellulose (HPMC) was a product of Fluka Chemical (Ronkonkoma, NY). *N,N,N',N'*-Tetramethylethylenediamine (TEMED), γ-(methacryloyloxy)propyltrimethoxysilane (bind-silane), ammonium persulfate, and acrylamide were purchased from Pharmacia Biotech (Piscataway, NJ). All reagents were at least of analytical grade.

Solutions

All aqueous solutions were prepared in the Type I deionized water from a Barnstead NANOpure deionization system (Barnstead/Thermolyne, Dubuque, IA). Buffer I consisted of 300 mM Tris and glycine, and 5 mM $MgCl_2$ at pH 8.2. Buffer II was Buffer I containing 0.2% HPMC (e.g., 0.2 g HPMC/100 mL

buffer solution). These buffers were filtered through 0.45 μm cellulose-acetate-membrane filters (Alltech, Deerfield, IL) and stored at 4°C until use. The working solutions of ALP, pNPP, and pNP were prepared daily in Buffer I and equilibrated to the assay temperature (25°C) in a thermostatic water bath prior to the analysis.

CE Conditions

Separation of pNP and pNPP

A mixture of pNP and pNPP in Buffer I was injected into the capillary by pressure at 3.4 x 10^3 N/m^2 (0.50 psi) for 3 s. The electrophoresis ran at a constant voltage of 8 kV in Buffer II and generated a current of approximately 22 μA. Between runs, the capillary was rinsed with Buffer II at 1.4 x 10^5 N/m^2 (20 psi) for 2 min.

ALP assay

A sample solution containing enzyme ALP and internal standard pNP in Buffer I was injected by pressure at 3.4 x 10^3 N/m^2 for 3 s into the capillary equilibrated with Buffer II. ALP and pNP were electrophoresed at 8 kV for 6 min to separate pNP from ALP, and to provide a volume of buffer prior to the injection of enzyme substrate. Enzyme substrate pNPP in Buffer I was then injected by voltage at 5 kV for 3 s. The electrophoresis ran at 8 kV to complete elution of the enzyme substrate and product (pNP). Between runs, the capillary was rinsed with Buffer II at 1.4 x 10^5 N/m^2 for 2 min.

RESULTS AND DISCUSSION

Plug-Plug Reaction Inside Electrophoretic Capillary

In the plug-plug reaction approach (Figure 1), a plug of slowly migrating enzyme (ALP) together with an internal standard (pNP) is hydrodynamically injected into a polyacrylamide-coated capillary. After initial separation of the internal standard from the enzyme plug, a plug of substrate (pNPP) is introduced by electrokinetic injection into the capillary. Since the enzyme and substrate have different apparent mobilities, the contact of ALP and pNPP is achieved by adjusting the timing of injection and the strength of electric field. In this study, the polyacrylamide-coated capillary was used to suppress electroosmotic flow; therefore, only anionic species were to migrate toward the anode and the detector.

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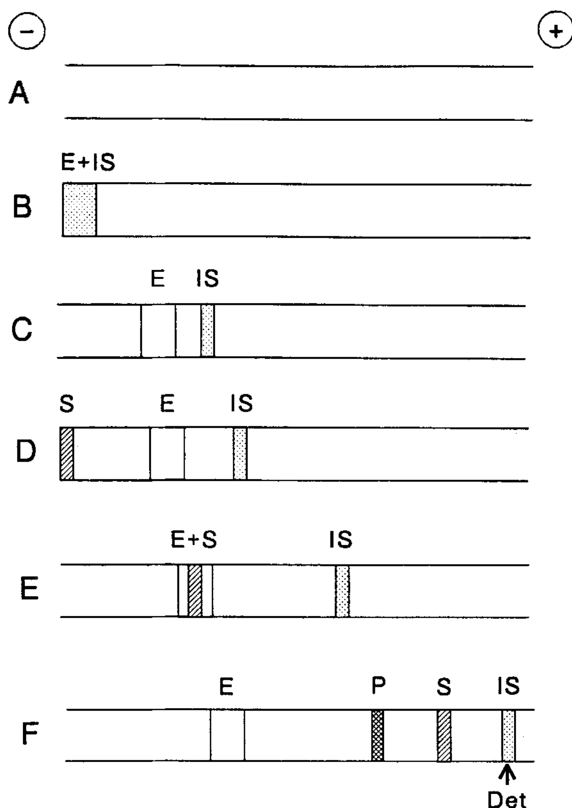

