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Notes

Determination of Cellular Aminopropyltransferase Activity Using Precolumn Fluorescent Etheno-Derivatization with High-Performance Liquid Chromatography

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Polyamines such as spermidine (Spd) and spermine (Spm), produced by aminopropyltransferase (Apt), play roles in cell growth and differentiation. A sensitive and simple fluorometric high-performance liquid chromatographic determination for Apt activity of spermidine synthase (Spdsyn) and spermine synthase (Spmsyn) was developed in order to examine cellular functions of polyamine synthesis. The derivatization procedure for methylthioadenosine (MTA) produced from decarboxylated *S*-adenosylmethionine by Apt was the reaction with 2-chloroacetaldehyde to give fluorescent 1, N^6-etheno methylthioadenosine. The reaction conditions for derivatization were optimized. A calibration curve was established, ranging from 0.01 to 25 pmol. Quantification of derivatized MTA was confirmed to be identical to Spd or Spm production. The developed method determined Spdsyn and Spmsyn activities in HepG2 cells treated with oleic acid as a cellular lipid accumulation model.

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Introduction

Polyamines such as spermidine (Spd) and spermine (Spm), produced by aminopropyltransferase (Apt), are widely distributed in eukaryote and most prokaryote cells, and are involved in cell proliferation and differentiation.[1,2] Spermidine is synthesized by transfer of the aminopropyl group from decarboxylated *S*-adenosylmethionine (dcSAM) to putrescine by spermidine synthase (Spdsyn) with a concomitant production of methylthioadenosine (MTA). Spermine synthase (Spmsyn) produces spermine by attaching the aminopropyl group of dcSAM to the N8-side of spermidine with a concomitant MTA synthesis.

Some biological significances of Apt were reported. Spdsyn gene deletion in *Escherichia coli* (*E. Coli*) caused the decrease in its growth rate,[3] and Spdsyn is reported to be essential for maintenance of a robust infection in mammals, indicating that pharmacologic inhibition of Spdsyn is a valid therapeutic strategy for the treatment of visceral forms of leishmaniasis.[4] Spdsyn is also suggested to be induced by Myc transcription factors in a direct manner, whose overexpression of which is a hallmark of cancer. Treatment of Myc-induced cancer with inhibition of Spdsyn could be an additional chemopreventive strategy.[5] Deletion of Spmsyn gene and of a part of phosphate-regulating gene with homologies to endopeptidases on the X chromosome gene in mice produces a phenotype of underweight, circling behavior and sterility, which is recovered by Spmsyn gene transfection.[6,7] Mutations in the human Spmsyn gene lead to the Snyder–Robinson syndrome with mental retardation, hypotonia, osteoporosis and leanness.[8] These symptoms seem to be caused by decreased Spmsyn activity, leading to a reduction in its product Spm and an increase in the precursor Spd. However, the biological functions of both Spdsyn and Spmsyn have not yet been fully elucidated.[9] The development of a convenient and highly sensitive method for the enzyme activity is necessary and important in order to reveal the function of polyamine synthesis.

Several assays for Apt activity have been reported. These use radioisotope (RI),[10] high-performance liquid chromatography (HPLC),[11,12] homogeneous time-resolved fluorescence[13] or mass spectrometry[14] methods. The RI method with ^{35}S-dcSAM as a substrate for Apt activity measurement needs preparation of ^{35}S-dcSAM by enzymatic reaction with S-adenosylmethionine (SAM) synthase, ATP, radio isotope-labeled methionine and SAM decarboxylase, followed by manual column chromatographic purification because dcSAM is not commercially available. Fluorescent HPLC determination for Spd or Spm contents produced by Spdsyn or Spmsyn, respectively, is commonly carried out by derivatization such as precolumn dansylation or postcolumn *o*-phthalaldehyde reaction with amines.[15] These methods measure the incremental increase of Spd or Spm produced by cellular Spdsyn or Spmsyn activity over that already present in the extract. Since high levels of polyamines exist endogenously in organisms, such assays need large amounts of tissues, longer enzymatic reaction time or dialysis to remove endogenous polyamines. Here, precolumn

