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Title:

Kinetic analysis of substrate competition in enzymatic reactions with *β*-D-galactosidase by capillary electrophoresis / dynamic frontal analysis

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Abstract

Competitive inhibition between two substrates with an enzyme is investigated by capillary electrophoresis/dynamic frontal analysis (CE/DFA). Enzymatic hydrolyses of *o*-nitrophenyl *β*-D-galactopyranoside and *p*-nitrophenyl *β*-D-galactopyranoside with *β*-D-galactosidase were examined as a model competitive reaction. A sample solution containing the two substrates was injected into a capillary filled with a separation buffer containing an enzyme. Enzymatic hydrolysis occurred during the electrophoresis, and the products of *o*-nitrophenol and *p*-nitrophenol were continuously formed and resolved from the sample zone. Two-steps plateau signal was detected with the two-substrate solutions based on the difference in the effective electrophoretic mobility of *o*-nitrophenol and *p*-nitrophenol. Michaelis-Menten constants and inhibition constants were determined with the plateau heights. Usefulness of CE/DFA on competitive inhibition analysis is demonstrated in this study.

Keywords

Capillary electrophoresis, dynamic frontal analysis, *β*-D-galactosidase, substrate competition, Michaelis-Menten constant, inhibition constant

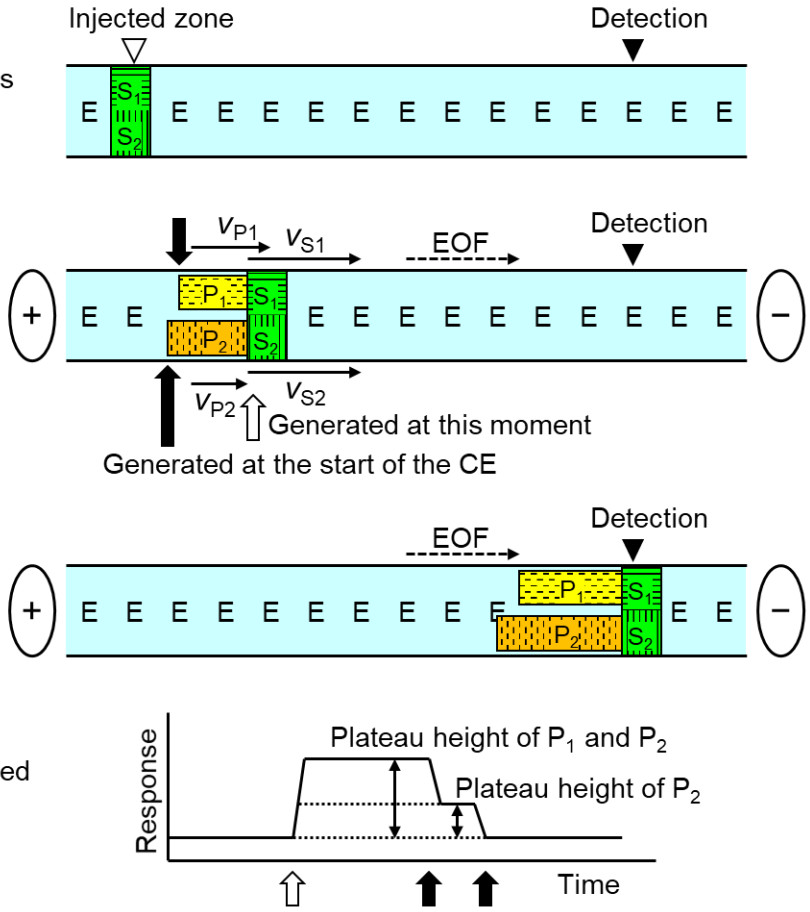
Graphical Abstract

A solution containing two substrates of S_1 and S_2 is injected into the capillary.

Two substrates are enzymatically hydrolyzed, and anionic products of P_1 and P_2 are resolved from the sample zone by CE at different velocity.

Continuous detection of the products results in plateau signals.

Two-steps plateau signal is detected in the electropherogram



1. Introduction

Substrate competition is found in various biochemical processes [1], including signal transduction pathways [2] and gene regulation [3]. In cases of one enzyme reacting with two substrates or two enzymes reacting with one substrate, such enzymatic activities are different from a single substrate reaction, due to the competition [1]. Substrate competition is comparable to the enzyme inhibition, because one of the substrates works as an inhibitor against another substrate [1]. Therefore, the substrate competitions have recently been investigated on oxidative metabolism [4], ester prodrug [5], regulatory network motifs [6], glycogen synthetic kinase-3β activity [7], and multiple volatile fatty acids [8]. Michaelis-Menten analysis have commonly been made in the kinetic analysis of enzymatic reactions [9].

