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Electrophoretically mediated microscale reaction of glycosidases: kinetic analysis of some glycosidases at the nanoliter scale☆

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Abstract

Capillary electrophoresis (CE) is one of the extremely important analytical techniques known for its high sensitivity and resolution. We have investigated electrophoretically mediated microanalysis (EMMA) for the assay of some native glycosidases. Under optimized conditions, the enzymatic reactions of α-glucosidase, β-galactosidase and β-*N*-acetylglucosaminidase were carried out, and the Michaelis constants were obtained. The current method may have advantages over traditional assay methods, especially in terms of the amount of enzyme and substrate required for a reaction. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Microreaction; Electrophoretically mediated microanalysis; PNP, glycosides; Glycosidases, kinetic analysis

1. Introduction

Capillary electrophoresis is extremely useful because of its high sensitivity and resolution, and its importance as a microanalytical method has been demonstrated. This largely relies on recent advances in automated systems that make reproducible injections possible. An approach in which the capillary is used not only as isolation field, but also as a field for the reaction is recognized to be highly valuable.[1–17] In particular, electrophoretically mediated microanalysis (EMMA)[6–17] is advantageous because the amounts of reactants, enzyme and substrate, which are often extremely valuable and expensive, can be minimized.

In general, there are two ways for mixing two components in a capillary under electrophoretic conditions. One is the continuous format,[6–10,12,13] which uses the capillary filled with one of the reactants, and then another reactant is injected. The other is plug–plug format,[11,12,14–17] which relies on a plug–plug interaction. When two compounds forming individual plugs have different electrolytic mobility, the reaction proceeds while one of the components is passing the other. The reaction product is then resolved based on the individual electromobilities in the electric field and passes through the detector on the way to the end of the capillary.

Oligosaccharides exist on the cell surface and in the extracellular matrix as a part of glycoproteins and glycolipids, and they play important roles in biological phenomena.[18] To investigate the functions of oligosaccharides, methodological investigations, especially ones that are applicable to the microscale experiment is of extreme importance. We have been interested in carbohydrate-related enzymes, especially oligosaccharide-processing enzymes, and reported some utilities of capillary zone electrophoresis (CZE) for the analysis of these enzymatic reactions.[19–23] In our previous papers, we have analyzed the reaction kinetics of glycosyltransferase and glycosidase based on isolation of the substrate and the product, which was possible using borate buffer to introduce negatively charged functionality based on the formation of borate ester of hydroxyl groups.[24] Through these studies, we realized the necessity to carry out enzymatic reactions in a capillary in order to minimize consumption of the reaction mixture

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because the reaction volume could not be reduced less than ~20 μL in our hands due to the experimental errors associated with surface tension problems when handling a tiny amount of liquid.

We have addressed this issue using the plug–plug format under electrophoretically mediated microscale reaction conditions. α-Glucosidase (EC 3.2.1.20), β-galactosidase (EC 3.2.1.23) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) catalyzed reactions were selected to show eligibility of the method. The analytical processes were carried out in an automated manner in order to speed-up the laborious handling.

2. Experimental

Materials.—The source of enzymes and substrates are as follows. α-Glucosidase (α-Glc-ase; EC 3.2.1.20) from *Saccharomyces* sp.: Toyobo Co., Ltd. (Osaka, Japan), β-galactosidase (β-Gal-ase; EC 3.2.1.23) from *Aspergillus oriza* and β-*N*-acetylglucosaminidase (β-GlcNAc-ase; EC 3.2.1.30) from bovine kidney: Sigma Chemical Co. (St. Louis, MO, USA); *p*-nitrophenyl-α-D-glucopyranoside (PNP-α-Glc): Nacalai Tesque., Inc. (Kyoto, Japan); *p*-nitrophenyl-β-D-galactopyranoside (PNP-β-Gal) and *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (PNP-β-GlcNAc): Sigma Chemical Co. (St. Louis, MO, USA); uridine: Kohjin Co. Ltd. (Tokyo, Japan). Double-deionized water was prepared from a Milli-Q system from Millipore Corp. (Milford, MA, USA).

