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A New Assay Method for Spermidine and Spermine Synthases Using Antibody Against MTA

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Abstract : We have developed a novel method for assays of spermidine and spermine synthase (aminopropyltransferase) activities using antibody against 5'-deoxy-5'-methylthioadenosine (MTA). A new assay is reported here which is based on the observation that MTA is formed as a stoichiometric by-product of the spermidine and spermine synthases reactions. In order to determine MTA, a radioimmunoassay method with sensitivity and rapidity was used. (Lee and Cho, 1997). In this assay, adenine must be added in the reaction mixture, since it effectively inhibits the action of MTA phosphorylase by which MTA is metabolized. This assay is a improvement in term of sensitivity and time saving, compared to the currently used methods. It has a level of sensitivity (100 fmol) sufficient to monitor aminopropyltransferase activities in incubations containing as little as 10 μg protein prepared from rat tissue homogenate. The results obtained showed that this method is particularly useful for cultured cells with low enzyme concentration. Moreover, this assay has the advantage which allows studies using alternative substrates (other amines). Spermidine synthase activity was high in rat liver, but low in rat kidney. The activity of spermine synthase was in most rat tissues very low as compared to that of spermidine synthase, but was high in brain.
Key words : 5'-deoxy-5'-methylthioadenosine, enzyme assay, radioimmunoassay, spermidine synthase, spermine synthase

Studies of polyamine biosynthetic pathway in certain micro-organisms, plant, and mammalian cells have established that aminopropyltransferases play essential roles in this process (Tabor and Tabor, 1976; Jänne *et al.*, 1978; De Rosa *et al.*, 1978). Mammalian cells contain two distinct aminopropyltransferases that transfer propylamine group from decarboxylated S-adenosylmethionine to amine acceptors leaving 5'-deoxy-5'-methylthioadenosine (MTA). Spermidine synthase uses putrescine as an acceptor and forms spermidine (Hannonen *et al.*, 1972; Hibasami *et al.*, 1980), while spermine synthase uses spermidine as acceptor producing spermine (Pajula *et al.*, 1979; Hibasami *et al.*, 1980). Spermidine synthase was first discovered in *Escherichia coil* and was later purified (Tabor *et al.*, 1958; Bowman *et al.*, 1973). This enzyme has been also purified from rat (Samejima and Yamanoha, 1982; Yamanoha and Samejima, 1984), bovine sources (Raina *et al.*, 1984) and human spleen (Kajander, 1989), and spermine synthase only from bovine brain (Pajula *et al.*, 1979). However, the work on these enzymes in plants is insufficient. These synthases from plants have not been purified to homogeniety yet. Although polyamines are essential for growth in a number of organisms and have been implicated in the regulation of a wide variety of physiological functions, these aminopropyltransferases in the pathway for polyamine synthesis are much less studied. In general, assay methods of these enzymes are complicated, tedious, and thus not suitable for the assay of a large number of samples. Therefore, the development of rapid and sensitive assay methods for aminopropyltransferase has made it possible to characterize these enzymes in greater detail and study on regulation mechanism of polyamine metabolism. In most papers reporting studies on spermidine and spermine synthases, radioisotopic methods were used; ^{14}C-labelled putrescine or ^{14}C-labelled decarboxylated S-adenosyl-methionine was incubated, followed by the separation of radioactive spermidine, spermine, or MTA by thin-layer chromatography, paper electrophoresis, and ion-exchange paper chromatography (Jänne *et al.*, 1971; Hannonen *et al.*, 1972; Raina *et al.*, 1976; Hibasami and Pegg, 1978; Tabor *et al.*, 1986).

In this paper, we describe a rapid and sensitive immunological method for the assay of spermidine and spermine synthases. The method is based on the meas-

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urement of amount of MTA stoichiometrically formed by these enzymes reaction.

Materials and Methods

Materials

Putrescine, spermidine, spermine, 5'-deoxy-5'-methylthioadenosine (MTA), bovine serum albumin, DTT, sodium periodate, sodium borohydride, ethylene glycol, Freund's complete adjuvant, and Freund's incomplete adjuvant were purchased from Sigma chemical Co. (St. Louis, USA). S-adenosyl-L-[methyl-^{3}H]methionine (91 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Decarboxylated S-adenosylmethionine was kindly supplied by Dr. Kijiro Samejima (Faculty of Pharmacentical Sciences, Josai Univ., Saitama, Japan). All other reagents used were obtained from commercial sources and were of analytical grade.

