ARTICLE

Kinetic analysis of an enzymatic hydrolysis of *p*-nitrophenyl acetate with carboxylesterase by pressure-assisted capillary electrophoresis/dynamic frontal analysis

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Masanori Mine, [a] Naoya Matsumoto, [b] Hitoshi Mizuguchi [c] and Toshio Takayanagi *[c]

An enzymatic hydrolysis of *p*-nitrophenyl acetate with carboxyesterase was analyzed by capillary electrophoresis/dynamic frontal analysis (CE/DFA). A plateau signal was expected with the anionic product of *p*-nitrophenol by the CE/DFA applying in-capillary reaction and the continuous CE resolution of the product from the substrate zone. However, the plateau height was not sufficient, and/or the plateau signal fluctuated and drifted. Therefore, a pressure assist was utilized in the CE/DFA to detect the product zone fast and to average the fluctuated plateau signal by mixing in a laminar flow. The plateau signal became relatively flat and its height was developed by the pressure-assisted capillary electrophoresis/dynamic frontal analysis (pCE/DFA). The plateau height was used for the Michaelis-Menten analysis, and a Michaelis-Menten constant was determined as K_M = 0.83 mmol L^{-1}. An enzyme inhibition was also examined with bis (*p*-nitrophenyl) phosphate by adding it in the separation buffer. The height of the plateau signal decreased by the inhibition, and a 50% inhibitory concentration was determined as IC_{50} = 0.79 μmol L^{-1}. The values of K_M and IC_{50} obtained in this study agreed well with the reported values. Since the proposed pCE/DFA includes electrophoretic migration of the substrate zone in a capillary, it is also noticed that the deactivation of the enzyme by ethanol on the preparation of the substrate solution can be avoided, as well as the exclusion of the inhibition by the product.

Introduction

Enzyme inhibition controls the mechanism of the body, and most small-molecule drugs work by inhibiting a specific enzyme.[1] Analyses of the inhibition reactions contribute to the drug discovery research based on various information about enzyme mechanisms.[2] The inhibition assays have conventionally been made in homogeneous systems. Recently, capillary electrophoresis (CE) has been considered to be an alternative analysis method, and it is widely used for the enzyme activity studies.[3] The advantages of the CE analysis are the small sample consumption and the measurements without any reaction terminator.[4] The in-capillary analyses of enzyme assays are popularly called as: electrophoretically mediated microanalysis (EMMA),[5-7] immobilized enzyme reactor (IMER),[8-10] pressure mediated microanalysis (PMMA),[11] or transverse diffusion of laminar flow profiles (TDLFP).[12,13] The EMMA format is broadly divided into a continuous mode and a plug-plug mode. While the reaction products are detected as peak signals by the plug-plug mode, box-sharped signals are often detected by the continuous mode.[5,14] Inhibition analyses by CE are reported with theophylline on alkaline phosphatase,[14] D-galactal[15] or L-ribose[16] on galactosidase, and argatroban on thrombin.[17] In PMMA, two zones of a substrate and an enzyme form laminar flow, and they are mixed by longitudinal and transversal diffusion.[11]

The present authors have recently developed an enzyme assay of capillary electrophoresis/dynamic frontal analysis (CE/DFA)[18-20] as a technique to monitor the dynamic enzymatic reaction in a capillary. The CE/DFA has common experimental procedure to one of the continuous modes of EMMA, because EMMA includes various formats. In most of the EMMA methods, the reaction product is generally detected as a peak signal and analyzed by area. On the contrary, the CE/DFA includes the following steps; 1: the reaction product is continuously formed in the substrate zone and it is immediately resolved from the substrate zone by electrophoresis, 2: the product is detected as a plateau signal by a zero-order kinetic reaction, and 3: the height of the plateau signal reflects the reaction rate and it can directly be used for the analysis of the zero-order kinetic reaction.[18] Items 1 and 2 correspond to the frontal analysis under a dynamic reaction. The detection of plateau signals based on zero-order kinetic reactions is a characteristic of CE/DFA, and therefore, the CE/DFA is not limited to enzymatic reaction; any zero-order kinetic reaction including homogeneous catalysts would be applied to the proposed CE/DFA. Additionally, the elimination of the product inhibition is also a characteristic of CE/DFA, because the reaction product is continuously resolved from the substrate zone by electrophoresis. The latter characteristics is not noticed or