Capillary electrophoresis (CE) is widely used for the analysis of enzymatic reactions owing to the small amount of sample used for the measurements [10]. The CE analyses have been made in electrophoretically mediated microanalysis (EMMA) [11-15], in-capillary immobilized enzyme reactor (IMER) [14], and transverse diffusion of laminar flow profiles (TDLFP) [15]. The EMMA format is broadly divided into a continuous mode and a plug-plug mode [12]. The enzymatic reaction proceeds in the entire capillary in the continuous mode or at a part of the capillary in the plug-plug mode. While the reaction products are detected as peak signals by the plug-plug mode, box-shaped signals are often detected by the continuous mode [10,16]. On the other hand, IMER [17,18] or TDLFP [19] formats have been used on the analysis of inhibition reaction. The products generated from the substrate are resolved by CE, and they are detected as corresponding peak signals due to the differences in the electrophoretic mobility. One of major advantages on using CE is the coupling of the kinetic reaction with the electrophoretic separation; enzymatically formed product is electrophoretically resolved from the substrate. Based on the advantage of the CE analysis, the present authors utilized the CE separation of the product from the substrate, and a plateau signal was detected based on the zero-order kinetic reaction of an enzyme. Since a plateau signal is detected by the dynamic reaction of an enzyme, the analysis method is named as capillary electrophoresis/dynamic frontal analysis (CE/DFA) [20]. The height of the plateau signal is directly related to the reaction rate, and the enzyme assay is analyzed with the height of the plateau signal. Because the reaction products are electrophoretically resolved from the reaction mixture of the substrate zone immediately after the generation, a prominent characteristic can be noticed on the CE/DFA that the enzymatic reaction would proceed without any inhibition by the product [20]. When a substrate and an inhibitor zones are tandemly injected into the capillary in the CE/DFA format,

overlapping of the two zones occurs on the basis of the different electrophoretic mobility between the substrate and the inhibitor, and the enzymatic reaction is inhibited over the overlapping period. A depressed plateau can be detected at the plateau signal as a result of the inhibition, and the inhibition constant can be determined through the depressed plateau signal [21].

β-D-Galactosidase (EC 3.2.1.23) is an enzyme that hydrolyzes glycosidic bonds, and it is present in cells of various microorganisms, plants, and animals [22]. It is used as a biocatalyst in the production of dairy products, and lactose degradation by *β*-D-galactosidase is regarded as one of the important processes [23]. Craig, *et al.* have reported the monitoring methods for *β*-D-galactosidase activity by in-capillary reaction [24-26]. The methods are based on EMMA by filling a capillary with a substrate solution and performing electrophoresis using an enzyme solution as a sample solution. One enzyme molecule is introduced into the capillary, and the differences in the activity and the electrophoretic mobility of one enzyme molecule are discussed. The product was detected as a box signal as a trace of the passage of the enzyme. However, the competition analysis of the substrates was not discussed.

In this study, a direct analysis of substrate competition is demonstrated by a CE/DFA format. *β*-D-Galactosidase was used as an enzyme, and *o*-nitrophenyl *β*-D-galactopyranoside (ONPG) and *p*-nitrophenyl *β*-D-galactopyranoside (PNPG) were used as competitive substrates. Reaction products of *o*-nitrophenol (ONP) and *p*-nitrophenol (ONP) were detected as plateau signals in the CE/DFA format. Two-steps plateau signal was detected with the mixed sample solution of ONPG and PNPG on the basis of the different electrophoretic mobility of the products. Michaelis-Menten analysis and inhibition analysis were made with the two-steps plateau signal. This report would be the first analysis method for the substrate competition by in-capillary reaction.

2. Reaction and detection schemes in the two-substrates competition reaction in CE/DFA

The substrate competition reaction in CE/DFA is schematically illustrated in Fig. 1, related to the electrophoretic migration of the substrates and the products. In this format, a sample solution containing two substrates of S_1 and S_2 is introduced into the capillary filled with a separation buffer containing an enzyme (E), as is illustrated in Fig. 1a. When a DC voltage is applied to the capillary for the electrophoresis, the substrates migrate in the capillary and react with the enzyme during the electrophoretic migration. To realize the enzymatic reaction to be competitive, it is essential for the two substrates that they co-migrate in the capillary,

i.e., they should possess an identical electrophoretic mobility. When the substrates of S_1 and S_2 are electrically neutral, their electrophoretic mobility is equally zero and this requirement is satisfied. When the products of P_1 and P_2 possess different electrophoretic mobility from the substrates, they are electrophoretically resolved from the corresponding substrate, and a long zone of the product is formed and it would be detected as a plateau signal on the basis of the zero-order enzymatic reaction [20]. It is also essential for the products of P_1 and P_2 that they possess different electrophoretic mobility with each other. A zone length of one of the products is different from the zone length of another product, on the basis of the different electrophoretic mobility, as is illustrated in Fig. 1b. The long zone of the product is detected by a fixed detector, and a plateau signal can be recorded with the product during the reaction period from immediately generated moment to the start of the electrophoresis, as is illustrated in Figs. 1c and 1d. In case two substrates with identical electrophoretic mobility (S_1 and S_2) are injected as a zone, both products of P_1 and P_2 would simultaneously be detected but with different zone length. And therefore, a two-steps plateau signal would be detected as shown in Fig. 1d. The total height of the two-steps plateau signal includes the height of the two products, while the height of the lower plateau includes only one of the two products, P_2 in this case. The plateau height of anther product would be obtained by subtracting the lower plateau height from the total plateau height. The enzymatic reaction and the inhibition reactions are analyzed through the plateau heights.