Preparation of the immunogen and antiserum

Antibodies against MTA were raised in white rabbits according to Lee and Cho (1997). Initial injection of 1 mg of conjugates of MTA with BSA dissolved in PBS containing 1 mM DTT and emulsified with complete Freund's adjuvant was followed by 2 injections (1 mg MTA-BSA in PBS containing 1 mM DTT/incomplete Freund's adjuvant) at 4 week intervals. The rabbits were bled 10 days after the last injection. The collected fluid was immediately centrifuged and stored at -70°C.

Preparation of rat tissue extracts

Sprague-Dawley strain rats (200~300 g body weight) were killed by decapitation. The tissues were rapidly removed, washed in cold PBS. All subsequent operations were performed at 4°C. The tissue was miniced with scissors and then homogenized with approximately 4 volumes of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 0.1 mM DTT. The homogenates were centrifuged at 15,000×g for 20 min. The supernatant was used as an enzyme source.

Plant materials

Soybean seeds (*Glycine max*) were grown at 25°C in the dark and harvested as described by Kang and Cho (1990).

Enzymes assay

The assay mixture for spermidine and spermine synthases contained, 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM putrescine or spermidine, 0.04 mM decarboxylated S-adenosylmethionine, 5 mM DTT, 0.2 mM adenine, and the enzyme extract in a total volume of 0.2 ml. After incubation at 37°C for 20 min, the reaction was stopped by adding 20 μl of 1.2 M perchloric acid and centrifuged. Precipitates were discarded. The radioimmunoassay (RIA) for MTA was performed as previously described (Lee and Cho, 1997). Before RIA, the pH was adjusted to 7.0 by addition of 1 M Tris buffer (pH 9.0) to supernatant. In RIA, the antiserum was diluted to 10 fold and 20 μl was added to the reaction. The standard solutions or neutralized enzyme reaction mixtures were added to reaction mixture (total volume 190 μl) and incubated at 37°C for 30 min followed by addition of 10 μl of [methyl-^{3}H] labelled MTA (100 fmol, 10 nCi/ μl), prepared by acid hydrolysis of S-adenosyl-L-[methyl-^{3}H]methionine (91 Ci/mmol). After 30 min, 200 μl of 100% ammonium sulfate solution was added to each sample and left at 4°C for 20 min. The tubes were centrifuged at 13,000×g for 15 min and the supernatant was discarded carefully. The tubes were washed once with 50% ammonium sulfate solution and centrifuged. The precipitate was suspended with distilled water and radioactivity was measured.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) with BSA as a standard.

HPLC analysis

A Whatman Partisil 10 SCX (25×4.6 Cm) column was used in conjunction with a Waters HPLC apparatus. The volume of sample applied was 50 μl. The samples were eluted with 0.5 M formate buffer (pH 4). The flow rate was 1 ml/min. Peaks were detected at 254 nm.

Results and Disccusion

In the present paper, we report a new assay method for spermidine and spermine synthases activities, which employs antibody against MTA. This new method is based on the detection of MTA formed as a stoichiometric byproduct of the these enzymatic reactions (Pegg and Williams-Ashman, 1969) (Fig. 1). In order to identify MTA of the reaction, an incubated enzyme reaction mixture was subject to a Partisil 10 SCX HPLC column for HPLC analysis, performed as above described. Retention times (min) for MTA and decarboxylated S-adenosylmethionine were 3.1 and 5.8, respectively. Fig. 2B showed the formation of MTA by the reaction of spermidine synthase.