[a] *Graduate School of Advanced Technology and Science, Tokushima University, 2-1 Minamijyousanjimacho, Tokushima 770-8506, Japan*
[b] *Faculty of Science and Technology, Tokushima University, 2-1 Minamijyousanjimacho, Tokushima 770-8506, Japan*
[c] *Graduate School of Technology, Industrial and Social Sciences, Tokushima University, 2-1 Minamijyousanjimacho, Tokushima 770-8506, Japan.*
E-mail: toshio.takayanagi@tokushima-u.ac.jp

discussed in the general EMMA. Thus, the proposed CE/DFA would be a novel analysis method for the zero-order kinetic reactions. The CE/DFA has been applied to the inhibition assay by tandem injections of a substrate and an inhibitor solutions.[19] 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. The enzymatic reaction is inhibited over the overlapping period, and a depressed plateau can be detected at the plateau signal.[19] The substrate competition has also been analyzed by CE/DFA through the different electrophoretic mobility of the products.[20] When a sample solution containing two substrates is introduced into the capillary filled with a separation buffer containing an enzyme, a substrate competition occurs during the electrophoretic migration of the substrates. A two-steps plateau signal can be detected based on the different electrophoretic mobility of the two products, and the two plateau heights are used for the analysis of the competitive inhibition.[20]

Mammalian carboxylesterase (CES, EC.3.1.1.1) is a type of serine hydrolase that contains multiple gene families, and it is localized in the lumen of the endoplasmic reticulum.[21,22] It is divided into five gene families; CES1 being expressed in the liver, CES2 in the intestine, and CES3 in the brain, liver, and colon.[22] Because CES participates in the hydrolysis reactions of many substances with ester, thioester, amide, or carbamate moieties, it plays an important role *in vivo* including metabolism of xenobiotics, detoxification and metabolic activation of environmental toxins and carcinogens.[23] CES1 and CES2 are widely used in studies on xenobiotic metabolism.[24] These two enzymes differ significantly with each other in the hydrolysis of certain drugs, in addition to the differences in tissue distribution.[23] Since CES will continuously be used for the design of pro-drugs and soft drugs,[24-26] it is important to develop analysis methods for the CES activity.

In this study, the present authors propose a novel mode of CE/DFA applying an enzymatic hydrolysis of *p*-nitrophenylacetate (NPA) with CES as a model reaction. Pressure assist has been utilized to develop both the reaction efficiency and the mixing in the product zone based on the laminar flow. Although the CES activity has been analyzed by CE,[27,28] they are off-line analyses and any on-line monitoring has not been reported yet. Usefulness of pressure-assisted capillary electrophoresis (pCE) is reported with the MS analyses of heparin depolymerized disaccharides,[29] multivalent anions,[30] and metabolomics anions,[31] drug discovery,[32] analysis of acid dissociation constants,[33] frontal analyses of interactions between methoxan and human serum albumin[34] and between benzoate and hydroxypropyl-β-cyclodextrin.[35] A pressure assist was also used in the electrokinetic injection for the sensitivity development of haloacetic acids.[36] By using the pressure assist, the substrate and product migrated in the capillary as laminar flow profiles, and an averaged plateau signal of the product of *p*-nitrophenol (NP) was obtained by the pressure-assisted capillary electrophoresis/dynamic frontal analysis (pCE/DFA). A Michaelis-Menten constant (K_M) was successfully determined by the proposed pCE/DFA.

Inhibition assay was also examined by the proposed pCE/DFA. Bis (*p*-nitrophenyl) phosphate (BNPP) has been reported as an irreversible inhibitor of CES, and it is often used as a model inhibitor for the inhibition screening.[37,38] The height of the plateau signal was reduced by the inhibition reaction by the addition of BNPP in the separation buffer. Decrease in the plateau height was subjected to the inhibition analysis, and a 50% inhibitory concentration (IC_{50}) was also determined.

Reaction scheme in CE/DFA under pressure assist

As previously pointed out in PMMA, a laminar flow profile is generated by the pressure assist, and the flow profile develops the boundary area between the adjacent zones.[11] Mixing of the zones is also promoted by the laminar flow. The characteristics of the pressure assist were utilized in this study as pCE/DFA. Fig. 1 schematically illustrates the stepwise stages of the enzymatic reaction in pCE/DFA. Firstly, a separation capillary is filled with a separation buffer containing an enzyme (E). A substrate solution (S) is then introduced into the capillary hydrodynamically (Fig. 1a). A DC voltage is applied to the capillary for the electrophoresis, and a settled air pressure is simultaneously applied to the inlet vial over the separation period. The enzymatic reaction proceeds during the migration of the substrate in the capillary under the applied voltage and the pressure. The reaction product (P) is continuously formed and resolved from the substrate zone as in CE/DFA. Different from the conventional CE/DFA, mixing of the zones is

