Original Paper

Kinetic Analyses of Two-steps Enzymatic Oxidation from Hypoxanthine to Uric Acid with Xanthine Oxidase by Capillary Electrophoresis/Dynamic Frontal Analysis

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Abstract

Two steps of enzymatic oxidations from hypoxanthine to uric acid with xanthine oxidase (XOD) were kinetically analyzed by capillary electrophoresis/dynamic frontal analysis. When a substrate solution of hypoxanthine was introduced into a capillary with a separation buffer containing XOD, the enzymatic reaction continuously proceeded during the electrophoresis and a product of xanthine was continuously resolved from the substrate zone. A plateau signal of the product xanthine was detected based on the constant reaction rate with XOD. The plateau height was directly related with the reaction rate, and a Michaelis-Menten constant $K_{M,HXA}$ was successfully determined as 770±40 μmol L^{-1}. When xanthine was used as a substrate, a slope response of uric acid was obtained because of the low concentrations of the substrate and its significant decrease. However, the Michaelis-Menten constant was successfully determined by using the initial reaction rate, and a Michaelis-Menten constant of $K_{M,XA}$ was determined as 85±6 μmol L^{-1}.

Keywords: Xanthine oxidase; Hypoxanthine; Xanthine; Capillary electrophoresis; Dynamic frontal analysis

1. Introduction

Xanthine oxidase (XOD) is recognized as a terminal enzyme of purine catabolism [1-5]. It catalyzes hypoxanthine (HXA) to xanthine (XA) and XA to uric acid (UA) by oxidation, as shown in Fig. 1. The reaction kinetics have been examined by spectrophotometry [2,3,5], LC-MS/MS [6], and HPLC-DAD [7]. Two steps of Michaelis-Menten constants have also been determined by the kinetic analyses.

Recently, kinetic analyses of enzymes have popularly been made by capillary electrophoresis (CE) [8,9]. The CE analyses are broadly divided into two formats: off-line analysis and in-capillary analysis. In off-line analysis, the enzymatic reaction is done in a vial, and the reaction solution is subjected to the CE separation and determination. On the other hand, a substrate and an enzyme are introduced into a separation capillary, and the enzymatic reaction is done in the capillary by incubation. The reaction product is subsequently resolved and determined by CE. The in-capillary format is further classified as electrophoretically mediated microanalysis (EMMA) [10-15], transverse diffusion of laminar flow profile (TDLFP) [16-18], and immobilized enzyme reactor (IMER) [19-26]. Three formats are different in the merging technique between a substrate and an enzyme. A substrate and an enzyme solutions are

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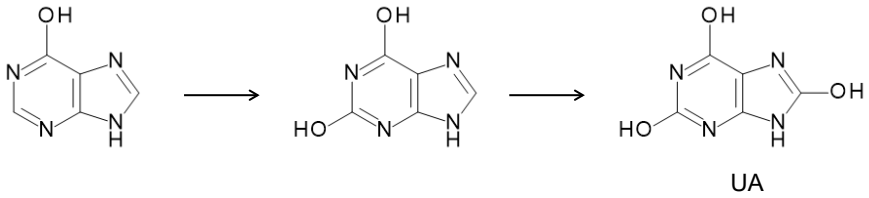

Fig. 1. Two-steps enzymatic oxidation of hypoxanthine with xanthine oxidase.

tandemly injected into a capillary and they are merged by electrophoretic migration (EMMA) or by hydrodynamic propelling (TDLFP). In IMER, an enzyme is immobilized on inner wall of a capillary and a substrate solution is held at the enzyme portion of the capillary. After the incubation for the enzymatic reaction, the substrate zone is electrophoretically resolved. The reaction product is detected as a peak signal, and it is determined for the kinetic analysis.

