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Michaelis–Menten analysis of immobilized enzyme by affinity capillary electrophoresis*

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Abstract: Michaelis constant of enzymatic reaction was evaluated by affinity capillary electrophoresis using β-galactosidase as a model enzyme and *o*- and *p*-isomers of nitrophenyl-β-galactoside as substrates. The enzyme was immobilized on the inner surface of a fused-silica capillary by the covalent bonding through a bridging group, and the substrates were introduced into the capillary. The reaction products migrated electrophoretically toward the detection side (anodic side), while the unreacted substrates moved toward the injection side (cathodic side) on a slow electroosmotic flow generated by the weak negative charge of the immobilized enzyme. The initial velocity of the enzymatic reaction was estimated from the peak height of the product, and the Michaelis constant was calculated according to Lineweaver–Burk equation. The results (K_m, 2.34 mM for *o*-isomer and 1.09 mM for *p*-isomer) were reproducible (RSD < 11.8%, $n = 5$). Although the estimated Michaelis constants were larger than the reported values measured in homogeneous solution, the ratio of the Michaelis constants of *o*-/*p*-isomers was in good agreement with the literature value. The present method required as low as a few microgram amount of enzyme and nanogram amount of substrate which is far smaller than those required in a conventional affinity HPLC.

Keywords: *High-performance capillary electrophoresis; β-galactosidase; o-nitrophenyl-β-galactoside; p-nitrophenyl-β-galactoside.*

Introduction

High-performance capillary electrophoresis (HPCE) has been appreciated in a wide variety of analytical fields because of high separation efficiency, rapid analysis and small sample size [1–3]. In biological analysis, HPCE can be successfully applied not only to the separation of biopolymers such as proteins and nucleotides but also to the estimation of the biological affinity. So far, capillary electrophoretic methods have been proposed for the estimation of bioaffinity such as drug–protein binding [4–6] and the interaction between antibiotics and peptides [7]. However, these reversible interactions are different from the enzymatic reaction presented here in that the latter involves chemical conversion of substrate into product. Recently, the capillary zone electrophoresis using tandem capillary enzyme reactor [8] and the ultramicro enzyme assay using capillary electrophoretic system [9] were proposed. In the former method, enzyme reaction was carried out in the enzyme-immobilized capillary, and the products were analysed by use of the coupled capillary zone electrophoresis. In the latter method, the free enzyme solution and the substrate solution were introduced into a capillary. The mixing of these solutions, the subsequent enzymatic reaction, and the determination of the product were carried out in the same capillary.

The present paper demonstrates another approach to apply HPCE to estimate kinetics of enzymatic reaction. Enzyme (β-galactosidase) was immobilized onto the capillary wall, and the substrate (*o*- and *p*-nitrophenyl-β-galactosides) solutions of different concentrations were injected into the capillary. The enzymatic reaction occurred in a limited zone of the substrate solution in the capillary, and the initial reaction velocity was estimated from the peak height of the product (*o*- and *p*-nitrophenol). Based on the data analysis using Lineweaver–Burk equation, the Michaelis–Menten constants were calculated.

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Experimental

Materials and reagents

β-Galactosidase, *o*-nitrophenyl-β-galactoside, *p*-nitrophenyl-β-galactoside, *p*-nitrophenyl-α-glucoside, and *m*-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) were purchased from the Sigma Chemical Company (St Louis, MO, USA). (3-Aminopropyl)triethoxysilane and 3-(N-morpholino) propanesulphonic acid (MOPS) were purchased from Wako Pure Chemicals (Osaka, Japan). Protein assay kit (kit II, cat No. 500-0002) was purchased from BIO-RAD Laboratories Inc. (Harbour Way South Richmond, CA, USA).

Apparatus

The instrument used was a CAPI-3000 (Otsuka Electronics Co., Ltd, Osaka, Japan) equipped with a photodiode array detector. Fused-silica capillaries (375-μm o.d., 75-μm i.d. total length 42 cm, effective length to detector 30 cm) were purchased from GL Sciences Inc. (Tokyo, Japan).