Most assay methods of these enzymes that were reported previously estimate [^{14}C] labelled spermidine and spermine formed (Jänne *et al.*, 1971; Hannonen *et al.*, 1972; Raina *et al.*, 1976; Hibasami and Pegg, 1978; Tabor *et al.*, 1986). In our method, MTA was deteted

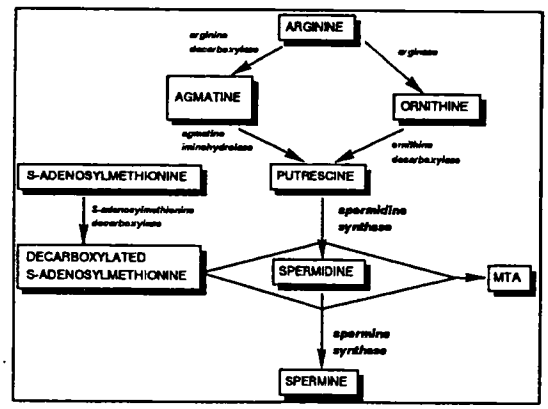

Fig. 1. The biosynthetic pathway of polyamine metabolism. Two separate aminopropyltransferases are present in mammalian and plants cells. Spermidine synthase catalyzes the transfer of an aminopropyl group from decarboxylated S-adenosylmethionine to putrescine, forming spermidine and MTA. Spermine synthase uses spermidine as the aminopropyl acceptor and produces spermine and MTA. All known aminopropyltransferases produce MTA, which is the basis of this assay.

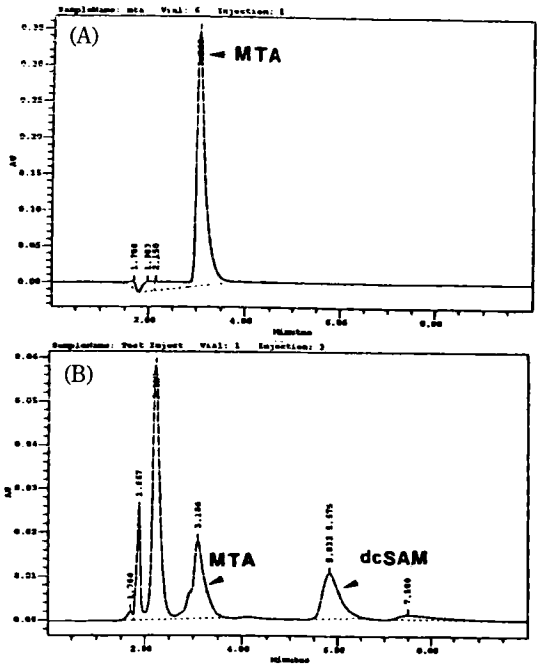

Fig. 2. High-performance liquid chromatographic separation of MTA fromed from enzyme reaction mixture. (A) HPLC peak showing the standard MTA. (B) HPLC peak showing the MTA produced by spermidine synthase from soybean and decarboxylated S-adenosylmethionine as substrate. Column, Partisil 10 SCX; eluent, ammonium formate, 0.5 M, pH 4.0; flow rate, 1 ml/min.

by the radioimmunoassay developed by Lee and Cho (1997). The antibody used here showed no cross-reactivity with other similar compounds, including decarboxylated S-adenosylmethionine and adenine in assay mixture. Fig. 3 illustrates that unlabelled decarboxylated S-adenosylmethionine did not inhibit the binding of

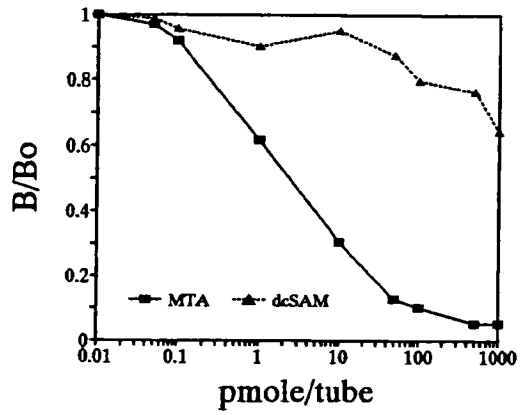

Fig. 3. Affinities of MTA and decarboxylated S-adenosylmethionine (dcSAM) for anti-MTA antiserum using RIA method. The mixture for the RIA contained PBS, 20 μl antiserum (1:200 dilution), and 100 μl MTA or dcSAM solution with various concentration in a total volume of 190 μl. After 30 min at 37°C, 10 μl of [methyl-^{3}H] labeled MTA (100 fmol, 10 nCi/μl). The curve showed the amount of bound radioactivity (B) for various doses of unlabeled compounds (MTA or dcSAM) as a ratio of that bound using [methyl-^{3}H] MTA alone (Bo).

