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Assay for spermidine synthase activity by micellar electrokinetic chromatography with laser-induced fluorescence detection

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Abstract

An assay for spermidine synthase (SPDS) activity in rat liver has been developed using micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection to enable the discovery of SPDS inhibitors. The assay was established by estimating the amount of spermidine (SPD) produced from the putrescine (PUT) present by SPDS. The SPD in an enzyme reaction mixture of homogenized rat liver could directly react with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescence derivatization reagent. The NBD derivatives of SPD and PUT could be separated and detected by MEKC-LIF detection within 15 min. The IC_{50} value measured for SPDS inhibitor, 4-methylcyclohexylamine, in rat liver by this assay was consistent with published data. Our SPDS assay using MEKC-LIF is simple and allows easy determination of SPDS activity in homogenized samples without troublesome procedures such as preparation of antibody or fluorescence-labeled substrate. The assay should be effective for discovering the SPDS inhibitors using biological samples.
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1. Introduction

The aliphatic polyamines, putrescine (PUT), spermidine (SPD), and spermine (SPM) are present in all eukaryotic cells, playing a significant role in cell proliferation and differentiation as polycationic mediators [1–3]. The biosynthesis of polyamine is carried out by three conserved enzymes: ornithine decarboxylase, spermidine synthase (SPDS), and spermine synthase (SPMS). SPDS catalyzes transfer of the propylamine group derived from decarboxylated *S*-adenosylmethionine (dcSAM) to the acceptor PUT to yield SPD, and also regulates the intracellular concentration of SPD. Their polyamines such as SPD regulate DNA replication, gene expression and protein synthesis at physiological condition. In pathological conditions, such as with cancers, higher levels of polyamines have been found [3–5]. These observations indicate that being able to estimate polyamine synthase activity would be very significant for the development of antiproliferative drugs.

In order to measure the concentrations of polyamine and the activities of polyamine synthases in biological samples, various methods have been reported [6]. For the determination of polyamines, there are thin-layer chromatography, gas chromatography, high-performance liquid chromatography, capillary electrophoresis and immunoassay. The HPLC methods, which include a polyamine derivatization step by a fluorescent-labeling reagent such as dansyl chloride, are widely used for determining the concentrations of polyamines in various matrices [7–9]. However, HPLC assays are often cumbersome, time-consuming and sample-consuming for enzyme activity assay in order to discover inhibitors. Thus, immunoassay methods are often applied for high throughput assay (HTS) as they are easy to use and rapid. Enzyme activity assay are not successful because antibodies against polyamines do not have high enough specificity [10,11].

Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection has been reported as an excellent and powerful analytical tool [12–15], having many advantages over its counterpart, HPLC, such as higher efficiency, lower sample volume, and high sensitivity. Also, the CE-LIF method is useful as a highly sensitive enzyme assay, requiring a smaller amount

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of enzyme for the assay. This method could be applicable to HTS using a capillary array system which can simultaneously measure 96 or 384 samples.

In this study, we developed a new method using CE-LIF for SPDS activity assay that can be readily applied to the examination of rat liver. Our method involves reacting SPD with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as a fluorescence labeling reagent [16–19], separating and detecting within an analysis time of 15 min by micellar electrokinetic capillary chromatography (MEKC). The SPD generated by SPDS was determined by directly adding NBD-F into the enzyme reaction mixture of homogenized rat liver. The assay was used to determine the IC_{50} value of SPDS inhibitor, 4-methylcyclohexylamine. The obtained value was consistent with a previously reported value [20]. Satisfactory validation of the method using rat liver homogenate suggests that this method should be useful and reliable for assessing the inhibitory activity of drug candidates.

2. Experimental

2.1. Reagents and materials

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and dithiothreitol (DTT) were obtained from Sigma–Aldrich Co. (St. Lous, MO). 7-Fuloro-4-nitrobenzo-2-oxa-1,3-diazone (NBD-F) was purchased from Dojindo Labs (Kumamoto, Japan), sodium dodecylsulfate (SDS) from Wako Pure Chemical Industries Ltd. (Osaka, Japan), disodium 1,2-ethanedisulfonate (DSEDS) from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and 4-methylcyclohexylamine (4-MCHA) from Nacalai Tesque (Kyoto, Japan). Decarboxylated *S*-adenosylmethionine (dcSAM) was synthesized as described previously [21].