Amidation of capillary

The fused silica capillary was cleaned by perfusing (by vacuum suction) with 1 N NaOH for 30 min followed by a 10 min rinse with distilled water. The capillary was dried and (3-aminopropyl) triethoxysilane solution was continuously passed through the capillary for 30 min at room temperature. (3-Aminopropyl)triethoxysilane solution was used without dilution. One end of the capillary was sealed by melting and the opposite end was attached to a vacuum line. After evacuation of the capillary for about 20 min in order to remove excess (3-aminopropyl)triethoxy silane, the other end of the capillary near the vacuum line was sealed and kept at 45°C overnight.

Immobilization of β-galactosidase on a capillary

A 0.5 mg ml^{-1} solution of β-galactosidase in 20 mM MOPS/NaOH buffer (pH 7.1) containing 20 mM magnesium chloride was mixed with a solution of MBS (final concentration, 40 μg ml^{-1}). The capillary was rinsed with buffer (20 mM MOPS/NaOH, pH 7.1) and perfused with the mixed solution for approximately 60 min at room temperature and rinsed with buffer for 3 min. The decrease in the enzyme concentration in the solution after reaction was determined by using protein assay kit. The amount of immobilized enzyme thus estimated was about 3.5 μg per one capillary.

Evaluation of Micahelis constants

A mixture of 20 mM MOPS and 20 mM magnesium chloride (pH 7.1) was used as a running buffer solution. The running buffer solution and the capillary were kept in a thermostatic bath at 37°C until the beginning of the analysis. After each electrophoretic analysis, the capillary was washed with the buffer solution for 3 min. The sample solutions containing 0.5, 1, 5 and 10 mM substrate were prepared by dissolving known amounts of the substrates (*o*-nitrophenyl-β-galactoside, *p*-nitrophenyl-β-galactoside and their mixture) in the running buffer solution. The sample solution was hydrostatically introduced into the capillary for 20 sec (estimated volume, about 7 nl), and a high voltage (−10 kV, the injection side was cathodic) was applied. The reaction products, *o*-nitrophenol and *p*-nitrophenol, migrated toward the anodic side and were detected at their maximum UV absorption wavelength (410 nm).

The kinetics of the enzymatic reaction was analysed according to Lineweaver–Burk equation

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \qquad (1)$$

where V_0 and V_{max} are initial and maximal velocities of the reaction, respectively, $[S]$ is concentration of substrate, K_m is Michaelis constant, and V_{max} is the maximal velocity of the enzymatic reaction. When the reaction time is short and constant, the peak height can be proportional to the initial velocity. Thus, equation (1) is rewritten as

$$\frac{1}{H} = \frac{K_m}{A[S]} + \frac{1}{A} \qquad (2)$$

where H is the peak height of the product, and A is a constant proportional to V_{max}. In this study, the products gave somewhat scattered migration times (RSD, 23% for *o*-isomer, and 15.6% for *p*-isomer, $n = 20$). This was due to the variation in the electroosmotic flow rate, while the electrophoretic migration velocity of the product was considered to be constant. Since the unreacted substrate was pushed out from the capillary by the electroosmotic flow,

the variation in the electroosmotic flow causes the scatter of the enzymatic reaction time. In addition, the peak height of the product obtained from the same volume of substrate solution decreased with time, because the enzyme activity decreased gradaully as the time. Therefore, the peak height of the product (H_o) should be corrected by the following equation

$$H = H_0 \times \frac{T_i + (L'/V_{eo_1})}{T_i + (L'/V_{eo_n})} \times \exp[\lambda(t_n - t_1)]. \quad (3)$$