Instrumentation.—EMMA assays. EMMA assays were performed on a Beckman P/ACE System 5500 (USA), which was equipped with a 75 μm i.d. (r) uncoated fused silica capillary with 37 cm total length (L). Detection was carried out by on-column measurement of UV absorbance at 214 and 405 nm with photodiode array (PDA) at 7.0 cm from the cathode. Electrophoresis was performed at 18 kV using a 40 mM borate buffer (pH 9.2) as an electrolyte.

Photometric assays. Assay solutions were analyzed by a Waters Quanta 4000E capillary electrophoresis system, which was equipped with a 75 μm i.d. (r) uncoated fused silica capillary with 37 cm–L. Detection was carried out by on-column measurement of UV absorption at 405 nm at 7.5 cm from the cathode. Samples were loaded by means of hydrostatic pressure at 10 cm height for 30 s (ca. 38.4 nL). Electrophoresis was performed at 15 kV using 40 mM sodium borate (pH 9.4) as an electrolyte at a constant temperature of 37 °C. Electropherograms were recorded on a Millennium 2010 system from Millipore Corp. The capillary used was pretreated or regenerated with 0.1 M NaOH (2 min) and elution with buffer before each injection.

Parameters.—The parameters used to estimate volume (V) of each plug are: $\Delta P = 34{,}475$ (dyne cm^{-2}) according to the equipment's specification. The viscosity (η) was measured using a viscometer (Ubbelohde) and shown to be $\eta = 0.00756$ and 0.00810 (Poise) for the enzyme plug and the substrate plug, respectively.

Procedures for EMMA

Obtaining kinetic parameter of α-Glc-ase. A solution containing α-Glc-ase (0.05 mg/mL) in 40 mM phosphate buffer (sodium phosphate, pH 7.0) was introduced by pressure injection for 3 s into the capillary equilibrated with 40 mM borate buffer. Then a solution of PNP-α-Glc at various concentrations in 200 mM phosphate buffer (pH 7.0) containing uridine (0.2 mM) as an internal standard was introduced by means of pressure injection for 5 s, followed by introduction of 200 mM phosphate buffer (pH 7.0) for 1 s. The assay was carried out by applying an electric field at a controlled temperature of 37 °C. EMMA was carried out under conditions where the reaction conversion was exceeding 10%.

Obtaining the kinetic parameter of β-Gal-ase. Conditions for EMMA were identical except for the following parameters. β-Gal-ase (0.5 mg/mL) was used. The pH of phosphate buffers to dissolve enzyme and substrate were 4.5 and 4.2, respectively.

Obtaining the kinetic parameter of β3-G1cNAc-ase. Conditions for EMMA were identical except for the following parameters. The enzyme, β-GlcNAc-ase (0.4 mg/mL) was dissolved in 40 mM phosphate buffer (pH 4.5), and the substrate was dissolved in 80 mM phosphate buffer (pH 4.4).

Automation of analytical process.—The automation cycle consists of (1) washing with 0.1 N NaOH (2 min), [and regenerated with water (1 min)]; (2) equilibration of electrolyte buffer (2 min); (3) injection of a solution containing enzyme in 40 mM phosphate buffer (sodium phosphate) (3 s); (4) introduction of 200 mM or 80 mM phosphate buffer for 1 s and; (5) injection of a solution of substrate at a various concentration in 200 mM or 80 mM phosphate buffer containing uridine (0.2 mM) (5 s). Each electropherogram was recorded over 4 min. One cycle took about 12 min including recalibration of PDA. The stock solutions of enzyme, substrate and buffers used in the EMMA were kept at 20 °C at the autosampler reservoir because large difference in temperature between stocks and the capillary (when stored enzyme at 4 °C) affected the enzymatic reaction. At this temperature, enzyme activity remained unaffected during ca. 5 h required for collecting a series of the EMMA data.