2.2. Apparatus

A P/ACE 5510 capillary electrophoresis system equipped with an argon ion laser emitting 488 nm and an LIF detector (emission wavelength filter of 560 nm) (Beckman Coulter Inc., Fullerton, CA) was used. An uncoated fused-silica capillary (Beckman Coulter Inc., Fullerton, CA) of 75 μm i.d. 360 μm o.d. and 47 cm (40 cm to detector window) was assembled in the LIF cartridge format, to which constant voltage (−12 kV) was applied for 15 min at 20 °C for the MEKC separation. The running buffer used was 50 mM phosphate buffer (pH 2.0) containing 20 mM SDS and 20 mM DSEDS-acetonitrile (4:1, v/v). Samples were injected under pressure for 10 s at 0.5 psi. Prior to injection, the capillary was washed with 0.1 M hydrochloride for 1.5 min, 2 g dL^{-1} SDS for 1.5 min, water for 1 min and finally running buffer for 1.5 min for each run. The electropherographic data were analyzed with Beckman P/ACE Station Software (version 1.0).

2.3. Preparation of rat liver sample

Male Sprague-Dawley rats were killed by decapitation. All operations thereafter were carried out at 0–4 °C. The liver was perfused with ice-cold saline and homogenized in four volumes of 0.1 M DTT, 0.05 M EDTA, 0.05 M phosphate buffer (pH 7.2) using a glass-homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 15,000 g for 15 min and the supernatant was recentrifuged at 15,000 g for 15 min. The supernatant fraction was stored at −40 °C until use as a rat liver homogenate sample for SPDS assay. To remove polyamines, part of the homogenate sample was dialyzed in 0.1 M DTT, 0.05 M EDTA, 0.05 M phosphate buffer (pH 7.2) using Slid-A-Lyzer® Dialysis Cassette 10,000 MW Cut-off (Pierce Biotechnology Inc., Rockford, IL) according to the manufacturer's protocol. The dialysis sample was using to confirm enzyme activity and assay specificity. The protein concentrations of homogenate and dialysis sample were analyzed using the BCA™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL).

2.4. SPDS assay

Ten micro liters of substrate mixture (0.5 mM DTT, 0.1 mM dcSAM and 1.5 mM PUT in 50 mM phosphate buffer (pH 7.2)) was added to 10 μL of rat liver sample in 50 mM phosphate buffer (pH 7.2) followed by incubation for 2 h at 37 °C. To the mixture, 2.5 μL of 50 mM NBD-F in acetonitrile was added followed by heating for 5 min at 37 °C. Forty micro liters of 50 mM hydrogen chloride was added to the reaction mixture, which was centrifuged at 3000 g for 3 min. The supernatant was subjected to analysis by the MEKC-LIF system. The peak areas of the NBD-derived SPD were monitored as an indicator of SPDS activity.

3. Results and discussion

3.1. Derivatization

We previously reported NBD-F to be useful fluorescence-labeling reagent for enzyme activity assay with MEKC-LIF [22]. NBD-F offers the advantages amine group derivatization being rapidly completed under the enzyme reaction conditions and the simultaneous occurrence of the derivatization and enzyme deactivation. Therefore, we employed NBD-F as a fluorescence derivatization reagent.

To optimize the derivatization of SPD with NBD-F, the effect of reaction time and amount of the NBD-F reagent on the derivatization were examined at 37 °C using rat liver homogenate (approximately 1 mg protein mL^{-1}) spiked with 5 μM of SPD (1 μL of 100 μM SPD was added to 20 μL of rat liver homogenate). As shown in Fig. 1, the maximum of the peak area of the NBD derivative of SPD (NBD-SPD) was achieved by incubation for 5 min with a 2.5-μL portion of 50 mM NBD-F. A long reaction time and a large additional amount of NBD-F decreased the peak area of NBD-SPD. This finding may indicate the formation of by-products undetectable in MEKC-LIF which have weak fluorescence intensity or ultraweak electrokinetics forward to the detection window under the MEKC condition. The optimal derivatization condition were incubation for 5 min at 37 °C with a 2.5-μL portion of 50 mM NBD-F.