In equation (3), V_{eo_n} is the velocity of the electroosmotic flow in the nth run, and V_{eo_1} is that in the first run. In every series of four analyses of 0.5–10 mM substrate solutions, the first run was the highest substrate concentration (10 mM). t_n is the time when the nth run started, and t_1 is the time when the first run started. Therefore, $(t_n - t_1)$ represents the time interval between the first reaction and the nth reaction. λ is the inactivation rate constant of the immobilized enzyme. Ti is the injection time (20 sec). L' means the zone length of the capillary occupied by the substrate solution. Since it took about 62 min to fill the effective length of the capillary (30 cm), L' was estimated as 0.16 cm. The denominator and the numerator of the middle term in equation (3) indicate the first and the nth enzymatic reaction time, respectively, and, therefore, the middle term in equation (3) corrects the difference in the reaction time at the first reaction and the nth reaction, which was due to the electroosmotic flow. The last term corrects the inactivation of the enzyme [10]. V_{eo_1} and V_{eo_n} were calculated from equations (4) and (5), respectively.

$$V_{eo_1} = \frac{L}{T_1} - V_{ep} \quad (4)$$

$$V_{eo_n} = \frac{L}{T_n} - V_{ep}, \quad (5)$$

where L is the effective capillary length (30 cm) to the detector, T is the migration time of the product, and T_1 is the migration time of the product in the first run. V_{ep} is the electrophoretic migration velocity of the product, which was measured previously by use of a non-coated fused silica capillary (o-nitrophenol; 1.707 cm min^{-1}, p-nitrophenol; 1.865 cm min^{-1}). At the end of every series of the analyses, the sample solution of the first run was analysed again, and the λ value was estimated from equation (6), where H_1 and H_f indicate the peak height of the product in the first run and in the final run, respectively, and t_f is the time when the final run started. V_{eo_f} is the velocity of the electroosmotic flow in the final run.

$$H_f = H_1 \times \frac{T_i + (L'/V_{eo_f})}{T_i + (L'/V_{eo_1})} \times \exp[-\lambda(t_f - t_1)]. \quad (6)$$

The λ value was estimated in every series of the analyses, and the average was 0.07 h^{-1}.

Evaluation of electric charge of the protein in the immobilized enzyme capillary

The electrophoresis was carried out with 40 mM phosphate/NaOH buffer at pH 4.1 and 20 mM MOPS and 20 mM Mg^{++} buffers at pH values 6.3, 7.1 and 7.9. p-Nitrophenyl-α-glucoside was used as the marker to monitor electroosmosis, because this compound has no electric charge and does not react with β-galactosidase. The applied voltage was +10 kV (the injection side was anodic) at pH 6.3, 7.1 and 7.9, and −10 kV (the injection side was cathodic) at pH 4.1. The samples were injected for 20 s. The detection was achieved with absorption at 410 nm.

Result and Discussion

Figure 1 illustrates the schematic diagram of the present HPCE method. The products having negative charge migrate toward the detector, while the substrates having substantially no electric charge gradually moved out of the capillary from the injection end by the slow electroosmotic flow of the running buffer. Although the treatment of the inner-wall of the capillary by (3-aminopropyl)triethoxysilane suppressed the electroosmotic flow to substantially zero, the subsequent immobilization of the enzyme generated the electroosmotic flow because of the slightly negative charge of the enzyme. Since the substrate was pushed out by the electroosmotic flow within about 10 s, the reaction time is very short, and consequently the peak height of the product reflects the initial reaction rate. Our preliminary experiment revealed that when the substrate solution (10 mM o-nitrophenyl-β-galactoside)

