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Short communication

Capillary electrophoresis apparatus equipped with a bioluminescence detector using a batch- or flow-type detection cell

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Abstract

We developed a capillary electrophoresis (CE) apparatus equipped with a batch- or flow-type bioluminescence (BL) detection cell. Firefly luciferin–luciferase BL reaction was used to analyze samples of nucleotides, such as ATP, dATP, ADP, GTP, UTP, CTP, ITP, and TTP. In the CE apparatus with the batch-type cell, ATP was detected at concentrations of 5–100 μM, while the other nucleotides were not detected at concentrations less than 500 μM. The electropherogram of ATP included two BL peaks; the latter peak showed peculiar broadening, which continued up to ca. 2.5 h. In the CE apparatus with the flow-type cell, ATP, dATP, and ADP were detected with single peaks with detection limits of 1, 75, and 100 μM, respectively. The other nucleotides, GTP, UTP, CTP, ITP, and TTP, were not detected at concentrations less than 0.5 mM. A mixture of 10 μM ATP and 100 μM dATP was examined using the CE apparatus with the flow-type BL detection cell. ATP and dATP were separated using running buffer at pH 10 containing 1 mM phenylboronic acid. The interaction between ATP and phenylboronic acid delayed the migration time of ATP.

Keywords: Capillary electrophoresis; Bioluminescence; Nucleotides; Phenylboronic acid

1. Introduction

Capillary electrophoresis (CE) has become one of the most powerful and conceptually simple separation techniques for the analysis of complex mixtures [1–3]. One of the major areas of research into CE involves the development of detection methods. Chemiluminescence (CL) has been examined as an attractive detection scheme in CE [4–11]. The interface device between CE and CL detector, i.e., the CL detection cell, is a very important factor in the development of CE with a CL detector. The CL detection cells developed to date can be classified mainly into two types, i.e., batch-type and flow-type [12,13].

On the other hand, one recent application in biochemical analysis is based on the highly efficient firefly luciferin–luciferase reaction. In the bioluminescence (BL) reaction, luciferin reacts with ATP and magnesium ions in the presence of luciferase to form adenylluciferin that transfers to oxylufirin, AMP, CO_2, and light. This BL reaction can be used to determine ATP either directly or coupled with other enzymatic systems. Although reagents related to the BL reaction are generally expensive, biochemical analysis would be easily applicable to CE, as it requires only small amounts of reagents as compared with flow injection analysis (FIA) and HPLC.

However, there have been few reports concerning CE with a BL detector. Dadoo et al. developed a CL or BL detector interface that can be used with CE, which involved the use of an optical fiber and three-dimension positioner [14]. The firefly luciferase reaction was chosen in this previous study because of its high CL quantum efficiency (which can reach 90%) and its relatively fast reaction kinetics as compared with other bioluminescent reactions. However, the detector was specific and complicated. In addition, they examined a single sample of ATP using the CE apparatus with a BL detector; and did not examine mixed samples with the apparatus. Recently, Liu et al. developed a microchip CE with BL detector for detection of ATP, which they used for ATP-conjugated metabolite analysis [15]. However, they also reported no information concerning separation of analytes despite use of the CE system.

In the present study, we developed a CE apparatus equipped with a batch- or flow-type BL detection cell. The firefly luciferin–luciferase BL reaction was used for the apparatus to analyze model nucleotide samples. We demonstrated the sepa-

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ration and detection of a mixture of ATP and dATP in the CE apparatus with a flow-type BL detection cell. It was necessary to consider the uses and applications of the BL reaction based on the data obtained in the CE with the BL detector using the two detection systems.

2. Experimental

2.1. Reagents

All reagents used were commercially available and of analytical grade. Ion-exchanged water was distilled for use. Freeze-dried firefly luciferin–luciferase mixture, guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), and phenylboronic acid were purchased from Wako (Osaka, Japan). Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), deoxyadenosine 5′-triphosphate (dATP), inosine 5′-triphosphate (ITP), uridine 5′-triphosphate (UTP), and thymidine 5′-triphosphate (TTP) were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA).

The freeze-dried firefly luciferin–luciferase mixture (100 mg) was dissolved in 10 mM phosphate buffer (pH 7.8) (5.4 ml) to prepare a BL stock solution. The stock solution was diluted with 10 mM phosphate buffer (pH 7.8) as needed for each experiment. The freeze-dried firefly luciferin–luciferase mixture included luciferin, luciferase, EDTA, magnesium acetate, and human serum albumin. However, quantitative information regarding the components was not reported by the manufacturer.