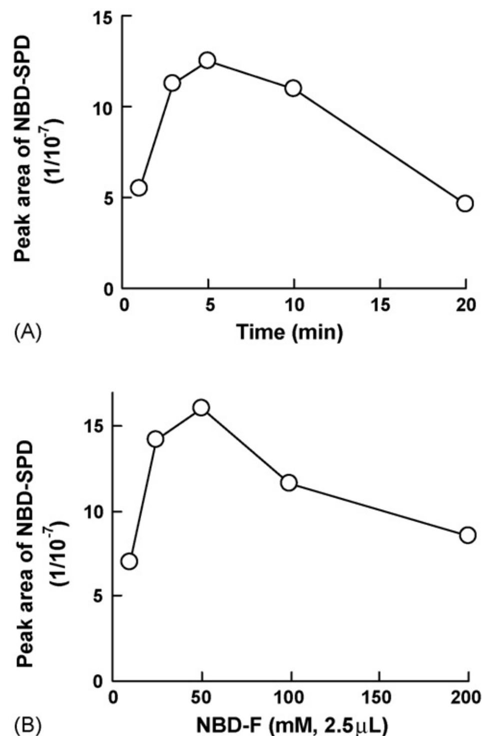

Fig. 1. Effect of reaction time (A) and amount of NBD-F reagent (B) on the formation of SPD derivatives in rat liver homogenate.

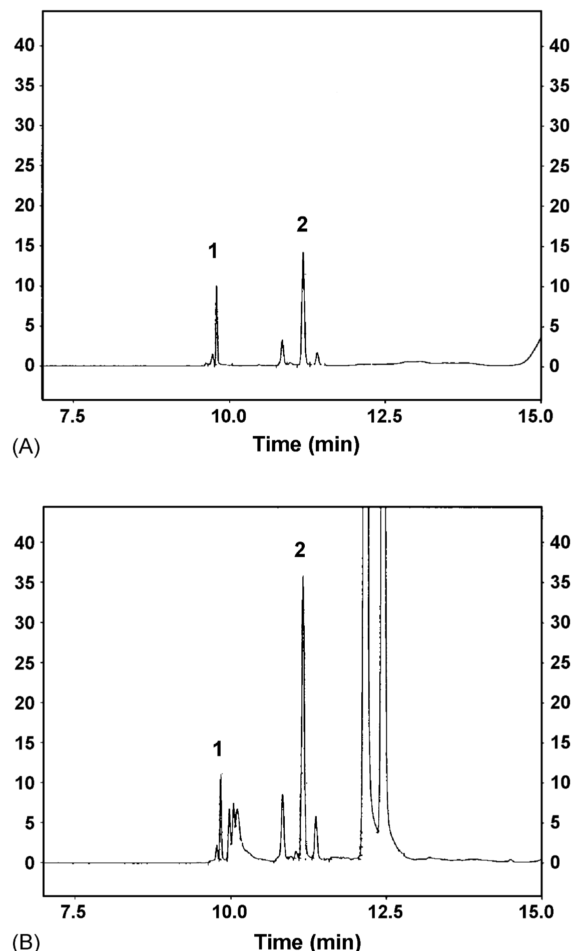

Fig. 2. MEKC-LIF detection of SPD in rat liver homogenate without SPDS reaction (A) and with SPDS reaction and the addition of substrate mixture (B). Capillary, fused-silica capillary (75 μm i.d., 40/47 cm); running buffer, 50 mM phosphate buffer (pH 2.0) containing 20 mM SDS and 20 mM DSEDS-acetonitrile (4:1, v/v); voltage, −12 kV; injection, 10 s at 0.5 psi; detection, Ex: 488 nm, Em: 560 nm. Peaks: (1) NBD-SPM; (2) NBD-SPD.

3.2. CE separation

Sufficient separation of the three polyamines (PUT, SPD and SPM) is necessary to carry out specific SPDS activity assay, because spermine synthase can generate SPM from SPD under SPDS reaction conditions.

Direct injection of sample into the CE system is likely to be employed in the MEKC mode without sample pretreatment when the polyamines in a biological sample are to be determined, because the biological matrices would not affect the CE separation in the MEKC mode [23,24]. Hence, we investigated the CE separation for the NBD-polyamine derivatives using the MEKC mode.

Initially the phosphate buffer (pH 9.0) containing SDS as a running buffer that could bring about an electro-osmotic flow was examined using an uncoated fused-silica capillary. However, no peaks of the NBD derivatives of polyamines could be detected, probably due the derivatives were unstable in the running buffer or were adsorbed onto the capillary wall.