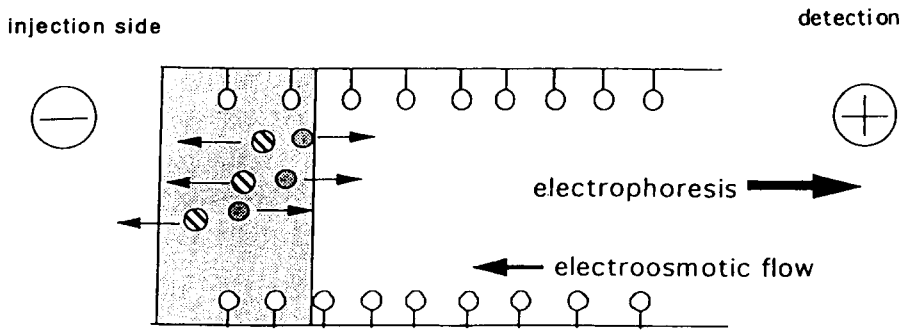

Figure 1
Scheme of the reaction between the enzyme and the substrate inside a fused-capillary. Symbols; ● substrate, ◍ product, ○ enzyme.

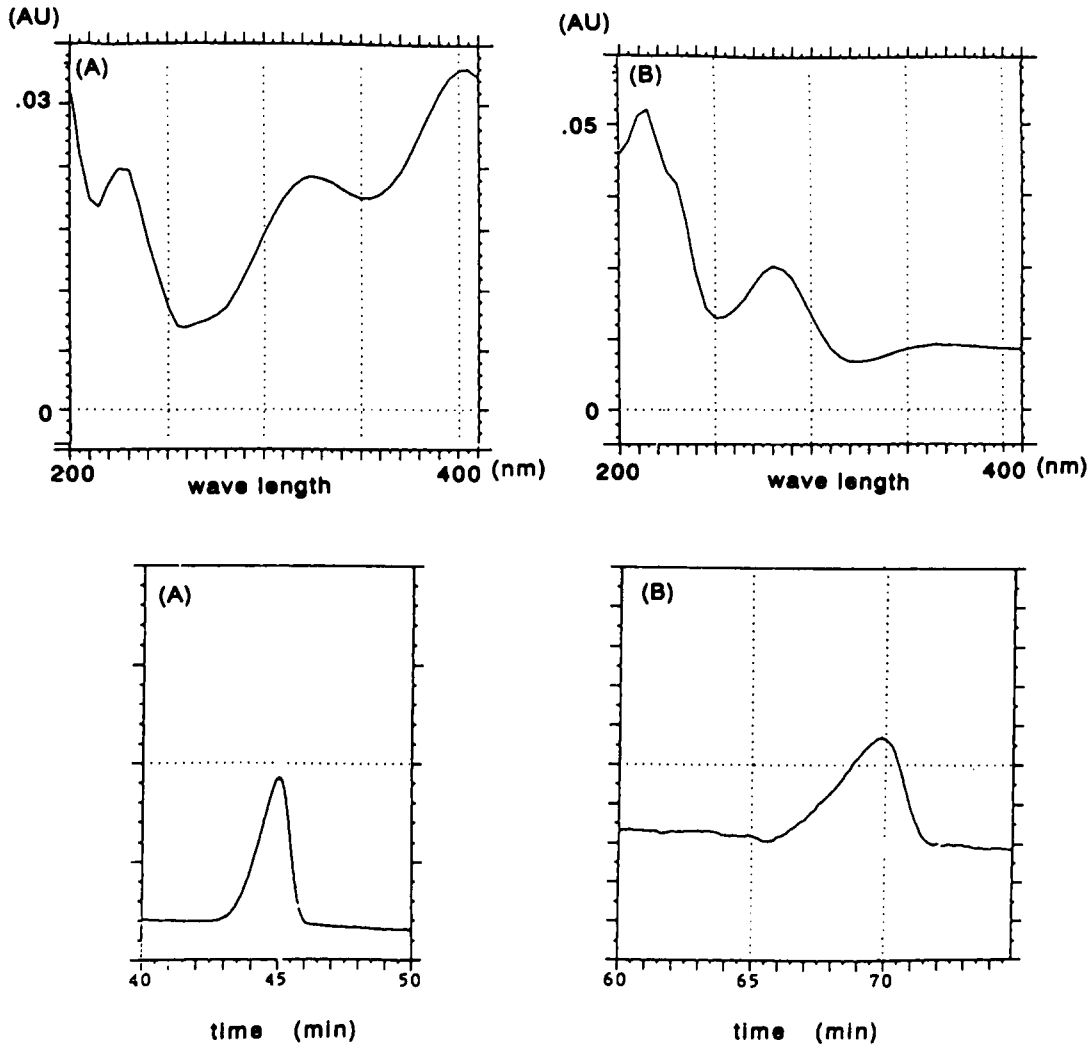

Figure 2
Electropherograms and UV spectra of the products, (A) *p*-nitrophenol and (B) *o*-nitrophenol by the enzyme reaction. Conditions; capillary, 42 cm long (effective length 30 cm), 75 μm i.d. Applied voltage, −10 kV. Current, 60 μA. Temperature, 37°C. Detection, 410 nm. Injection, 20 s. Buffer; 20 mM MOPS/NaOH (pH 7.1) including 20 mM $MgCl_2$.

was injected into the enzyme-immobilized capillary for 20, 30, 60, 120 s, the peak height of the product increased almost proportionally with the injection time (for example; the peak height in the case of 20, 30, 60, 120 s was 4.00×10^{-3}, 6.50×10^{-3}, 1.20×10^{-2}, 2.42×10^{-2} AU). This indicates that the reaction velocity was constant regardless of the injection time, and this velocity can be considered to be the initial velocity of the enzymatic reaction. Based on this result, the injection time was selected as 20 s.

Figure 2(a) shows the electropherogram and the spectrum of *p*-nitrophenol produced from *p*-nitrophenyl-β-galactoside. Figure 2(b) shows those of *o*-nitrophenol. *p*-Nitrophenol migrated faster than *o*-nitrophenol, which indicates that *p*-nitrophenol has stronger charge than *o*-nitrophenol because of the difference in the dissociation constant of the phenolic OH group.