When a separation capillary is filled with a substrate solution and an enzyme solution is injected into the capillary [9,27,28], or a separation capillary is filled with an enzyme solution and a substrate solution is injected into the capillary [29-34], the enzymatic reaction proceeds by the migration of a substance in the injected zone in the separation buffer. When any electrophoretic resolution of the product is involved, the product is continuously resolved and a plateau response of the product is obtained. In the latter case of the plateau response, the response is based on the continuous resolution of the product from the substrate zone, and it was named as capillary electrophoresis/dynamic frontal analysis (CE/DFA) by the present authors [29-34]. Since the product is promptly taken away from the substrate zone, the enzymatic reaction at the substrate zone is not inhibited with the product. The kinetic analyses were successfully made with alkaline phosphatase [29,30], β-D-glucosidase [31], esterase [32], creatine kinase [33], and tyrosinase [34].

In this study, two-steps kinetic analyses of XOD, shown in Fig. 1, were successively analyzed by CE/DFA. The substrate concentrations were examined at mmol L^{-1} level or less, and thus the generated products were very low concentrations. The plateau response is based on the formation rate of the product, and it was not sufficiently high. However, the Michaelis-Menten constants were successfully determined by the CE/DFA.

2. Theory

It is essential in CE/DFA that a substrate and a product possess different electrophoretic mobility (μ), because the product should electrophoretically be resolved from the substrate zone. The electrophoretic migration of the substrate and the products with the enzymatic reactions were examined prior to the practical CE/DFA. The reagents of HXA, XA, and UA are negatively charged at a reaction condition of pH 7.4, but the net charge of the reagents is varied based on their acid dissociation constant (pK_a). The pK_a values for HXA, XA, and UA are 8.9, 7.4, and 5.4, respectively [35]. The pK_a values suggest that HXA is the least charged at pH 7.4, and UA is the most anionic at the pH condition. Accordingly, degree of the effective electrophoretic mobility of the substances are: $\mu_{HXA} < \mu_{XA} < \mu_{UA}$.

When a sample solution containing a substrate of XA is introduced into a separation capillary filled with an enzyme solution, as in Fig. 2(a), the XA is simply oxidized to form a product of UA by the second-step enzymatic reaction. The effective electrophoretic mobility of the anionic UA is larger than XA, and net velocity under an electroosmotic flow (EOF) is faster with XA. Thus, UA migrate slower than XA.

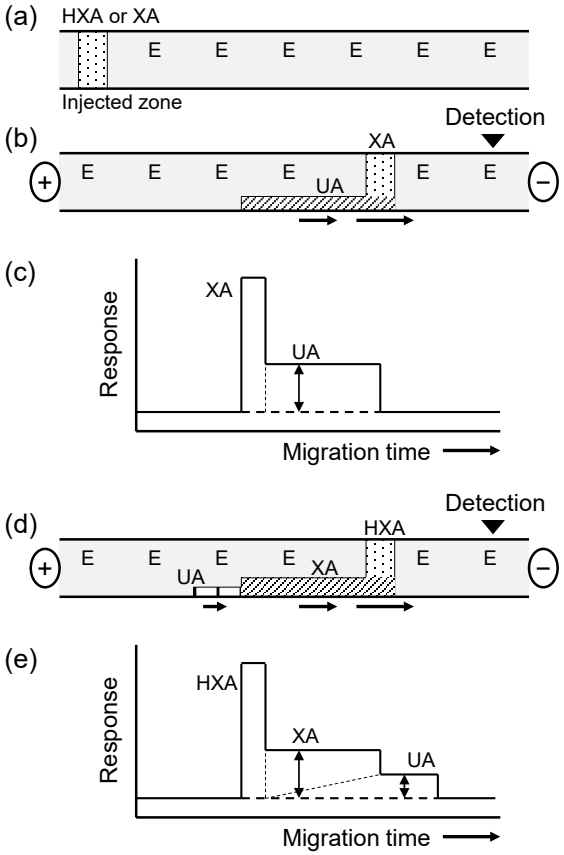

Fig. 2. Schematic illustration on enzymatic oxidation with XOD by CE/DFA. A substrate solution of HXA or XA is injected into a capillary as a short plug (a). An enzyme XOD (E) is contained in the separation buffer, and the substrate is oxidized to the product(s) during the electrophoretic migration in a capillary (b) and (d). Continuously generated product(s) would be detected as a plateau signal(s) as illustrated in the electropherograms (c) and (e).

