Direct measurement of initial rate of enzyme reaction by electrokinetic filtration using a hydrogel-plugged capillary device

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Abstract:

A novel electrokinetic filtration device using a plugged hydrogel was developed to directly measure the initial rate of enzyme reactions. In the proposed method, the enzyme reaction proceeded only for a short time when substrate passed through a thin layer of enzyme trapped by the hydrogel without any lag times for mixing and detection. In experimental, alkaline phosphatase (enzyme) was filtrated at a cathodic-side interface of the plugged hydrogel by molecular sieving effect, providing the thin enzyme zone whose thickness was approximately 100 \( \mu \)m. When 4-methylumberiferyl phosphate (substrate) was electrokinetically introduced into the device after trapping the enzyme, 4-methylumberiferone (product) was generated by the enzyme reaction for only 1.26 s as the substrate passed through the trapped enzyme zone. As a result, the initial rate of the enzyme reaction could be directly calculated to 31.0 \( \mu \)M/s by simply dividing the concentration of the product by the tunable reaction time. Compared to the initial rate obtained by mixing the enzyme and substrate solutions, the value of the maximum velocity of the enzyme reaction was 30-fold larger than that in the mixing method due to the preconcentration of the enzyme by trapping. The Michaelis-Menten constant in the proposed method was 2.7-fold larger
than that in the mixing method, suggesting the variation of changes in the equilibrium of complex
formation under the experimental conditions.

**Keywords** Enzyme, initial rate of enzyme reaction, electrokinetic filtration, gel electrophoresis
1. Introduction

Enzymes are proteins with catalytic activity for specific substrates; they are utilized in various fields such as diagnosis, engineering, and synthesis. The kinetic analyses of their catalytic activities are exceedingly crucial to evaluate their performance and achieve a highly effective enzyme reaction. The initial rate ($v_0$) of an enzyme reaction is one of the most important factors to understand its kinetics. In conventional assays, microplates are often employed to measure enzymatic activity, but this involves an inevitable lag time from mixing solutions of enzymes and substrates in each microwell.\textsuperscript{1-4} Therefore, the direct measurement of $v_0$ is difficult in conventional microplate assays, and $v_0$ must be estimated mathematically from the time interval required to yield the amounts of products.

To overcome this problem, a stopped flow system was employed to evaluate the $v_0$ values immediately after mixing the solutions containing the enzyme and substrate.\textsuperscript{5-8} In the stopped flow assays, the enzyme and substrate solutions are rapidly injected into a mixing chamber by applying high pressure, resulting in effective mixing for several milliseconds. Immediately after, the mixed solution is transferred to an optical cell for detection. Wei et al. revealed the inhibition mechanism of an enzyme reaction by investigating the pre-steady-state kinetics using the stopped flow method.\textsuperscript{5} Alexandra \textit{et al.} studied an endonuclease whose specificity is changed by the sequence of nucleotide.\textsuperscript{6} The stopped flow systems revealed the kinetics of the enzyme reaction immediately after mixing due to the transportation of the mixed solution from the mixing chamber to the optical cell in just milliseconds. Meanwhile, this method includes a dead time. Additionally, detection of the product immediately after arrival at the optical cell sometimes encounters an experimental limitation, owing to the mixing efficiency of the enzyme/substrate solutions. Thus, the
stopped flow system often requires a theoretical calculation/mathematical fitting by software to evaluate $v_0$.