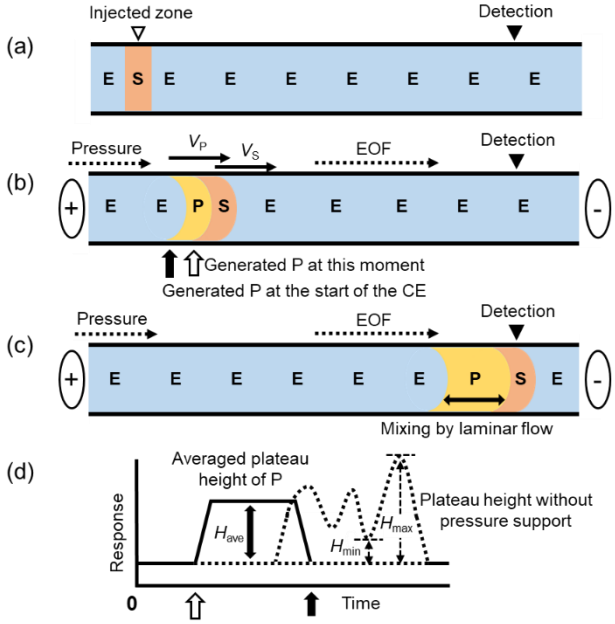

Fig. 1 Schematic diagram of an enzymatic hydrolysis of NPA with CES by pCE/DFA. (a)-(c) show the migration of the substrate (S) and the product (P) in a laminar flow profile in a capillary, where an enzyme (E) is contained in the separation buffer. A box-shaped plateau signal would be detected by the pCE/DFA as in solid line in (d) based on the diffusion by the laminar flow, while the fluctuated signal (dotted line) might be detected without pressure assist. H_{max}, H_{min} and H_{ave} denote the maximum, minimum, and average heights of the plateau signal.

accompanied due to the laminar flow (Fig. 1b). In the reaction system forming monoanionic NP from electrically neutral NPA, the substrate NPA reaches the detector position fast and a wide zone of the product NP follows (Fig. 1c). During the migration in the capillary, the formation of product may not be at a constant rate and the detected plateau signal might be fluctuated as in dotted line in Fig. 1d. However, the diffusion by the laminar flow mixes the fluctuated concentrations of the products, and the fluctuated response would be averaged, as in the solid lines in Fig. 1d. Thus, an averaged plateau height, H_{ave}, would be detected. Detection of the plateau signal would also be shortened by the pressure assist, which would suppress the adsorption of the enzyme to the inner wall of the capillary and reduce the shot signals.

Experimental

Apparatus

All CE experiments were performed by a ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) quipped with a photodiode array detector. A fused-silica capillary (GL Sciences, Tokyo, Japan) was set in a cassette cartridge, and the cartridge was installed in the CE system. Dimensions of the 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. 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. An Agilent Technologies ChemStation software (Ver. B04.02) was used for the control of the CE system, the data acquisition, and the data analysis.

Chemicals

An enzyme of carboxylesterase (CES1; Carboxylesterase 1 isoform b human, EC: 3.1.1.1) was obtained from Sigma-Aldrich (St. Louis, MO, USA). A substrate of NPA was from Tokyo Chemical Industry (Tokyo, Japan). The NPA was dissolved in ethanol and used after the dilution with purified water. NP was from FUJIFILM Wako Pure Chemical (Osaka, Japan). An inhibitor of BNPP was from Sigma-Aldrich. All other reagents were of analytical grade. Water used was purified by a Milli-Q Gradient A10 (Merck Millipore, Milford, MA, USA).

Procedure

A phosphate buffer solution (10 mmol L^{-1}, pH 7.4) was used as a separation buffer; the separation buffer also contained 2.0 U mL^{-1} CES. After the separation capillary being equilibrated with the separation buffer by flushing for 2 min, a substrate solution was hydrodynamically injected into the capillary from the anodic end by applying a pressure at 50 mbar for 3 s. Both ends of the capillary were dipped in the separation buffer vials, and a DC voltage of 3 kV and an air pressure at 15 mbar were applied to the inlet vial for the pCE. The substrate NPA was detected at 270 nm, while the product NP was detected at 400 nm. Separation buffers containing the inhibitor BNPP were used in the inhibition assay at its concentration range from 0.01 to 10 μmol L^{-1}.

