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

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

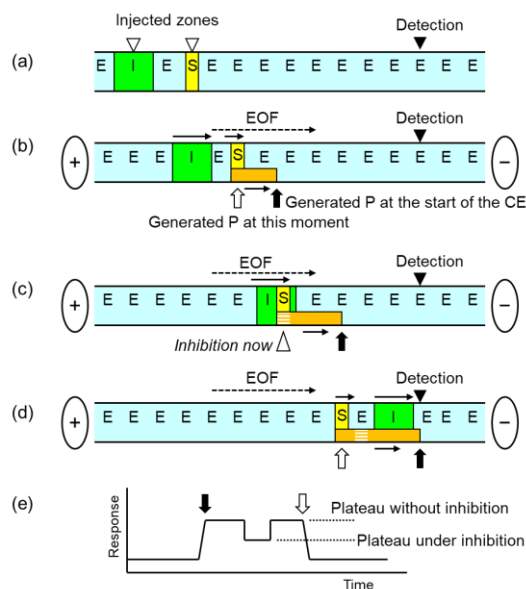
12 **Keywords:** Capillary electrophoresis, Alkaline  
13 phosphatase, Inhibition assay  
14

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

34 The present authors have proposed a different format of  
35 EMMA.<sup>19</sup> A substrate solution is injected into a separation  
36 capillary, where the separation buffer contains an enzyme.  
37 Along with the electrophoretic migration of the substrate  
38 zone in the separation buffer, the substrate continuously  
39 reacts with the enzyme and the formed product is  
40 continuously resolved from the substrate zone. The product  
41 is kinetically formed at a constant reaction rate, and thus, the  
42 product is detected as a plateau signal from the start of the  
43 reaction to the detection time of the substrate. The height of  
44 the plateau signal is directly related with the reaction rate, and  
45 it is used for the enzyme assay. The plateau signal is based on  
46 the continuous resolution of the product from the substrate  
47 zone under the zero-order kinetic reaction of an enzyme, and  
48 the analysis method is named as capillary  
49 electrophoresis/dynamic frontal analysis (CE/DFA).<sup>19</sup> A

50 main advantage of CE/DFA is the continuous resolution of  
51 the product from the substrate-enzyme zone, which  
52 eliminates the interference from the product on the enzyme  
53 reaction.

54 In this study, the present authors propose a novel  
55 inhibition assay using CE/DFA. The schematic diagram of  
56 the inhibition by CE/DFA is shown in Figure 1. A substrate  
57 solution (S) and an inhibitor solution (I) are tandemly  
58 introduced into the capillary filled with a separation buffer  
59 containing an enzyme (Figure 1a). Both zones of the substrate  
60 and the inhibitor electrophoretically migrate in the separation  
61 buffer at different velocity, and the inhibition occurs when  
62 the two zones overlap (Figure 1c). While the reaction product  
63 (P) continuously generated is detected as a plateau signal, the  
64 height of the plateau signal is suppressed by the inhibition  
65 with the reduced amount of the product. Consequently, an  
66 electropherogram of two-steps plateau signal can be detected,  
67 as shown in Figure 1e. The characteristic in the CE/DFA  
68 format is that both the inhibition and no inhibition can  
69 simultaneously be detected in one electropherogram.  
70



71

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

77

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

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

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

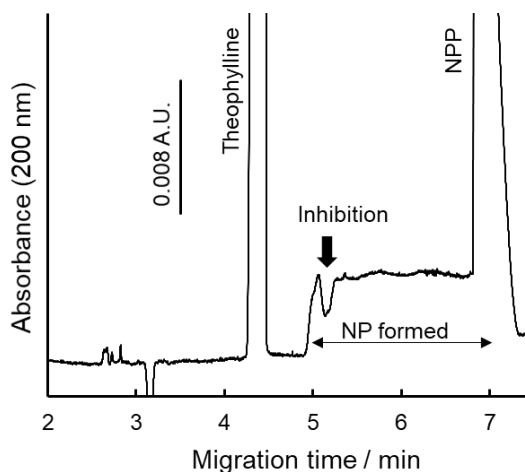
18 A <sup>3D</sup>CE system (Agilent Technologies) equipped with a  
 19 photodiode array detector was used as a CE system. A fused  
 20 silica capillary held in a capillary cartridge was used as a  
 21 separation capillary with its dimensions of 75  $\mu\text{m}$  i.d., 375  
 22  $\mu\text{m}$  o.d., 64.5 cm in total length, and 56 cm in effective length  
 23 from the injection end to the detection point. A ChemStation  
 24 program (Agilent Technologies, Ver. B04.02) was used for  
 25 the control of the CE system and the data analysis.