The electrophoretic migration of XA and UA are schematically illustrated in Fig. 2 (b). Continuously generated UA would be detected as a plateau signal subsequent to the detection of the substrate XA peak, as is illustrated in Fig. 2 (c).

When HXA is used as a substrate, a product of XA is generated by the enzymatic reaction. A plateau signal for XA would be detected, as a similar manner to the XA oxidation. The second-step oxidation of XA would further proceed at the XA plateau zone, because the XA at the plateau zone migrate in the separation buffer containing XOD. The contact time of the generated XA with the XOD increases along with the migration in the capillary, and consequently, the concentration of the generated UA would increase. Therefore, the concentration of the generated UA would be the highest at the start of the CE (late migration time) and the lowest at the detection of the substrate HXA (early migration time). The effective electrophoretic mobility of the final product UA is larger than HXA or XA, and UA would go out from the XA plateau. After the UA going out from the XA plateau, the substrate of XA does not exist and the UA would be detected as a second plateau, as illustrated in Figs. 2 (d) and (e).

3. Experimental

3.1. Apparatus

An Agilent Technologies (Waldbronn, Germany) ^{3D}CE equipped with a photodiode array detector was used as a CE system. A fused silica capillary purchased from GL Sciences (Tokyo, Japan) was cut in an adequate length and it was packed in a cassette cartridge; the cartridge was installed in the 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. A ChemStation software (Agilent Technologies, Ver. B04.02) was used for the control of the CE system and the data analysis. A Horiba (Kyoto, Japan) F-71s pH meter, calibrated with standard pH solutions, was used for adjusting the pH of the separation buffers.

3.2. Reagents

An enzyme of xanthine oxidase (from bovine milk, EC 1.17.3.2) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Substrates of hypoxanthine and xanthine, as well as a reaction product of uric acid, were also from Sigma-Aldrich. Other reagents were of analytical grade. Water used was purified by PURELAB Chorus (Veolia Water STI, Saint-Maurice, France).

3.3. Procedure for the pre-capillary reactions of the enzyme

An enzyme solution of XOD was added to a sample solution containing a substrate HXA or XA with its temperature controlled at 37 °C. After the enzymatic reaction with a reaction time of 10 or 15 min, the reactant solution was subjected to the measurement by capillary zone electrophoresis (CZE). A separation buffer contained 10 mmol L^{-1} phosphate buffer (pH 7.4). The reactant solution was hydrodynamically injected into the capillary from anodic end at 50 mbar for 3 s, and a CZE separation was made by applying a voltage of 20 kV. The substrate and the product were photometrically detected at 200 nm. During the CZE, the temperature of the capillary was thermostated at 37 °C.

3.4. Procedure for the CE/DFA of the enzymatic reactions

In the CE/DFA format, the enzymatic reaction continuously proceeds in a separation capillary, and the separation buffer contains the enzyme XOD. Control of the pH of the separation buffer is also essential to give stable EOF and the adequate reaction conditions of the enzyme. Thus, the separation buffer contained a phosphate buffer (pH 7.4) and an enzyme of XOD. A sample solution containing a substrate, HXA or XA, was hydrodynamically injected into the capillary from the anodic end at 50 mbar for 3 s, and a CE/DFA was made by applying a voltage of 15 or 20 kV. Both the substrate and the reaction product were photometrically detected at 210 nm. During the CE/DFA, the temperature of the capillary was thermostated at 37 °C.