Figure 3 shows the typical Lineweaver–Burk plot. It is found that β-galactosidase has the higher affinity with *p*-nitrophenyl-β-galactose than *o*-nitrophenyl-β-galactoside. Table 1 gives the result for Michaelis constant for each substrate calculated from equation (2). The value of Michaelis constant, K_m, for *o*- and *p*-nitrophenyl-β-galactosides are 2.34 mM (RSD = 7.21%, n = 5) and 1.09 mM (RSD = 11.8%, n = 5), respectively. These values are significantly larger than those obtained from the corresponding reactions in the homogeneous solutions (K_m = 0.950 mM and 0.445 mM for *o*- and *p*-nitrophenyl-β-galactoside, respectively) [11]. The pH (7.1) and temperature (37°C), which influence the enzymatic reaction [12], were almost the same with those in the literature (pH 7.3, 4°C) [11]. Therefore, the decrease in the enzymatic affinity by the immobilization of enzyme is possibly due to conformational change in the higher structure of the enzyme during immobilization. In addition, the substrate solution was introduced into the capillary column by siphoning, and this could result in axial dilution of the substrate solution by a laminar flow profile. The diffusional or mass-transfer effect of the substrates from the bulk solution inside the capillary to the inner surface could also make the K_m values apparently larger. However, the ratio of the Michaelis constants for the isomers (*o*-/p- = 2.15) remained same as that of the literature values (*o*-/*p*- = 2.13). This indicates the enzyme did not loose its specificity. Therefore, the present method is applicable to the comparison of the enzyme rection between a series of substrates such as steric isomers. Furthermore, the on-line three dimensional detection is beneficial to the simultaneous identification of the products.