Fig. 1

3. Material and methods

3.1 Apparatus

All CE experiments were performed by a ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a photodiode array detector. A fused-silica capillary (GL Sciences, Tokyo, Japan) was set in a cassette cartridge, and it was installed in the CE system. Dimensions of the separation capillary were 75 μm i.d., 375 μm o.d., 48.5 cm in total length, and 40 cm in effective length from the injection end to the detection point. The capillary cartridge was thermostat at 37 °C by circulating constant temperature air. A ChemStation program (Ver. B04.02, Agilent Technologies) was used for the control of the CE system, the data acquisition, and the data analysis. The inner wall of the capillary was refreshed daily by flushing with 0.1 mol L^{-1} NaOH for 2 min followed with purified water for 2 min.

3.2 Chemicals

An enzyme of *β*-D-galactosidase (from *Escherichia coli*, EC: 3.2.1.23) was obtained from Fujifilm Wako Pure Chemical (Osaka, Japan). The substrates of *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) and *p*-nitrophenyl-*β*-D-galactopyranoside (PNPG) were from Fujifilm Wako Pure Chemical and Tokyo Chemical Industry (Tokyo, Japan), respectively. Other reagents used were of analytical grade. All solutions were prepared with purified water prepared by a Milli-Q Gradient A10 (Millipore, Milford, MA, USA).

3.3 Procedure for the CE/DFA on two-substrates competition

An enzyme solution of *β*-D-galactosidase was prepared at 1.22 unit mL^{-1} in a phosphate buffer (pH 6.78); it was used as a separation buffer in CE. Since high concentrations of the buffer components may induce excess Joule's heat and serious temperature rise, the concentration of the phosphate buffer was set at 10 mmol L^{-1}. After the separation capillary filled with the separation buffer, a substrate solution containing the two substrates of ONPG and PNPG was hydrodynamically injected into the capillary from the anodic end by applying a pressure at 50 mbar for 2 s. After both ends of the capillary were dipped in the separation buffer vials, a DC voltage of 15 kV was applied to the capillary for the electrophoresis. Enzymatically hydrolyzed products of ONP and PNP were photometrically detected at both 200 and 400 nm. The plateau heights of the products in the electropherograms were used for the analysis of the enzyme assay.

3.4 Determination of the Michaelis-Menten constant and the inhibition constant

Michaelis-Menten kinetic analysis is generally done on enzymatic reactions. The reaction rate is monitored at several substrate concentrations, and the Michaelis-Menten constant (K_M) is determined by the Lineweaver-Burk plots as in Equation (1) [9].

$$\frac{1}{v} = \frac{K_M + [S]}{V_{max}[S]} = \frac{K_M}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where [S] is the initial concentration of the substrate and v is the reaction rate with the substrate concentration. A series of the pairs of [S] and v are used for the analysis, and values of V_{max} and K_M are determined. In CE/DFA,

the height of the plateau signal directly corresponds to the reaction rate, and the plateau height is used for the Michaelis-Menten analysis instead of the reaction rate [20]. On the analysis of the substrate competition reactions, the plateau heights of the two products of ONP and PNP can be obtained through the electrophoretic separation. In the substrate competition, the substrates compete with the binding site of an enzyme with each other, and thus, they can be regarded as reversible competitive inhibitors. Equation (2) can be used in the analysis of the competitive inhibition [9].

$$K_{\mathrm{M}}{}' = K_{\mathrm{M}}\left(1 + \frac{[\mathrm{S}_2]}{K_{\mathrm{I,S2}}{}^{\mathrm{S1}}}\right) \quad (2)$$

In Equation (2), K_M' denotes the apparent K_M of a substrate S_1 determined by the Lineweaver-Burk plots in the presence of a constant concentration of an inhibitor, S_2 in this case, and $K_{I,S2}{}^{S1}$ denotes the inhibition constant of substrate S_2 against a single substrate reaction of S_1. The inhibition constant of $K_{I,S2}{}^{S1}$ is determined through the K_M' values at some S_2 concentrations.

4. Results and Discussion

4.1 Analysis of single-substrate hydrolysis of ONPG or PNPG with β-D-galactosidase

In the CE/DFA format, the enzymatic hydrolysis occurs in the capillary during the migration of the substrate in the separation buffer [20]. A plateau signal is obtained in the electropherogram based on the zero-order reaction of an enzyme and the continuously formed product. The height of the plateau signal is subjected to the Michaelis-Menten analysis, because the plateau height is directly related to the reaction rate. A sample solution containing a single substrate of ONPG or PNPG was injected into the capillary, and the enzymatic hydrolysis was made by in-capillary reaction. Figs. 2A and 2B show the electropherograms obtained with ONPG or PNPG over the concentrations range from 0.5 to 3.0 mmol L^{-1}. Monoanionic ONP or PNP is continuously generated from the corresponding substrate of ONPG or PNPG by the enzymatic hydrolysis. Therefore, the detection order of the substances under the fast EOF is: the neutral substrate of ONPG or PNPG at the migration time of the EOF, followed by a plateau signal of the product of anionic ONP or PNP continuously generated. The descending of the plateau signal to the baseline is apparently two steps at the end