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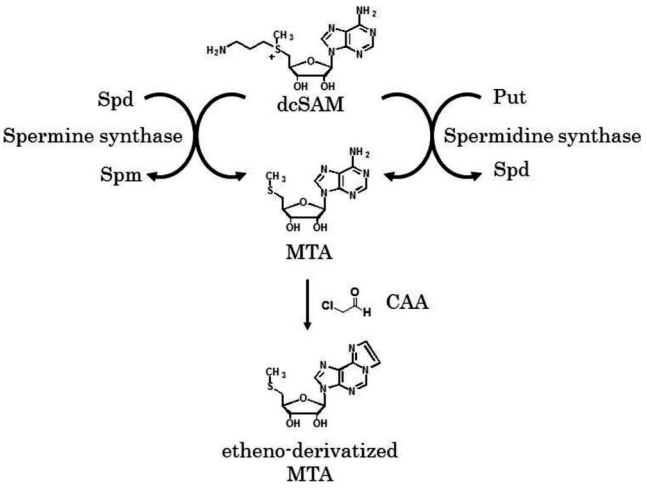

Fig. 1 Derivatization reaction scheme for aminopropyltransferase activity measurements.

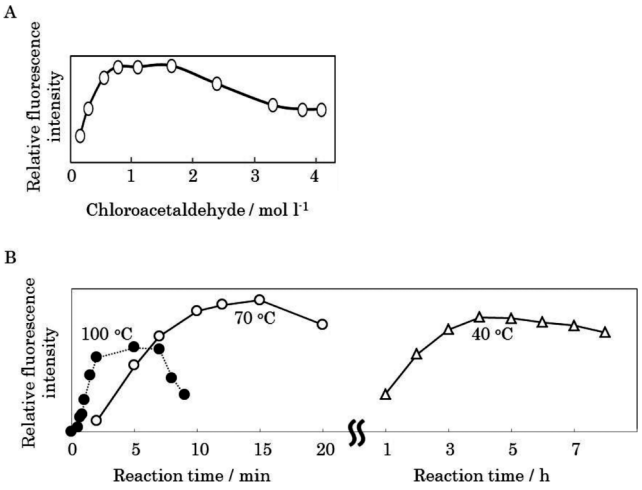

Fig. 2 Optimization for etheno-derivative reaction. (A) Effect of chloroacetaldehyde concentration on fluorescence intensity. (B) Effect of reaction temperature and time on fluorescence intensity. Results are shown for reaction temperature at 100°C (●), 70°C (○), and 40°C (△).

derivatization of MTA, which is another product of Apt which is present in much lower endogenous amount because of its metabolism, has been chosen for the assay because it is fully sensitive and convenient and easy to handle. An adenine analog of MTA was determined by etheno-derivatization with chloro- or bromo-acetaldehyde,[16,17] indicating that an adenine analogue of the MTA produced by Spdsyn or Spmsyn could be also etheno-derivatized to be fluorescent. In this report, cellular Spdsyn and Spmsyn activitiy assay methods were developed by using etheno-derivatization with chloroacetaldehyde (CAA) (Fig. 1). The developed method determined Spdsyn and Spmsyn activities in HepG2 cells treated with oleic acid as a cellular lipid accumulation model[18] in order to examine the relationship between lipid accumulation and polyamine synthesis.