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Figure 1. An illustration of ALP assay by CE using plug-plug reaction approach. (A) A polyacrylamide-coated capillary is initially filled with the enzyme reaction buffer; (B) a plug of enzyme, E, together with an internal standard, IS, is hydrodynamically injected into the capillary; (C) an electric field is applied to separate the internal standard from the enzyme plug; (D) a plug of enzyme substrate, S, is introduced into the capillary by electrokinetic injection; (E) the fast migrating substrate plug traverses across the slow migrating enzyme plug under an electric field; (F) a product, P, is generated during the enzyme-substrate reaction and the subsequent migrating bands are measured by the detector, Det.

The order of migration times (Figure 2) was pNP (internal standard, IS) < pNPP < pNP (enzyme product) << ALP (the migration time of ALP was 3172.9 s and the electropherogram was not shown). The extent of conversion of substrate pNPP to product pNP was a function of the amount of ALP in the enzyme plug.

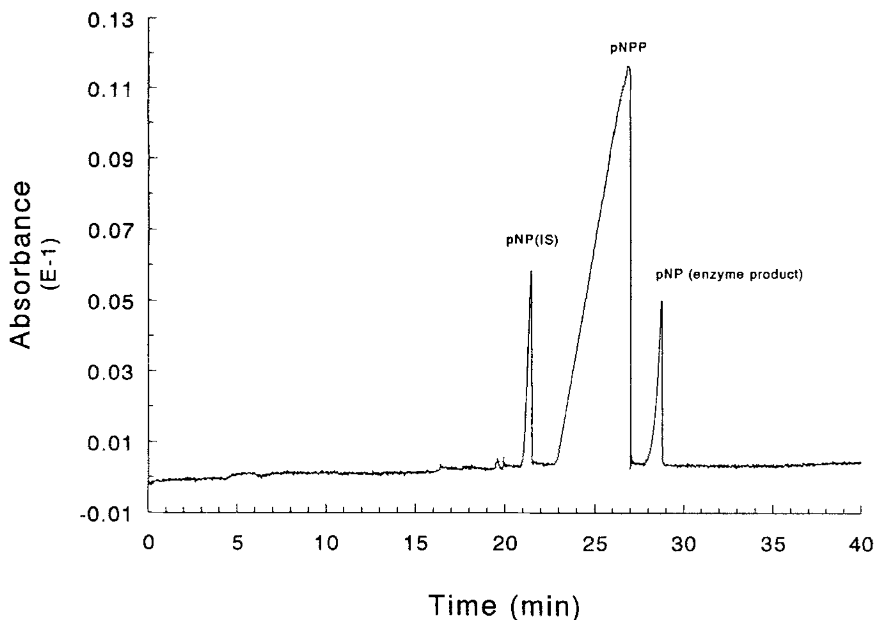

Figure 2. Electropherogram of ALP assay. ALP, 10 IU/mL; pNP (internal standard, IS), 300 μM; pNPP, 20 mM. See EXPERIMENTAL section for CE conditions.

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In comparison to the more frequently used zonal injection EMMA,[12-20] the merits of the plug-plug reaction approach are at least twofold. Firstly, the electropherogram of the plug-plug reaction is similar to that of the conventional CE. There is no signal stepping or artifact peak; therefore, there is no need for baseline correction. Secondly, the electrophoretic process prior to the mixing of enzyme and substrate permits the separation of potential interferents (e.g., enzyme inhibitors or activators) in sample from enzyme of analytical interest. This feature may be advantageous when assaying enzyme activity in crude biological sample.