Determination of the Michaelis-Menten constant

Michaelis-Menten kinetic analysis is popularly made with Lineweaver-Burk plots as in equation (1). A Michaelis-Menten constant (K_M) can be determined with several data sets of the substrate concentration, [S], and the reaction rate, v. In this study, the plateau height was used instead of the reaction rate, since the plateau height directly related with the reaction rate.[18,19]

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

The inhibition assay was made at several BNPP concentrations, and a 50% inhibitory concentration (IC_{50}) was determined with the results.

Results and discussion

Optimization of the assist pressure

The substrate NPA is electrically neutral and the product NP possesses anionic charge of −1. Thus, NPA would be detected first and the NP continuously produced would follow as a plateau signal, as is schematically illustrated in Fig. 1. When a solution containing 3.0 mmol L^{-1} of NPA was used as a sample solution in a CE/DFA, electropherograms were obtained as shown in Fig. 2. The residual NPA was detected at the migration time of the electroosmotic flow (EOF). An obvious peak was also detected at the end of the raised response; the peak is attributed to the NP degraded prior to the CE analysis. When a CE/DFA was examined at 15 kV applied voltage (Fig. 2A), the plateau signal of the product NP was not sufficiently high and some shot signals were detected in the electropherogram. Decreasing the applied voltage is one of the choices to improve the plateau height; the product NP would slowly be resolved from the substrate zone. The result at 3 kV applied voltage is shown in Fig. 2B. Although the detection of the plateau signal took very long time up to 70 min, the height of the plateau signal was developed and the shot signals fortunately

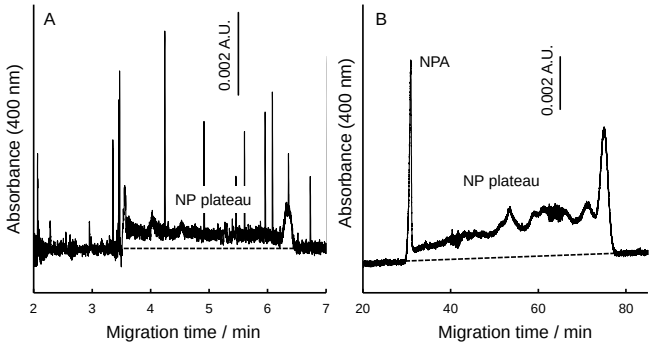

Fig. 2 CE/DFA electropherograms of NPA at (A) 15 kV and (B) 3 kV applied voltage. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) with 2.0 U mL^{-1} CES. The sample solution contained 3.0 mmol L^{-1} NPA and the phosphate buffer. CE conditions except the applied voltage are written in the text.

disappeared. However, the plateau signal fluctuated; the fluctuated response suggests that the reaction rate would not be constant. The fluctuation of the raised response was not developed even by changing the applied voltage. Additionally, the height of the plateau signal gradually decreased along with the course of the reaction from the late detection time to the early detection time. A long measurement time is equivalent to a long reaction time in CE/DFA, and the descending is because of the decreased concentration of the substrate NPA along with the reaction time. The measurement time of up to 70 min is not practical, and the fluctuating response seriously interferes with the kinetic analysis of the enzymatic hydrolysis. Therefore, pressure assist was utilized to shorten the measurement time, as well as to improve the contact time between the substrate and the enzyme through the zone dispersion of the substrate zone by the laminar flow profile.

Fig. 3 shows the effect of the assist pressure during the electrophoresis; the assist pressure was examined in the range between 5 and 20 mbar. Both the detection time and the plateau width were significantly reduced by the pressure assist. The result is attributed to the faster migration of the separation buffer toward the detection point. Both the fluctuation and the drift of the plateau signal were also developed by the pressure assist, owing to the mixing in the product zone. The height of the plateau signal, however, changed little by the assist pressure. The reaction rate with the enzyme was not affected by the changes in the flow profile under pCE. This result suggests that the substrate zone is diluted little by the applied pressure and that a settled concentration of the enzyme is kept constant during the pCE. Assist pressure above 20 mbar was not suitable because the plateau signal overlapped with the degraded NP before the CE analysis. On the other hand, assist pressure below 5 mbar was not practical due to the long measurement time. Therefore, an assist pressure of 15 mbar was adopted in the pCE/DFA, compromising the short analysis time and the wide plateau width.