Recently, many researchers have focused on microfluidic devices because they allow direct monitoring of various chemical processes, such as mixing, reactions, and separations.\textsuperscript{9-19} These devices have numerous advantages, e.g., low consumption of samples/reagents, short analysis time, and large surface-to-volume ratio, which is beneficial for bioassays that require small amounts of samples.\textsuperscript{15-19} Thus, various enzyme reactions have been analyzed using these microfluidic devices. Hughes et al. developed a microfluidic device for zymography using a gradient gel.\textsuperscript{17} The proposed method allowed measurement of the fluorescent product from the reaction between the immobilized enzyme and the electrokinetically introduced substrate without any dead time/volume. However, the value of $v_0$ had to be estimated by theoretical calculations after carrying out prolonged fluorescence imaging near the immobilized enzyme. In addition, the value of $K_M$ in the method was larger than that in the free solution because of the limitation of diffusion in the hydrogel. In other cases, microfluidic droplet devices were reported for monitoring enzyme reactions.\textsuperscript{20-23} Helen et al. developed a method for investigating millisecond kinetics using a microdroplet device.\textsuperscript{20} This device allows the rapid and highly effective mixing of reagents by an eddy flow in the microdroplets, thus circumventing the issues pertaining to the dead time and mixing efficiency in the stopped flow method described above. David et al. demonstrated the high-throughput enzyme kinetic analysis using a stroboscopic camera.\textsuperscript{21} Sequential fluorescence images of the product in microdroplets containing the enzyme and substrate could be analyzed individually by the developed method, realizing the rapid and precise measurement of $v_0$. However, both devices required a mixing channel before monitoring
the fluorescence of the products. Thus, these methods could not measure the value of $v_0$ directly.

In other technologies for the measurement of enzymatic activity, capillary electrophoresis is often used due to its low sample consumption and high separating ability. In enzyme assay with capillary electrophoresis, enzymes are often immobilized onto an inner surface of open-tubular/monolithic capillaries or beads filled in capillaries, which is called immobilized enzyme reactors (IMERs). A substrate is introduced into the capillary and reacts with the immobilized enzyme, providing a product of enzyme reaction. The IMERs have many advantages such as quantitative determination, reusability and less requirement of sample work-up. Thus, IMERs has advanced the various application of pharmaceutical industry.

Mohamed et al. demonstrated an analysis of enzyme kinetics which exhibited good stability and reproducibility. Jing and coworkers developed an enzyme reactor using magnetic beads. However, IMERs must require the immobilization of enzyme, which might alter the conformation and activity of them. In contrast, Sakai-Kato et al. developed the non-covalently immobilized enzyme reactor using microsomes encapsulated by a sol-gel matrix. The method allowed to measure $v_0$ which was closed to $v_0$ in the free solution. However, there still remains the problem of mixing efficiency as same as the stopped flow methods and the other IMERs. Therefore, the calculation of an accurate value of $v_0$ is also difficult in IMERs. Recently, Mine and Takayanagi et al. reported a measurement of $v_0$ based on a frontal analysis in capillary electrophoresis. In the proposed method, a short plug of substrate solution was introduced into the capillary filled with a background solution containing an enzyme. During the migration of a sufficient amount of substrate molecules in the enzyme solution, the plateau signal of the product was observed at a constant rate. The proposed method exhibited fine fitting curves and comparable parameters pertaining to enzyme
reactions by employing automated CE systems. However, some issues still remain, such as the decrease in the plateau signal with a decrease in the concentration of the substrate, difficulty in the analysis of the reaction with low-concentration enzyme solutions, and the possibility of the concentration of the products changing due to diffusion during migration. In other words, the lag time between the onset of the reaction and the detection may affect the results and the evaluated parameters. Thus, the direct measurement of $v_0$ has been difficult using the previously reported methods.

In this study, to resolve the problems related to the direct measurement of $v_0$, a trapping technology by filtration using a capillary partially filled with a hydrogel is applied to study the kinetics of enzyme reactions. Based on the molecular sieving effect of a plugged hydrogel, the proposed device allows the filtrate of enzymes near the upstream interface of the plug.35 Following this, the substrate molecules pass through the interface and react with the trapped enzymes. As a result, the time for enzyme reaction can be controlled by changing the electrophoretic migration velocity of the substrates, thus enabling the direct measurement of $v_0$ (Fig. 1). In this manuscript, the filtration of an enzyme and the enzyme reaction based on the concept described in the next paragraph were confirmed by microscopic fluorescence imaging. The proposed method, which measures $v_0$ directly, was evaluated in comparison to the conventional mixing method by Michaelis-Menten fitting. The control performance of the enzyme reaction time was also investigated to confirm the proposed concept.