Quantitation of pNP

The CE system used in this work contains built-in 200, 214, 230, 254, 260, 280, and 300 nm narrow-band filters for on-line detection. We have measured the absorbances of pNP and pNPP under the aforementioned wavelengths (Figure 3). The wavelength chosen for the enzyme assay detection was 230 nm.

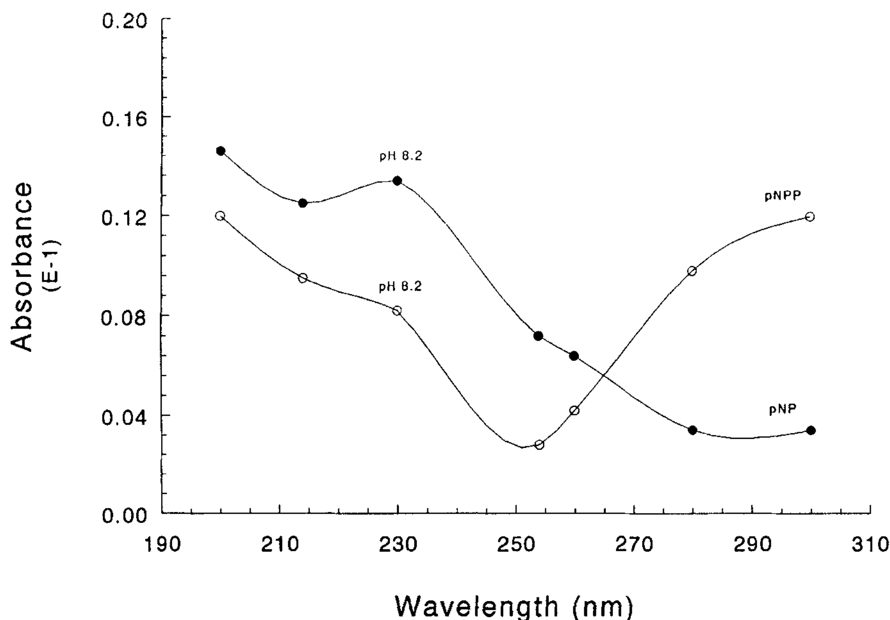

Figure 3. Absorption spectra of pNP and pNPP in CE. UV filters, 200, 214, 230, 254, 260, 280, and 300 nm; pNP, 614 μM; pNPP, 510 μM. Conditions for separation of pNP and pNPP were the same as those in the EXPERIMENTAL section. Absorbance represents the peak absorbance of pNP or pNPP.

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Although both pNP and pNPP absorb at this wavelength, the resolving power of CE enable us to separate the product of enzyme reaction from the substrate and detect them sequentially. This feature of the method permits the use of substrate and product with similar spectral characteristics, which is prevented by the standard spectrophotometric assays.

Quantitation of ALP activity can be achieved by measuring the concentration of pNP generated by the enzyme reaction. To determine the linear range of pNP detection, a calibration curve was constructed (Figure 4), where the concentrations of pNP from 11.2 to 614 μM were plotted against the peak areas of pNP (A_{pNP}). The detection of pNP by CE showed to be linear over the tested pNP concentration range with a regression coefficient of 1.00. The limit of detection (LOD) for pNP, defined as the ratio of the triple average area of 30 baseline peaks to the area of pNP (signal/noise = 3) was found to be 0.534 μM. According to our calculation, 44.4 nL (less than 1% of the total volume of the capillary) of pNP were injected into the capillary by a 3-s injection at 3.4 x 10^3 N/m^2. This LOD corresponded to 23.7 fmol of pNP.