4. Results and discussion

4.1. Kinetic analyses by precapillary reactions with XOD

Prior to the CE/DFA with XOD, the enzymatic reactions were examined in a precapillary format. The enzymatic reaction was carried out in a sample vial for a fixed reaction time of 10 or 15 min, and the reactant solution was subjected to the CZE analysis.

A typical electropherogram with HXA substrate is shown in Fig. 3A. The substrate HXA is less charged at pH 7.4, and it migrated just after the EOF marker. A major product of XA and a minor product of UA were detected. The migration order agreed with the effective charge of the products: UA is the most anionic and it migrated the slowest at the CZE conditions. Calibration graphs for HXA, XA, and UA by CZE analysis were linear in their concentration range from 0 to 1.0 mmol L^{-1} ($r > 0.996$). The concentrations of XA and UA detected in Fig. 3A were approximately 50 μmol L^{-1} and 2 μmol L^{-1}, respectively. Thus, the second-step conversion from XA to UA was little in the reaction vial. Considering the linear calibration graph for XA and the little conversion from XA to UA, the peak area for XA was directly used for the Michaelis-Menten analysis.

When XA was used as a substrate in the enzymatic reaction, a reaction product of UA was resolved from XA by a CZE analysis, as in Fig. 3B. The peak area for UA was also used for the Michaelis-Menten analysis.

Michaelis-Menten plots for HXA and XA are shown in

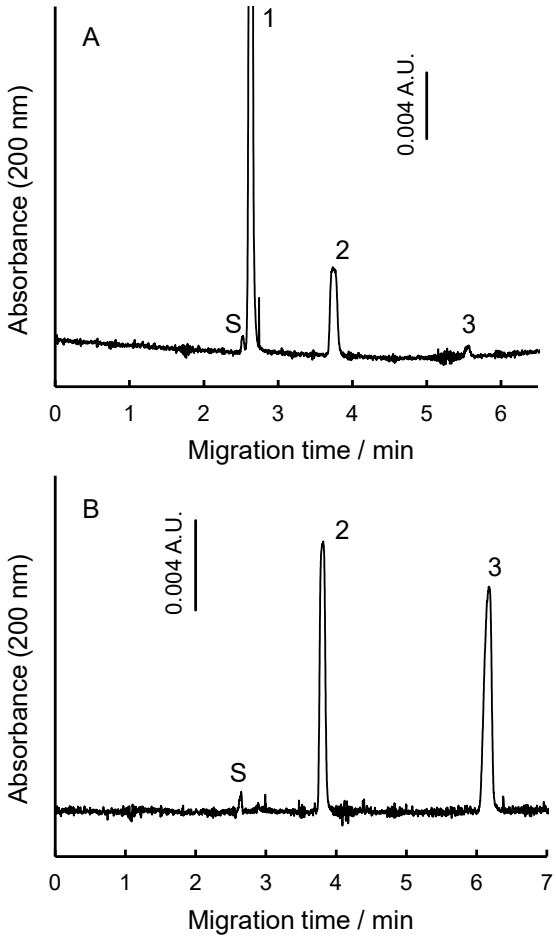

Fig. 3. Typical electropherograms for A: HXA and B: XA under enzymatic reaction with XOD by a precapillary reaction. Sample solution contained 0.8 mmol L^{-1} HXA or 0.16 mmol L^{-1} XA with 0.47 unit mL^{-1} XOD. Enzymatic reaction was done for 15 min at 37 °C. Separation buffer contained 10 mmol L^{-1} phosphate buffer (pH 7.4). Signals: 1, HXA; 2, XA; 3, UA. S: solvent (EOF). CZE conditions: 20 kV applied voltage, 37 °C capillary temperature, 50 mbar × 3 s sample injection, and 200 nm detection wavelength.

Figs. 4A and 4B, respectively. The generation of the products, XA from HXA and UA from XA, was developed with the longer reaction time from 10 min to 15 min.

