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PARTIAL PURIFICATION AND CHARACTERIZATION OF SPERMINE SYNTHASE FROM RAT BRAIN

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SUMMARY

Spermine synthase, the enzyme catalyzing the formation of spermine from spermidine and 5′-deoxy-5′-*S*-(3-methylthiopropylamine)sulphonium adenosine (decarboxylated *S*-adenosylmethionine) has been purified more than 100-fold from rat brain cytosol fraction. Spermine synthase activity can be resolved from the other polyamine-synthesizing enzyme activites, *i.e.* from *S*-adenosylmethionine decarboxylase and spermidine synthase activities, by a single chromatography run on DEAE-cellulose.

The purified spermine synthase, free of any *S*-adenosylmethionine decarboxylase or spermidine synthase activity, showed a broad pH optimum between 7.5 and 8.1 and an acidic isoelectric point at pH 5.0. Spermine synthase appeared to have a high affinity for decarboxylated *S*-adenosylmethionine, the apparent K_m value being below 0.005 mM. The K_m for spermidine was 0.07 mM. Putrescine was shown to be a competitive inhibitor with respect to spermidine, and spermine, the product of the reaction, was also inhibitory. No metal or other cofactor requirements for spermine synthase were found.

INTRODUCTION

A number of experiments *in vivo* with chick embryos[1], rat liver[2,3] and Ehrlich ascites cells[4] have suggested that spermidine acts as a natural precursor for spermine. Pegg and Williams-Ashman[5,6] showed that cell-free preparations from rat ventral prostate catalyzed the synthesis of spermine from spermidine in the presence of *S*-adenosylmethionine or decarboxylated *S*-adenosylmethionine. Even after considerable purification it was not possible to resolve the spermine synthesizing activity from *S*-adenosylmethionine decarboxylase and spermidine synthase activities[5,6]. Recently, however, evidence has accumulated indicating that in the enzymic synthesis of spermidine and spermine in mammalian tissues, at least three separable proteins are needed: (i) a putrescine-activated *S*-adenosylmethionine decarboxylase[5,7], (ii) spermidine synthase[8,9] and (iii) spermine synthase[9,10].

In the present communication we report a simple purification procedure for rat brain spermine synthase resulting in an enzyme preparation free of any *S*-adenosylmethionine decarboxylase and spermidine synthase activities. Some of the properties of the refined spermine synthase are also described.

MATERIALS AND METHODS

Animals

Female rats of the Wistar strain weighing 150–180 g were used.

Chemicals

Unlabelled putrescine, spermidine and spermine as their HCl salts were purchased from Calbiochem. [1,4-^{14}C]Putrescine·2 HCl (spec. act. 17.5 mCi/mmole), [1,4-^{14}C]spermidine·3 HCl (spec. act. 10.22 mCi/mmole) and DL-[*carboxy*-^{14}C]methionine (spec. act. 3.54 mCi/mmole) were obtained from the New England Nuclear Corporation. The radioactive putrescine as well as the unlabelled putrescine and spermidine were purified on a Dowex 50-H^+ column before use[11]. Labelled and unlabelled *S*-adenosylmethionine were synthesized enzymically and purified as described earlier[5]. Decarboxylated *S*-adenosylmethionine was synthesized using *S*-adenosylmethionine decarboxylase[5] from *Escherichia coli* and purified on a Dowex 50-H^+ column followed by preparative paper electrophoresis[9,12].

Analytical procedures

Protein was measured by the method of Lowry *et al.*[13]. Quantitative polyamine measurements were carried out by a modification of the method of Raina and Cohen[14].

Isoelectric focussing was performed with an LKB 8101 apparatus (110 ml, LKB Produkter, Stockholm, Sweden) with a double cooling jacket using 1% ampholine (pH 3–10) and a linear sucrose gradient from 5 to 50% as stabilizer. After a constant current was reached (after about 60 h) fractions of 4 ml were collected from which the pH value and spermine synthase activity were determined.

The molecular Stokes radius of spermine synthase was calculated by the method of Ackers and Steere[15] using Blue Dextran 2000, potassium dichromate, yeast alcohol dehydrogenase and bovine serum albumin as standards for the calibration of the Sephadex G-200 column.