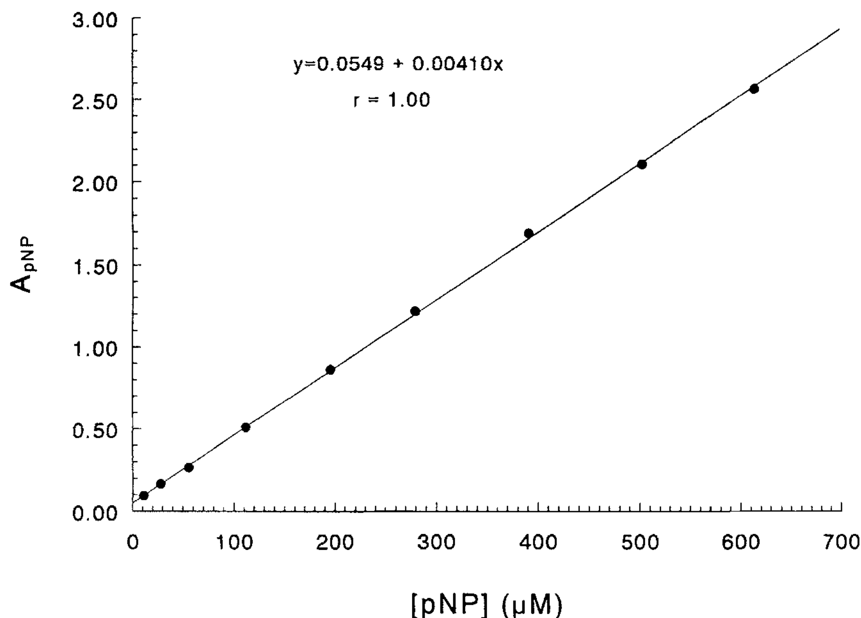

Figure 4. The calibration curve of pNP. CE conditions were the same as those in Figure 2.

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Estimation of Enzyme-Substrate Reaction Time

In ALP activity assay, a plug of enzyme containing internal standard was first injected into the capillary by pressure injection, and Equation (1)[34] was used to calculate the length of the plug (L_1):

$$L_1 = \frac{\Delta P r^2 t_1}{8\eta L_t} \qquad (1)$$

where ΔP (N/m^2) is the pressure difference between the ends of the capillary, r (cm) is the inner radius of the capillary, t_1 (s) is the injection time, η (Ns/m^2) is the viscosity of the medium, and L_t (cm) is the total length of the capillary. After the initial separation of the internal standard from the enzyme plug, a plug of substrate (pNPP) was introduced by electrokinetic injection into the capillary. By knowing the electrophoretic mobilities of pNPP, pNP, and ALP, and assuming no enzyme adsorption on the polyacrylamide-coated capillary[33] and negligible magnitude of longitudinal diffusion of pNPP, pNP, and ALP in the

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Table 1

The Calculated Parameters of ALP Assay by CE Using Plug-Plug Reaction

Compound	Electrophoresis[a] (8kV, 3.0 s)	Pressure Injection[b] ($3.4x10^3$ N/m², 3.0 s)	Initial Separation[c] (8kV, 360.0 s)		Electrokinetic Injection[c] (5kV, 3.0 s)	
	μ_{app} (cm²/Vs)	L_1 (cm)	$v_{app,s}$(cm/s)	L_2(cm)	$v_{app,I}$(cm/s)	L_3(cm)
pNPP	3.5×10^{-4}	NA[d]	4.9×10^{-2}	NA[d]	3.1×10^{-2}	9.3×10^{-2}
PNP (IS)	2.9×10^{-4}	1.4×10^{-3}	4.1×10^{-2}	15	2.5×10^{-2}	7.5×10^{-2}
ALP	1.1×10^{-4}	1.4×10^{-3}	1.5×10^{-2}	5.4	9.6×10^{3}	2.9×10^{-2}