of the plateau signal. The apparently two-step signal with the single substrate is because of the two reaction conditions. One is the formation at the boundary between the injected solution and the separation buffer during the injection period of a solution, which corresponds to the late detection time. The other is the formation during the electrophoresis, and this reaction period corresponds to a wide plateau signal. The migration time of the EOF got longer a little along with the increase in the measurement number. The EOF delay would be attributed to the adsorption of the enzyme to the inner wall of the capillary. However, the separation buffer in the capillary is equilibrated, and the enzymatic reaction proceed at its constant concentration. Even if the migration time may change, the plateau height is slightly affected in this detection format through a certain reaction rate. The plateau signal gradually increased with the migration time at lower concentrations of the substrate. Because the concentration of the substrate was not sufficiently enough and the concentration of the substrate gradually decreased along with the reaction progress, the formation rate of the product got lower. The result is reflected in the electropherograms; the reaction rate is the highest at the start of the electrophoresis, corresponding to the late detection time of the plateau signal. Therefore, the highest response of the plateau signal at the start of the enzymatic reaction was used for the Michaelis-Menten analysis. The insufficiency was not serious at higher concentrations of the substrate, and the plateau signal was flat at 3.0 mmol L^{-1} ONPG, as in Fig. 2c. On the contrary, relatively flat plateau signals were obtained with PNPG over the concentration range examined.

Fig. 2

The Michaelis-Menten constants (K_M) of the enzymatic hydrolysis with β-D-galactosidase were determined through the Lineweaver-Burk plots; the results are shown in Fig. 3. The K_M values were determined with the x-intercept, and $K_{M,ONPG}$ = 0.31 mmol L^{-1} and $K_{M,PNPG}$ = 0.27 mmol L^{-1} were obtained with ONPG and PNPG, respectively. The K_M values obtained in this study are close to the previously reported values of $K_{M,ONPG}$ = 0.12 mmol L^{-1} and $K_{M,PNPG}$= 0.033 mmol L^{-1} [27]. The results are summarized in Table 1. Although the K_M values obtained in this study are somewhat larger than the reported values, the degree of the K_M values are equally $K_{M,ONPG} > K_{M,PNPG}$. On the other hand, the obtained $K_{M,ONPG}$ value is comparable or somewhat smaller than the reported value of $K_{M,ONPG}$ = 0.60 mmol L^{-1} [28]. Because the product is electrophoretically resolved from the substrate zone, the inhibition by the product would be excluded by the CE/DFA format.

When the product is eliminated by the CE separation and the inhibition by the product is excluded, the Michaelis-Menten constant, a dissociation constant, would become smaller by the acceleration of the complex formation reaction from the substrate and the enzyme.

Fig. 3

Table 1

4.2 Evaluation of each plateau height from two-substrate reaction

In a former section, two-substrates reaction in a capillary is schematically illustrated in Fig. 1 with its descriptions. When the electrophoretic mobility of the two substrates are identical and the effective electrophoretic mobility (μ_{eff}) of the corresponding products are different from each other, two-steps plateau signal would be detected as shown in Fig. 1d. The products of ONP and PNP suit the conditions of the μ_{eff} values. The acid dissociation constants (pK_a) of ONP and PNP are 7.23 and 7.15 [29], respectively, and they would possess different μ_{eff} values at certain pH conditions. A smaller pK_a value of PNP suggests that the net charge of PNP is larger than that of ONP and that the negative μ_{eff} value of PNP would also be larger than that of ONP. The larger μ_{eff} value of PNP results in faster backward velocity of the formed PNP and wider plateau signal of PNP. Therefore, the two-steps plateau signal would be detected with lower plateau height as the formed PNP and the total plateau height as the total of the formed ONP and PNP, as illustrated in Fig. 1d.

A practical electropherogram is shown in Fig. 4 with two-substrate reactions of ONPG and PNPG by CE/DFA. The photometric detection was made at two wavelengths of (a) 400 nm and (b) 200 nm for the resolution of the two plateau heights. PNP possesses an absorption maximum at 400 nm, while ONP possesses little absorption at this wavelength. Thus, most of the plateau signal detected at 400 nm (Fig. 4a) can be assigned to PNP. In this way, it is helpful to monitor at the two wavelengths for the discrimination of the plateau signals. It can be noted from Fig. 4b that roughly two-steps plateau signal was detected; the formed PNP was detected at a wider detection period than the formed ONP. The widths of the plateau signals fairly correspond to the anionic character of ONP and PNP; PNP is more anionic. The lower plateau height (arrow y) at the migration time around 6.6 min corresponds to the formed PNP, and the total plateau height (arrow y + arrow z) over the migration time ranging from 4.8 min to 6.3 min corresponds to the total of the formed ONP and

PNP. Therefore, electropherograms detected at 200 nm were practically used for the identifications of the plateau heights and the reaction analysis in the further experiments.

Fig. 4

4.3 Inhibition analysis of substrate competition: Determination of $K_{I,\ PNPG}^{ONPG}$ *and* $K_{I,\ ONPG}^{PNPG}$

Both ONPG and PNPG act as the substrate in the enzymatic reaction of β-D-galactosidase, and therefore, one of the substrates works as a competitive inhibitor against another substrate. Conventionally, substrate competitions have been analyzed through the correction formula proposed by Chou and Talaly [30]. Or, shorter reaction time or initial reaction speed was used for the competitive inhibition. In this study, the substrate competition was examined by the CE/DFA format. Both Michaelis-Menten constants and the inhibition constants are the analysis subjects in this CE/DFA, and the concentration of a substrate was changed in its concentration range from 0.5 mmol L^{-1} to 3.0 mmol L^{-1}. The concentration of an inhibitor was set at 0.5 mmol L^{-1} or 1.0 mmol L^{-1} for the inhibition analysis.