Photometric assay by CE.—Kinetic analysis of α-Glc-ase. In order to confirm reliability of data obtained by the EMMA method, a traditional photometric assay was carried out. Incubations were performed in a total volume of 20 μL. Unless otherwise stated, reaction mixtures contained 40 mM phosphate buffer (pH 7.0), various amount of PNP-α-Glc (0.1–1.0 mM)

with 5 mU of α-Glc-ase. The reaction was started by the addition of α-Glc-ase, and the reaction mixture was incubated for 5 min at rt. Then, the reaction was terminated by addition of 40 μL of 0.2 M Na_2CO_3.

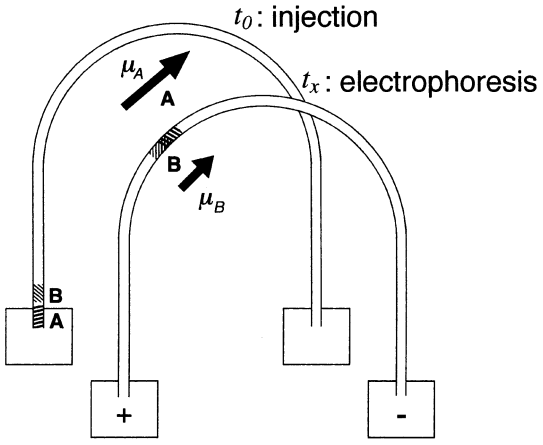

Fig. 1. Schematic representation of a microreaction in a capillary under electrophoretic conditions. The isolated plugs of A and B are introduced into capillary. The order of the injection is determined based on the relative electromobility, μ_A and μ_B, as shown by the arrows. The reaction proceeds during plug–plug mixing, and the individual plugs of substrate, enzyme and products are separated.

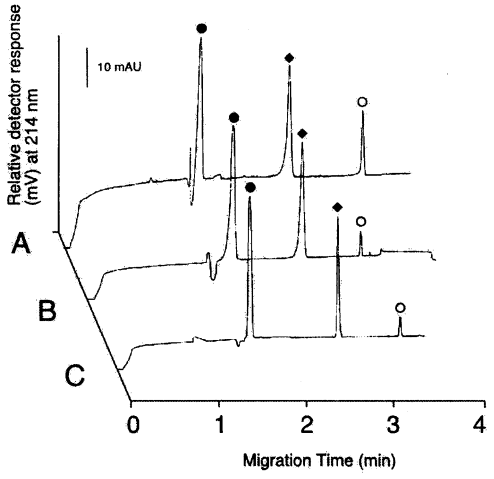

Fig. 2. Electropherograms of enzymatic reactions of glycosidases. (A) Injection of 1.0 mM PNP-α-Glc in 200 mM phosphate buffer (pH 7.0) after injection of 0.05 mg/mL α-Glc-ase in 40 mM phosphate buffer (pH 7.0) (3 s) and 200 mM phosphate buffer (pH 7.0) (1 s). (B) Injection of 1.0 mM PNP-β-Gal in 200 mM phosphate buffer (pH 4.2) after injection of 0.1 mg/mL β-Gal-ase in 40 mM phosphate buffer (pH 4.5) (3 s) and 200 mM phosphate buffer (pH 4.2) (1 s). (C) Injection of 1.0 mM PNP-β-GlcNAc in 80 mM phosphate buffer (pH 4.4) after injection of 0.05 mg/mL β-GlcNAc-ase in 40 mM phosphate buffer (pH 4.2) (3 s) and 80 mM phosphate buffer (pH 4.4) (1 s). ●, substrate; ○, product (PNP-ol); ◆, internal standard (uridine). Electrophoresis conditions were as described in the Experimental section (absorbance: 214 nm). Each peak in the electropherogram was identified by analyzing individual material prior to the experiments.

Kinetic analysis of β-Gal-ase. The procedure is the same as that described for the analysis of α-Glc-ase except for the pH of reaction mixtures (pH 4.5), except for one pH of reaction mixtures (pH 4.5), the substrate PNP-β-Gal (0.2–2.0 mM), and the enzyme β-Gal-ase (4 mU).