[a] Experimental conditions were the same as those in Figure 2.
[b] Assuming that the viscosity of Buffer II was 4.00×10^{-1} Ns/m².
[c] Experimental conditions were the same as those in Figure 2.
[d] NA -- Not applicable.

capillary filled with Buffer II, we could calculate the migration distances of pNP and ALP during the initial separation step (L_2) and during the electrokinetic injection (L_3), as well as the length of substrate plug injected electrokinetically (L_3) by Equation (2) or Equation (3):[34]

$$L_2 = \upsilon_{app,s} t_2 = \mu_{app} \frac{V_s}{L_t} t_2 \tag{2}$$

$$L_3 = \upsilon_{app,I} t_3 = \mu_{app} \frac{V_I}{L_t} t_3 \tag{3}$$

where μ_{app} (cm²/Vs) is the apparent mobility of the species being studied in Buffer II; $\upsilon_{app,S}$ (cm/s) is the apparent velocity of the species under the separation conditions, t_2 (s) is the initial separation time, V_S (V) is the separation voltage, and L_t (cm) is the total length of the capillary; and $\upsilon_{app,I}$ (cm/s) is velocity of the species during the electrokinetic injection, t_3 (s) is the time of electrokinetic injection, and V_I (V) is the injection voltage.

With the knowledge of the experimental conditions and the calculated assay parameters (Table 1), the starting time (t_s) and the ending time (t_e) of the on-capillary enzyme reaction after the injection of enzyme substrate could be calculated by the following Equations:

$$v_{app,S}(pNPP)t_s + L_3(pNPP) = v_{app,S}(ALP)t_s + L_2(ALP) + L_3(ALP) \tag{4}$$

$$t_3 = \frac{L_2(ALP) + L_3(ALP) - L_3(pNPP)}{\nu_{app,S}(pNPP) - \nu_{app,S}(ALP)} = 157s \quad (5)$$

$$\nu_{app,S}(pNPP)t_e = \nu_{app,S}(ALP)t_e + L_1(ALP) + L_2(ALP)$$
$$L_3\,(ALP) \quad (6)$$

$$t_e = \frac{L_1(ALP) + L_2(ALP) + L_3(ALP)}{\upsilon_{app,S}(pNPP) - \upsilon_{app,S}(ALP)} = 160\,s \quad (7)$$

From the values of t_e and t_s, the total enzyme-substrate reaction time (t_e - t_s) could be calculated to be 3 s. Since the longitudinal diffusion of pNPP was apparent (Figure 2), the calculated enzyme-substrate reaction time was not the actual enzyme-substrate reaction time. Therefore, it could only serve as a rough estimate.

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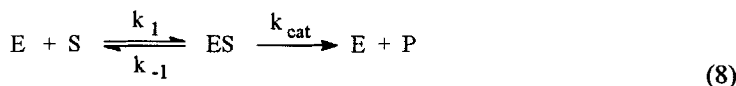
Michaelis-Menten Analysis of ALP

For enzyme-catalyzed reactions that employ a single substrate or the hydrolysis of a single substrate, the following postulated reaction scheme may be used:



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \quad (8)$$

where E is enzyme, S is substrate, and P is product. The reaction proceeds through an intermediate enzyme-substrate complex (ES). When all of the enzyme is in the ES state (i.e., the enzyme is saturated with substrate), the initial reaction velocity (υ_0) reaches its maximal (V_{max}).

The relation between initial reaction velocity (υ_0) and substrate concentration ([S]) can be described mathematically by the Michaelis-Menten equation:

$$\upsilon_0 = \frac{V_{max}[S]}{K_M + [S]} \quad (9)$$

In this expression, K_M is the Michaelis constant and equal to $(k_{-1} + k_{cat})/k_1$, where the k values are the specific rate constants of Equation (8). K_M is equivalent to the substrate concentration that yields an initial reaction velocity

(υ_0) equal to one-half the maximum initial reaction velocity (V_{max}), and has the dimensions of mol/L. Although K_M is not the true dissociation constant of the enzyme-substrate complex, the value of K_M reflects the stability of the enzyme-substrate interaction and is of great practical value.