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

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

44 Both the injection sequence and the migration order of  
 45 the substrate and the inhibitor are the key factors on  
 46 examining the inhibition assay by CE/DFA. The enzymatic  
 47 hydrolysis continuously proceeds during the migration of the  
 48 substrate zone in the separation buffer, providing plateau  
 49 signal of the product, as shown in Figure 1b.<sup>19</sup> The  
 50 electrophoretic mobility of a substrate NPP and a product of  
 51 NP are different with each other, and the injected zones  
 52 migrate in the capillary at the different velocity. Theophylline  
 53 as an inhibitor and the substrate of NPP possess the charge of  
 54 -1 and -2 respectively under the enzymatic reaction  
 55 conditions of pH 9.8. The backward velocity of NPP is faster  
 56 than theophylline, and the order of the forward velocity is in  
 57 the order of theophylline > NPP under a fast electroosmotic  
 58 flow (EOF). Thus, the injection sequence is in the order of a  
 59 substrate NPP, the separation buffer to divide the two zones,

60 and an inhibitor theophylline, as shown in Figure 1a. The  
 61 inhibition with the inhibitor occurs in the CE/DFA, when the  
 62 inhibitor zone is overlapping on the substrate zone, as is  
 63 shown in Figure 1c; the passing of the inhibitor zone over the  
 64 substrate zone is essential for the inhibition assay by CE/DFA.  
 65 An electropherogram was obtained with such an injection  
 66 sequence, as shown in Figure 2. In this electropherogram, the  
 67 detection wavelength was set at 200 nm to detect all of the  
 68 substrate NPP, the product NP, and the inhibitor theophylline.  
 69 It can be seen from the electropherogram that the  
 70 theophylline as an injected zone is firstly detected as a peak  
 71 signal, then the product NP detected as a plateau signal, and  
 72 finally the injected NPP detected as a peak signal. A plateau  
 73 signal of NP suggests that NPP is continuously hydrolyzed  
 74 with ALP during the electrophoresis. A dipped plateau is  
 75 detected in the electropherogram with an arrow. The  
 76 enzymatic hydrolysis would be inhibited with theophylline  
 77 during the electrophoretic migration of the injected zones and  
 78 their overlapping. A dipped signal of the product has been  
 79 detected by an enzymatic reaction of ALP by injecting an  
 80 enzyme zone and an inhibitor zone, where a fluorescent  
 81 substrate has been contained in the running buffer.<sup>17</sup> The  
 82 inhibition occurred when the enzyme zone and the inhibitor  
 83 zone overlap. However, the dipped signal was not plateau and  
 84 simultaneous determinations of  $K_M$  and  $K_I$  were not achieved  
 85 by such sequence.<sup>17</sup> Although  $K_M$  and  $K_I$  were simultaneously  
 86 determined by Crawford, *et al.*, it was necessary to stop the  
 87 electrophoresis once for the enzymatic reaction.<sup>25</sup>  
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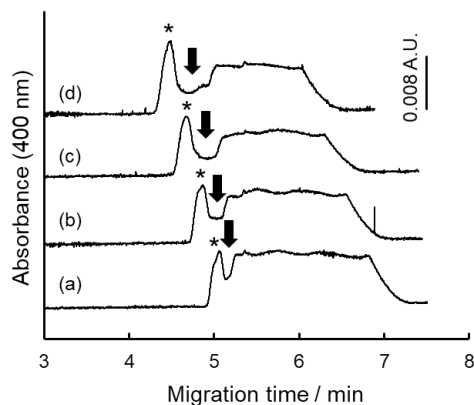


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90 **Figure 2.** Typical electropherogram in CE/DFA accompanying  
 91 inhibition. A substrate of NPP and an inhibitor of theophylline were  
 92 tandemly injected into the capillary. A dipped plateau by the inhibition  
 93 is detected in the plateau range of the formed NP. Concentration of NPP  
 94 in the injected zone: 1.0 mmol L<sup>-1</sup>. Concentration of theophylline in the  
 95 injected zone: 1.0 mmol L<sup>-1</sup>.

