

# Capillary Electrophoresis/Dynamic Frontal Analysis for the Enzyme Assay of 4-Nitrophenyl Phosphate with Alkaline Phosphatase

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A substrate of 4-nitrophenyl phosphate was enzymatically hydrolyzed by alkaline phosphatase (ALP) in a capillary tube, while an injected zone of the substrate was electrophoretically migrating in the separation buffer containing the enzyme by capillary electrophoresis (CE). During CE migration of the substrate from the start time of the electrophoresis to the detection time of the substrate, the substrate was continuously hydrolyzed by ALP to form a product of 4-nitrophenolate, and a plateau signal of 4-nitrophenolate was detected as a result of the zero-order kinetic reaction. The height of the plateau signal was directly related to the reaction rate, and it was used for the determination of a Michaelis-Menten constant through Lineweaver-Burk plots. Since the plateau signal is attributed to the dynamic formation of the product by the enzymatic reaction in CE, this analysis method is named as capillary electrophoresis/dynamic frontal analysis (CE/DFA). In CE/DFA, the CE separation is included on detecting the plateau signal, and the hydrolysis product before the sample injection is resolved from the dynamically and continuously formed product. The inhibition of the enzyme with the product is also eliminated in CE/DFA by the CE separation.

**Keywords** Dynamic frontal analysis, capillary electrophoresis, enzymatic reaction, Michaelis-Menten constant, alkaline phosphatase, 4-nitrophenyl phosphate, 4-nitrophenolate

(Received December 16, 2019; Accepted January 9, 2020; Advance Publication Released Online by J-STAGE January 17, 2020)

## Introduction

Capillary electrophoresis (CE) is one of the established separation techniques; ionic substances are resolved through their electrophoretic mobility. When a sample solution containing equilibrium species is introduced into a capillary with a relatively long plug, one of the equilibrium species, usually unbound species of a small ion, electrophoretically migrates in the capillary and a long zone of the equilibrium species is detected as a plateau signal as a result of the bound/free (B/F) separation. This format of CE is called as capillary electrophoresis/frontal analysis (CE/FA); the height of a plateau signal is used for quantification of the unbound species, and CE/FA has been utilized for equilibrium or affinity analysis.<sup>1-4</sup> Binding equilibria of drug-protein complexes,<sup>5,6</sup> peptide complexes,<sup>2</sup> liposome-analyte interactions,<sup>3</sup> and alkaloid-DNA complexes<sup>7</sup> have been investigated by CE/FA. Inhibitors of protein-protein interaction were also investigated by CE/FA.<sup>8</sup> CE/FA is applicable to both fast and slow equilibria.<sup>9-11</sup> Simulation of the plateau signal was also studied with the binding of ibuprofen to hydroxypropyl- $\beta$ -cyclodextrin.<sup>12</sup> A simulation program of Simul 5 Complex is applicable to CE/FA.<sup>13,14</sup>

As for the enzyme assays, CE is widely used in the dynamic

or kinetic reactions of enzymes. The utilization of CE on enzyme assays is divided broadly into two categories. One is the pre-capillary reaction format and the other is an in-capillary reaction format. The latter is commonly called as electrophoretically mediated microanalysis (EMMA).<sup>15</sup> In the pre-capillary format, the enzymatic reaction is done in a sample vial containing both an enzyme and a substrate, and a small portion of the reaction solution is periodically analyzed and the product is quantified by CE or micellar electrokinetic chromatography. Michaelis-Menten constants have been determined by the pre-capillary format for GTPase activity,<sup>16</sup> urease assay,<sup>17</sup> sirtuin assay,<sup>18</sup> hydrolysis of bovine  $\kappa$ -casein,<sup>19</sup> methionine sulfoxide reductase,<sup>20</sup> and *N*-demethylation of ketamine.<sup>21</sup>

To investigate the reaction dynamics of an enzyme, the format of an in-capillary reaction (EMMA) is more attractive. This is because the reaction conditions are controllable on the bases of the injection sequence, the length of the injected zones, and the electrophoretic migration of the substances. One of the first EMMA was reported with glucose-6-phosphate dehydrogenase.<sup>22</sup> Both an enzyme solution and a substrate solution, which contained a substrate of glucose-6-phosphate and a coenzyme of NADP, were tandemly injected into the capillary. When a DC voltage was applied to the capillary, the two zones electrophoretically counter-migrated in the capillary and a zone of the enzyme passed through the sample zone. In a period of the enzyme zone passing through the substrate zone, the enzymatic reaction proceeded and the products of 6-phosphogluconate and NADPH were constantly formed.