Enzymatic reaction and a Michaelis-Menten constant are defined as in Reaction (1) and Eq. (2), respectively.

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_\mathrm{M}}{\rightleftarrows}} \mathrm{ES} \longrightarrow \mathrm{E} + \mathrm{P} \qquad (1)$$

$$K_\mathrm{M} = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]} \qquad (2)$$

where E, S, P, and ES are an enzyme, a substrate, a product, and a complex, respectively, and K_M is the Michaelis-Menten constant. The reaction rate of the product, v, is written as in Eq. (3) with the Michaelis-Menten constant and a maximum rate, V_{max}.

$$v = \frac{V_{max}[\mathrm{S}]}{K_\mathrm{M}+[S]} \qquad (3)$$

Both Michaelis-Menten constants from HXA to XA, $K_{\mathrm{M,HXA}}$, and from and from XA to UA, $K_{\mathrm{M,XA}}$, were analyzed through the results in Figs. 4A and 4B, respectively. A software of R program (Ver. 4.2.1) [36] was used for the analysis. A series of pairs of substrate concentration and peak area of the product were input in Eq. (3), and values of the V_{max} and the K_M were optimized by a non-linear least-squares analysis. Curves in Figs. 4A and 4B are drawn with the optimized values. Determined K_M values are summarized in Table 1. The reaction time affected little on the K_M values.

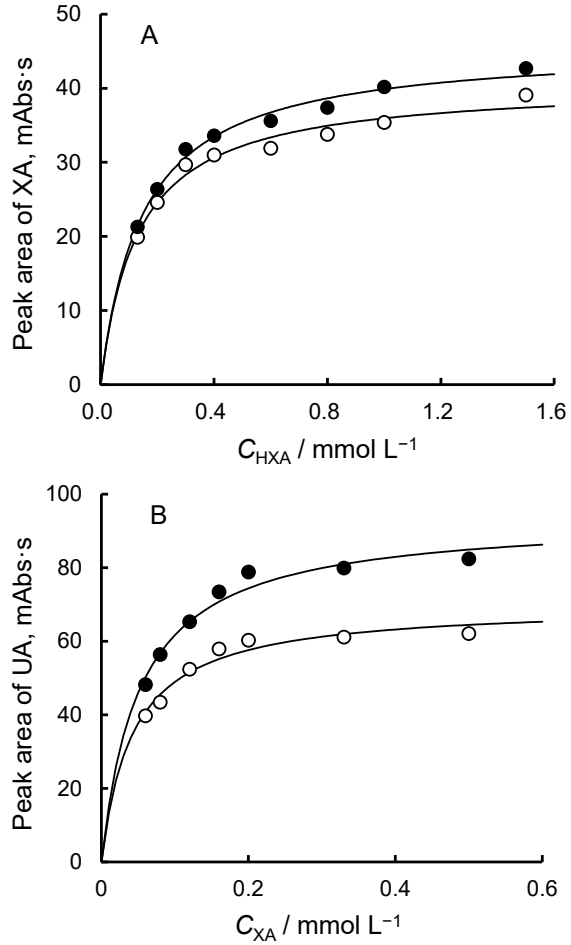

Fig. 4. Michaelis-Menten plots for the enzymatic reactions of A: HXA and B: XA. Rection time: ○, 10 min; ●, 15 min. Reaction conditions and CE conditions are the same as in Fig. 3.

Table 1. Michaelis-Menten constants determined by the pre-capillary reaction.

Reaction time	Michaelis-Menten constant, μmol L^{-1}	
	$K_{\mathrm{M,HXA}}$	$K_{\mathrm{M,XA}}$
10 min	130±20*	43±7*
15 min	150±10*	52±7*

* Error: standard error.