Firstly, ONPG was examined as a substrate and PNPG was set as an inhibitor. Both ONPG and PNPG were contained in the sample solution, where the concentration of PNPG was set at constant and the concentration of ONPG was changed. The Electropherograms are shown in Fig. 5A. It can be read from the electropherograms that the plateau height of ONP increased with increasing concentrations of ONPG. Conversely, the plateau height of PNP decreased with increasing concentrations of ONPG. The results correspond to the fact that excess of one of the substrates reduces the reaction of another substrate by competitive inhibition. Fig. 5B shows Lineweaver-Burk plots of ONPG; single-substrate reaction in Fig. 3 is also included. It is noted in the Lineweaver-Burk plots that all three lines intersect at the y-axis position in spite of the different K_M' values (reciprocal of x intercept). The inhibition pattern can be classified to be competitive inhibition [9]. The inhibition constant was analyzed through Equation (2), and an inhibition constant of PNPG against ONPG was determined as $K_{I,\ PNPG}^{ONPG} = 0.25$ mmol L^{-1} on both PNPG concentrations of 0.5 mmol L^{-1} and 1.0 mmol L^{-1}.

ONPG was also examined as an inhibitor against a substrate PNPG in an opposite way. The electropherograms are shown in Fig. 6A. The plateau height of PNP increased with increasing concentrations of the substrate PNPG, as in the case of Fig. 5A. The plateau height of ONP, a product from the inhibitor,

similarly decreased with increasing concentrations of the substrate. Fig. 6B shows Lineweaver-Burk plots of PNPG; single-substrate reaction in Fig. 3 is also included. The three lines of the Lineweaver-Burk plots also intersect at the y-axis position, and the competitive inhibition is also suggested with ONPG. The competitive inhibition constant of ONPG against PNPG, $K_{\mathrm{I,\ ONPG}}^{\mathrm{PNPG}}$ = 0.30 mmol L^{-1}, was obtained on both ONPG concentrations of 0.5 mmol L^{-1} and 1.0 mmol L^{-1} through Equation (2).

Results on K_I are also summarized in Table 1. Since both K_M and K_I values are related to the affinity of the substrate or the inhibitor toward the enzyme, they link with each other. The K_I values of ONPG or PNPG fairly agrees well with the corresponding K_M values. It is noted from the K_M and K_I values that PNPG possesses higher affinity than ONPG; the result on selective affinity is consistent with the literature values [27].

Fig. 5

Fig. 6

5. Conclusions

An analysis method of two-substrates competition in the enzymatic reaction is proposed in this study based on CE/DFA. This method revealed a prominent feature that a competitive reaction can directly be measured with a solution containing two substrates. ONPG and PNPG were adopted as model competitive substances, and the corresponding products of ONP and ONP were detected as a two-steps plateau signal as a result of the enzymatic hydrolysis with β-D-galactosidase. The heights of two-steps plateau signal were subjected to the Michaelis-Menten kinetic analysis. Lineweaver-Burk plots of the results suggested the competitive inhibition. The K_I value of the inhibitor linked to its K_M value as a substrate, and both values showed that PNPG had higher affinity than ONPG. The usefulness of CE/DFA is demonstrated on the analysis of competitive inhibition on enzyme assay. The proposed CE/DFA analysis would be applicable to other enzymes including multi-substrates reactions and/or competitive inhibitions. Applicability of multi-substrates analysis by CE/DFA will be discussed in the forthcoming study with the degree of the difference in the effective electrophoretic mobility of the products.

Acknowledgements

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Table 1 Micahelis-Menten constants and inhibition constants for ONPG and PNPG determined by CE/DFA

Substrate	K_M / mmol L^{-1}	K_M / mmol L^{-1} [Ref.]	K_I* / mmol L^{-1}
ONPG	0.31	0.12 [27] 0.60 [28]	0.30
PNPG	0.27	0.033 [27]	0.25

*: Average at the substrate concentrations of 0.5 and 1.0 mmol L^{-1}.