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A plateau signal of the formed NADPH was detected and used for the enzyme assay.<sup>22</sup> The EMMA was examined in various formats including the in-capillary dynamic reactions. A Michaelis-Menten constant of alkaline phosphatase (ALP) was determined on the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol by the tandem injections of the enzyme and the substrate solutions.<sup>23</sup> An enzyme inhibition of ALP was examined with theophylline by an EMMA format by a sequential injections of the inhibitor solution and the enzyme solution to a separation buffer containing a fluorogenic substrate of AttoPhos.<sup>24</sup> An EMMA format was also applied to the oxidative deamination of aromatic primary amine by amine oxidase.<sup>25</sup> While the plateau signal was instable at the early stage of the EMMA, the stability of the plateau signal was developed by thermostating,<sup>26</sup> and the enzyme-inhibitor assay was also studied with sodium vanadate, sodium arsenate, and EDTA.<sup>26</sup> Although plateau signals were sometimes detected by these EMMA analyses, the plateau signals were not connected with CE/FA. The EMMA assay was also studied with immobilized capillary.<sup>27-31</sup> A silica capillary was dynamically coated with polybrene to immobilize ALP.<sup>28</sup> Acetylcholinesterase was immobilized on polyethylenimine-coated capillary.<sup>29</sup> Immobilized enzyme in a capillary was also used for the assays of trypsin<sup>30</sup> and tyrosinase inhibitors.<sup>31</sup> This type of EMMA is classified as immobilized capillary enzyme reactor (ICER).<sup>28</sup> Single enzyme molecule assay was studied by incubating the enzyme molecule in the substrate solution filled in the capillary.<sup>32-35</sup> The inhibitor dissociation constant of an enzyme molecule of  $\beta$ -galactosidase was also studied by stopping the applied voltage and incubation.<sup>36</sup>

In this study, a different format of EMMA was examined. A capillary was filled with an enzyme solution of alkaline phosphatase, and a substrate solution of 4-nitrophenyl phosphate was introduced into the capillary. In this CE format, a sufficient amount of the substrate was migrating in the enzyme solution, and a product of 4-nitrophenolate was continuously generated by the enzymatic reaction during the CE. A plateau signal was detected with the product. This plateau signal of the product was pulled out from the short zone of the substrate by the dynamic enzymatic reaction, and this format is comparable to CE/FA. While the ordinary CE/FA is based on the separation of the equilibrium species from the equilibrium mixture, the EMMA-based plateau signal is a result of the dynamic reaction of the enzyme. Therefore, the proposed EMMA format would be called as capillary electrophoresis/dynamic frontal analysis (CE/DFA).

## Experimental

### Reagents and chemicals

An enzyme of alkaline phosphatase (ALP; from bovine intestinal mucosa, EC: 3.1.3.1) was obtained from Sigma-Aldrich (St. Louis, MO, USA). A substrate of disodium 4-nitrophenyl phosphate (NPP) was from Kanto Chemical (Tokyo, Japan). 4-Nitrophenol (NP) as a reaction product and Methyl Orange (MO) as an internal standard were from FUJIFILM Wako Pure Chemical (Osaka, Japan). All other reagents were of analytical grade. Water used was purified by a Milli-Q Gradient A10 (Merck Millipore, Billerica, MA, USA). The separation buffer was prepared with 10 mmol L<sup>-1</sup> sodium tetraborate (borax) + NaOH (pH 9.8). Alkaline phosphatase was also contained in the separation buffer at a concentration of 0.87 unit mL<sup>-1</sup>.

### Apparatus

An Agilent Technologies (Waldbronn, Germany) <sup>3D</sup>CE was

used as a capillary electrophoresis system equipped with a photodiode array detector. A fused silica capillary purchased from GL Sciences (Tokyo, Japan) was cut to an adequate length, and packed in a capillary cartridge; the cartridge was attached to the CE system. The dimensions of the capillary were 64.5 cm in the total length, 56 cm in the separation length from the injection end to the detection point, 75  $\mu$ m in the inner diameter, and 365  $\mu$ m in the outer diameter. The capillary cartridge was thermostat at 37°C by circulating constant-temperature air.