Experimentally, we may measure the value of K_M by the Michaelis-Menten plot (υ_0 versus [S]); however, it is far more accurate and convenient to use the Lineweaver-Burk plot to determine K_M, which has a linear relation between $\frac{1}{\upsilon_0}$ and $\frac{1}{[S]}$, and can be obtained by inversion of the Michaelis-Menten equation:

$$\frac{1}{\upsilon_0} = \frac{K_M}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{10}$$

In Lineweaver-Burk plot, initial reaction velocities at different substrate concentrations are plotted as reciprocals. Values for K_M and V_{max} are obtained from extrapolation of the line, where $\frac{1}{V_{max}}$ is the ordinate intercept, $-\frac{1}{K_M}$ is the abscissa intercept, and $\frac{K_M}{V_{max}}$ is the slope.

In this experiment, since a constant potential electrophoresis was used for the on-capillary enzyme assay, the fast migrating substrate plug traversed through the slow migrating enzyme plug in a short period of time (3 s), and the enzyme concentration was considerably low compared to that of substrate; therefore, the loss of substrate during the period of assay was negligible and the initial reaction velocity (υ_0) could be measured directly from the peak area of the enzyme product. For calibration purposes, an internal standard (pNP) was used, which was injected into the capillary together with the enzyme and separated from the enzyme plug prior to the introduction of enzyme substrate. The peak area ratio (A_P/A_{IS}) of the enzyme product (pNP) to the internal standard (pNP) was used as initial reaction velocity for constructing the Lineweaver-Burk plot. The use of peak areas instead of peak heights eliminates the error caused by the longitudinal diffusion, and the use of internal calibration corrects the deviations in enzyme injection, migration time, and detector response during the enzyme assay.

Figure 5 shows the typical Lineweaver-Burk plots for ALP-catalyzed reactions that hydrolyze pNPP to pNP in Buffer II (pH 8.2, 25°C) at *four* different ALP concentrations (3.9, 7.8, 16, and 31 IU/mL). Linear regression

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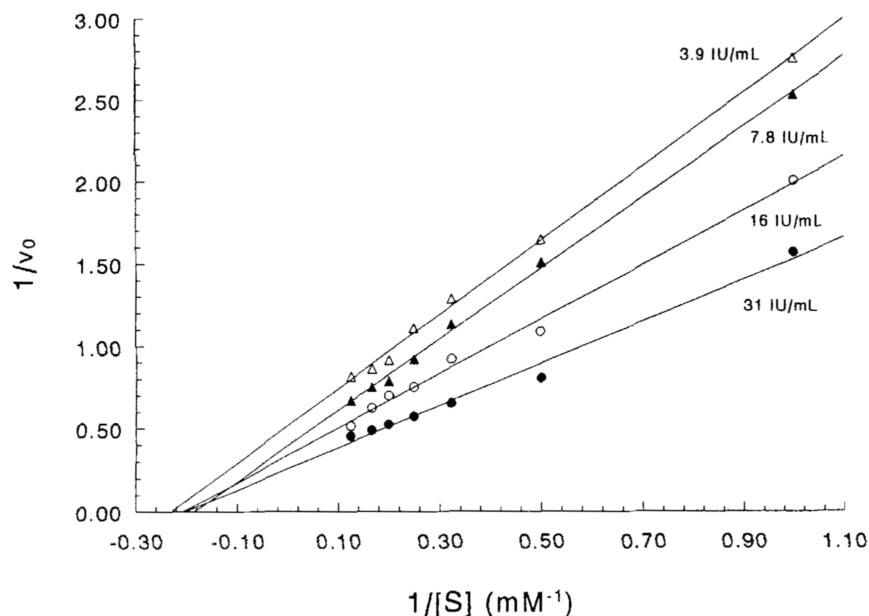

Figure 5. The Lineweaver-Burk plot. Experimental conditions were the same as those in Figure 2. $\frac{1}{\upsilon_0} = \frac{1}{A_P/A_{IS}}$, where A_P is the peak area of enzyme product (pNP) and A_{IS} is the peak area of internal standard (pNP). $\frac{1}{[S]}$ is the reciprocal concentration of enzyme substrate (pNPP).