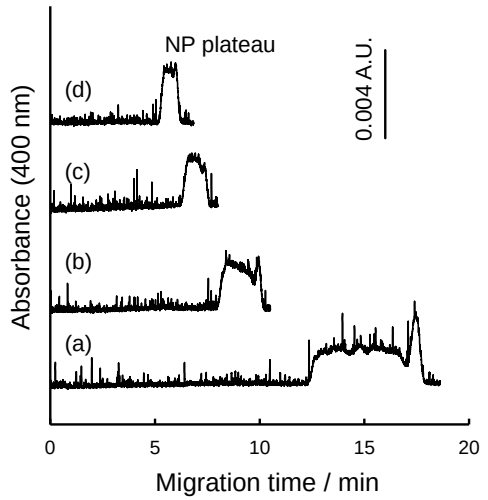

Fig. 3 Effect of the assist pressure on pCE/DFA. The assist pressure was: (a) 5 mbar, (b) 10 mbar, (c) 15 mbar, (d) 20 mbar. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) with 2.0 U mL^{-1} CES. The sample solution contained 3.0 mmol L^{-1} NPA and the phosphate buffer. pCE conditions are written in the text.

Michaelis-Menten analysis of CES by pCE/DFA

Michaelis-Menten analysis was made in a series of the substrate concentrations ranging from 0.5 to 3.0 mmol L^{-1}; the height of the plateau signal was monitored. Typical electropherograms are shown in Fig. 4A. The height of the plateau signal increased with increasing substrate concentrations. The ending edge of the plateau signal overlapped with the degraded NP before the CE analysis, and a time-lag also exists on complete contact between the sample zone and the enzyme in the separation buffer. Because of the time-lag, the plateau height gradually increased with the reaction progress from the later to the earlier detection time. Since the starting edge of the plateau signal was a little higher over the range of the plateau, the height of the midpoint of the plateau signal was used for the analysis. The Michaelis-Menten constant (K_M) of the enzymatic hydrolysis of NPA has been determined by Lineweaver-Burk plots; the results are shown in Fig. 4B. A straight line was obtained by the analysis at an assist pressure of 15 mbar, suggesting that the reaction rate was successfully measured under a steady state. A K_M value of 0.83 mmol L^{-1} was obtained. Lineweaver-Burk plots were also made at the assist pressure of 5 or 10 mbar (Fig. 4B), and almost identical K_M values were obtained within ±0.02 mM; the certainty of the proposed pCE/DFA method was thus confirmed. The obtained K_M value agreed well with a literature value of 0.822 mmol L^{-1}.[39] The product NP is continuously resolved from the NPA zone in pCE/DFA, and therefore, the inhibition from the product is excluded, as previously noticed.[18,19]

The height of the NP plateau signal was the same for four repeated measurements. The apparent electrophoretic mobility of the NP may differ under the conditions of the inner wall of the capillary tube and the time range of the plateau signal may change as well, the height of the plateau signal changed little.

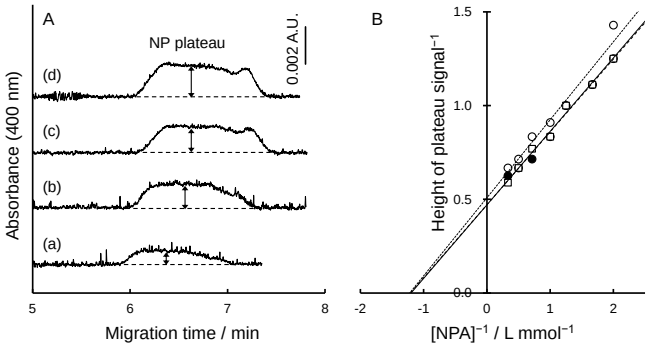

Fig. 4 Typical electropherograms (A) and Lineweaver-Burk plots (B) of NPA in pCE/DFA. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) with 2.0 U mL^{-1} CES. Sample solutions contained (a) 0.5 mmol L^{-1}, (b) 1.0 mmol L^{-1}, (c) 2.0 mmol L^{-1}, (d) 3.0 mmol L^{-1} NPA and the phosphate buffer. The assist pressure in (B) was ○ 5 mbar, ● 10 mbar, and □ 15 mbar. Other pCE conditions are the same as in Fig. 3c.

