PURIFICATION OF SPERMINE SYNTHASE FROM BOVINE BRAIN BY SPERMINE–SEPHAROSE AFFINITY CHROMATOGRAPHY

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1. Introduction

We have demonstrated [1–3] that spermine synthase which catalyses the synthesis of spermine from spermidine and decarboxylated *S*-adenosylmethionine (*S*-methyladenosylhomocysteamine) can be separated by conventional chromatographic methods from other enzymes involved in the biosynthesis of polyamines. However, due to a tedious and time-consuming assay method this enzyme has not been extensively purified and characterized. An approx. 100-fold purification of spermine synthase from rat brain has been reported [3]. Development of a rapid and sensitive isotopic method [4] has considerably facilitated the assay of spermidine and spermine synthase activities. We describe here a method for the isolation of highly-purified spermine synthase from bovine brain using spermine–Sepharose affinity chromatography.

2. Materials and methods

D,L-[2-^{14}C]methionine (spec. act. 4.08 mCi/mmol) and [1,4-^{14}C]spermine tetrahydrochloride (18.75 mCi/mmol) were purchased from the New England Nuclear Corp. Labelled *S*-adenosylmethionine was synthesized from D,L-[2-^{14}C]methionine essentially as in [5]. Radioactive decarboxylated *S*-adenosylmethionine was prepared from *S*-adenosylmethionine labelled at the C-2 position of the methionine moiety using *S*-adenosylmethionine decarboxylase from *E. coli* purified through step 3 by the method in [6], as the enzyme. The product was purified on a Dowex 50-H^+ column and finally freed on any contaminating *S*-adenosylmethionine by preparative paper electrophoresis as in [2]. The unreacted *S*-adenosylmethionine was isolated in the same purification procedure and could be used as the substrate in a further incubation.

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and dithiothreitol were products of Calbiochem. Whatman phosphocellulose paper P81 was obtained from Reeve Angel and Co., London. CH-Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala.

In preparing spermine–Sepharose the general instructions of the manufacturer were followed. CH-Sepharose 4B, 1 g dry wt, was swollen in 0.5 M NaCl overnight, washed with 200 ml the same solution and then with distilled water. The washed gel (~4 ml) was mixed with 500 μmol spermine in 5 ml water. Radioactive spermine (0.5 μCi) was added as a marker and the pH adjusted to 5.5. The mixture was stirred slowly and 230 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) in 1 ml water was added dropwise. The mixture (in a glass-stoppered tube) was slowly rotated for 24 h at room temperature. The gel was then washed several times alternately with 0.05 M formic acid buffer (pH 3.0)–1 M NaCl and with 0.1 M Tris–HCl (pH 8.0)–1 M NaCl, and finally with water and stored in 50 mM Tris–HCl (pH 7.5) at 5°C until used. About 8–10 μmol of spermine was bound/ml gel as judged from the radioactivity determinations.

Fresh bovine brain (about 900 g) obtained from a local slaughterhouse was immediately cooled in

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ice. All subsequent operations were performed at 0–4°C. The brain was freed of membranes, washed in 0.25 M sucrose and homogenized in 2 vol. 0.25 M sucrose–0.1 mM EDTA–1 mM 2-mercaptoethanol–1 mM dithiothreitol with an Ultra-Turrax (Janke and Kunkel) homogenizer. The homogenate was centrifuged at 23 000 × *g* for 60 min. The supernatant fraction was removed, made up to 5 mM with dithiothreitol and treated with solid ammonium sulphate (Mann, special enzyme grade) as in [2]. The proteins which precipitated between 60% and 75% saturation were dissolved in 50 mM Tris–HCl (pH 7.5, at 5°C)–0.1 M NaCl–1 mM 2-mercaptoethanol–0.1 mM dithiothreitol and dialysed for 15 h against 200 vol. the same solution. The dialysed sample (about 1.2 g protein) was applied to a DEAE–cellulose column (Whatman DE 52, 5 × 36 cm) pre-equilibrated with the dialysis buffer containing 0.5 mM dithiothreitol, and eluted with the same solution. Spermine synthase was eluted between 530 ml and 630 ml. The most active fractions were pooled (90 ml) and brought to 80% saturation with ammonium sulphate to concentrate the enzyme.