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analysis was used for plotting the straight lines, and the regression coefficients for these four lines were 1.0, 1.0, 0.99 and 1.0, respectively. K_M values obtained from the intercepts on the abscissa were 4.4, 4.9, 4.9 and 5.2 mM, representing an average K_M value of 4.8 ± 0.3 mM (n = 4, CV = 6%).

ALP Activity Assay

The catalytic activity of ALP can be measured by Michaelis-Menten equation derived from the steady-state reaction conditions:[35]

$$v_0 = \frac{V_{max}[S]}{K_M + [S]} = \frac{k_{cat}[E_T]\cdot[S]}{K_M + [S]} \tag{11}$$

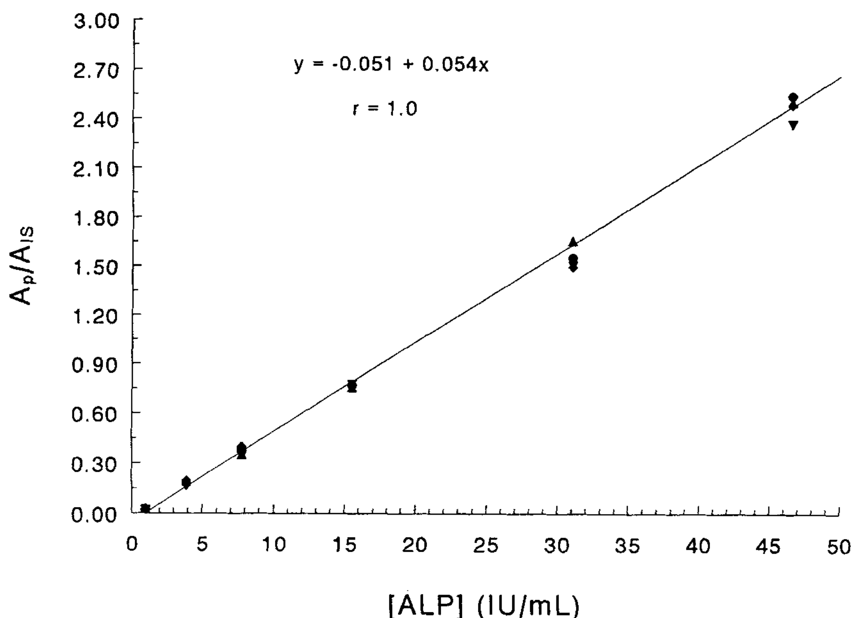

Figure 6. The calibration curve of ALP activity. Experimental conditions were the same as those in Figure 2.

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In this formulation, [S] is the initial substrate concentration and $[E_T]$ is the total enzyme concentration ([E] + [ES]). Because k_{cat} and K_M are constants of the system and [S] is known from the assay conditions, the initial reaction velocity (υ_0) is directly proportional to $[E_T]$. If such high concentration of substrate is used that only a negligible loss occurs during the assay, a linear relationship of υ_0 to $[E_T]$ can be derived. Figure 6 shows such a linear calibration plot for ALP by constant potential electrophoresis (8 kV) using pNP as internal standard and 20 mM pNPP as substrate (> 4 times K_M).