Experimental

Reagents and chemicals

Methylthioadenosine (MTA), spermidine trihydrochloride (Spd), spermine tetrahydrochloride (Spm) and *N*-(3-aminopropyl)cyclohexylamine (APCHA) were obtained from Sigma (Tokyo, Japan), and were recrystallized with hydrochloric acid before use. Sodium acetate, sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate 12 water, and chloroacetaldehyde (CAA) were obtained from Wako (Osaka, Japan). Putrescine dihydrochloride (Put) and 4-methylcyclohexylamine (MCHA) (*cis*- and *trans*-mixture) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and *trans*-MCHA was purified from the mixture by recrystallization.[19] Adenine was purchased from Kohjin (Tokyo, Japan). Perchloric acid (PCA) was obtained from Kanto Kagaku (Tokyo, Japan). Decarboxylated *S*-adenosylmethionine (dcSAM) was prepared in our laboratory.[20]

Activity assay for Apt

Human Spdsyn and Spmsyn were purified according to the report by Wu.[21,22] Spdsyn activity assays for purified protein and cellular supernatant were carried out in a reaction buffer containing 0.1 M sodium phosphate pH 7.5, 0.1 mM dcSAM, 1 mM Put, 0.6 mM adenine, and 0.05 mM APCHA, which was added for cellular activity measurement to block Spmsyn, another endogenous Apt.[23] Each reaction mixture was incubated at 37°C for 1 h and the reaction was stopped by the addition of 1.1 M PCA, followed by centrifugation at 16000*g* for 20 min. Then, 1.5 M CAA and 0.2 M sodium acetate was added to the supernatant to adjust the pH to around 4.5, and the mixture was incubated for derivatization reaction at 70°C for 15 min. Etheno-derivatizated MTA was determined by reversed-phase HPLC: column, TOSOH TSK-gel ODS-80Ts (TOSOH), 4.6 mm i.d. × 150 mm; mobile phase, 6% (v/v) isopropanol; flow rate, 0.5 mL min^{-1} (pump, LC-10A, Shimadzu); detection, excitation wavelength 270 nm, emission wavelength 410 nm (RF-10A, Shimadzu).

Spmsyn activity assay for purified protein and cellular supernatant was carried out in a reaction buffer containing 0.1 M sodium phosphate pH 7.5, 0.1 mM dcSAM, 5 mM Spd, 0.6 mM adenine with 0.05 mM MCHA, which was added to inhibit Spdsyn, another Apt. The following procedure for etheno-derivatization was similar to that of the activity assay for Spdsyn.

Spd and Spm contents after centrifugation of PCA-treated reaction mixture were determined. Samples were injected onto an anion-exchange HPLC by using postcolumn fluorescence derivatization with *o*-phthalaldehyde.[23]

HepG2 cells treated with oleic acid

HepG2 cells were disseminated at 1.0×10^4 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM) (GIBCO, USA) containing 10% fetal bovine serum (FBS) (GIBCO, USA) and incubated for 3 days. DMEM was replaced with DMEM containing 5% bovine serum albumin (BSA) (Wako, Osaka, Japan), and on the next day the medium was exchanged to DMEM containing 1 mM oleic acid (Wako, Osaka, Japan) and 5% BSA to induce lipid accumulation.[24,25] The medium was replaced with fresh material every day.

Results and Discussion

Optimization conditions for etheno-derivatization

Spdsyn or Spmsyn produce MTA and Spd or Spm from dcSAM and Put or Spd, respectively. In this study, in order to examine the Apt activity, the fluorescence intensity of etheno-derivatized MTA was determined by reverse-phase