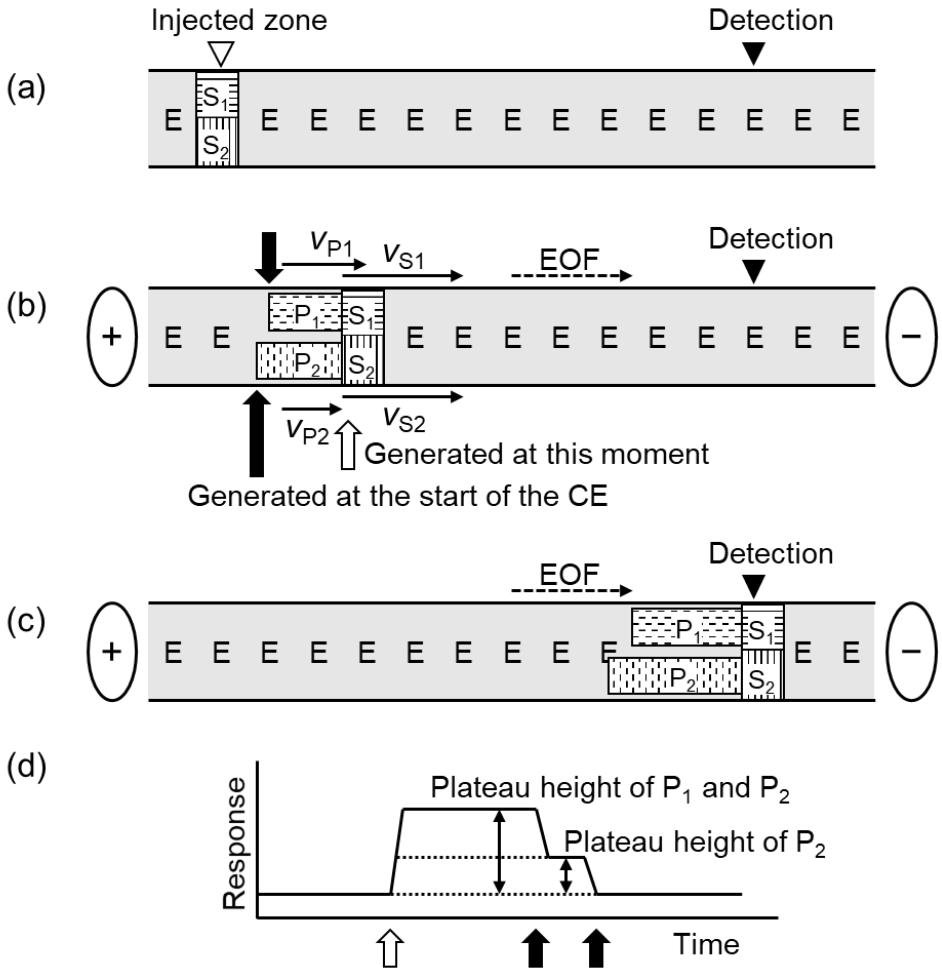

Fig. 1 Schematic migration diagram of two-substrates competitive reaction in CE/DFA. (a) – (c) Two products of P_1 and P_2 are formed from two substrates of S_1 and S_2 during the electrophoresis. An electropherogram (d) is expected by the competitive reaction; two products of P_1 and P_2 are detected as two-steps plateau signals by the enzymatic reaction.

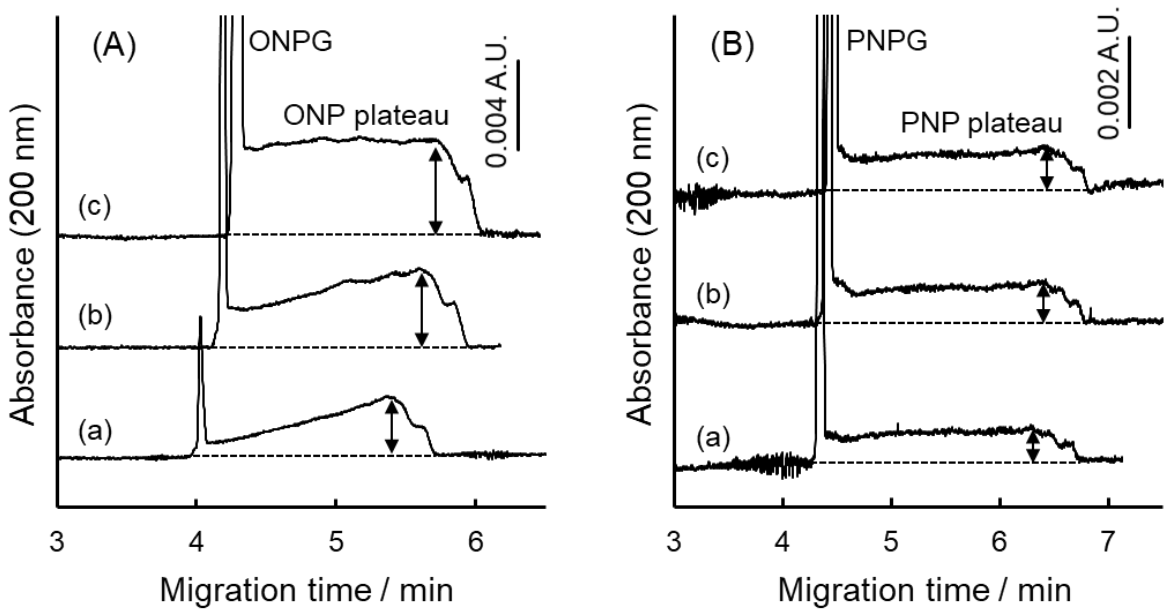

Fig. 2 Electropherograms of (A) ONPG and (B) PNPG under CE/DFA. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 6.78) with 1.22 U mL^{-1} β-D-galactosidase. Concentrations of the substrate in the sample solution: (a) 0.5 mmol L^{-1}, (b) 1.0 mmol L^{-1}, (c) 3.0 mmol L^{-1}. CE conditions are written in the text.