Analysis of BNPP inhibition in pCE/DFA

BNPP has been reported as an irreversible inhibitor of CES,[37] and it is often used as a model inhibitor of CES.[38] In this study, BNPP was also examined as an inhibitor in pCE/DFA. Separation buffers containing BNPP and CES were prepared in the pCE/DFA, and the decrease in the plateau height was measured as activity

decrease (%). Electropherograms under the BNPP inhibition are shown in Fig. 5A. The height of the plateau signal decreased with the increasing concentrations of BNPP. The enzymatic hydrolysis was continuously inhibited with BNPP during the migration of the substrate NPA in the separation buffer. The inhibition results based on the decrease in the plateau height are shown in Fig. 5B. A 50% inhibitory concentration was obtained as IC_{50} = 0.79 μmol L^{-1} from the intersection value with a straight-line crossing y = 50. Reference values of IC_{50} = 0.60 μmol L^{-1} [40] and 1.8 μmol L^{-1} [41] have been reported with hCES1 and porcine liver CES1, respectively. The IC_{50} value determined in this study is close to the values. Therefore, the proposed pCE/DFA would be useful for the inhibition analysis of enzymes.

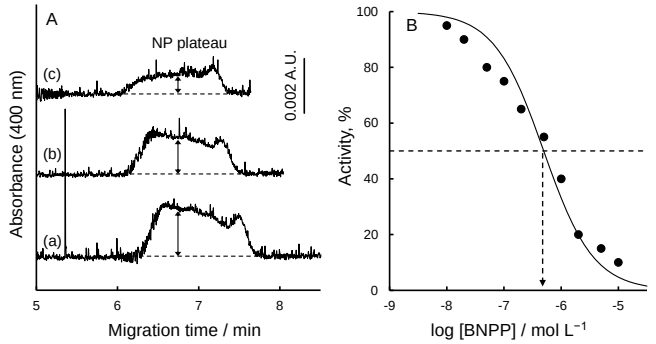

Fig. 5 Typical pCE/DFA electropherograms (A) and inhibition plots (B) of NPA under the BNPP inhibition. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) + 2.0 U mL^{-1} CES + BNPP. Concentrations of BNPP: (a) 0.01 μmol L^{-1}, (b) 0.1 μmol L^{-1}, (c) 1.0 μmol L^{-1}. Sample solutions contained 2.0 mmol L^{-1} NPA and the phosphate buffer. pCE conditions are the same as in Fig. 3c. A curve in (B) is drawn with $100/(1+[BNPP]/IC_{50})$.

Avoiding the deactivation with ethanol in pCE/DFA

Under the investigation of the inhibition analysis, another advantage was noticed in pCE/DFA. Deactivation of the enzyme with ethanol can be avoided by the in-capillary reaction and the electrophoretic migration. In a practical enzymatic analysis in a homogeneous solution, the reaction batch sometimes contains organic solvent to dissolve the substrate. Since the addition of organic solvent causes irreversible deactivation of the enzyme, its use should be kept to a minimum in the sample preparation.[10] It is also necessary to optimize the addition amount of the solvent.[10] In the pCE/DFA format, ethanol is also used on the preparation of the NPA solution; the NPA solution is injected as a sample zone. The NPA zone migrates in a capillary and the NPA reacts with a fresh enzyme in the separation buffer. Thus, the deactivation of the enzyme would be minimized.

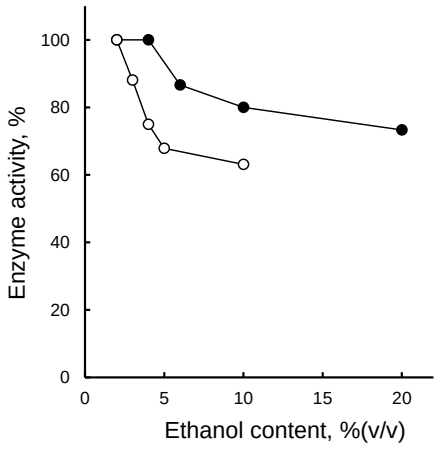

Fig. 6 Relationship between the ethanol content in the sample solution and the enzyme activity. ●, pCE/DFA; ○, pre-capillary reaction. Conditions for the pre-capillary reaction: 2.0 mmol L^{-1} NPA and 2.0 U mL^{-1} CES in 10 mmol L^{-1} phosphate buffer (pH 7.4). Incubation time: 6 min. CE conditions for the pre-capillary reaction: 25 kV applied voltage, 50 mbar × 3 s sample injection, detection wavelength at 400 nm, and 37 °C capillary temperature. The separation buffer was 10 mmol L^{-1} phosphate buffer (pH 7.4).

