Inhibition Assay of Theophylline by Capillary Electrophoresis/Dynamic Frontal Analysis on the Hydrolysis of *p*-Nitrophenyl Phosphate with Alkaline Phosphatase

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A novel inhibition assay is proposed by capillary electrophoresis/dynamic frontal analysis (CE/DFA). When a substrate of *p*-nitrophenyl phosphate and an inhibitor of theophylline were tandemly introduced into the capillary containing alkaline phosphatase as an enzyme, two plateau signals were detected in the electropherogram. A higher plateau is based on the CE/DFA without inhibition, and a suppressed plateau is formed under the inhibition while the substrate zone passing through the inhibitor zone. Inhibition constant was successfully determined through the two plateau heights.

Keywords: Capillary electrophoresis, Alkaline phosphatase, Inhibition assay

Enzyme assays including reaction kinetics have been analyzed in a homogeneous batch solution for a long time. Michaelis-Menten kinetic analysis is one of the major targets, and Michaelis-Menten constant (K_M) is an essential parameter for enzymes. Recently, capillary electrophoretic methods have been proposed for the enzyme assays by in-capillary reactions.[1-4] The analysis methods by capillary electrophoresis (CE) are classified in electrophoretically mediated microanalysis (EMMA),[5-11] in-capillary immobilized enzyme reactor (IMER),[11-15] and transverse diffusion of laminar flow profiles (TDLFP).[8,10] In most of the analyses, the substrate and the product are electrophoretically resolved and the peak signals have been used for the quantifications and for the analysis of the enzyme assays. When a zone of an enzyme solution was introduced into the separation buffer containing a substrate, a plateau signal was detected as the result of the footprint of the enzyme passing through the substrate solution.[16-18] The plateau heights were used for the enzyme assays.

The present authors have proposed a different format of EMMA.[19] A substrate solution is injected into a separation capillary, where the separation buffer contains an enzyme. Along with the electrophoretic migration of the substrate zone in the separation buffer, the substrate continuously reacts with the enzyme and the formed product is continuously resolved from the substrate zone. The product is kinetically formed at a constant reaction rate, and thus, the product is detected as a plateau signal from the start of the reaction to the detection time of the substrate. The height of the plateau signal is directly related with the reaction rate, and it is used for the enzyme assay. The plateau signal is based on the continuous resolution of the product from the substrate zone under the zero-order kinetic reaction of an enzyme, and the analysis method is named as capillary electrophoresis/dynamic frontal analysis (CE/DFA).[19] A main advantage of CE/DFA is the continuous resolution of the product from the substrate−enzyme zone, which eliminates the interference from the product on the enzyme reaction.

In this study, the present authors propose a novel inhibition assay using CE/DFA. The schematic diagram of the inhibition by CE/DFA is shown in Figure 1. A substrate solution (S) and an inhibitor solution (I) are tandemly introduced into the capillary filled with a separation buffer containing an enzyme (Figure 1a). Both zones of the substrate and the inhibitor electrophoretically migrate in the separation buffer at different velocity, and the inhibition occurs when the two zones overlap (Figure 1c). While the reaction product (P) continuously generated is detected as a plateau signal, the height of the plateau signal is suppressed by the inhibition with the reduced amount of the product. Consequently, an electropherogram of two-steps plateau signal can be detected, as shown in Figure 1e. The characteristic in the CE/DFA format is that both the inhibition and no inhibition can simultaneously be detected in one electropherogram.