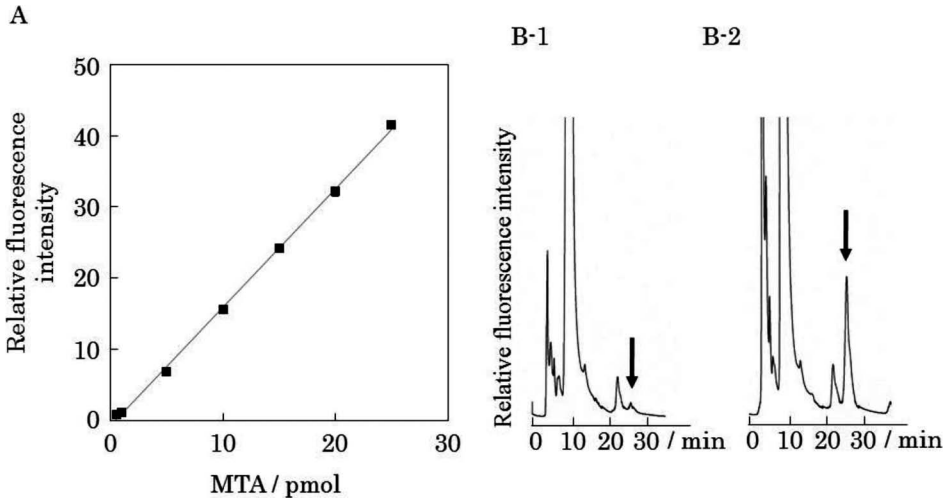

Fig. 3 Standard curve and HPLC chromatograms for etheno-derivatized MTA. (A) Standard curve for etheno-derivatized MTA. (B) HPLC chromatograms for SpdSyn activity in HepG2 cell. Results are shown for etheno-derivatized MTA (↓). (B-1) Reaction at zero time point, (B-2) after reaction for 1 h.

HPLC. This derivatization reaction is based on etheno cyclization of adenine moiety with CAA.[16] The reaction conditions such as the concentration of CAA, the reaction temperature and the time for a maximal etheno-derivatized MTA production have been optimized. During the examination on the effect of CAA concentration on etheno-derivatization, the fluorescence intensity of etheno-derivatized MTA increased up to 1.0 M, although the value decreased beyond 1.5 M (Fig. 2A). As a result of the effects of reaction temperature and time on the etheno-derivatized MTA synthesis shown in Fig. 2B, a good reaction efficiency in relatively shorter time was obtained under the conditions of 70°C for 15 min. In this experiment, 1.5 M CAA for 15 min at 70°C as a reaction condition was chosen for the etheno-derivatization. By using this derivatization, we found that a standard curve for etheno-derivatized MTA in the range of 0.01 – 1 pmol and 0.5 – 25 pmol gave excellent linearity at $r = 0.9999$ for the respective ranges. The detection limit was 100 fmol ($S/N = 5$).

In order to confirm the accuracy of Spdsyn or Spmsyn activity, the correlation between an activity determined by this etheno-derivatization for MTA production and that by postcolumn derivatization with OPA for Spd or Spm synthesis was examined and the effect of potent inhibitors was determined. The IC50 values of MCHA to recombinant Spdsyn and that of APCHA to recombinant Spmsyn, were 2 and 6 μM respectively, which were the same as those measured by OPA determination.

Measurement of Apt activity in HepG2 cells

This etheno-derivatization method was applied to measurements for Spdsyn and Spmsyn activities in HepG2 cells as a biological sample. Because intracellular MTA is metabolized rapidly by MTA phosphorylase (MTAP) in mammalian cells, adenine which is known to be a potent MTAP inhibitor,[26,27] was added to the reaction mixture to block endogenous MTAP in this experiment. Etheno-derivatized MTA and adenine were detected at 25 and 22 min, respectively, as shown in the HPLC chromatograms for Spdsyn activity assay in supernatant protein of HepG2 cells (Figs. 3B-1 and 3B-2). Determination of cellular MTA produced by Spdsyn or Spmsyn showed good linearity in the time range of 15 – 180 min and in the tested range of 10 – 50 μg protein in HepG2 cells (data not shown). Comparison of this etheno-derivatizated MTA measurement with Spd or Spm quantification by postcolumn OPA fluorescence HPLC method for cellular Spdsyn and Spmsyn assay produced similar results. The etheno-derivatized MTA quantification gave 25.7 ± 0.5 pmol mg protein^{-1} h^{-1} as Spdsyn activity and 4.07 ± 0.04 pmol mg protein^{-1} h^{-1} as Spmsyn activity, and the OPA method gave 27.0 ± 0.9 pmol mg protein^{-1} h^{-1} and 4.39 ± 0.4 pmol mg protein^{-1} h^{-1}, showing that there were no significant differences between the two methods. This demonstrates that the method developed here produces results in good accord with the methods used commonly. The OPA method, however, required approximately 10 times more cellular protein because the Spd or Spm amounts produced in an activity assay need to exceed the endogenous polyamine contents significantly. A tissue or cellular sample contains high levels[28] of endogenous polyamines despite a low Apt activity but has a low amount of endogenous MTA, suggesting that this developed method could be a useful assay for a small amount of sample such as is the case in biogenic samples.