### Procedure

The CE capillary was refreshed daily with 0.1 mol L<sup>-1</sup> NaOH for 2 min by applying a pressure to the inlet end of the capillary, followed with purified water for 2 min. After that, the capillary was filled with the separation buffer and equilibrated. The separation buffer was also held in both an anodic buffer vial and a cathodic buffer vial. A sample solution containing NPP and MO, as well as 4% (v/v) ethanol to monitor the electroosmotic flow, was injected into the capillary from the anodic end by applying a pressure at 50 mbar for 5 s. Then, a DC voltage of 25 kV was applied to the capillary, and a CE separation was made. Both NPP as a substrate and NP as a product, as well as MO as an internal standard, were photometrically detected at 400 nm. The electropherograms were recorded and analyzed by an Agilent ChemStation (Ver. B.04.02).

### Evaluation of the reaction rate of NPP to NP

A plateau signal would be detected by the CE/DFA, and the height of the plateau signal is used for the analysis of the enzymatic reaction. The continuous formation of NP in the capillary results in a plateau signal in an electropherogram, as is described in the next section, and the height of the plateau signal is directly related to the formation rate of the NP. Therefore, the plateau signal would be quantified to evaluate the reaction rate of the formed NP. The amount of the substrate NPP introduced into the capillary may change by the pressure injection. Thus, an internal standard of MO was used to correct the height of the plateau signal by dividing the plateau height by the height of the MO peak signal, because both NP and MO are photometrically detected at 400 nm and MO is stably monoanionic at a pH of around 10. Methyl Orange was coexisted in the sample solution at a concentration of  $1 \times 10^{-4}$  mol L<sup>-1</sup>.

## Results and Discussion

### Detection mechanism of plateau signal by capillary electrophoresis/dynamic frontal analysis

The detection mechanism of CE/DFA is schematically illustrated in Fig. 1. Firstly, the capillary is filled with a separation buffer containing an enzyme (E), alkaline phosphatase in the present case. Then, a sample solution containing a substrate (S), NPP in this case, is injected into the capillary from the anodic end (Fig. 1a). When a DC voltage is applied to the capillary and the electrophoresis starts, an analyte of the anionic substrate migrates toward cathodic end, since a fast electroosmotic flow (EOF) is generated in the silica capillary. However, the net velocity of the anionic substrate is slower than the EOF. The electrophoretic migration of the substrate S into the separation buffer promotes the enzymatic hydrolysis of NPP forming NP over the substrate zone. Although NP is less anionic than NPP and the effective electrophoretic mobility of NP is smaller than that of NPP, the fast EOF directs both of the anionic species toward the cathodic end of the capillary; the apparent electrophoretic mobility of NP toward the detection

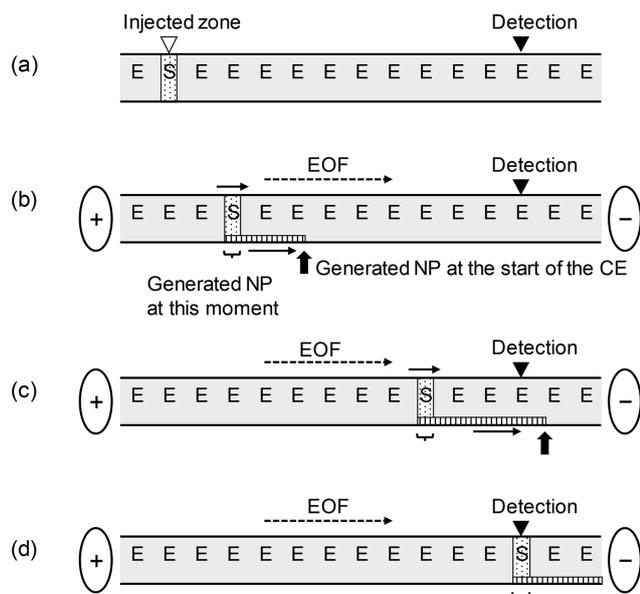


Fig. 1 Schematic illustration for the detection mechanism of CE/DFA. (a) A sample plug containing a substrate of NPP (S) is injected into the capillary. (b) A substrate of NPP electrophoretically migrates in the enzyme solution under applied DC voltage, where the NPP is continuously hydrolyzed to form NP (a rectangle with vertical lines). The reaction rate is constant, and the concentration of NP is definite over the rectangle length. (c) Continuously generated NP is detected, providing a plateau signal. (d) Finally, the residual substrate is detected at the end of the plateau signal. Solid arrows on the substrate S and the product NP indicate their gross velocity in the capillary.