The peaks of NBD derivatives could be individually detected using phosphate buffer (pH 2.0) containing SDS as a running buffer with a negative charge voltage, however, the three peaks of the derivatives could not be separated. For sufficient separation, acetonitrile was added to the running buffer in order to weaken the interaction between the analytes and SDS micelle and to delay the migration of SDS micelle. As a result, the migration time of the NBD derivative of PUT (NBD-PUT) changed notably from those of the other NBD derivatives, and the peak of NBD-PUT could be completely separated from the other peaks. However, the peaks of the NBD derivative of NBD-SPD and the NBD derivative of SPM (NBD-SPM) still could not be separated. This is because the ionic interaction of the NBD-SPD or the NBD-SPM to the SDS micelle surface in the running buffer was very strong and the two interactions were almost the same. In order to weaken the ionic interaction and improve the separation of the two peaks, the addition of divalent anionic reagent DSEDS to the running buffer was investigated, retaining the status of the negative charge in the capillary and the separation of NBD-PUT peak from other peaks. This enabled successful separation of the three derivative's peaks, and NBD-SPD was selectively detected within 15 min using 50 mM phosphate buffer (pH 2.0) containing 20 mM SDS and 20 mM DSEDS-acetonitrile (4:1, v/v) as a running buffer as shown in Fig. 2. Under this condition, the migration time of NBD-PUT was more than 15 min.

3.3. SPDS activity assay

According to a previously described method [25], the reaction conditions for SPDS assay were investigated using

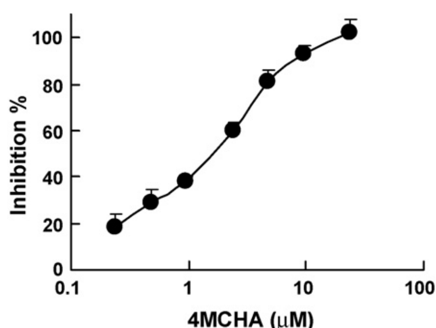

Fig. 3. Inhibition curve of 4-MCHA for SPDS activity. Each point represents a mean ± S.D. of six determinations.

rat liver dialysis sample without endogenous polyamines. The correlation between the incubation time of sample with substrate mixture and the peak area of NBD-SPD was examined. As the peak area of NBD-SPD increased linearly with an increase in the incubation time, a good correlation between the incubation time and the peak area of NBD-SPD was obtained up to the incubation time of 3 h (data not shown). As a result, the incubation time for the SPDS assay was set at 2 h.

Next, the correlation between the protein concentration of dialysis samples and the peak area of NBD-SPD was examined. A good linearity of the peak area of the NBD-SPD was obtained over the range from 0.065 to 1.3 mg protein mL^{-1} ($y \times 10^{-7} = 10.1x - 0.1$, $r = 0.9996$). Thus, this established method can measure SPDS activity in the range of the SPD generation activity corresponding to the above protein concentration.

3.4. Evaluation of inhibitor

In order to use dimethylsulfoxide (DMSO) as an organic solvent for dissolving inhibitors, the effect of adding 2% DMSO to the assay solution was estimated prior to the evaluation of inhibitors according to a previously described procedure [22]. The results confirmed that addition of 2% DMSO did not affect these assays of enzyme activity and the MEKC-LIF separation was not affected by the addition of DMSO. The IC_{50} value of the known SPDS inhibitors 4-MCHA was determined by the established SPDS assays using rat liver homogenate (0.8 mg protein mL^{-1}). Fig. 3 shows the inhibition curve with each point representing the mean ± S.D. of six determinations. The IC_{50} value of 4-MCHA from SPDS assay was 1.4 μM, which was similar to the value obtained by other assay method reported by Shirahata et al. (1.7 μM) [20]. The between-run variation of IC_{50} values was a relative standard deviation of 11.8% ($n = 6$). Thus, our assay method enables ready estimation of SPDS inhibitors without purified enzyme and antibody.

4. Conclusion

We developed the MEKC-LIF method to enable determination of SPDS activity by estimating the response of the fluorescence-labeling SPD produced from PUT by an enzyme reaction. The present MEKC-LIF method should aid in the rapid establishment of SPDS activity assays. Furthermore, this work showed that the MEKC-LIF analysis methods have the potential of serving as tools for enzyme activity assays for drug discovery when no highly-specific antibody is available and there is difficulty in using labeled substrates due to enzyme reactivity.

The assay system described here can measure 80 samples a day including MEKC analysis and sample preparation such as SPDS enzyme reaction and NBD-derivatization. If a 384-capillary array system were used, it should be possible to examine more than 30,000 samples per day. We foresee MEKC-LIF with a capillary array system as being useful for developing high throughput assay system for the discovery of inhibitors.

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