Changes in Apt activity in oleic acid-treated HepG2 cells

Spmsyn gene deletion in a male mouse, called Gy, causes lower weight and shorter size than those of a normal mouse and also induces sterility. Spmsyn gene-overexpressed mice are heavier by about 20% than a normal mouse.[29] Inhibitors of Apt have a crucial influence in differentiation of preadipocyte 3T3-L1 cells.[30] In order to investigate the relationship between lipid accumulation and polyamine synthesis, we measured cellular Apt activity in the adipogenesis process in HepG2 cells treated with oleic acid as a hepatic steatosis model.[17] Spdsyn activity and Spmsyn activity in oleic acid-treated cells were found to increase by 50 and 15%, respectively, compared to the activities in non-treated cells at 2 days after treatment using this method (Figs. 4A and 4B). The contents of both Spd and Spm in oleic acid-treated cells were found to increase at 3 days (Figs. 4C and 4D), indicating that the Spdsyn and Spmsyn activity values augmented in response to oleic acid would elevate intracellular polyamine contents during lipid accumulation. However, the mechanism where polyamine might be involved in lipid accumulation in HepG2 cells treated with oleic acid remains to be resolved.

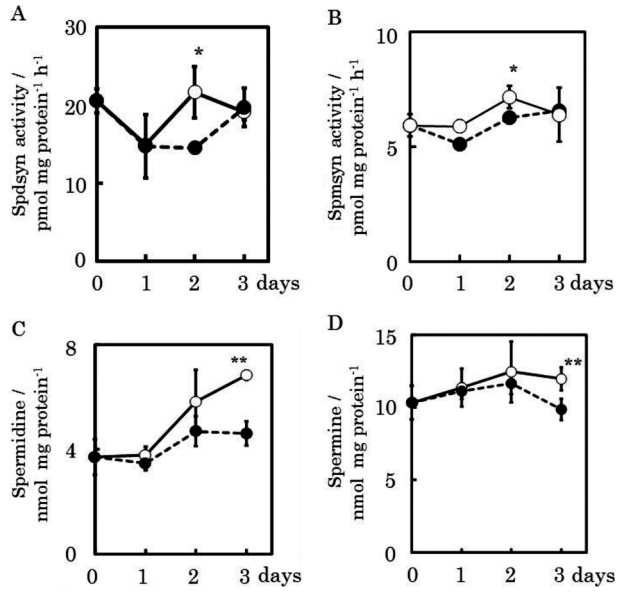

Fig. 4 Apt activity and polyamines contents in oleoic acid-treated HepG2. (A) Spdsyn activity. (B) Spmsyn activity. (C) Spd content. (D) Spm content. Results are shown for oleic acid-treated group (○), and control group (●). Results represent the mean ± S.E. *, p <0.05, **, p <0.01 compared with control.

Conclusion

In this study, we have shown that this etheno-derivatization could be a useful method for Spdsyn and Spmsyn activity measurements during research for new functions of cellular polyamines. This derivatization for the measurement of cellular Apt activity was simple and convenient: preparations of radioactive-labeled substrates such as ^{35}S-AdoMet or more procedures like dansylation or other derivatization were not needed; another pump for postcolumn HPLC was also not needed. These results illustrated that this method also could be applied usefully to the activity measurement for biological samples with even lower activity. There results also suggested that Spmsyn might be involved in lipid accumulation in HepG2 cells treated with oleic acid

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