2.2. CE apparatus with batch-type BL detection cell

A quartz cuvette (inner diameter ca. 5 mm and inner volume ca. 0.7 ml) was used as a BL detection cell. The BL stock solution was diluted three-fold with 10 mM phosphate buffer (pH 7.8) to obtain the BL reagent, which was then added to the batch-type BL detection cell. A fused-silica capillary (50 μm I.D. and 50 cm in length) and platinum wire (500 μm I.D.) as a grounding electrode were also inserted into the cell. The detection cell was put in front of the face of the photomultiplier tube (R-464, Hamamatsu Photonics, Hamamatsu, Japan). Samples of nucleotides that prepared in 10 mM phosphate buffer (pH 7.8) were injected into the capillary inlet by siphoning (for 15 s from a height of 20 cm; ca. 10 nl). The capillary was filled with a running buffer consisting of 10 mM phosphate buffer (pH 7.8) in advance. A high voltage of 12 kV was applied between the capillary ends. Samples migrated electrophoretically toward the capillary outlet and mixed with the reagent at the tip of capillary to produce BL.

The resulting BL was detected with the photomultiplier tube, measured by a photon counter (C1230, Hamamastu Photonics), and treated with an integrator (Chromatopac C-R6A, Shimadzu, Kyoto, Japan).

2.3. CE apparatus with a flow-type BL detection cell

The flow-type cell was made using a three-way joint and a PTFE tube (500 μm I.D.). The detection cell was placed in front of the face of the photomultiplier tube. A fused-silica capillary tube (50 μm I.D., 50 cm in length) and a platinum wire (500 μm I.D.) were inserted into the three-way joint. The BL reagent, which was obtained by diluting the BL stock solution two-fold with 10 mM phosphate buffer (pH 7.8), was delivered toward the tip of capillary outlet at a flow rate of 2.5 μl min^{-1} by a syringe pump. Samples of nucleotides prepared in 10 mM phosphate buffer (pH 7.8) were injected into the capillary by siphoning (for 15 s from a height of 20 cm; ca. 10 nl). The capillary was filled with a running buffer consisting of 10 mM phosphate buffer (pH 7.8) in advance. A high voltage of 12 kV was applied between the capillary ends. Samples migrated electrophoretically to the capillary outlet and mixed with the reagent to produce BL. The obtained BL was detected with the photomultiplier tube.

3. Results and discussion

3.1. BL profiles of ATP in CE apparatus using batch-type BL detection cell

We developed a CE apparatus equipped with a batch-type BL detection cell as described in Section 2, and used it to examine ATP, dATP, ADP, GTP, UTP, CTP, ITP, and TTP. ATP was detected at concentrations of 5–100 μM, while the other nucleotides were not detected at concentrations of less than 500 μM. The electropherogram of ATP is shown in Fig. 1. We observed two BL peaks on the electropherogram; the latter peak showed peculiar broadening, which continued up to ca. 2.5 h. The reason for the two peaks has not yet been determined. ATP reacted with the BL reagents at the tip of capillary outlet, i.e., a specific microenvironment, in the batch-type cell. The specific microenvironment may cause such an electropherogram with two peaks. The first peak appeared immediately when ATP emerged from the capillary outlet and reacted with the reagent. The BL that formed the first peak may be observed around the tip of the capillary outlet. On the other hand, the second peak occurred for a long time during the dispersion of ATP into the

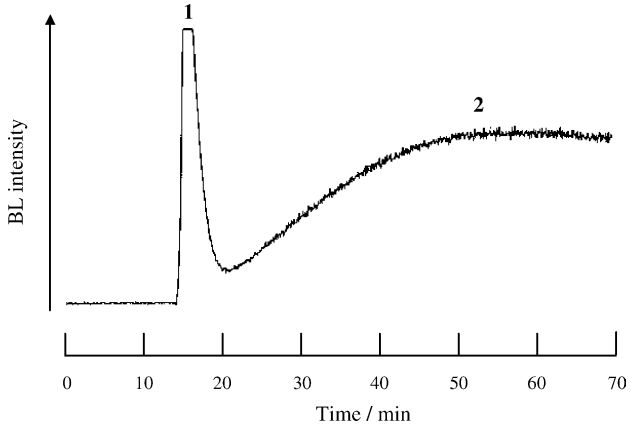

Fig. 1. Electropherogram obtained for ATP in the CE apparatus with a batch-type BL detection cell. *Conditions*: fused-silica capillary, 50 μm I.D. and 50 cm in length; applied voltage, 12 kV; running buffer, 10 mM phosphate buffer (pH 7.8); BL reagent, prepared as described in Section 2; and sample, 0.1 mM ATP.

cell. The BL that formed the second peak was observed from the whole volume of the cell.

Tentatively, we examined the calibration curve of ATP based on the first peak; ATP was determined over the range of 5–100 μM with a detection limit of ca. 50 fmol (S/N = 3). The relative standard deviation and correlation coefficient were 2.4–3.8% (n = 8) and 0.999, respectively. The BL reagent in the cell and the running buffer in the capillary were exchanged for each experiment.

We examined the effects of magnesium sulfate concentration and pH value in the detection cell on the electropherogram. We hypothesized that it may be possible to change the electropherogram of ATP with two peaks to that with only a single peak. A maximum BL intensity was observed around an added magnesium sulfate concentration of 0.5 μM and pH 7.8. However, the two BL peaks were always observed on the electropherograms without any changes throughout the experiments.