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

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

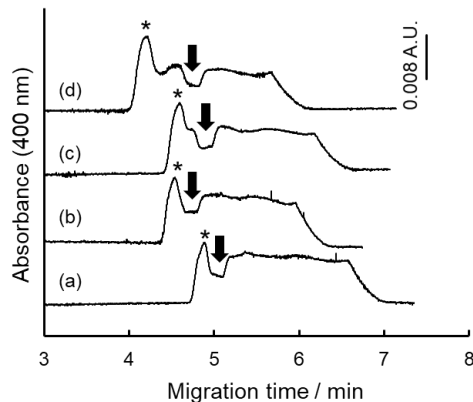


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11 **Figure 3.** CE/DFA electropherograms of NPP with ALP under tandem  
 12 injections of NPP and theophylline. The injection order of the solutions  
 13 was: 2.0 mmol L<sup>-1</sup> NPP as a substrate for 5 s, the separation buffer for  
 14 10 s, and 1.0 mmol L<sup>-1</sup> theophylline as an inhibitor. Injection period of  
 15 theophylline: (a) 10, (b) 20, (c) 30, and (d) 40 s. Black arrows indicate  
 16 the inhibition with theophylline. \*: NP generated in the NPP solution  
 17 before the electrophoresis. The separation buffer and the CE conditions  
 18 are written in the text.

19 The injection period of the separation buffer dividing  
 20 the substrate zone and the inhibitor zone was also examined.  
 21 Aim of dividing the substrate zone and the inhibitor zone is  
 22 to delay the overlapping time from the start of the  
 23 electrophoresis. Because the substrate NPP sometimes  
 24 degraded to form NP before the CE/DFA measurements, as  
 25 well as by the contact of substrate plug and electrophoretic  
 26 buffer just before applying the voltage, the contaminated NP  
 27 would interfere with the dipped plateau as shown in the  
 28 asterisks in the electropherograms. The injection period of the  
 29 separation buffer was examined in the range between 5 s and  
 30 40 s; the results are shown in Figure 4. With the extension of  
 31 the injection period of the separation buffer, it is possible to  
 32 resolve the contaminated NP (\*) and the dipped plateau.  
 33 Longer injection period of the separation buffer would  
 34 promote the dispersion of the injected zones, and an injection  
 35 period of 10 s was chosen for the separation buffer. In this  
 36 way, a clear suppressed plateau is detected by controlling the  
 37 injection sequence and the injection periods.

38



39

40 **Figure 4.** CE/DFA electropherograms of NPP with ALP under tandem  
 41 injections of NPP and theophylline. The injection order of the solutions  
 42 is: 2.0 mmol L<sup>-1</sup> NPP for 5 s, the separation buffer, and 1.0 mmol L<sup>-1</sup>  
 43 theophylline for 20 s. Injection period of the separation buffer: (a) 5, (b)  
 44 10, (c) 20, and (d) 40 s. Black arrows indicate the inhibition with  
 45 theophylline. \*: NP generated before the electrophoresis. The separation  
 46 buffer and the CE conditions are the same as in Figure 3.

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

52 Michaelis-Menten kinetic analysis was examined for  
 53 the determinations of  $K_M$  and  $K_I$  values through the plateau  
 54 signals. The height of the product plateau was used for the  
 55 analysis instead of the reaction rate.<sup>19</sup> The product plateaus  
 56 were obtained at different NPP concentrations from 0.4 mmol  
 57 L<sup>-1</sup> to 3.0 mmol L<sup>-1</sup>, where the concentration of an inhibitor  
 58 theophylline was set at 0.5 mmol L<sup>-1</sup> or 1.0 mmol L<sup>-1</sup>. The  
 59 electropherograms obtained with 1.0 mmol L<sup>-1</sup> theophylline  
 60 are shown in Figure 5. Two-steps of the plateau height were  
 61 detected in the electropherograms. The plateau height  
 62 without inhibition (higher plateau) increased with the  
 63 increase in the NPP concentrations, as previously reported.<sup>19</sup>  
 64 The plateau height under inhibition (lower plateau) also  
 65 increased with the increase in the NPP concentrations, but the  
 66 height is lower than the higher plateau because of the  
 67 inhibition.