Table 1. Spermidine and spermine synthases activities in various rat tissues

Tissue	Spermidine synthase	Spermine synthase	Spermidine synthase (no addition of adenine)
	(pmol/mg protein per 30 min)		
Liver	45,250±1030	530±20	580±20
Brain	25,760±870	8,250±410	3,040±120
Kidney	7,280±360	780±40	650±20
Spleen	31,060±930	1,420±60	4,316±130
Testis	9,190±410	20	2,790±80
Prostate	25,110±1050	5,970±240	4,420±180
Lung	9,580±410	750±30	1,239±50

Tissues of male rats (200 g body weight) were used for analysis. The values for enzyme activities represent the means (±SD) of three determinations.

[methyl-^{3}H]MTA with specific antibody.

In this assay, 0.2 mM adenine were added in the assay mixture because adenine is a strong potent inhibitor of MTA phosphorylase by which MTA was degraded to methylthioribose-1-phosphate and adenine (Pegg and Williams-Ashman, 1969). The breakdown of MTA by MTA phosphorylase interfered with precise measurement of spermidine and spermine synthases activities in this present assay. Table 1 shows that the formation of MTA was signiqicantly decreased when adenine was not added. It indicates that MTA phosphorylase exist in all tissues and adenine is effective for the inhibition of MTA phosphorylase activity. Spermidine and spermine synthases activities in the various rat tissues were det-

ermined by the present assay. Spermidine synthase activity was high in the liver, but low in the kidney. The spermine synthase activity was in most tissues very low as compared to the activity of spermidine synthase, but was high in the brain. Raina et al. (1976) reported the distribution of spermidine and spermine synthases in various rat tissues using a radioisotopic assay. When compared, our results are basically similar to theirs. However, there is a remarkable difference in the activity of spermidine synthase in the rat liver. Accoring to Raina *et al.* (1976), the activity of spermidine synthase in the prostate was 6-fold higher than that in the liver, while our result showed the activity of this enzyme in the prostate was low compared to that in the liver.

Unlike our assay method, the radioisotopic method reported by Raina et al. was based on the isolation of the radioactive polyamines formed from radioactive decarboxylated S-adenosylmethionine labelled in the propylamine moiety by using phosphocellulose ion exchange paper. But, polyamines are catabolized by polyamine oxidase which is present in all mammalian tissues. If polyamine oxidase is not blocked during the assay, the measurement of spermidine synthase activity is hindered by the action of this oxidase. In the method reported by Raina *et al.* (1976), spermidine synthase was assayed without any inhibitor of polyamine oxidase. In addition the activity of polyamine oxidase is very high in the liver compared with that in other tissues (Seiler *et al.*, 1980). In constrast, our method is not affected by polyamine oxidase because it employs the measurement of MTA instead of spermidine. Thus, considering polyamine oxidase, we think that our data are more resonable than previous ones.

Fig. 4 shows changes in spermidine synthase activity during the seed germination and the early growth period of soybean (*Glycine max*). The enzyme activity reached a maxium value for 1 day after germination and declined steadily as germination progressed.

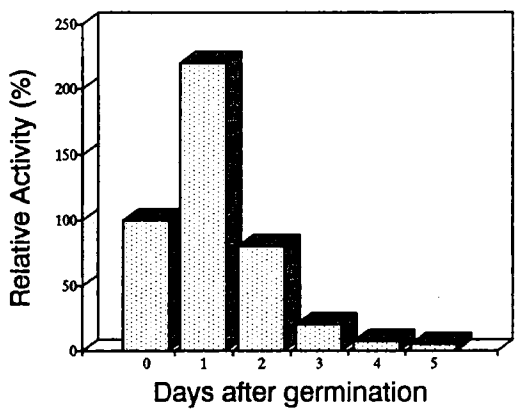

Fig. 4. Changes of spermidine synthase activity from soybean (*Glycine max*) after germination. The activity for germiation was shown as 100%.

Fig. 5 shows the synthases activities in rat prostate as a function of enzyme concentration. A linear relationship was observed in the range from 0 to 130 μg of enzyme protein. The detection limit was 0.1-100 pmol of MTA. Fig. 6 shows the synthases activities in rat prostate as a function of incubation time. Linearity was obtained for 30 min in spermidine and spermine synthases.