2. Materials and Methods

   Concept

   A microfluidic method for an enzyme reaction under electrophoresis is proposed to realize the direct measurement of a product due to a short-period enzyme reaction (Figure 1). In the
proposed concept, the enzyme is electrokinetically introduced into a gel-plugged capillary. The enzyme molecules can migrate in the hollow capillary filled with a buffer solution. They are then stopped near the interface of the hydrogel plug because the large molecules of the enzyme cannot penetrate the small pores in the 3D network of the polymer hydrogel. As a result, the enzyme molecules accumulate near the interface of the hydrogel plug and form a thin layer, as shown in Fig. 1a. The thickness of the enzyme layer ($\Delta L$) depends on the electrophoretic mobility, diffusion coefficient of the enzyme molecule, size distribution of the pores of the hydrogel, and the time and strength of the applied electric field. After the formation of the thin layer of enzyme, substrate molecules are electrokinetically introduced into the capillary in a similar way (Fig. 1b). When the substrate molecules enter the enzyme zone and start migrating, they immediately react with the trapped enzyme molecules, providing a fluorescent product. In this case, the reaction time can be described as follows:

$$\Delta t = \Delta L / v_{ep}$$ (1)

where $v_{ep}$ is the electrophoretic velocity of the substrate molecules. Thus, the short reaction time when the substrate overtakes the enzyme can be easily controlled by $v_{ep}$ and $\Delta L$. After the enzyme reaction, both the unreacted substrate and the generated product can pass through the hydrogel plug because these molecules are smaller than the smallest pore of the hydrogel. This penetration provides a rapid separation of these molecules from the enzyme zone, so that substrate molecules are not converted to product after separation. Therefore, the fluorescent product generated only in the enzyme zone can be observed downstream of the hydrogel plug. In this scheme, not the substrate solution, but the substrate molecules are rapidly transported to the constant volume/concentration of the enzyme zone by electrophoresis, resulting in an enzyme reaction that suppresses the changes in the enzyme/substrate concentration. The mixing process, based on electrophoresis, starts
immediately after the substrate molecules invade the enzyme zone, which requires very few dead volumes/time. These characteristics are advantageous for investigating enzyme reactions with small amounts of samples, compared to the conventional methods based on molecular diffusion, that require mixing the substrate and enzyme solutions while changing their volume/concentration. In addition, the short reaction time in Eq. (1) can be accurately controlled by changing only the applied voltage. Therefore, the proposed method can measure $v_0$ directly without any dead volume/time.

Chemical reagents

Alkaline phosphatase (ALP) from calf intestinal (~140 kDa), 30% (w/w) acrylamide/bis-acrylamide solution (37.5:1), 4-methylumbelliferyl phosphate (4-MUP), and 4-methylumbelliferone (4-MU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(dimethylsiloxane) (PDMS) prepolymer (SILPOT 184) and curing agent (SILPOT184 CAT) were purchased from Dow Corning Toray (Tokyo, Japan). Sodium hydroxide was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Hydrochloric acid and 2-amino-2-hydroxymethyl-1,3-propanediol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The reagent 2-hydroxy-2-methylpropiophenone (HOMPP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). HiLyte Fluor™ 555 Labeling Kit-NH₂ was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Deionized water ($18 \times 10^7$ S/cm) was prepared by Milli-Q (direct-Q UV 3, Merck, KGaA, Darmstadt, Germany).

Apparatus

A fluorescence microscope (VB-S20, Keyence Corp., Osaka, Japan) equipped with a mercury light (VB-L10, 120 W, Keyence Corp., Osaka, Japan) and a battery (PA250-0.25B,
TEXIO Corp., Kanagawa, Japan) was employed to observe the fluorescent product generated by the enzyme reaction in the capillary. Mask aligner (UV-CL251S, 250 W) was obtained from San-Ei Electric, Osaka, Japan.

**Preparation of the hydrogel-plugged capillary**

After the capillaries (inner diameter: 500 µm, length: 2 cm) were cut from the long one, they were modified by the following procedure to immobilize a short plug of polyacrylamide hydrogel onto the inner surface of the capillary. The capillaries were washed with 1 M NaOH and then replenished with deionized water. After drying, they were immersed in a mixture containing 3-(triethoxysilyl) propyl methacrylate and 100 mM HCl [4:1 (vol/vol)] for 3 h. In the preliminary study, it was confirmed that a protein with molecular weight of 130~150 kDa could not pass through a polyacrylamide hydrogel with a concentration of more than 20wt%.