4.2. Reaction analysis from HXA to XA by a CE/DFA

Enzymatic reactions with XOD were applied to a CE/DFA format. Characteristics of CE/DFA is that the product is generated from the substrate zone at a constant rate and a plateau signal is obtained with the product when the substrate is sufficiently excess. A major advantage on CE/DFA is that the product is continuously resolved from the substrate zone, and the enzymatic reaction is not inhibited with the product [29,31-34]. Results on the formation of XA from HXA are shown in Fig. 5. Detection wavelength was set at 210 nm to reduce the baseline noise. In the electropherograms, HXA is less charged at pH 7.4, and it migrated just after the EOF marker. Plateau signal of XA was detected after the HXA peak. The migration behavior and the detected response are as expected in Fig. 2. The end edge of the plateau signal corresponds to the generated XA at the start of the electrophoresis and the enzymatic reaction. The effective electrophoretic mobility calculated from the end edge is close to that of XA, and the plateau signal would be attributed to XA. The second-step oxidation from XA to UA is involved in this CE/DFA. However, the second plateau, expected in Fig. 2(e), was not detected in the electropherograms, and the formation of UA would not be sufficient. Results on the precapillary reaction described above also supports the formation of little amount of UA. The reaction time, CE separation time, is also shorter than the precapillary reaction. Therefore, contribution of UA to the plateau signal would be negligible in this practical CE/DFA.

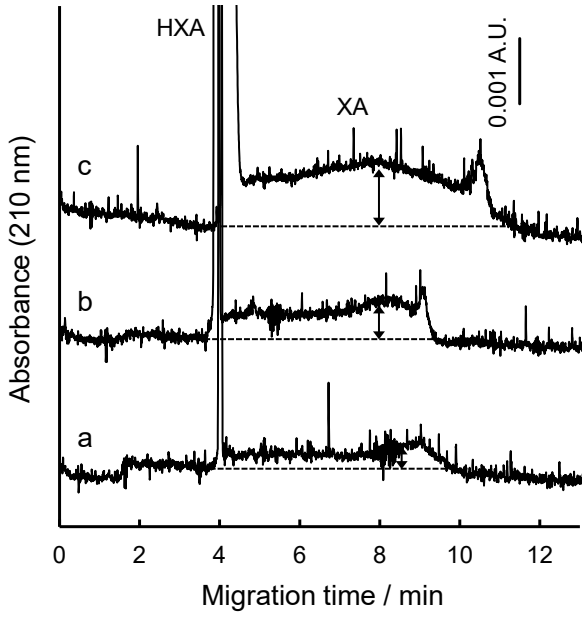

Fig. 5. Typical electropherogram for the enzymatic reaction of HXA with XOD under CE/DFA. Concentration of HXA in the sample solution: a, 0.4 mmol L^{-1}; b, 1.6 mmol L^{-1}; c, 3.0 mmol L^{-1}. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) + 0.04 unit mL^{-1} XOD. CE conditions: 15 kV applied voltage, 37 °C capillary temperature, 50 mbar × 3 s sample injection, and 210 nm detection wavelength.

The height of the plateau signal developed with increasing concentrations of HXA, although the plateau response was not sufficiently high and thus the baseline is relatively noisy. The effectiveness of the plateau height is discussed later. Since the height of the plateau signal is directly related with the reaction rate, the plateau height was used for the Michaelis-Menten analysis [29]. The plateau signal fluctuated a little, and the plateau height at the maximum was used for the analysis. Because the substrate HXA is less charged at the CE/DFA conditions, its migration from the injected plug zone would take a while, and time-lag to the reaction maximum would have been taken.

The Michaelis-Menten plots for HXA by the CE/DFA are shown in Fig. 6. A series of pairs of substrate concentration and plateau height were input in Eq. (3), and values of the V_{max} and the $K_{M,HXA}$ were optimized by a non-linear least-squares analysis, as in a similar manner with the precapillary reactions. A $K_{M,HXA}$ value of 770±40 μmol L^{-1} was determined; a curve in Fig. 6 is drawn with the optimized values. The value is somewhat larger than the one determined by the precapillary reaction. The $K_{M,HXA}$ value determined by CE/DFA is also summarized in Table 2.