point is larger than that of NPP (shown with the solid arrows in Fig. 1b). Therefore, the formed NP is to be resolved from the NPP zone by the electrophoresis, and it is pulled out from the NPP zone (Fig. 1b). The enzymatic hydrolysis continuously proceeds in the capillary tube and a constant amount of NP is continuously generated. As long as the concentration of the substrate NPP is sufficient against the enzymatic consumption, the concentration of the formed NP in the fronting zone is inevitably the same, as is shown in the rectangle with vertical lines in Fig. 1b. When the fronting zone of the continuously generated NP reaches to the detector, a constant concentration of NP is to be detected continuously (Fig. 1c). As a result of the continuous generation of NP with the enzyme, the long NP zone is detected as a plateau signal. Finally, the NPP zone passes the detection point (Fig. 1d), and the CE response comes back to the background level. In this way, dynamically and continuously generated NP is detected as a plateau signal.

Since the kinetically generated NP is pulled out from the substrate zone by CE and a plateau signal is observed, the detection style would be equivalent to the conventional CE/FA. A difference with the conventional CE/FA is that the plateau zone is the result of the dynamic enzymatic reaction; the hydrolysis of NPP forming NP, while a plateau signal is formed by an electrophoretic separation of an unbound species from an equilibrium mixture of a long injection-plug by the conventional CE/FA. Therefore, this detection sequence would be called as CE/DFA.

#### Detection of a plateau signal of NP by the enzymatic reaction in the capillary

The migration behavior of NPP and NP was firstly examined preceding to the in-capillary enzymatic reaction. When a

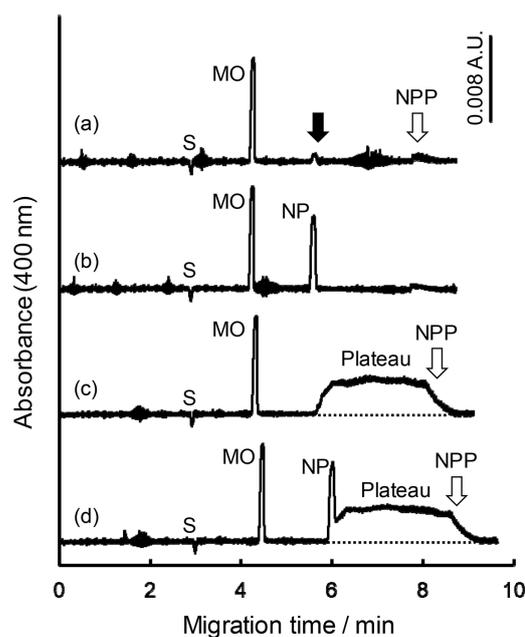


Fig. 2 Detection of a plateau signal of NP by the enzymatic reaction of NPP with ALP. (a) Detection of  $1 \times 10^{-3}$  mol L<sup>-1</sup> NPP without ALP. (b) Detection of  $1 \times 10^{-4}$  mol L<sup>-1</sup> NP without ALP. (c) Detection of  $1 \times 10^{-3}$  mol L<sup>-1</sup> NPP in the presence of ALP in the separation buffer. (d) Detection of  $1 \times 10^{-3}$  mol L<sup>-1</sup> NPP and  $1 \times 10^{-4}$  mol L<sup>-1</sup> NP in the presence of ALP in the separation buffer. Separation buffer: (a) and (b), 10 mmol L<sup>-1</sup> borax + NaOH (pH 9.8); (c) and (d), 10 mmol L<sup>-1</sup> borax + NaOH (pH 9.8) + 0.87 U mL<sup>-1</sup> ALP. MO: Methyl Orange as internal standard. S: Solvent (EOF). CE conditions: 25 kV applied voltage, detection wavelength at 400 nm, sample injection at 50 mbar for 5 s, capillary cartridge temperature at 37°C.