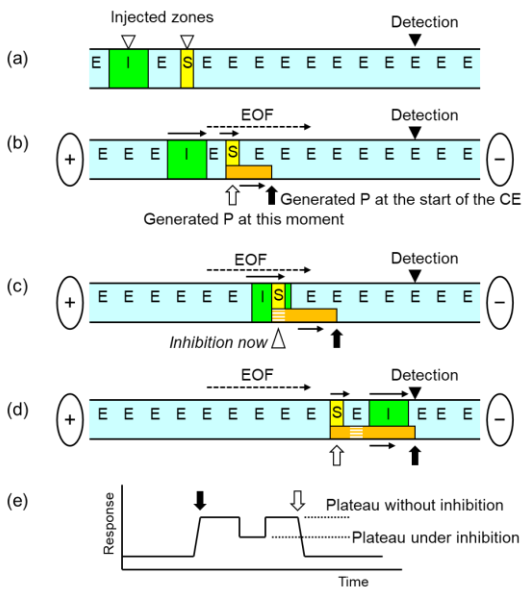

Figure 1. Schematic diagram of the inhibition in CE/DFA by the tandem injections of a substrate and an inhibitor solutions (a) − (d), and a typical electropherogram (e). The inhibition occurs when the zones of a substrate (S) and an inhibitor (I) overlap by the electrophoretic migration. E: an enzyme contained in the separation buffer.

Alkaline phosphatase (ALP, EC 3.1.3.1) catalyzes the hydrolysis of phosphoric monoester to give phosphoric acid

and an alcohol. ALP plays an important role in human skeletal mineralization,[20] and ALP assays have intensely been studied.[21,22] Theophylline is a well-known inhibitor of ALP, and it is often used as a model inhibitor for ALP assays.[16,23,24] Since the enzymatic reaction of ALP and the inhibition of theophylline are well established, they are adopted in this study. *p*-Nitrophenyl phosphate (NPP) was used as a substrate, and the hydrolysis product of *p*-nitrophenolate (NP) was photometrically detected.

Alkaline phosphatase from bovine intestinal mucosa was purchased from Sigma-Aldrich. A substrate of NPP as disodium salt hexahydrate, and a hydrolysis product of NP were from Fujifilm Wako Pure Chemical. An inhibitor of theophylline was from Tokyo Chemical Industry. Other reagents used were of analytical grade. All solutions were prepared with deionized water purified by Milli-Q Gradient A10.

A ^{3D}CE system (Agilent Technologies) equipped with a photodiode array detector was used as a CE system. A fused silica capillary held in a capillary cartridge was used as a separation capillary with its dimensions of 75 μm i.d., 375 μm o.d., 64.5 cm in total length, and 56 cm in effective length from the injection end to the detection point. A ChemStation program (Agilent Technologies, Ver. B04.02) was used for the control of the CE system and the data analysis.

A separation buffer was prepared with 0.01 mol L^{-1} borax with its pH controlled at 9.8 with NaOH. An enzyme of ALP was contained in the separation buffer at 0.55 unit mL^{-1}. After the capillary filled with the separation buffer, solutions of a substrate NPP, the separation buffer, and an inhibitor of theophylline were tandemly injected into the capillary from the anodic end by applying pressure, as shown in Figure 1a. Both ends of the capillary were dipped in the buffer vials, and a DC voltage of 25 kV was applied to the capillary for the electrophoresis. The hydrolysis product of NP was photometrically detected at 400 nm. During the experiments, the capillary, as well as the buffer vials were thermostat at 37 °C.

Michaelis-Menten kinetic analysis was made with the plateau heights of the generated NP in the electropherograms. Lineweaver-Burk plots were made to determine the Michaelis-Menten constant (K_M) and the inhibition constant (K_I).