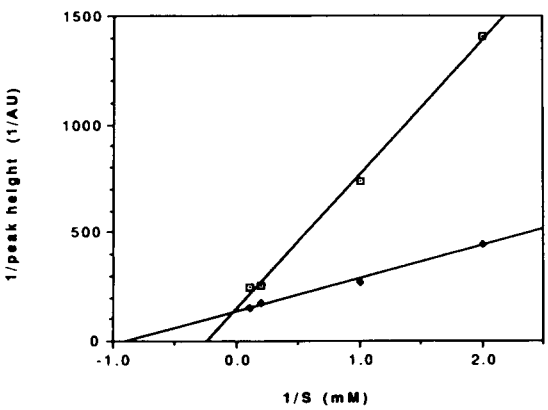

Figure 3
Lineweaver–Burk plot of the *o*- or *p*-nitrophenyl-β-galactoside and β-galactosidase reaction. Symbols; (-□-) *o*-nitrophenyl-β-galactoside, (-●-) *p*-nitrophenyl-β-galactoside.

The effect of pH on the migration time of *p*-nitrophenyl-α-glucoside was investigated to evaluate the electroosmotic flow. Since *p*-nitrophenyl-α-glucoside has no electric charge, the migration depends upon electroosmotic flow alone. At pH 4.1, the electroosmotic flow directs toward the anodic end, since the immobilized β-galactosidase has a positive charge (isoelectric point; 5.4). The electro-

Table 1
Michaelis constants of *o*-NPG and *p*-NPG for β-galactosidase*

		K_m (mM)	RSD (%) (n = 5)	*o*-/*p*-ratio
Present method	*o*-NPG	2.34	7.21	2.15
	p-NPG	1.09	11.8	
Reference data [11]	*o*-NPG	0.950	—	2.13
	p-NPG	0.445	—	

**o*-NPG; *o*-nitrophenyl-β-galactoside. *p*-NPG; *p*-nitrophenyl-β-galactoside.

osmotic mobility was 1.37×10^{-4} cm^2 V^{-1} s^{-1}. In contrast, the enzyme has a negative charge at pH 6.3, 7.1 and 7.9, respectively. Therefore, the electroosmotic flow directs toward the cathodic end. In addition, higher pH makes the enzyme more negatively charged, which results in the faster electroosmotic flow. The electroosmotic mobility at pH 6.3, 7.1 and 7.9 was 9.52×10^{-5}, 1.06×10^{-4} and 1.20×10^{-4} cm^2 V^{-1} s^{-1}, respectively. When pH of the buffer is near the isoelectric point of the enzyme, the electroosmotic flow is very slow. In such case, it is probably difficult to estimate the Michaelis constant accurately, because the reaction time increases and, hence, the peak height of the product does not reflect the initial velocity.

It is well known that intracapillary temperature is increased by Joule heating during electrophoresis [13–15]. The equipment used in this study controls the capillary temperature by air thermostating using Peltier cooler. According to the manufacturer (Otsuka Electronics), it was estimated that the intracapillary temperature increased from 25 to 37°C by applying 2 W m^{-1} of power density (10 kV and 100 μA; capillary size, 50 cm × 75 μm). In our present study, the applied voltage, current and capillary size were −10 kV, 60 μA and 42 cm × 75 μm, respectively. Assuming that the increase in intracapillary temperature is proportional to the power density, the increase in the temperature is estimated as 5.7°C. The enzymatic reaction time is the sum of the sample loading time (20 s) and the residence time of substrate in the capillary. The latter time was about 6 s, calculated from the electroosmotic flow (1.06×10^{-4} cm^2 V^{-1} s^{-1}) and the capillary zone length occupied by the substrate solution (0.16 cm). Joule heat was not generated during the sample loading, because no voltage was applied. The enzymatic reaction suffered from the Joule heat during only the latter period (6 s). In addition, the increase in the temperature during this short period would be less than the estimated value (5.7°C), because the temperature does not increase momentarily. Therefore, the effect of Joule heat on the enzymatic reaction is considered to be not serious.

Conclusions

The enzyme-immobilized capillary electrophoresis method has several advantages that (i) very small amounts of enzyme and substrate are required, (ii) Michaelis–Menten constants of immobilized enzymes can be evaluated easily by on-line analyses, and (iii) the enzymatic reaction of a series of substrate such as steric isomers can be easily compared.

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