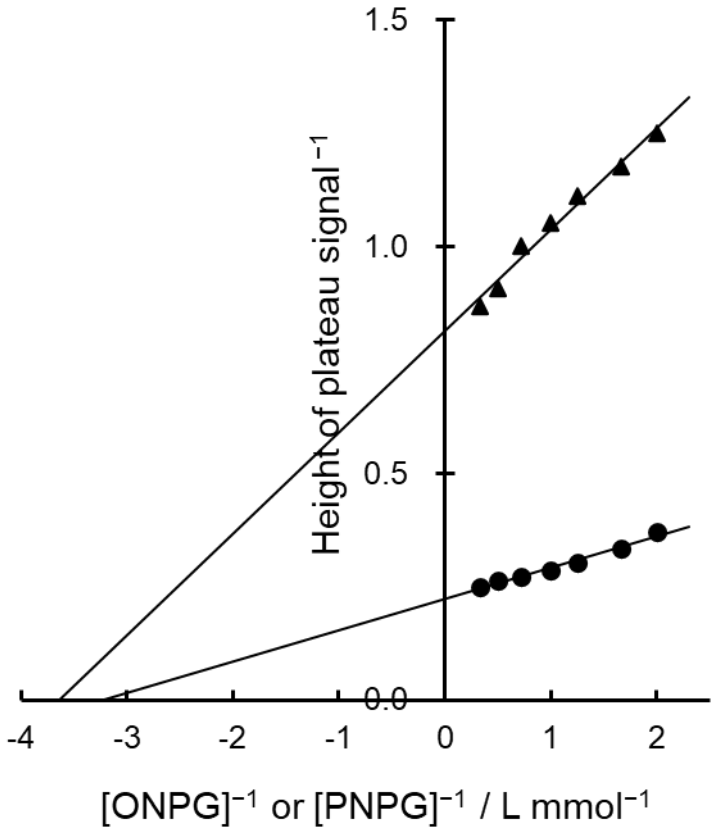

Fig. 3 Lineweaver-Burk plots of the enzymatic reactions of ONPG (●) and PNPG (▲). The separation buffer, the sample solution, and the CE conditions are the same as in Fig. 2.

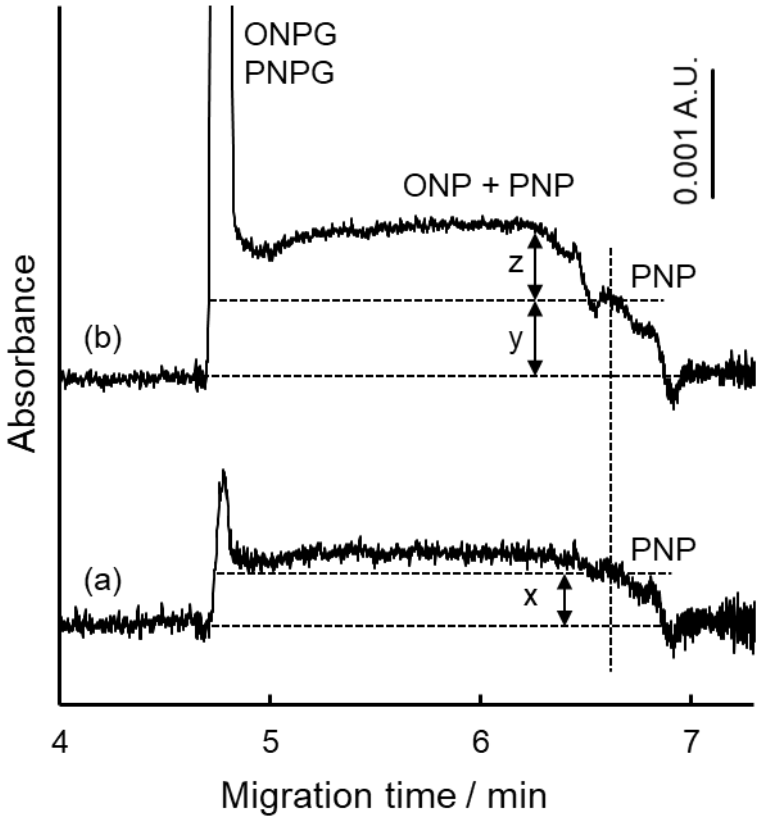

Fig. 4 An electropherogram of two-substrates competitive reaction by CE/DFA detected at two wavelengths of (a) 400 nm and (b) 200 nm. Double-headed arrows x and y correspond to the height of plateau signal of PNP and that of z corresponds to the height of plateau signal of ONP.

Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 6.78) with 1.22 U mL^{-1} β-D-galactosidase. Both concentrations of ONPG and PNPG in the sample solution were 1.0 mmol L^{-1}. CE conditions are written in the text.