Kinetic analysis of β-GlcNAc-ase. The procedure is same as that described for the assay of α-Glc-ase, except for the pH of reaction mixtures pH 4.5), the substrate PNP-β-GlcNAc (0.5–3.0 mM), and the enzyme β-GlcNAc-ase (1.3 mU).

3. Results and discussion

General aspects.—Among many important carbohydrate processing enzymes, several glycosidases namely, α-G1c-ase, β-Gal-ase and β-GlcNAc-ase in the current study to demonstrate the feasibility of our method. The series of enzymes hydrolyze the corresponding *p*-nitrophenyl D-glycosides as the substrates to yield *p*-nitrophenol (PNP-ol).

Several issues have to be addressed for this EMMA technique. In order for the enzymatic reaction to take place in EMMA using the plug–plug format, one of the reactants must pass the other (Fig. 1). The fast-moving plug (A) electrophoretically migrates to the end boundary of slow-moving plug (B) where interaction of two substances such as an enzyme and its substrate starts. The reaction finishes when the end boundary of the plug A reaches front boundary of the plug B. Furthermore, it is important to select a certain plug format to enable both successful enzymatic reaction and isolation of essentially non-charged compounds such as carbohydrates. For this, we decided to use a combination of a phosphate buffer with pH close to the optimal pH of the hydrolyases for the reactions, and basic borate buffer pH 9.2, as it is commonly used for the analysis of carbohydrates using capillary zone electrophoresis (CZE) for the purpose of isolation of the reactants and product, as well as the detection of the PNP-ol.

Determining the order of injection.—The order of injection was first determined. Each enzyme migrated slower than its substrate in borate buffer (pH 9.2) (data not shown). This suggested that the analysis could be performed under these conditions where the slow-moving enzyme is injected first and the fast-moving substrate next injected to enable plug–plug interaction to occur. It was anticipated that accuracy and reproducibility would be an issue in this microscale reaction; therefore, as an internal standard, uridine, was used. Typical electropherograms of individual enzyme reactions are shown in Fig. 2 where the substrate, uridine as an internal standard and PNP-ol are clearly resolved.

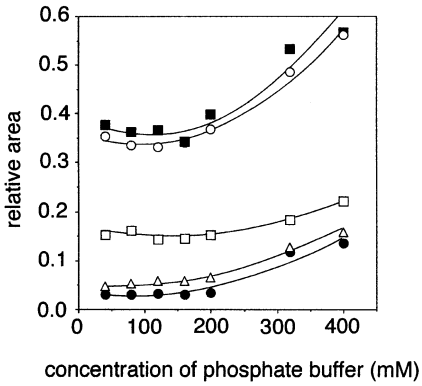

Fig. 3. The effect of concentration of the phosphate buffer on relative area of PNP-ol against uridine. Result of α-Glc-ase (■). α-Glc-ase (0.1 mg/mL) in 40 mM phosphate buffer (pH 7.2) was injected (3 s), followed by injection of various concentrations of phosphate buffer (pH 7.2) (1 s) and 0.5 mM PNP-α-Glc in various concentrations of phosphate buffer (pH 7.2) (5 s). Results of β-Gal-ase: (△) β-Gal-ase (0.1 mg/mL) in 40 mM phosphate buffer (pH 4.5) was injected (3 s), followed by injection of various concentrations of phosphate buffer (pH 4.2) (1 s) and 1.0 mM PNP-β-Gal in various concentrations of phosphate buffer (pH 4.2) (5 s). Results of β-GlcNAc-ase: (□) β-GlcNAc-ase (0.1 mg/mL) in 40 mM phosphate buffer (pH 4.5) was injected (3 s), followed by injection of various concentrations of phosphate buffer (pH 4.2) (1 s) and 1.0 mM PNP-β-GlcNAc in various concentrations of phosphate buffer (pH 4.2) (5 s). 0.05 mM PNP-ol (○), 0.005 mM PNP-ol (●) (absorbance: 214 nm).