In summary, we have doveloped a new assay based on the measurement of amount of MTA replaced as spemidine and spermine. MTA formed by synthases was estimated by radioimmunoassay (Lee and Cho, 1997). Our immunological method is rapid and extremely sensitive. The lower limit of detection by this assay was 100 fmol of MTA. In addition, the method can be used with amines other than putrescine or spermidine to test whether these are substrates for the reaction, since any aminopropyl transfer reaction will generate MTA. The radio immunological assay method for spermidine and spermine synthases described in this

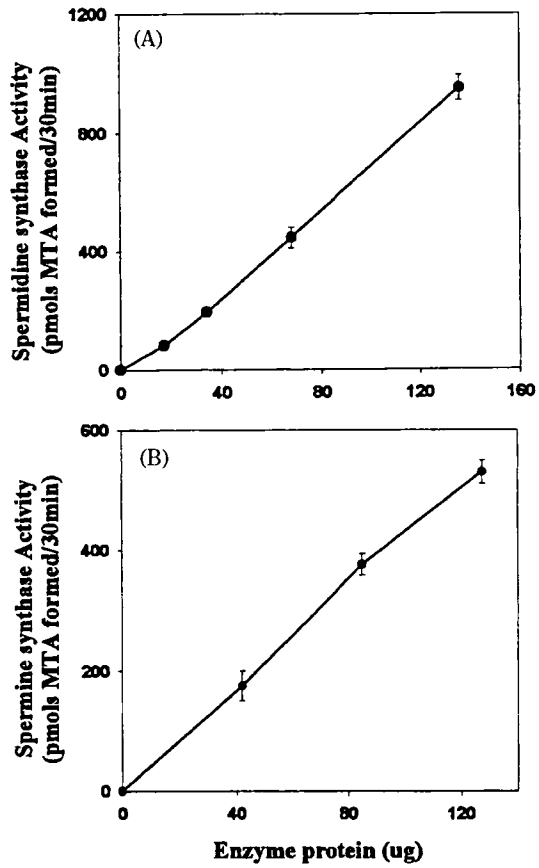

Fig. 5. Spermidine (A) and spermine (B) synthases activities as a function of enzyme concentration. Homogenate of rat prostate was used as an enzyme source. Reaction mixture was incubated for 15 min. Data are mean±SD (bar) for three experiments.

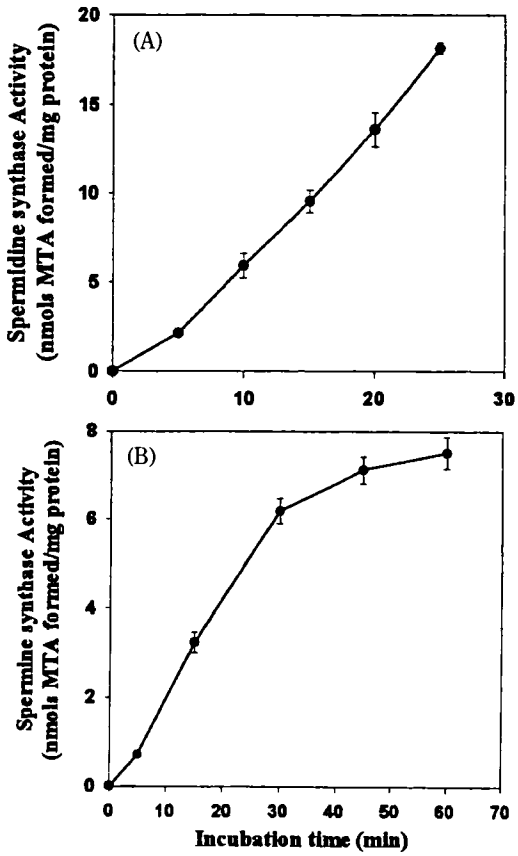

Fig. 6. Spermidine (A) and spermine (B) synthases activities as a function of incubation time. The reaction mixture contained 85 μg protein of homogenate of rat prostate. Each point represents the mean obtained from three experiments.

paper makes it more feasible to study these enzymes and polyamine metabolism.

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