Both the injection sequence and the migration order of the substrate and the inhibitor are the key factors on examining the inhibition assay by CE/DFA. The enzymatic hydrolysis continuously proceeds during the migration of the substrate zone in the separation buffer, providing plateau signal of the product, as shown in Figure 1b.[19] The electrophoretic mobility of a substrate NPP and a product of NP are different with each other, and the injected zones migrate in the capillary at the different velocity. Theophylline as an inhibitor and the substrate of NPP possess the charge of −1 and −2 respectively under the enzymatic reaction conditions of pH 9.8. The backward velocity of NPP is faster than theophylline, and the order of the forward velocity is in the order of theophylline > NPP under a fast electroosmotic flow (EOF). Thus, the injection sequence is in the order of a substrate NPP, the separation buffer to divide the two zones, and an inhibitor theophylline, as shown in Figure 1a. The inhibition with the inhibitor occurs in the CE/DFA, when the inhibitor zone is overlapping on the substrate zone, as is shown in Figure 1c; the passing of the inhibitor zone over the substrate zone is essential for the inhibition assay by CE/DFA. An electropherogram was obtained with such an injection sequence, as shown in Figure 2. In this electropherogram, the detection wavelength was set at 200 nm to detect all of the substrate NPP, the product NP, and the inhibitor theophylline. It can be seen from the electropherogram that the theophylline as an injected zone is firstly detected as a peak signal, then the product NP detected as a plateau signal, and finally the injected NPP detected as a peak signal. A plateau signal of NP suggests that NPP is continuously hydrolyzed with ALP during the electrophoresis. A dipped plateau is detected in the electropherogram with an arrow. The enzymatic hydrolysis would be inhibited with theophylline during the electrophoretic migration of the injected zones and their overlapping. A dipped signal of the product has been detected by an enzymatic reaction of ALP by injecting an enzyme zone and an inhibitor zone, where a fluorescent substrate has been contained in the running buffer.[17] The inhibition occured when the enzyme zone and the inhibitor zone overlap. However, the dipped signal was not plateau and simultaneous determinations of K_M and K_I were not achieved by such sequence.[17] Although K_M and K_I were simultaneously determined by Crawford, *et al.*, it was necessary to stop the electrophoresis once for the enzymatic reaction.[25]

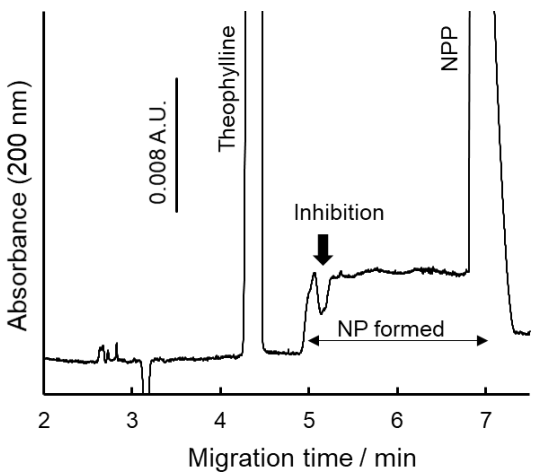

Figure 2. Typical electropherogram in CE/DFA accompanying inhibition. A substrate of NPP and an inhibitor of theophylline were tandemly injected into the capillary. A dipped plateau by the inhibition is detected in the plateau range of the formed NP. Concentration of NPP in the injected zone: 1.0 mmol L^{-1}. Concentration of theophylline in the injected zone: 1.0 mmol L^{-1}.

It is essential for the inhibition assay to detect the dipped region as a clear plateau. The injection period of the theophylline zone was examined in the range between 10 s and 40 s to control the overlapping time on the NPP zone. The results are shown in Figure 3. The detection wavelength was set at 400 nm, and only the product of NP was detected. The difference in the detection time of the plateau signal is

due to the injection period. It is noticed from Figure 3 that the dipped plateau came to be wide by extending the injection period of the theophylline solution. However, the dipped plateau with theophylline inhibition is not suitable over the injection period of 30 s. It would be because the precedent injected zone of the substrate dispersed seriously by the long-time pressure injection. Therefore, the injection time of theophylline was set at 20 s.

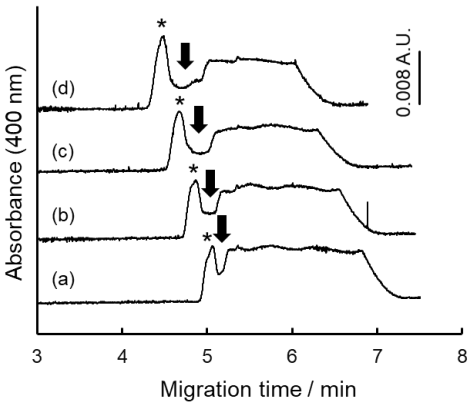

Figure 3. CE/DFA electropherograms of NPP with ALP under tandem injections of NPP and theophylline. The injection order of the solutions was: 2.0 mmol L^{-1} NPP as a substrate for 5 s, the separation buffer for 10 s, and 1.0 mmol L^{-1} theophylline as an inhibitor. Injection period of theophylline: (a) 10, (b) 20, (c) 30, and (d) 40 s. Black arrows indicate the inhibition with theophylline. *: NP generated in the NPP solution before the electrophoresis. The separation buffer and the CE conditions are written in the text.