sample solution containing NPP was measured by the CE, NPP was dianionic and it was detected at around 8 min (Fig. 2a). The detection wavelength was set at 400 nm, which fitted to detect NP, and the NPP was detected as a very small peak. However, a sufficient height of a CE peak was detected with NPP, when it was detected at 360 nm (Fig. S1a). Monoanionic NP was also examined in the CE, and the result is shown in Fig. 2b; the NP was detected at around 6 min. In Fig. 2a, a small peak at around 6 min (black arrow) is also detected; the peak is attributed to the hydrolyzed NP from NPP in an aqueous solution under the storage conditions. An internal standard of MO is also detected as an ordinary sharp signal both in Figs. 2a and 2b.

When a sample solution containing NPP was measured with a separation buffer containing an enzyme of alkaline phosphatase, a plateau signal was detected, as in Fig. 2c. The rising and falling times of the plateau signal corresponded to the migration time of NP and NPP, respectively. When NP was added in the sample solution, the spiked NP was detected as an ordinary CE signal at the start of the plateau signal. It suggests that the NP detected at the rising time is generated soon after the start of the electrophoresis. The formed NP generated at around the inlet position of the capillary was electrophoretically resolved from the substrate NPP and electrophoretically migrated to the detector. Negatively charged NPP in the sample plug moved into the separation buffer from the start of the electrophoresis, and the hydrolysis of NPP with the ALP continued. When the substrate NPP is sufficiently excess against the enzyme ALP, the formation rate of NP is constant over the reaction time and the

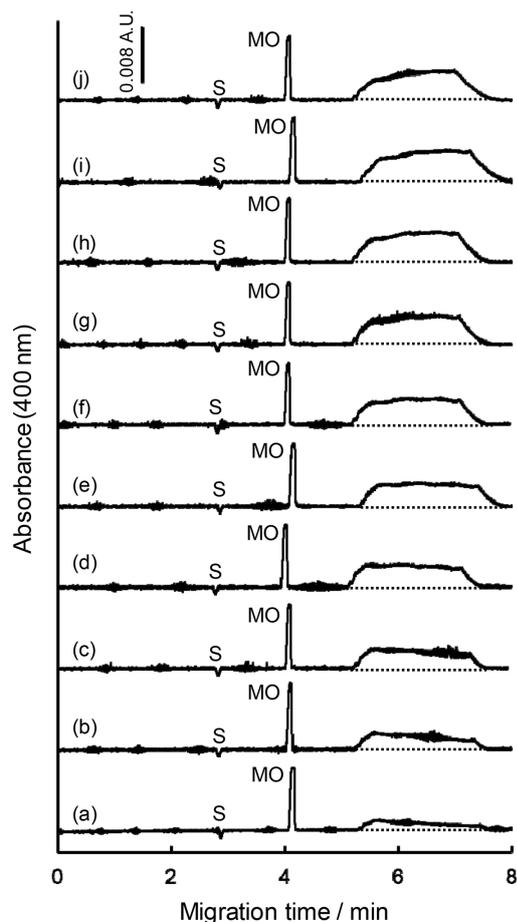


Fig. 3 Typical electropherograms in CE/DFA. Concentrations of NPP in the sample solution in  $\text{mmol L}^{-1}$ : (a), 0.2; (b), 0.4; (c), 0.6; (d), 0.8; (e), 1.0; (f), 1.2; (g), 1.4; (h), 1.6; (i), 1.8; (j), 2.0. Separation buffer: 10  $\text{mmol L}^{-1}$  borax + NaOH (pH 9.8) + 0.87  $\text{U mL}^{-1}$  ALP. MO: Methyl Orange as internal standard. S: Solvent (EOF). CE conditions: 25 kV applied voltage, detection wavelength at 400 nm, sample injection at 50 mbar for 5 s, capillary cartridge temperature at 37°C.

constantly formed NP is detected as a plateau signal. The height of the plateau signal is directly related to the reaction rate of the enzymatic hydrolysis. The falling time of the plateau signal agreed with the migration time of NPP, as in Fig. S1. This means that the detected NP at the migration time of NPP is formed just before the detection of NPP and that the enzymatic reaction has continued in the capillary, as shown in Fig. 1d. The enzymatic hydrolysis would continue even after the detection of the NPP zone, but the NPP is the slowest substance migrating in the capillary and the enzymatically formed NP is not detected any more. In this way, a plateau signal of the enzymatically formed NP is detected ranging from the start of the electrophoresis to the detection of NPP.