Enzyme assays

The assay conditions for *S*-adenosylmethionine decarboxylase and spermidine synthase have been recently described elsewhere[7–9]. The standard incubation mixture for spermine synthase contained 100 mM potassium phosphate (pH 7.5), 0.1 mM decarboxylated *S*-adenosylmethionine, 0.5 mM [1,4-^{14}C]spermidine, 5 mM dithiothreitol and the enzyme protein in a final volume of 0.15 ml. Under these conditions the reaction was linear for at least 30 min and proportional to the amount of the enzyme protein added. Radioactive spermine was isolated after the incubation as follows: the incubation was halted with 1 ml of 1.5 M HCl containing 0.2 μmole of unlabelled spermine as carrier. After centrifugation the supernatant fraction was applied to a small (0.2 cm × 5 cm) Dowex 50-H^+ column previously equilibrated with 1.5 M HCl. Radioactive spermidine was washed out with 150 ml of 1.5 M HCl

and spermine was eluted with 15 ml of 6 M HCl[5]. The eluate was evaporated to dryness under reduced pressure, the residue dissolved in a small volume of 0.1 M HCl and subjected to paper electrophoresis[1] (this treatment completely resolves radioactive spermine from spermidine). The recovery of spermine was checked by quantitative measurements as described above using the spermine carrier added after the incubation.

Alcohol dehydrogenase activity was assayed essentially as described by Racker[16].

Purification of spermine synthase

After decapitation of the rats, the brains were removed and immediately homogenized with 2 vol. of 0.25 M sucrose containing 0.3 mM EDTA and 1 mM 2-mercaptoethanol in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged for 60 min at 100 000 × g_{max} and the supernatant fraction was used for further purification. The crude extract (Fraction 1) was fractionated with solid $(NH_4)_2SO_4$ (Mann, special enzyme grade) at 0 °C. The proteins precipitated between 0.55 and 0.75 saturation of $(NH_4)_2SO_4$ were dissolved in a small volume of 25 mM sodium phosphate buffer (pH 7.2), containing 0.3 mM EDTA and 0.5 mM dithiothreitol and dialyzed overnight against 2 l of the same buffer, except that dithiothreitol was replaced by 5 mM 2-mercaptoethanol. The dialyzed $(NH_4)_2SO_4$ fraction (Fraction 2) was applied to a DEAE-cellulose column (Whatman DE 52, microgranular, 3 cm × 32 cm) equilibrated with the above buffer containing 0.5 mM dithiothreitol. The column was washed with 120 ml of the equilibration buffer and connected to a linear gradient of 0.1 to 0.4 M NaCl in the equilibration buffer (total gradient volume was 1000 ml). Spermine synthase activity was eluted between 390 and 470 ml. To avoid contamination with *S*-adenosylmethionine decarboxylase activity, fractions between 420 and 470 ml were pooled (Fraction 3), concentrated in a pressurized ultrafiltration cell (Amicon Corp.) to 5 ml and applied to a Sephadex G-200 column (Pharmacia, Sweden, 2.5 cm × 30 cm) previously equilibrated with 25 mM Tris–HCl (pH 7.2), containing 50 mM KCl, 0.2 mM EDTA and 0.5 mM dithiothreitol. The most active fractions, eluted between 108 and 125 ml, were pooled and designated as Fraction 4.

RESULTS

Table I summarizes the purification procedure used. *S*-Adenosylmethionine decarboxylase and spermidine synthase activities were already partially resolved from spermine synthase activity by the $(NH_4)_2SO_4$ fractionation (*S*-adenosylmethionine decarboxylase and spermidine synthase activities were precipitated below 0.6 saturation of $(NH_4)_2SO_4$). After DEAE-cellulose chromatography the pooled spermine synthase fraction (Fraction 3) did not contain any detectable *S*-adenosylmethionine decarboxylase or spermidine synthase activity. The overall purification through this four-step procedure was about 100-fold with a yield of about 6%.