In this plot, each of the six ALP concentrations was run in triplicate and linear regression analysis was used for drawing the straight line (r = 1.0). Over the calibration range, CVs varied from 1.1 to 10%. The enzyme assay has a LOD of 0.99 IU/mL or 4.4 x 10^{-5} IU (signal/noise = 3) and a linear range up to 47 IU/mL or 2.1 x 10^{-3} IU. If the separation voltage is turned off (V_S = 0 V) after the substrate plug completely migrates into the enzyme plug, the total enzyme reaction time can be extended and a lower LODs of the enzyme may be achieved.

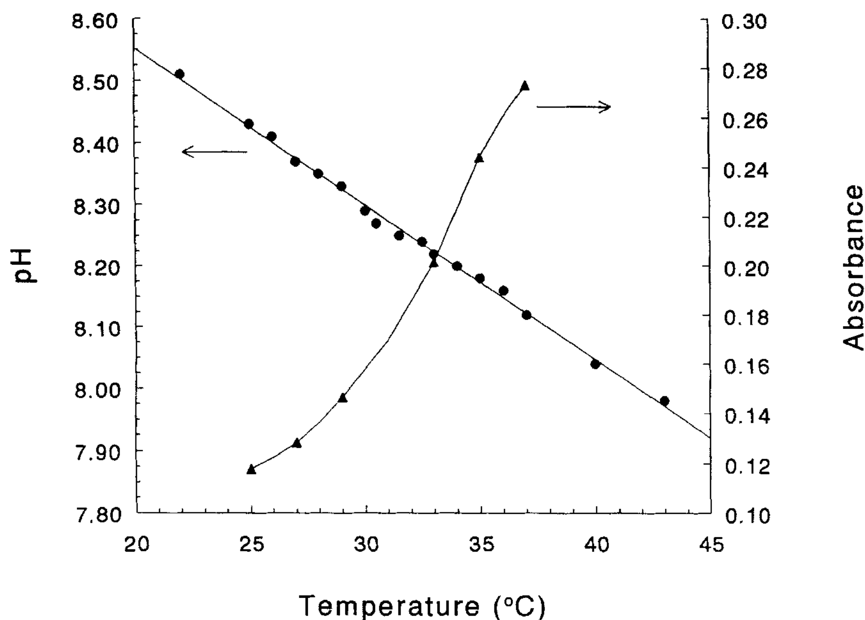

Figure 7. The effects of temperature on ALP activity and buffer pH. Experimental conditions were the same as those in Figure 2. Absorbance represents the peak absorbance of the enzyme product (pNP).

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Effect of Temperature on ALP Activity

The effect of temperature on enzyme activity in electrophoretic capillary is shown in Figure 7. Higher assay temperatures resulted in higher ALP activities that were expressed in terms of peak absorbances of the enzyme product in the electropherograms. When the assay temperature increased from 25 to 37°C, the enzyme activity increased more than two times.

In CE, it is relatively easy to maintain an assay temperature that is near the room temperature; therefore, 25°C was used as the assay temperature. It should be noted that the pH values of enzyme reaction buffer could also be affected by temperature.

In this assay, a linear decrease in pH of Buffer II was observed as increasing temperature (Figure 7). However, the change of pH caused by temperature did not override the effect of temperature on ALP activity.

CONCLUSION

This report has demonstrated that it is practical to use the plug-plug reaction approach in capillary electrophoresis for Michaelis-Menten analysis of ALP. The assay procedure can quantitatively measure K_M value and ALP activity. Compared to bulk-solution enzyme assays, this procedure uses far less reagents and analysis time, and can detect substrate and product simultaneously. It can also eliminate the artifact peaks resulting from the continuous enzyme-substrate reaction in the zonal injection EMMA procedure, and make signal integration simple and precise. The use of polyacrylamide coating on the inner wall of capillary and soluble polymer in the assay buffer prevent the possible enzyme adsorption and reduce the diffusion inside the capillary. This method has the potential to be applied to other enzyme-substrate systems that exhibit differential electrophoretic mobilities among enzyme, substrate, and product.

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