While a plateau signal is detected in both Figs. 2c and 2d, the origin of the plateau signals is different from that of the conventional CE/FA. In the conventional CE/FA, a sample solution is introduced into the capillary as a relatively-long plug. One of the equilibrium species, usually unbound species, electrophoretically moves out from the long zone of the equilibrium mixture, and the equilibrium species is detected as a plateau signal. On the contrary in CE/DFA, a sample solution is introduced into the capillary as a short plug; the signals of NP and MO are detected as usual sharp peaks. A plateau signal can

be detected even with the short injection plug, and the plateau signal is thus the result of the continuous formation of the hydrolysis product by the enzyme; result of the dynamic reaction.

#### Analysis of Michaelis-Menten constant from the plateau signal

Michaelis-Menten equation is popularly used to evaluate the reaction kinetics of enzymes. The formation rate of a product P,  $v$ , depends on the concentration of the substrate S, as in

$$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]}, \quad (1)$$

where  $V_{\max}$  is the maximum reaction rate of the enzymatic reaction, and  $K_m$  is a Michaelis-Menten constant. The Michaelis-Menten constant is experimentally determined by Lineweaver-Burk plots (or double reciprocal plots) as in

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}}. \quad (2)$$

In Eq. (2), the x-intercept of the Lineweaver-Burk plots represents  $-1/K_m$ . The enzymatic hydrolysis of NPP was examined by the proposed CE/DFA. The concentration of an enzyme ALP in the separation buffer was set at 0.87  $\text{U mL}^{-1}$ , and a series of the concentration of the substrate NPP was examined for the enzymatic hydrolysis. Changes in the plateau signal with different concentrations of the substrate NPP are shown in Fig. 3. It can be seen from Fig. 3 that the height of the plateau signal increased with increasing concentrations of NPP in the sample solution. The shape of the plateau signals, however, is not uniform; the plateau signal is ideally flat, but a little inclined. When the concentration of the substrate is low, the plateau signal is highest at the early detection time (typically in Fig. 3a). The result suggested that the generation rate of NP was higher at the start of the enzymatic reaction. It would be because the concentration of the substrate NPP was not sufficient and the excess conditions was not assured thoroughly. On the contrary, the plateau signal is highest at the late detection time with the higher concentrations of the substrate (typically in Fig. 3j). The result suggested that the generation rate of NP increased with the reaction time. The reason is difficult to understand at this stage.

Since taking the highest point of the plateau signal even at the different migration time is willful, an average height of the plateau signal was read and used for the analysis. Fortunately, the slope of the plateau signal was not serious over the substrate concentrations examined. The Michaelis-Menten curve is shown in Fig. 4a, where the height of the plateau signal was standardized with the peak height of MO. The standardized plateau height is to be saturated at the higher concentrations of NPP. A Lineweaver-Burk plots was made as in Fig. 4b. A straight line was obtained and a Michaelis-Menten constant,  $K_m$ , was calculated to be 0.57  $\text{mmol L}^{-1}$  from the x-intercept. The  $K_m$  value determined in this study is comparable to the reported values of 0.1 – 0.2  $\text{mmol L}^{-1}$  (spectrophotometry),<sup>37</sup> 1.5  $\text{mmol L}^{-1}$  (pre-capillary reaction and CE),<sup>38</sup> or 4.8  $\text{mmol L}^{-1}$  (plug-plug reaction in CE).<sup>23</sup> Therefore, the proposed CE/DFA would be applicable to the enzyme assays.

When the enzyme assay was operated at a 20 kV applied voltage, both the time range and the height of the plateau signal changed. However, the determined  $K_m$  value was 0.52  $\text{mmol L}^{-1}$ ; it was slightly changed by the electrophoretic conditions.