The injection period of the separation buffer dividing the substrate zone and the inhibitor zone was also examined. Aim of dividing the substrate zone and the inhibitor zone is to delay the overlapping time from the start of the electrophoresis. Because the substrate NPP sometimes degraded to form NP before the CE/DFA measurements, as well as by the contact of substrate plug and electrophoretic buffer just before applying the voltage, the contaminated NP would interfere with the dipped plateau as shown in the asterisks in the electropherograms. The injection period of the separation buffer was examined in the range between 5 s and 40 s; the results are shown in Figure 4. With the extension of the injection period of the separation buffer, it is possible to resolve the contaminated NP (*) and the dipped plateau. Longer injection period of the separation buffer would promote the dispersion of the injected zones, and an injection period of 10 s was chosen for the separation buffer. In this way, a clear suppressed plateau is detected by controlling the injection sequence and the injection periods.

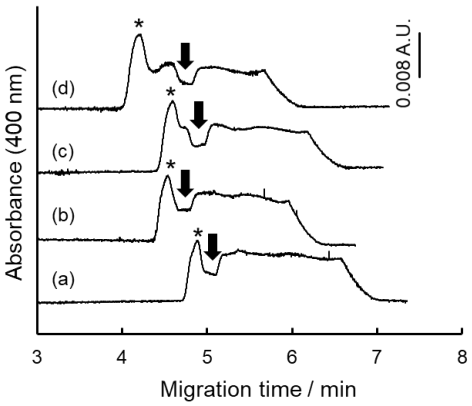

Figure 4. CE/DFA electropherograms of NPP with ALP under tandem injections of NPP and theophylline. The injection order of the solutions is: 2.0 mmol L^{-1} NPP for 5 s, the separation buffer, and 1.0 mmol L^{-1} theophylline for 20 s. Injection period of the separation buffer: (a) 5, (b) 10, (c) 20, and (d) 40 s. Black arrows indicate the inhibition with theophylline. *: NP generated before the electrophoresis. The separation buffer and the CE conditions are the same as in Figure 3.

The two plateau heights were reproducible with 7 repeated measurements, and no plateau response was detected with the separation buffer without the enzyme after several CE/DFA measurements. Thus, adsorption of the enzyme to the inner wall of the capillary is not significant.

Michaelis-Menten kinetic analysis was examined for the determinations of K_M and K_I values through the plateau signals. The height of the product plateau was used for the analysis instead of the reaction rate.[19] The product plateaus were obtained at different NPP concentrations from 0.4 mmol L^{-1} to 3.0 mmol L^{-1}, where the concentration of an inhibitor theophylline was set at 0.5 mmol L^{-1} or 1.0 mmol L^{-1}. The electropherograms obtained with 1.0 mmol L^{-1} theophylline are shown in Figure 5. Two-steps of the plateau height were detected in the electropherograms. The plateau height without inhibition (higher plateau) increased with the increase in the NPP concentrations, as previously reported.[19] The plateau height under inhibition (lower plateau) also increased with the increase in the NPP concentrations, but the height is lower than the higher plateau because of the inhibition.