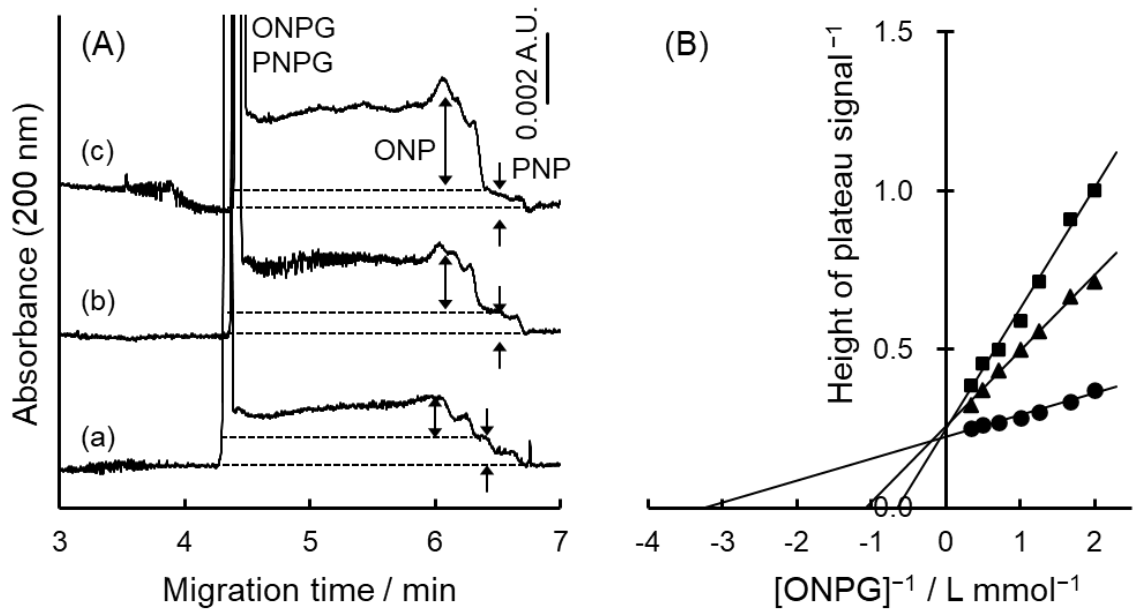

Fig. 5 Electropherograms (A) and Lineweaver-Burk plots (B) of ONPG under inhibition by PNPG.

(A) Concentration of an inhibitor PNPG: 0.5 mmol L^{-1}. Concentration of a substrate ONPG: (a), 0.5 mmol L^{-1}; (b), 1.0 mmol L^{-1}; (c), 3.0 mmol L^{-1}. (B) Concentration of an inhibitor PNPG: (●), none; (▲), 0.5 mmol L^{-1}; (■), 1.0 mmol L^{-1}. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 6.78) with 1.22 U mL^{-1} β-D-galactosidase. CE conditions are written in the text.

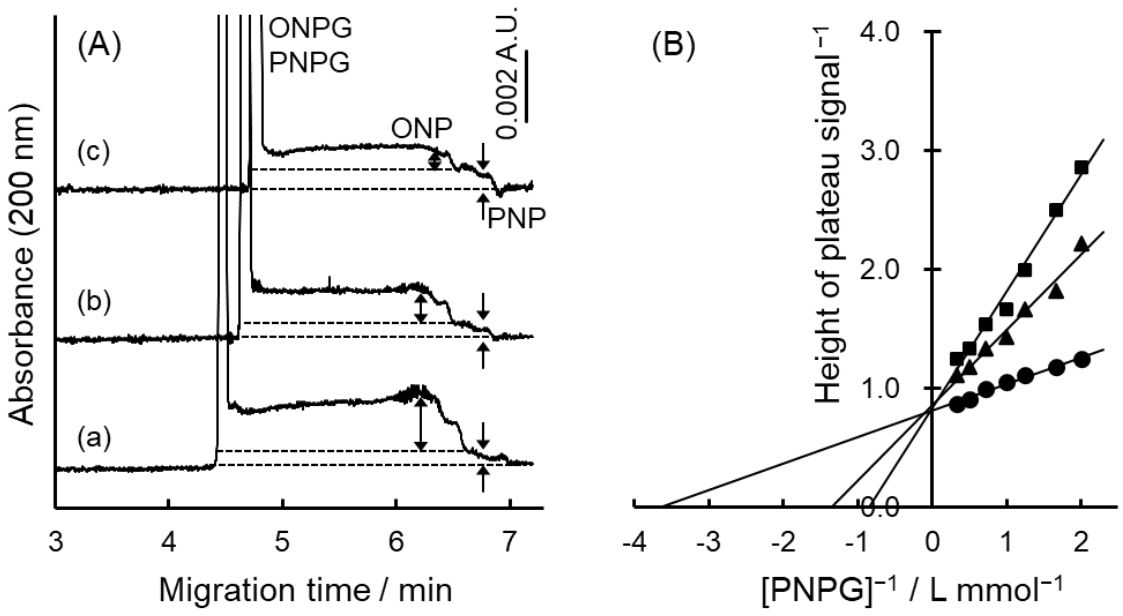

Fig. 6 Electropherograms (A) and Lineweaver-Burk plots (B) of PNPG under inhibition by ONPG.

(A) Concentration of an inhibitor ONPG: 0.5 mmol L^{-1}. Concentration of a substrate PNPG: (a), 0.5 mmol L^{-1}; (b), 1.0 mmol L^{-1}; (c), 3.0 mmol L^{-1}. (B) Concentration of an inhibitor ONPG: (●), none; (▲), 0.5 mmol L^{-1}; (■), 1.0 mmol L^{-1}. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 6.78) with 1.22 U mL^{-1} β-D-galactosidase. CE conditions are the same as in Fig. 5.