To ensure that ALP was filtrated by the hydrogel, a 0.4 µL prepolymer solution (50 mM Tris-HCl buffer (pH 9.0) containing 27 wt% acrylamide, N, N'-methylene-bis-acrylamide, 1 vol% HOMPP) was introduced from one end of the capillary (Fig. S1a). Then the prepolymer solution was moved to the center part of the capillary by sucking the air from the other end (Fig. S1b). The hydrogel of which length was approximately 2 mm was formed by photopolymerization using a UV lamp of a mask aligner for 2 min without a photomask (Fig. S1c). Finally, 100 mM Tris-HCl buffer (pH 9.0) was introduced with syringe into the hollow parts of the capillary (Fig. S1d), resulting in a capillary partially filled with a short immobilized-hydrogel plug (the hydrogel-plugged capillary) (Fig. S1e).

*Fabrication of electrophoresis device for measuring the $v_0$*
PDMS reservoirs (capacity: 500 µL) to contact the sample/buffer solutions were prepared by SILPOT 184 and its curing agent. The fabrication of the acrylic platform to hold the PDMS reservoirs was referred to in a previous report.\textsuperscript{36} The electrophoresis device to evaluate the direct measurement of $v_0$ based on the proposed concept was fabricated by combining the prepared hydrogel-plugged capillary with the PDMS reservoirs placed on the glass platform (Figure S1e). All measurements were carried out using this setup.

\textit{Confirmation of electrokinetic filtration of ALP}

ALP was fluorescently labeled using the HiLyte Fluor\textsuperscript{TM} 555 labeling kit. Briefly, 100 mg of ALP reacted with the labeling reagent in the kit. After the labeling reaction, the labeled ALP was separated from the reagents by centrifugation (8000 G) for 10 min using a filtration tube (MWCO = 10 kDa). The fluorescently labeled ALP (F-ALP) remaining on the filter was obtained by dilution using 100 mM Tris-HCl buffer solution (pH 9.0), and the diluted solution was stored at 4 °C. The anodic/cathodic reservoirs were filled with the buffer solution with/without F-ALP, respectively. After inserting the platinum electrodes into the reservoirs, F-ALP was introduced into the hydrogel-plugged capillary for 30 min by applying a voltage ($V_{FE}$) of 50 V to the filtrate enzyme. Fluorescence near the upstream interface of the hydrogel plug was observed using a fluorescence microscope equipped with excitation/emission filters (RFP for excitation, $540 \pm 25$ nm; RFP for emission, $> 572$ nm). From the obtained fluorescence images, the preconcentration rate and the thickness of $\Delta L$ were estimated using ImageJ software (NIH, Bethesda, MD, USA).

\textit{Confirmation of direct measurement of $v_0$ based on the proposed concept}
The model enzyme (ALP, 0.1 U/mL) solution was prepared by the approximately $2 \times 10^5$-fold dilution of the ALP solution commercially available ($1.84 \times 10^4$ U/mL) with 100 mM Tris-HCl buffer solution (pH 9.0). The model substrate solution (4-MUP, 200 µM) was also prepared with 100 mM Tris-HCl buffer solution (pH 9.0). After 30 min of filtering of ALP, the hydrogel-plugged capillary was washed with the buffer solution for 5 min to complete the filtering of ALP remaining in the hollow part of the capillary. The cathodic reservoir solution was then exchanged with the buffer containing 4-MUP, and the 4-MUP was electrokinetically introduced into the capillary by applying a voltage ($V_{MS}$) of 50 V for the migration of the substrate. The fluorescence of 4-methylumbelliferon (4-MU), which is the dephosphorylated product of the enzyme reaction, was observed downstream of the hydrogel plug. The fluorescence of the 4-MU was observed using a microscope equipped with filters (XF1001, 330 ± 80 nm; XF3097, > 400 nm). In contrast, in the mixing method, ALP and 4-MUP solutions were mixed in the sample tube. The final concentration of ALP after mixing was 1 U/mL. The lag time from mixing the solutions to the measurement of the fluorescence intensity was 45 s. The mixed solution was then introduced into the hollow capillary without a hydrogel plug, and the fluorescence of the product was observed over time to estimate the value of $v_0$.