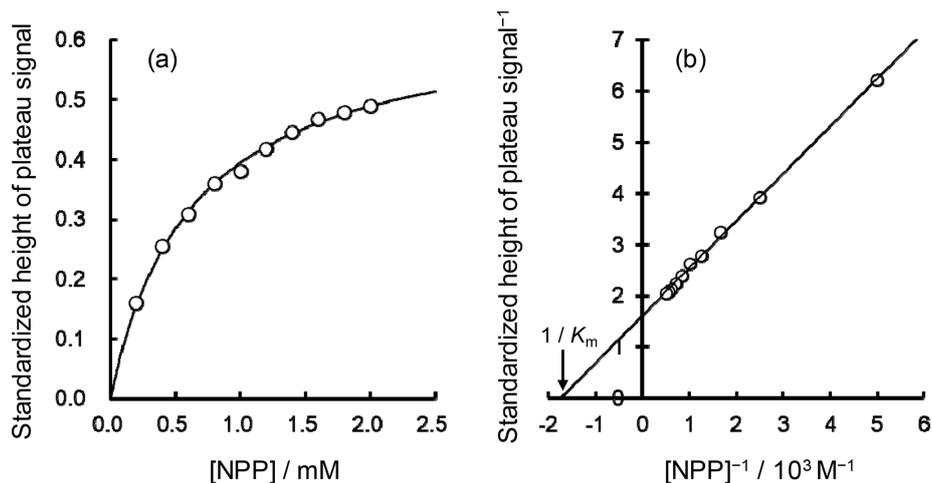


Fig. 4 (a) Michaelis-Menten curve and (b) Lineweaver-Burk plots for the analysis of the Michaelis-Menten constant. The CE conditions are the same as in Fig. 3.

#### Characteristics of CE/DFA for the enzyme assay

Plateau signals were detected by some EMMA studies.<sup>22,24,26</sup> In those studies, a separation capillary was filled with a separation buffer containing the substrate, and an enzyme solution was injected into the capillary as a short plug. When the enzyme was passing through the separation buffer by electrophoretic migration, a product was continuously formed at the enzyme zone and a plateau signal was generated by migration of the enzyme in the separation capillary. The plateau signal is thus the footprint of the enzyme passing through the substrate and, therefore, this EMMA format is not similar to the frontal analysis, because separation is not included. A plateau signal was also detected in this study by the dynamic reaction of the enzyme. The plateau signal is introduced by the separation of the continuously generated product from the substrate. The separation is similar to the conventional CE/FA. While the conventional CE/FA is simply the separation of unbound species from the equilibrium mixture, the proposed CE/DFA is the result of the dynamic reaction of the enzyme; a plateau signal is the result of the continuous pulling out of the product from the enzyme-substrate complex zone.

Since the proposed CE/DFA includes electrophoretic separation of the product from the substrate zone, two advantages can be pointed out. One is the separation of impurities from the substrate solution. The reaction substrates are often unstable, and a blank reaction may occur before examining the enzymatic reaction. In the case of 4-nitrophenyl phosphate, it is hydrolyzed in an aqueous solution to form 4-nitrophenol. As can be seen in Fig. 2a, the hydrolyzed NP was detected as a peak signal even in the absence of the ALP. Therefore, purification of the substrate is essential to examine the reaction dynamics in a homogeneous batch solution. However, the pre-hydrolyzed product of NP can be excluded from the plateau signal by CE separation, as can be seen in Fig. 2d; the spiked NP is resolved from the plateau signal. It is a matter of course that the substrate should be less contaminated to examine the Michaelis-Menten analysis. Another advantage is that the inhibition of the enzymatic reaction by the product is eliminated. Because the product is electrophoretically pulled out from the enzyme-substrate complex zone.

#### Conclusions

In the present study, a novel EMMA format is proposed for the enzyme assay; a substrate zone of NPP is passing through the separation buffer containing an enzyme of ALP. Enzymatic hydrolysis occurs by the migration of the substrate zone, and a product of NP is electrophoretically pulled out from the enzyme-substrate complex zone. As a result of the electrophoretic migrations, a plateau signal of the product is detected and the reaction dynamics of the ALP is analyzed by a Michaelis-Menten equation. As this EMMA format includes a continuous pulling out of the product from the enzyme-substrate complex zone, the analysis through the plateau signal would be called as CE/DFA. Since the proposed CE/DFA includes electrophoretic separation, the proposed CE/DFA possesses advantages in the separation of the contaminant from the sample zone and on eliminating the inhibition of the enzymatic reaction by the product.

#### Acknowledgements

This work was partly supported by JSPS KAKENHI [grant number 17K05903].

#### Supporting Information

This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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