Fig. 1 presents the elution of all three enzymes of crude brain extract from the DEAE-cellulose column. The elution pattern clearly demonstrates that *S*-adenosylmethionine decarboxylase, and spermidine and spermine synthase activities can be separated from each other by a single DEAE-cellulose chromatography even if a prior exposure to high ionic strength (*i.e.* $(NH_4)_2SO_4$ fractionation) is omitted. This result

TABLE I

PARTIAL PURIFICATION OF SPERMINE SYNTHASE FROM RAT BRAIN

The crude extract was prepared from 70 g of rat brain and processed as described in the text. The spermine synthase activity is expressed as nmoles of spermine formed per 30 min under standard assay conditions.

Fraction	*Total protein (mg)*	*Total activity (nmoles)*	*Specific activity (nmoles/mg protein)*	*Yield (%)*	*Purification (fold)*
1. Crude extract	1419	4060	2.87	100	1
2. $(NH_4)_2SO_4$ fraction (0.55–0.75 saturation)	326	2060	6.31	51	2.2
3. DEAE-cellulose	7.1	960	136	24	48
4. Sephadex G-200	0.78	237	306	6	107

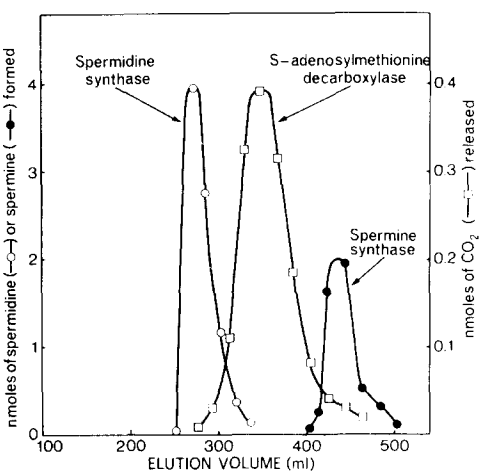

Fig. 1. Separation of *S*-adenosylmethionine decarboxylase, spermidine synthase and spermine synthase activities from crude brain extract on a DEAE-cellulose column. 60 ml (546 mg of protein) of brain cytosol fraction were applied to a DEAE-cellulose column and the column eluted as described in the text. Enzyme activities are expressed as nmoles of CO_2 released or spermidine (or spermine) formed per 0.1 ml of fraction per 30 min.

is in agreement with our earlier observation on rat liver enzymes but is in marked contrast to those reported by Feldman *et al.*[17,18].

Some properties of spermine synthase

The purified spermine synthase showed a rather broad pH optimum between 7.5 and 8.1 in phosphate buffer. In isoelectric focussing experiments spermine synthase activity appeared as a symmetrical peak at pH 5.0. Spermine synthase activity was eluted from Sephadex G-200 column between yeast alcohol dehydrogenase and bovine serum albumin. The apparent molecular Stokes radius was calculated to be 3.98 nm which is very close to the sizes of *S*-adenosylmethionine decarboxylase and spermidine synthase (unpublished results). Fig. 2 shows the effect of the decarboxylated *S*-adenosylmethionine concentration on the spermine synthase reaction. The reaction

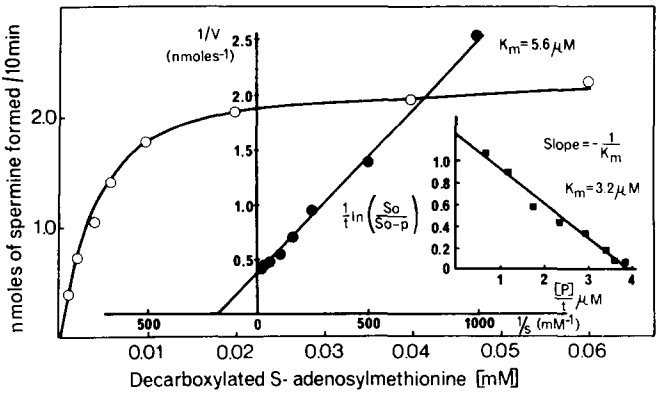

Fig. 2. Effect of decarboxylated *S*-adenosylmethionine concentration on spermine synthase activity. Fraction 4 enzyme preparation (16 μg) was incubated under standard incubation conditions and the concentration of decarboxylated *S*-adenosylmethionine was varied as indicated. S_0, the concentration of decarboxylated *S*-adenosylmethionine at the start of the incubation. P, the concentration of spermine at the end of incubation. The lines were plotted by computer by the least squares method.