**Analyses of kinetic parameters in the enzyme reaction**

In the proposed method, 4-MUP is introduced into the ALP-filtrated capillary, resulting in the formation of the complex (ES) by binding 4-MUP (S) with ALP (E). Subsequently, bound 4-MUP is converted to 4-MU (P) by ALP-catalyzed dephosphorylation. These processes are described as a typical Michaelis-Menten model:
\[ E + S \xrightarrow{k_1 \quad k_{-1}} ES \xrightarrow{k_2} E + P, \]  

(2)

where \(k_1\) and \(k_{-1}\) are the kinetic constants of the equilibrium reaction and \(k_2\) is the kinetic constant of the irreversible reaction. When the enzyme reaction is employed with a sufficient amount of 4-MUP, the value of \(v_0\) can be calculated directly by dividing the concentration of the product estimated from the calibration curve by \(\Delta t\), according to the proposed method. On the other hand, the values of \(v_0\) in conventional mixing were also estimated by variation of the product concentration over time, after the mixed solution was introduced into the hollow capillary.

The obtained values of \(v_0\) were analyzed by Michaelis-Menten fitting using Origin 2018b software, and the kinetic parameters were calculated from the Michaelis-Menten equation, which is described as follows:

\[ v_0 = \frac{v_{\text{max}} [S]}{K_M + [S]}, \]

(3)

where \([S]\) is the concentration of 4-MUP, \(v_{\text{max}}\) is the maximum velocity of the enzyme reaction, and \(K_M\) is the Michaelis-Menten constant. The values of \(v_{\text{max}}\) and \(K_M\) are also described as follows:

\[ v_{\text{max}} = k_2 \cdot [E], \]

(4)

\[ K_M = \frac{k_{-1} + k_2}{k_1}. \]

(5)
These parameters in the proposed method were compared with those obtained using the conventional mixing method.

_Evaluation of the effect of applied voltage_

To evaluate the effect of the applied voltage on the enzyme reaction, various voltages (25–150 V) were applied to both ends of the capillary via reservoirs to introduce the substrate. Other conditions were the same as described above. The kinetic parameters were also estimated for each condition.

3. Results and Discussion

_Confirmation of electrokinetic filtration of ALP_

When F-ALP was introduced into the gel-plugged capillary for 30 min, strong fluorescence was observed near the cathodic-side interface of the hydrogel plug (Figs. 2a-c). The fluorescence intensity profile was estimated along the white horizontal dotted line centered in the capillary using ImageJ software (Figure 2d). The obtained values of the fluorescence intensity were compared to that in the F-ALP solution only introduced into the hollow capillary to evaluate trapping efficiency (Fig. 2e and Figure S2, Supporting Information 2). As a result, the trapping efficiency of electrophoretic filtration for 30 min was calculated to be approximately 300-fold by comparing the fluorescence intensities with/without filtering. As shown in Figure 2b and c, the interface of the hydrogel immobilized onto the inner surface of the cylindrical capillary was slightly distorted by swelling, which appears to slightly increase the apparent width of the interface. The cylindrical capillary makes an apparent fluorescence of the center a slightly stronger than that near the inner surface due to the longer optical path length. On the other hand, the model
protein was considered to be evenly distributed on the interface by the electrophoresis at a uniform rate under the condition with the suppressed electroosmotic flow. In addition, the \( \Delta L \) was kept constant because the flux by electrophoresis toward the anode was much larger than that by molecular diffusion toward the cathode (see Supporting Information 3). On the above considerations, \( \Delta L \) was estimated to be approximately 100 \( \mu \text{m} \) from the full width at half maximum (FWHM) value that was converted from pixel to length (8.08 \( \mu \text{m/pixel} \)) in Figure 2d. Thus, it was confirmed that the developed device allows both filtering of enzymes and formation of a thin enzyme layer by the proposed electrokinetic filtration.