In this section, the effect of ethanol added under the sample preparation was compared between a pre-capillary reaction and a pCE/DFA. The concentration of ethanol in the reaction vessel was controlled at 2-10%(v/v) in the pre-capillary reaction, and the formed NP was determined by a practical CE analysis. On the contrary in pCE/DFA, a separation buffer containing an enzyme was prepared in an aqueous solution, while an NPA solution was prepared in ethanol and it was used as sample solutions after the dilution with water. The sample solutions practically contained 2 %(v/v) ethanol. Fig. 6 shows the decrease in the enzyme activity due to the ethanol. An enzymatic activity of 100% was the results of 2 %(v/v) ethanol, because the concentration was the least in a series of the measurements. It is noticed from Fig. 6 that the activity decreased by more than 15% in the pre-capillary reaction even at 3 %(v/v) ethanol. On the other hand, the deactivation with ethanol was avoided up to 4 %(v/v) ethanol in pCE/DFA. An isoelectric point (p*I*) of 6.2 is reported with human CES1,[42] and the CES is anionic in the separation buffer. Thus, the enzymatic hydrolysis occurs while the CES passing through the zone of the sample solution containing electrically neutral NPA. The enzyme is not deactivated with ethanol just before the enzymatic reaction in the NPA zone, and the enzyme is continuously fed into the NPA zone. Therefore, the deactivation with ethanol would be avoided in pCE/DFA.

Conclusions

A novel analysis method of an enzyme assay is proposed in pCE/DFA. An enzymatic hydrolysis of NPA with CES was used as a practical example, as well as the irreversible inhibition with BNPP. By using the pressure assist, the detection time of the plateau signal was shortened, and both the fluctuation and the drift of the plateau were developed. A K_M value was determined as 0.83 mmol L^{-1} from the Lineweaver-Burk plots and it agreed well with the literature value. An IC_{50} value was also determined with BNPP through the decrease in the plateau height; IC_{50} = 0.79 μmol L^{-1} was obtained. Usefulness of the pCE/DFA was noticed on avoiding the deactivation with ethanol. The proposed pCE/DFA would be utilized for other enzymatic assays, as well as zero-order kinetic reactions.

Conflicts of interest

There is no conflict of interest.

Acknowledgement

This work was partly supported by JSPS KAKENHI [grant number 20K05568].