The $K_{M,HXA}$ value of 770 μmol L^{-1} suggests that the enzymatic reaction should be examined at this concentration level of the substrate. The plateau height in Fig. 5 also suggests that the formed XA is 10^{-5} mol L^{-1} level or less. To obtain sufficient height of the plateau response, increasing the enzyme concentration in the separation buffer could be a solution, but higher concentration of the enzyme can consume the substrate much more and the concentration of substrate would seriously decrease during CE/DFA. In all cases, concentration level of the substrate and the reaction rate of the enzyme should be considered to obtain desirable height of the plateau response.

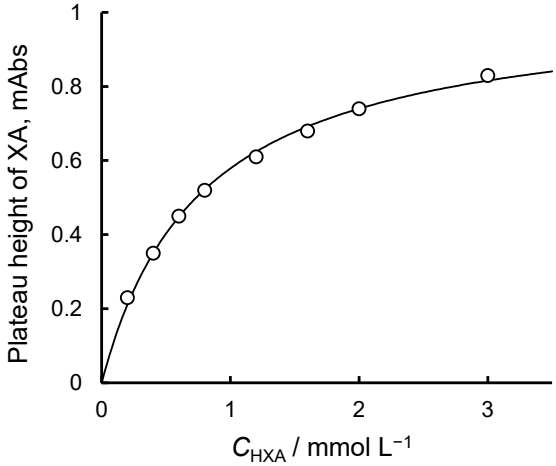

Fig. 6. Michaelis-Menten plots for the enzymatic reaction of HXA by a CE/DFA. CE conditions are the same as in Fig. 5.

4.3. Reaction analysis from XA to UA by a CE/DFA

The CE/DFA format was also applied to the second-step

enzymatic reaction of XOD from XA to UA. The results are shown in Fig. 7, where a substrate XA was contained in the sample solutions. Sloped signal of the product UA was detected after the peak of the substrate XA. The slope signal of UA, descending from its late migration time to the early migration time, suggests that formation rate of UA has been reduced by a meaningful consumption of the substrate. Because the substrate concentrations should be set at sub mmol L^{-1} level for the Michaelis-Menten analysis, significant decrease in the XA concentration is unavoidable. The formation rate of the product UA descended in the electropherograms, and therefore, a height of the UA response at the initial reaction rate, *i.e.*, at the late migration time, was used for the Michaelis-Menten analysis.

The Michaelis- Menten plots for XA by the CE/DFA are shown in Fig. 8. A Michaelis-Menten constant, $K_{M,XA}$, was also determined by an R program with a series of the data set. A $K_{M,HXA}$ value of 85±6 μmol L^{-1} was determined; a curve in Fig. 8 is drawn with the optimized values. The value is comparable with the one determined by the precapillary reaction. The $K_{M,XA}$ value is also summarized in Table 2.