Confirmation of direct measurement of \( v_0 \) based on the proposed concept

After the formation of the thin ALP layer by 30 min of filtration and 5 min of washing with the running buffer solution, 4-MUP was introduced into the capillary. Under these conditions, a constant concentration of the fresh substrate solution was continuously supplied to the thin enzyme layer, which allowed the continuous generation of the constant-concentration product by the enzyme reaction for \( \Delta t \). The fluorescence images obtained downstream of the hydrogel clarified that the fluorescence intensity profile along with the hollow part of the capillary was almost constant. Figure 3 shows the observed fluorescence at one of the detection points located downstream of the hydrogel plug. At first, fluorescence was not observed at the detection point because the products did not reach there by electrophoresis. After applying voltages for 7 min, the fluorescence intensity gradually increased, which suggested the concentration distribution of the frontal zone of both the substrate and product by diffusion during migration. The fluorescence intensity finally reached a plateau after 13 min, indicating that the electrokinetic supply of the substrate became constant. As Liu et al. reported, an enzyme activity was almost kept in the 100
consecutive measurements under a high electric field (280 V/cm). Thus, these results indicated that the product of the enzyme reaction was generated only during the migration of substrates in the thin ALP layer located near the upstream interface of the hydrogel plug without changing the enzyme activity by the applied electric field (75 V/cm). The concentration of the product under this condition was estimated by the calibration curve of the fluorescence intensity, resulting in a calculation of 39.1 µM (Fig. S3, Supporting Information 4). In preliminary study, the value of electrophoretic mobility of 4-MUP was estimated to be $3.35 \times 10^{-8}$ m$^2$·V$^{-1}$·s$^{-1}$ (see Supporting Information 5). The value of $\Delta t$ was also estimated as 1.26 s from Eq. (1) in the condition of $V_{MS}$ at 50 V. Therefore, the value of $v_0$ was calculated as 31.0 µM/s by only dividing the concentration of 4-MU by $\Delta t$. Thus, it was confirmed that the proposed method could evaluate the value of $v_0$ without any theoretical fitting by the mathematic software.

Analyses of kinetic parameters in the enzyme reaction

To evaluate the kinetic parameters, various concentrations of 4-MUP solutions were introduced into the ALP-filtrated capillary at an applied voltage of 50 V. After converting the measured fluorescence intensity to the concentration of the product, the obtained values were depicted in Figure 4, and the plots were then fitted based on Eq. (3). Figure 4 shows the typical plots and fitting curves based on the Michaelis-Menten model. From these fitting curves, the important values in enzyme reactions, $v_{max}$ and $K_M$, were calculated using Origin 2018b software. As a result, $v_{max}$ was calculated 1.08 ± 0.06 and 32.9 ± 2.20 µM/s in the mixing method described in the experimental section and proposed one. In the proposed method, the $v_{max}$ value was approximately 30-fold larger than that in the mixing method (1 U/mL ALP, lag time from mixing ALP with 4-MUP to measurement: 45 s) in spite of the 10-
fold dilution of the ALP solution in the proposed method as compared to that in the mixing method. As shown in Fig. 2, the filtration for 30 min provided a 300-fold enhancement of the fluorescence intensity. The concentration of filtrated ALP was still much lower than that commercially available ($1.84 \times 10^4 \text{ U/mL}$). Additionally, as Sakai-Kato reported, the encapsulation of the enzyme by the sol-gel matrix had few effect on the value of $v_0$. In the proposed method, therefore, the denaturation of enzyme due to the agglutination and/or the adsorption on the hydrogel is considered to have hardly occurred. Thus, the concentration of ALP after filtration was estimated to be 30-fold higher than that in the mixing method, which indicated comparable values of $k_2$ calculated from Eq. (4).