References

1 N. V. Bhagavan, C.-E. Ha, in "*Essentials of Medical Biochemistry with Clinical Cases*", *Chap. 6 Enzyme and Enzyme Regulation*, 2011, Academic Press, pp. 47-58, London UK.
2 L. Mazzei, S. Ciurli, B. Zambelli, *Method Enzymol.*, 2016, **567**, 215.
3 J. J. Bao, J. M. Fujima, N. D. Danielson, *J. Chromatogr. B*, 1997, **699**, 481.
4 Y. Fan, G. K. E. Scriba, *J. Pharm. Biomed. Anal*., 2010, **53**, 1076.
5 J. Bao, F. E. Regnier, *J. Chromatogr. A*, 1992, **608**, 217.
6 B. J. Harmon, D. H. Patterson, F. E. Regnier, *J. Chromatogr. A,* 1993, **657**, 429.
7 D. H. Patterson, B. J. Harmon, F. E. Regnier, *J. Chromatogr. A,* 1994, **662**, 389.
8 J. Iqbal, *Anal. Biochem*., 2011, **414**, 226.
9 J. Iqbal, S. Iqbal, C. E. Müller, *Analyst*, 2013, **138**, 3104.
10 M. Cheng, Z. Chen, *Electrophoresis*, 2017, **38**, 486.
11 H. Nehme, R. Nehme, P. Lafite, S. Routier, P. Morin, *Anal. Chim. Acta*, 2012, **722**, 127.
12 S. M. Krylova, V. Okhonin, S. N. Krylov, *J. Sep. Sci.*, 2009, **32**, 742.
13 E. Farcaş, L. Pochet, M. Fillet, *Talanta*, 2018, **188**, 516.
14 A. R. Whisnant, S. E. Johnston, S. D. Gilman, *Electrophoresis,* 2000, **21**, 1341.
15 D. B. Craig, T. T. Morris, C. M. Q. Ong-Justiniano, *Anal. Chem.*, 2012, **84**, 4598.
16 J. J. Crawford, J. W. Hollett, D. B. Craig, *Electrophoresis*, 2016, **37**, 2217.
17 L. Pochet, A.-C. Servais, E. Farcas, V. Bettonville, C. Bouckaert, M. Fillet, *Talanta*, 2013, **116**, 719.
18 T. Takayanagi, M. Mine, H. Mizuguchi, *Anal. Sci.*, 2020, **36**, 829.
19 M. Mine, H. Mizuguchi, T. Takayanagi, *Chem. Lett.*, 2020, **49**, 681.
20 M. Mine, H. Mizuguchi, T. Takayanagi, *J. Pharm. Biomed. Anal.*, 2020, **188**, 113390.
21 T. Satoh, M. Hosokawa, *Annu. Rev. Pharmacol.*, 1998, **38**, 257.
22 R. S. Holmes, M. W. Wright, S. J. F. Laulederkind, L. A. Cox, M. Hosokawa, T. Imai, S. Ishibashi, R. Lehner, M. Miyazaki, E. J. Perkins, P. M. Potter, M. R. Redinbo, J. Robert, T. Satoh, T. Yamashita, B. Yan, T. Yokoi, R. Zechner, L. J. Maltais, *Mamm. Genome*, 2010, **21**, 427.
23 D. Yang, R. E. Pearce, X. Wang, R. Gaedigk, Y.-J. Y. Wan, B. Yan, *Biochem. Pharmacol.*, 2009, **77**, 238.
24 D. Wang, L. Zou, Q. Jin, J. Hou, G. Ge, L. Yang, *Acta Pharm. Sin. B*, 2018, **8**, 699.
25 T. Imai, *Drug Metab. Pharmacok.*, 2006, **21**, 173.
26 L. D. Hicks, J. L. Hyatt, S. Stoddard, L. Tsurkan, C. C. Edwards, R. M. Wadkins, P. M. Potter, *J. Med. Chem*., 2009, **52**, 3742.
27 J. Lamego, A. S. Coroadinha, A. L. Simplício, *Anal. Chem.*, 2011, **83**, 881.
28 L. Lan, X. Ren, J. Yang, D. Liu, C. Zhang, *Bioorg. Chem.*, **94**, 2020, 103388.
29 V. Ruiz-Calero, E. Moyano, L. Puignou, M. T. Galceran, *J. Chromatogr. A*, 2001, **914**, 277.
30 T. Soga, Y. Ueno, H. Naraoka, K. Matsuda, M. Tomita, T. Nishioka, *Anal. Chem.*, 2002, **74**, 6224.
31 K. Harada, E. Fukusaki, A. Kobayashi, *J. Biosci. Bioeng.*, 2006 **101**, 403.
32 J. M. Miller, A. C. Blackburn, Y. Shi, A. J. Melzak, H. Y. Ando, *Electrophoresis*, 2002, **23**, 2833.
33 Z. Szakács, B. Noszál, *Electrophoresis*, 2006, **27**, 3399.
34 L. Zhao, D. Chen, *Biomed. Chromatogr.*, 2015, **29**, 123.
35 C. Qian, S. Wang, H. Fu, R. F. B. Turner, H. Li, D. D. Y. Chen, *Electrophoresis*, 2018, **39**, 1786.
36 H. Zhang, J. Zhu, R. Aranda-Rodriguez, Y.-L. Feng, *Anal. Chim. Acta*, 2011, **706**, 176.
37 S. Kehraus, S. Gorzalka, C. Hallmen, J. Iqbal, C. E. Müller, A. D. Wright, M. Wiese, G. M. König, *J. Med. Chem.*, 2004, **47**, 2243.
38 A. L. Simplício, A. S. Coroadinha, J. F. Gilmer, J. Lamego, *Methods Mol. Biol.*, 2013, **984**, 309.
39 M. J Hatfield, L. Tsurkan, J. L. Hyatt, X. Yu, C. C. Edwards, L. D. Hicks, R. M. Wadkins, P. M. Potter, *Br. J. Pharmacol*., 2010, **160**, 1916.
40 J. Shi, X. Wang, J. Nguyen, A. H. Wu, B. E. Bleske, H.-J. Zhu, *Drug Metab. Dispos.*, 2016, **44**, 554.
41 G. F. Makhaeva, S. V. Lushchekina, N. P. Boltneva, V. B. Sokolov, V. V. Grigoriev, O. G. Serebryakova, E. A. Vikhareva, A. Y. Aksinenko, G. E. Barreto, G. Aliev, S. O. Bachurin, *Sci. Rep.*, 2015, **5**, 13164.
42 R. S. Holmes, L. A. Cox, J. L. VandeBerg, *Com. Biochem. Physiol. D*, 2009, **4**, 209.