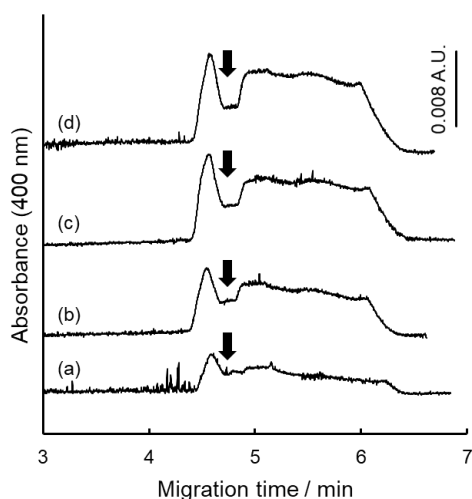
68 Lineweaver-Burk plots were made to determine the  
 69 Michaelis-Menten constant ( $K_M$ ) and the inhibition constant  
 70 ( $K_I$ ). Since the plateau height as absorbance response is  
 71 directly related with the kinetic reaction rate, it was used for  
 72 the analysis instead of the reaction rate. The plateau height,  
 73 however, gradually decreases with the reaction time, *i.e.*, the  
 74 late detection time. It is because of the gradually reduced  
 75 substrate concentration by the enzymatic reaction. Therefore,  
 76 the highest response of the plateau was used for the analysis.  
 77 The results are shown in Figure 6. The  $K_M$  value was  
 78 determined through the x-intercept of the signal heights of the  
 79 higher plateau (● in Figure 6); the  $K_M$  value was 1.59 mmol  
 80 L<sup>-1</sup>. The  $K_m$  value determined in this study agreed with the  
 81 reported ones; 0.4 mmol L<sup>-1</sup><sup>23</sup> or 1.5 mmol L<sup>-1</sup>.<sup>26</sup> It is noticed  
 82 from Figure 6 that the Lineweaver-Burk plots are parallel  
 83 between in the absence (higher plateau) and in the presence

1 (lower plateau) of theophylline. The parallel lines suggest  
 2 that the inhibition form of theophylline is uncompetitive. The  
 3 uncompetitive form of the inhibition agrees with the reported  
 4 result.<sup>23,24</sup> The inhibition constant of theophylline as an  
 5 uncompetitive inhibitor,  $K_I$ , can be determined using eq. (1).<sup>27</sup>

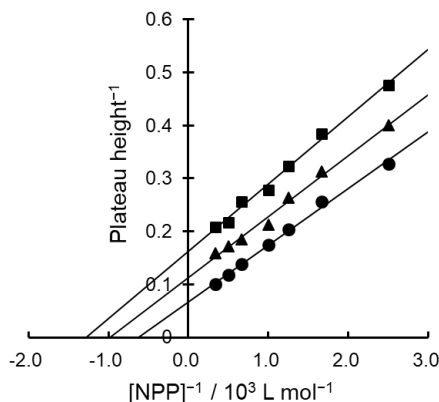
$$\frac{1}{v} = \frac{K_M}{V_{\max}[S]} + \frac{1}{V_{\max}} \left( 1 + \frac{[I]}{K_I} \right) \quad (1)$$

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

15 The  $K_I$  value determined in this study was  $0.72 \text{ mmol L}^{-1}$ .  
 16 The value also agreed with the reported values,  $0.1 \text{ mmol L}^{-1}$   
 17 <sup>17</sup> or  $0.69 \text{ mmol L}^{-1}$ .<sup>23</sup>



19  
 20 **Figure 5.** CE/DFA electropherograms of NPP with ALP under tandem  
 21 injections of NPP and theophylline at different concentrations of NPP.  
 22 Concentrations of NPP: (a) 0.4, (b) 1.0, (c) 1.5, (d) 2.0  $\text{mmol L}^{-1}$ .  
 23 Concentrations of theophylline:  $1.0 \text{ mmol L}^{-1}$ . The injection sequence of  
 24 the sample solutions is: substrate soln. for 5 s, separation buffer for 10 s,  
 25 and inhibitor soln. for 20 s. The separation buffer and the CE conditions  
 26 are the same as in Figure 3.



27

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

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

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