3.2. BL profiles and detection of nucleotides in CE apparatus using the flow-type BL detection cell

We developed a CE apparatus with a flow-type BL detection cell as described in Section 2, and used it to examine ATP, dATP, ADP, GTP, UTP, CTP, ITP, and TTP. ATP, dATP, and ADP were detected with a single peak; the second peak that was observed in the batch-type cell did not appear. In the flow-type cell, the confined BL was detected at the tip of the capillary outlet for the definite reaction time, which was defined with the flow rate of the reagent. The other nucleotides, GTP, UTP, CTP, ITP, and TTP, were not detected at concentrations of less than 0.5 mM.

ATP was determined over the range of 1–25 μM with the detection limit of ca. 10 fmol (S/N = 3). dATP was determined over the range of 75 μM–0.5 mM with the detection limit of ca. 800 fmol (S/N = 3). ADP was determined over the range of 0.1–0.5 mM with the detection limit of ca. 1 pmol (S/N = 3). Their relative standard deviations and correlation coefficients were 4.5–6.4% (n = 8) and 0.995–0.998, respectively. The effective mixing of sample with the reagent in the flow-type cell may improve sensitivity, as compared with the data obtained with the batch-type cell. Under these conditions, all were detected at ca. 12 min, i.e., they were not separated.

3.3. Analysis of the mixture of ATP and dATP in the CE apparatus using the flow-type BL detection cell

We attempted to separate the mixture of ATP and dATP by taking advantage of the interaction between the cis-diol group and phenylboronic acid. The cis-diol group of ATP interacts with phenylboronic acid through ester formation in alkaline solution [16,17]. The interaction puts the negative charge on the complex between ATP and phenylboronic acid and increases the molecular weight by the mass of phenylboronic acid. On the other hand, dATP without the cis-diol group does not show such an interaction with phenylboronic acid. The different behavior toward phenylboronic acid between ATP and dATP must result in electrophoretic separation on CE.

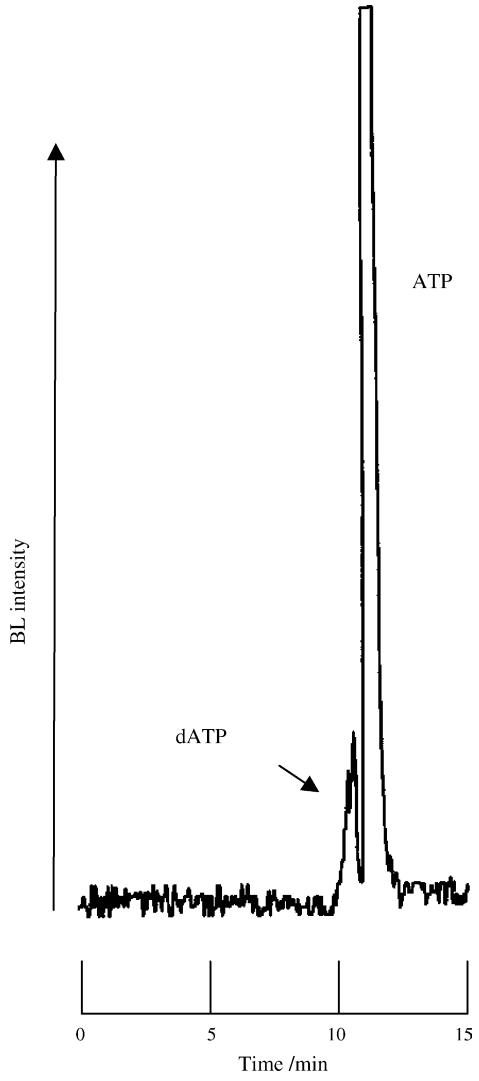

Fig. 2. Electropherogram obtained for a mixture of ATP and dATP in the CE apparatus with a flow-type BL detection cell. *Conditions*: fused-silica capillary, 50 μm I.D. and 50 cm in length; applied voltage, 12 kV; running buffer, 10 mM borate buffer (pH 10.0) containing 1 mM phenylboronic acid; BL reagent, prepared as described in Section 2; and sample, a mixture of 10 μM ATP and 100 μM dATP.

A mixture of 10 μM ATP and 100 μM dATP was examined using the CE apparatus with a flow-type BL detection cell. Borate buffer (10 mM, pH 8 or 10) containing 1 mM phenylboronic acid was used as a running buffer. As the pKa value of phenylboronic acid was reported to be 8.7, ATP would interact with phenylboronic acid in pH 10 running buffer of, but not that at pH 8. ATP and dATP were successfully separated when using the pH 10 running buffer containing 1 mM phenylboronic acid. The obtained electropherogram is shown in Fig. 2. The results indicated that the first peak was due to dATP and the second was due to ATP. The interaction between ATP and phenylboronic acid in alkaline solution at pH 10 delayed the migration time of ATP. This was supported by the experimental observation that they were not separated when using the pH 8 running buffer. The easy and rapid separation of ATP and dATP will be effective for studies concerning DNA and metabolic processes.

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