In contrast, the values of $K_M$ were calculated $15.1 \pm 5.2$ and $40.8 \pm 12.0 \mu\text{M}$ in the mixing method and proposed one, respectively. The value of $K_M$ obtained by the proposed method was 2.7-fold larger than that obtained by the mixing method. In the proposed method, it was observed that ALP was filtered near the upstream interface of the hydrogel plug by the molecular sieving effect. When the ALP molecules reached the interface, they could enter via the large pores of the hydrogel by electrophoresis and were trapped by the small pores because of the size distribution of the pores in the three-dimensional hydrogel structure.

Under the described conditions, ALP was located in the hydrogel near the interface because the electrophoretic velocity of ALP was faster than its molecular diffusion. As Hughes reported, the increased $K_M$ in the hydrogel without a drop in $k_2$ can be attributed to the reduced molecular diffusivity in the hydrogel matrix as compared to that in free solution. Thus, the value of $K_M$ increased by changing the values of $k_1$ and $k_{-1}$ (Eqs. 2 and 5), suggesting the variation of the complexation/dissociation constants. Consequently, it was clarified that the proposed method can evaluate enzymatic activity from the direct measurement of $v_0$ by detecting the product during the short and limited period of enzyme reaction. The period of
reaction could be defined as the electrophoresis of the substrate without the dead volume/time.

**Evaluation of the effect of applied voltage**

To confirm the controllability of the $\Delta t$ by tuning the $V_{MS}$, 4-MUP was also electrokinetically introduced into the ALP-filtrated capillaries using $V_{MS}$ values of 25, 30, 50, 75, 100, 120, and 150 V. The values of $v_{ep}$ were calculated as 41.9, 50.2, 83.7, 126, 168, 201, and 251 µm/s at the $V_{MS}$ described above, respectively (Table 1). When the 500 µM 4-MUP solution was introduced, the fluorescence of 4-MU decreased upon increasing $V_{MS}$ (Table 1). Figure 5 shows that the concentration of generated 4-MU linearly increased with increasing $\Delta t$, as calculated by Eq. 1 (Table 1). Therefore, it is clarified that the proposed method allows the measurement of $v_0$ directly by controlling the reaction time by only changing the applied voltage when a sufficient amount of substrate was supplied to the enzyme layer ([S] >> [E]).

In addition, various concentrations of 4-MUP were introduced into the capillary at a variety of voltages. As a result of Michaelis-Menten fitting from the values of $v_0$ obtained by the proposed method, the values of $v_{max}$ and $K_M$ were calculated as 36.9 ± 7.09 µM/s and 68.6 ± 42.9 µM, respectively (Table 1). Regarding the values of $v_{max}$ and $K_M$, these values fluctuated within a certain range, while there was no significant relationship with the values of $V_{MS}$. Furthermore, the linearity of the [4-MU]-$\Delta t$ plot decreased with decreasing [4-MUP], meaning that there is not enough substrate to meet the conditions for the Michaelis-Menten equation ([S] >> [E]). Thus, these comparable values of $v_{max}$ and $K_M$, obtained by adding different $V_{MS}$, suggested that the proposed method was not affected by changing $V_{MS}$. It was also suggested that the issue related to the fluctuation of the values was caused by the reproducibility of the fabricated test device, including the position of the hydrogel in the
capillary, pore-size distribution of the formed hydrogel, small differences in the setup in each experiment, and so on. This issue will be improved by the fabrication of a microfluidic device equipped with a tuning channel for mixing substrate and buffer solutions to change the concentration of the substrate in a single run.

4. Conclusion

In this study, a novel method for the direct measurement of \( v_0 \) in enzyme reactions was developed. The formation of the thin enzyme layer near the upstream interface of the hydrogel plug was confirmed, and the thickness of the layer, \( \Delta L \), was estimated to be approximately 100 µm. The fluorescent product of the enzyme reaction during the period when the substrate molecules pass through the enzyme zone could be observed downstream of the hydrogel plug. These results clarified that the proposed method allows the direct calculation of \( v_0 \) by only dividing the concentration of the product by the enzyme reaction time without the dead volume/time for the reaction. Compared to the conventional mixing method, the kinetic parameters obtained by the Michaelis-Menten fitting indicated that the proposed method could evaluate \( v_0 \) as in the previous method based on zymography.\(^{17}\) It was also confirmed that the developed method can control the reaction time by changing the applied voltage. The latter did not affect the enzyme reaction significantly, while further evaluation is required for confirmation because of the issue of poor reproducibility of the device. To clarify the issues of the applied voltage and reproducibility, further study will be required to obtain quantitative kinetic data using a microchip system combined with a tuning channel for changing the concentration of the substrate in a single run.