Optimization of EMMA conditions.—We examined the adsorption of the analytes to the capillary wall by using polyacrylamide coated and uncoated capillaries, but we could not find any marked difference in product formation during EMMA.[25] We, therefore, decided to use an uncoated capillary in this study for convenience.

The volume of the enzyme and the substrate involved in each reaction was estimated based on the equation of Poiseuille (Eq. (1))[26],

$$V = \Delta P \pi r^4 t / 8 \eta L \quad (1)$$

where ΔP is the pressure drop across the capillary during injection, r is a capillary radius, t is injection time, η is a viscosity of the buffer and L is total length of the capillary from inlet to outlet. The enzyme and substrate solutions were injected by pressure for 3 and 5 s, respectively. The internal diameter of capillary was 75 μm. EMMA was performed at 37 °C. The length and volume (V) of the enzyme and substrate plugs are estimated to be 6.5 and 10 mm and 29 and 45 nL, respectively, which were isolated by a 2 mm (9 nL) phosphate buffer plug to avoid cross contamination during injections.

It was important to use the proper concentration of phosphate buffer for the successful enzymatic reaction.[25] As shown in Fig. 3, the transformations in the case of α-Glc-ase (closed square) and β-Gal-ase (open triangle) were not affected significantly by the concentration of phosphate buffer as compared with the relative area obtained for the PNP-ol (open circle; 0.05 mM and closed circle; 0.005 mM). This indicates that the enzymatic reaction is independent of the phosphate concentrations. In the case of β-G1cNAc-ase (open square), however, the curve did not match the ones obtained for PNP-ol, which indicates the enzyme reaction was affected by the phosphate concentration and was efficient at lower concentrations. To obtain optimal results, we chose a concentration of 200 mM phosphate buffer for α-Glc-ase and β-Gal-ase and 80 mM buffer for β-GlcNAc-ase. The use of higher phosphate concentrations (> 300 mM) were basically avoided due to the inaccuracy associated with a baseline disturbance.[25] Changes in the referenced PNP-ol area at different buffer concentrations cannot be explained yet, but it might relate to the peak shape which occurs as the result of the concentration of substance.

Obtaining kinetic parameters.—Based on the conditions for EMMA of α-Glc-ase described above, β-Gal-ase and β-GlcNAc-ase catalyzed enzymatic reactions, we worked further to obtain K_m values of these reactions. The reaction time is not directly given due to the heterogeneous nature of the reaction during the plug–plug interaction, but a linear relationship was obtained for the product area during the experiments at a fixed concentration of phosphate buffer. Thus, we used the area as an equivalent value of v to obtain the constant. In the case of the α-Glc-ase catalyzed reaction, the capillary was filled with 40 mM borate buffer (pH 9.2) and sequentially injected with an enzyme plug in 40 mM phosphate buffer, 200 mM phosphate buffer (pH 7.2), and a plug containing various amounts of substrate in 200 mM phosphate buffer (pH 7.0), all by means of positive pressure at the anodic end. The capillary was then placed in the 40 mM borate buffer. The assay was performed in quadruplicate using a 37-cm capillary with a controlled capillary temperature at 37 °C and detection at 6.5 cm from the cathodic end. The reaction was monitored at 214 and 405 nm to eliminate ambiguity due to the possible effects on the PNP-ol peak by other factors.

The K_m values were obtained by a Lineweaver–Burk plot, where relative area was used to indicate product concentrations, which was used instead of v values (Fig. 4). Approximately a threefold improvement in sensitivity was observed for the data obtained at 405 nm. The K_m values thus obtained were $K_m = 0.9$ mM for α-Glc-ase ($n = 4$, each concentration, CV (214 nm) = 2.8–8.2%), $K_m = 1.0$ mM for β-Gal-ase ($n = 4$, each concentration, CV (214 nm) = 2.0–7.9%) and $K_m = 1.1$ mM for β-GlcNAc-ase ($n = 4$, each concentration, CV (214 nm) = 4.3–12.6%), which were in the same range as the values obtained by the photometric assay shown below. The results were also similar to the reported