Lineweaver-Burk plots were made to determine the Michaelis-Menten constant (K_M) and the inhibition constant (K_I). Since the plateau height as absorbance response is directly related with the kinetic reaction rate, it was used for the analysis instead of the reaction rate. The plateau height, however, gradually decreases with the reaction time, *i.e.*, the late detection time. It is because of the gradually reduced substrate concentration by the enzymatic reaction. Therefore, the highest response of the plateau was used for the analysis. The results are shown in Figure 6. The K_M value was determined through the x-intercept of the signal heights of the higher plateau (● in Figure 6); the K_M value was 1.59 mmol L^{-1}. The K_m value determined in this study agreed with the reported ones; 0.4 mmol L^{-1} [23] or 1.5 mmol L^{-1}.[26] It is noticed from Figure 6 that the Lineweaver-Burk plots are parallel between in the absence (higher plateau) and in the presence

(lower plateau) of theophylline. The parallel lines suggest that the inhibition form of theophylline is uncompetitive. The uncompetitive form of the inhibition agrees with the reported result.[23,24] The inhibition constant of theophylline as an uncompetitive inhibitor, K_I, can be determined using eq. (1).[27]

$$\frac{1}{v} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_I}\right) \quad (1)$$

$$\frac{1}{v} = \frac{K_M}{V_{max}\,[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_I}\right) \quad (1)$$

The K_I value determined in this study was 0.72 mmol L^{-1}. The value also agreed with the reported values, 0.1 mmol L^{-1} [17] or 0.69 mmol L^{-1}.[23]

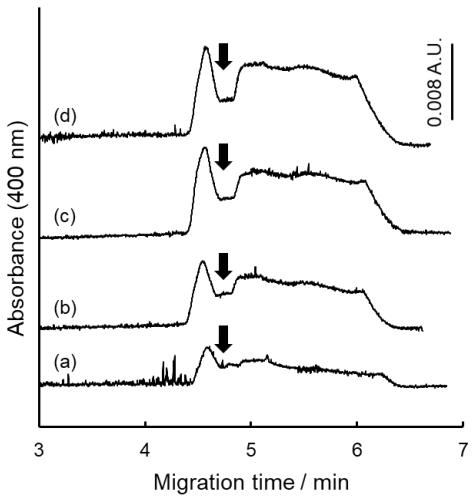

Figure 5. CE/DFA electropherograms of NPP with ALP under tandem injections of NPP and theophylline at different concentrations of NPP. Concentrations of NPP: (a) 0.4, (b) 1.0, (c) 1.5, (d) 2.0 mmol L^{-1}. Concentrations of theophylline: 1.0 mmol L^{-1}. The injection sequence of the sample solutions is: substrate soln. for 5 s, separation buffer for 10 s, and inhibitor soln. for 20 s. The separation buffer and the CE conditions are the same as in Figure 3.

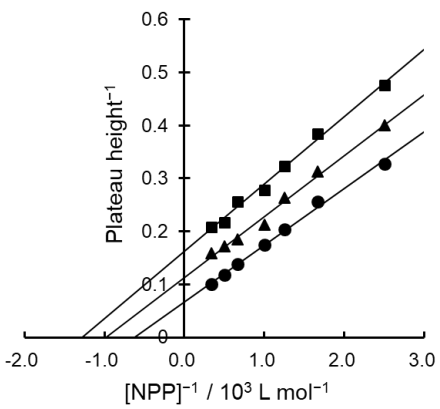

Figure 6. Lineweaver-Burk plots for the enzymatic hydrolysis of NPP with ALP by the inhibition with theophylline. Theophylline concentrations: ●, none (higher plateau); ▲, 0.5 mmol L^{-1}; ■, 1.0 mmol L^{-1}. The injection sequence, the separation buffer, and the CE conditions are the same as in Figure 5.

In conclusion, a novel inhibition assay of enzymatic reaction is proposed by CE/DFA. It is demonstrated that dynamic reactions in a capillary led to the plateau signals in CE/DFA. Two plateau signals are detected in this CE/DFA by the tandem injections of a substrate and an inhibitor solutions, as well as by their electrophoretic migration. By using the two plateau heights, both K_M and K_I values can simultaneously be determined by a series of the measurements. Zero-order kinetic reactions with an inhibition reaction would be analyzed by this format of CE/DFA.

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

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