showed typical Michaelis–Menten type kinetics, and an apparent K_m value of 5.6 μM for decarboxylated *S*-adenosylmethionine was obtained from the usual double reciprocals using the least squares plot (calculation of the apparent K_m value by the method of Wilkinson[19] gave a value of 4.8 μM). However, owing to the extensive consumption of the substrate at lower concentrations of decarboxylated *S*-adenosylmethionine, these values seem to be overestimations. The insert in Fig. 2 shows the apparent K_m value for decarboxylated *S*-adenosylmethionine using the integrated Michaelis–Menten equation[20]. The slope ($= -1/K_m$) of the line gives an apparent K_m value only of about half that obtained with the usual double reciprocal plot.

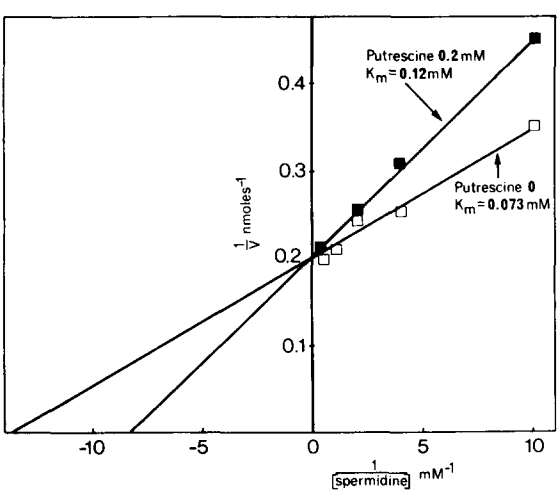

Fig. 3. Effect of putrescine and spermidine on spermine synthase activity. Spermine synthase activity (Fraction 4, 16 μg) was assayed under standard assay conditions by varying the concentration of spermidine in the absence or presence of 0.2 mM putrescine. The double reciprocal lines were plotted by computer by the least squares method.

The apparent K_m value for spermidine in the presence of saturating levels of decarboxylated *S*-adenosylmethionine was 0.07 mM (Fig. 3). Fig. 3 also shows that putrescine acted as a competitive inhibitor with respect to spermidine; the apparent K_i value being 0.3 mM. Spermine also had a slight inhibitory effect on the reaction although higher concentrations were needed.

Spermine synthase, apparently, was not dependent on any metal cofactor since the inclusion of EDTA, $CaCl_2$, $MgCl_2$ or $MnCl_2$ (all 5 mM) into the incubation mixture did not alter the catalytic activity. Isonicotinic acid hydrazide (10 mM), semicarbazide (5 mM) or canaline (5 mM) were also without any effect. Similarly 5 mM *N*-ethylmaleimide (in the absence of dithiothreitol) failed to decrease the activity.

DISCUSSION

The results of the present study establish that at least three different enzymes are needed for the synthesis of spermidine and spermine in rat brain. The properties of the partially purified spermine synthase from rat brain, free of *S*-adenosylmethionine decarboxylase and spermidine synthase activities, are in general agreement with those reported by Pegg and Williams-Ashman[6]. However, using an enzyme preparation purified some 15-fold and still containing both *S*-adenosylmethionine decarboxylase and spermidine synthase activities, they obtained a K_m value of 0.025 mM for decarboxylated *S*-adenosylmethionine and 1.1 mM for spermidine. As demonstrated by the present study, the purified spermine synthase from rat brain had a much higher affinity for both substrates. Pegg and Williams-Ashman[6] also found that putrescine was a competitive inhibitor with respect to spermidine; however, the K_i value was considerably lower as compared to the data of the present study.

The low apparent K_m value of spermine synthase for decarboxylated *S*-adenosylmethionine may explain the close stoichiometry between the release of CO_2 and formation of spermine from spermidine and *S*-adenosylmethionine by crude or partially purified prostatic[6], liver[11] and brain preparations[9]. In terms of specific activities it appears likely that the decarboxylation of *S*-adenosylmethionine is the rate limiting step in the formation of spermine in mammalian tissues. However, much work is still needed to solve the functional relationships between the three enzymes needed for the synthesis of spermidine and spermine in mammalian tissues.

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