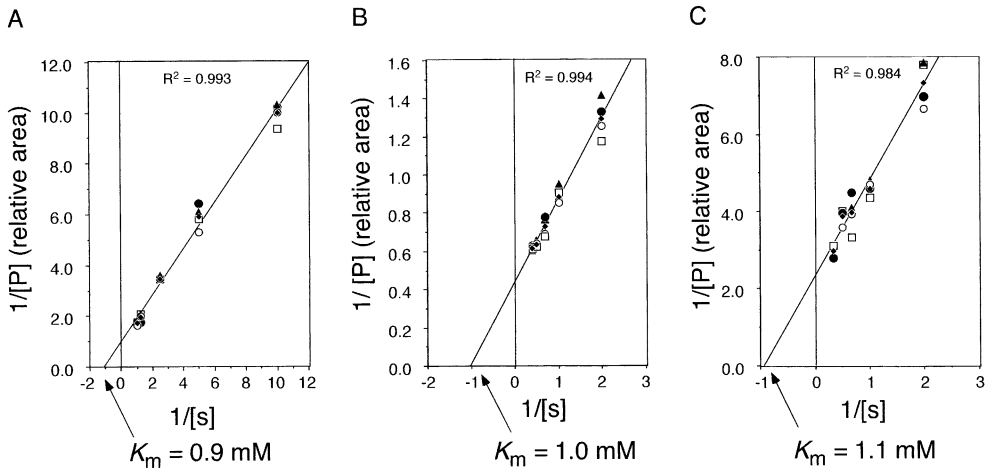

Fig. 4. Double-reciprocal plots of glycosidase assay. (A) α-Glc-ase assay results of the microreaction. (B) β-Gal-ase assay results of the reaction. (C) β-GlcNAc-ase assay results of the reaction. Concentration of the product [P] (relative area) was used instead of initial velocity (see text for explanation). Data are obtained in quadruplicate as shown in the figure. Curves were obtained using means (◆) of individual data sets.

value.[20,22] K_m values obtained by the photometric assay method are: α-Glc-ase: $K_m = 0.7 \pm 0.1$ mM ($n = 3$, each concentration, CV = 1.5–9.4%), $V_{max} = 1.5$ μM/s; β-Gal-ase: $K_m = 1.2 \pm 0.2$ mM ($n = 3$, each concentration, CV = 1.7–4.9%), $V_{max} = 2.5$ μM/s; and β-GalNAc-ase: $K_m = 1.8 \pm 0.3$ mM ($n = 3$, each concentration, CV = 3.2–10.0%), $V_{max} = 1.6$ μM/s, respectively. The V_{max} values were not obtained because it was impossible at this time to estimate reaction time due to the heterogeneity of the buffer system. Furthermore, the mobility of the entire electrolyte in the capillary would have been affected.

4. Conclusions

It was shown that the enzymatic transformation of carbohydrate-related native enzymes using the plug–plug format under electrophoretically mediated microanalysis conditions was possible. Furthermore, the kinetic analyses were achieved while the scale of the enzymatic reactions was radically reduced (1000-fold improvement when compared with the off-line analysis of the reaction carried out in a microcentrifuge tube or by a method using radiolabeled materials). Thus, we were able to perform enzymatic reactions in a reaction volume of a few nL, whereas a limit in reaction volume for the traditional methods was ~20 μL due to surface tension. This is a dramatic improvement in methodologies, because a traditional assay, a photometric assay for example, takes as much as 400 μL (4 × 20 μL/reaction × 5 points) to obtain the K_m value, while EMMA only requires a total of 20 μL. As a representative of the EMMA, enzyme reactions of α-glucosidase, β-galactosidase and β-*N*-acetylglucosaminidase were successfully analyzed, and K_m values similar to those obtained by photometric assay were obtained. This indicates that EMMA with plug–plug format can be used as a reliable method for the kinetic analysis of carbohydrate-related enzymes. Further, the process was carried out automatically using a temperature controlled autosampler in order to eliminate routine handling and to speed up the process.

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