5. Supporting Information
The fabrication of the hydrogel-plugged capillary and electrophoresis device, time course with generated 4-MU, calibration curve of 4-MU, and effect of the molecular diffusion are described in the Supporting Information. This material is available free of change on the Web at http://www.jsac.or.jp/analsci/.

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Table 1. The effect of the applied voltages on kinetic parameters ($v_{\text{max}}$ and $K_M$).

| Voltage (V) | 150 | 120 | 100 | 75  | 50  | 30  | 25  |
|------------|-----|-----|-----|-----|-----|-----|-----|
| $v_{\text{ep}}$ (µM/s)* | 251 | 201 | 168 | 126 | 83.7| 50.2| 41.9|
| $\Delta t$ (s) | 0.421 | 0.526 | 0.632 | 0.842 | 1.26 | 2.09 | 2.53 |
| [4-MU] (µM)** | 15.8 | 12.9 | 25.1 | 29.8 | 42.7 | 58.5 | 101 |
| $v_{\text{max}}$ (µM/s) | 29.2 | 21.4 | 42.3 | 38.1 | 32.9 | 43.6 | 40.7 |
| $K_M$ (µM) | 34.0 | 77.9 | 149 | 34.5 | 40.8 | 112 | 32.1 |

* $v_{\text{ep}} = \mu_{\text{ep}} \times E$; $\mu_{\text{ep}}$, electrophoretic mobility of 4-MUP ($3.35 \times 10^{-8}$ m²·V⁻¹·s⁻¹); $E$, electric field.

** Concentration of the substrate, [4-MUP], is 500 µM.
Figure Captions

Figure 1.

(a) Enzyme molecules are introduced into a gel-plugged capillary by electrophoresis and concentrated near the upstream interface of the hydrogel plug by the molecular sieving effect. (b) Substrate molecules are electrokinetically introduced into the capillary after the filtering enzymes. The substrate molecules entering the enzyme zone react with the enzyme for $\Delta t$, which is defined by dividing the thickness of the enzyme layer by the electrophoretic velocity of the substrate ($\Delta L / v_{ep}$). The substrate and product are separated from the enzyme layer, terminating the enzyme reaction. The generated products are detected downstream of the hydrogel. Between the applied voltages, fresh substrates are continuously introduced from the cathodic reservoir, thus enabling a continuous enzyme reaction.
Figure 2. Confirmation of the filtration of enzyme molecules by molecular sieving effect of the plugged hydrogel. (a) The Schematic illustration and fluorescence images obtained near the upstream interface of the gel (b) before and (c) after the filtration of fluorescently-labeled alkaline phosphatase (F-ALP). The white vertical broken lines represent the cathodic interface of the plugged hydrogel. Imaging conditions: exposure time, 1 s; filter, RFP for excitation, 540 ± 25 nm; RFP for emission, > 572 nm; applied voltage, 50 V. (d) Fluorescence intensity profile estimated along the white horizontal dotted line centered in the capillary by ImageJ software shown in (c). (e) Relationship between filtration time and trapping efficiency.
Figure 3. Schematics of the fluorescence measurement. (a) Schematic illustration of the detection of fluorescent product. (b) Fluorescence intensity of the enzyme reaction product, 4-methylumberiferon (4-MU) over time, when the concentration of 4-MUP was 300 µM.
Figure 4. The Michaelis-Menten plots and the fitting curves estimated by Origin 2018b software for (a) the conventional mixing method and (b) the proposed method.
Figure 5. Confirmation of the controllability of $v_0$ by tuning the applied voltages. The broken line was obtained by the linear fitting of the plots ($y = 36.0 x - 1.94$, $R^2 = 0.93$).