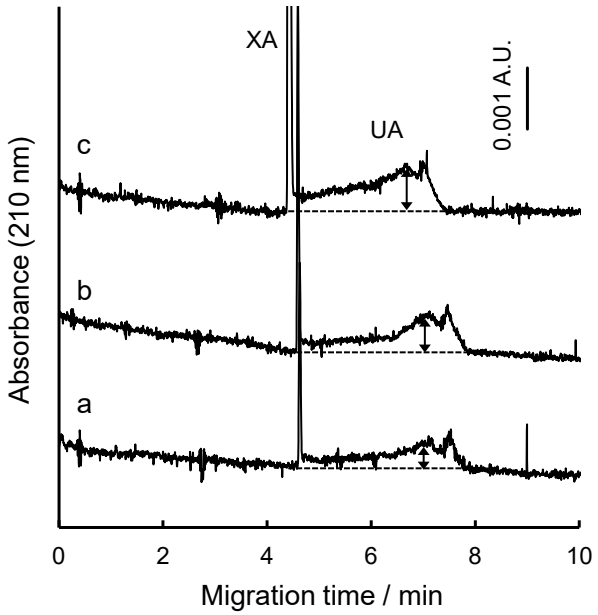

Fig. 7. Typical electropherogram for the enzymatic reaction of XA with XOD under CE/DFA. Concentration of XA in the sample solution: a, 0.08 mmol L^{-1}; b, 0.16 mmol L^{-1}; c, 0.33 mmol L^{-1}. Separation buffer: 10 mmol L^{-1} phosphate buffer (pH 7.4) + 0.04 unit mL^{-1} XOD. CE conditions: 20 kV applied voltage, 37 ºC capillary temperature, 50 mbar × 3 s sample injection, and 210 nm detection wavelength.

4.4. Comparison of the Michaelis-Menten constants

Michaelis-Menten constants determined by the present CE/DFA are compared with some reported values. It can be noted from Table 2 that both K_M values are in wide variations with the analysis methods. Results by the spectrophotometry [2,3,5] showed small K_M values. It would be because

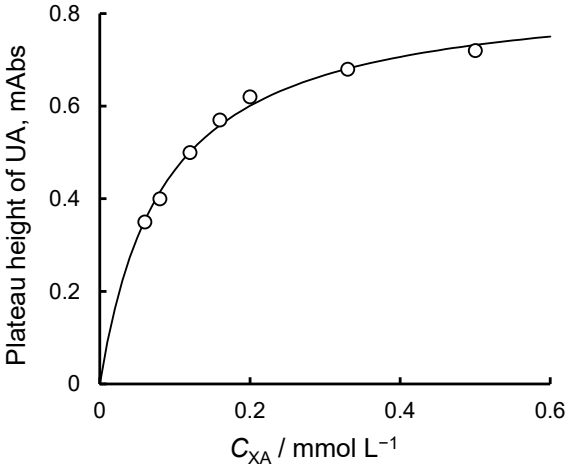

Fig. 8. Michaelis-Menten plots for the enzymatic reaction of XA by a CE/DFA. CE conditions are the same as in Fig. 7.

spectrophotometric analysis does not include separation between the substrate and the product. Results obtained by the precapillary reaction, summarized in Table 1, are comparable with the results by LC-MS/MS [6], HPLC-DAD [7], and IMER [26]. These analysis formats, including pre-capillary reaction in this study, commonly involve incubation for a settled reaction time. Results by the CE/DFA are also comparable with the reported values but somewhat larger than the results by LC-MS/MS [6] and HPLC-DAD [7]. CE/DFA does not involve incubation, and the product is promptly removed from the reaction mixture. Such difference in the reaction matrix would have affected the Michaelis-Menten constants.

Table 2. Michaelis-Menten constants determined by CE/DFA and reported values.

Analysis method	Michaelis-Menten constant, μmol L^{-1}		Ref.
	$K_{M,HXA}$	$K_{M,XA}$	
CE/DFA	770±40*	85±6*	This study
Spectrophotometry	6	8	2
Spectrophotometry	1.86±0.1	3.38±0.17	3
Spectrophotometry	52.7±5.7	–**	5
LC-MS/MS	–**	14.5±0.5	6
HPLC-DAD	250	–**	7
IMER	–**	390	26

* Error: standard error. ** Not reported.

5. Conclusions

In this study, two-steps of the kinetic analyses of XOD were successively analyzed by CE/DFA from HXA to XA and from XA to UA. Although the product responses are faint at the examined substrate concentrations, both Michaelis-Menten constants were successfully determined through the plateau height and the peak height at the initial reaction rate, respectively. The K_M values reported are in wide variations, however, results by the present CE/DFA are comparable with them.

Acknowledgements

This work was partly supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research C (20K05568).

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