A column (1 × 5 cm) was packed with Sepharose linked to spermine and equilibrated with 50 mM Tris–HCl (pH 7.5), containing 0.15 M NaCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 1 mM dithiothreitol (solution E). The precipitate obtained by ammonium sulphate after DEAE-cellulose fractionation was dissolved in solution E and dialysed overnight against the same solution. A portion of the dialysed sample was applied to the column at 10 ml/h flow rate. The column was washed with solution E, followed by solution E containing 0.3 M NaCl. Spermine synthase was eluted with solution E containing 0.3 M NaCl and 1 mM spermidine.

The assay of spermine synthase was performed using radioactive decarboxylated *S*-adenosylmethionine as substrate as detailed in [4]. The original procedure was modified as follows: After alkaline hydrolysis, a 20 μl aliquot of the acidified supernatant was applied to a single Whatman P81 disc, followed by a small amount of water. The numbered paper discs were then washed in a beaker using mechanical stirring and a wash solution of 10 mM Tris–HCl (pH 8.0) and 100 mM NaCl. A washing time of 30 min (3 × 10 min) has given satisfactory results. One unit of enzyme activity represents the formation of 1 nmol spermine in 1 min under the standard assay conditions.

Protein was measured by the method in [7] either directly or after precipitation with trichloroacetic acid, or by ultra violet absorption [8].

3. Results and discussion

In the rat, spermine synthase activity was highest in the brain tissue [4]. Bovine brain homogenates contained spermine synthase activity as high as that found in the rat and was therefore chosen as the enzyme source for large-scale purification. No gross differences were found in the enzyme activity between different parts of the brain (results not shown).

Several polyamines including putrescine, spermidine and spermine were tested as a ligand for affinity chromatography. Spermine–Sepharose showed a high affinity for spermine synthase, whereas no specific retention was observed with putrescine linked to Sepharose. Although spermidine–Sepharose also seemed to be useful for purification, spermine–Sepharose was chosen for further studies.

Application of crude supernatant or dialysed ammonium sulphate fraction to spermine–Sepharose column resulted in precipitation of some protein(s) which interfered with the binding of spermine synthase. However, with an enzyme preparation previously purified by fractionation with ammonium sulphate and DEAE-cellulose chromatography, an excellent purification was achieved using the spermine–Sepharose column. Tris–HCl buffer (50 mM) containing 0.3 NaCl efficiently removed contaminating proteins (fig.1). Spermidine synthase was eluted with the same solution added with 1 mM spermidine. Putrescine at 1 mM concentration did not elute the enzyme. As spermidine is a substrate of spermine synthase, no previous dialysis was needed for the enzyme assay. The recovery of spermine synthase activity in the spermine–Sepharose purification step was close to 100% in several experiments.

The results of a purification procedure involving ammonium sulphate precipitation, DEAE-cellulose fractionation and spermine–Sepharose affinity chromatography are summarized in table 1. An

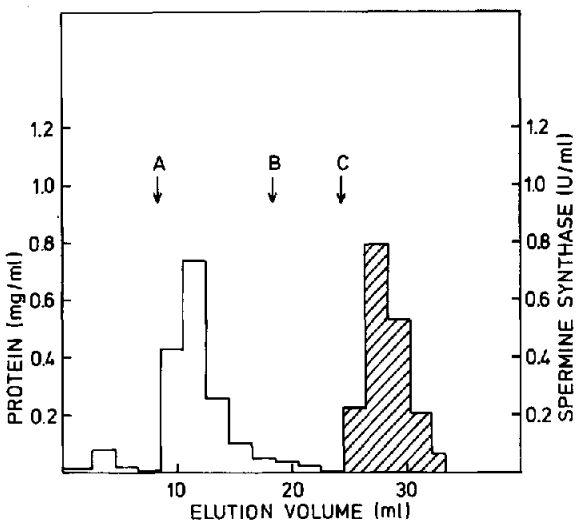

Fig.1. Purification of spermine synthase by spermine–Sepharose affinity chromatography. An aliquot of partially purified enzyme preparation (ammonium sulphate fraction obtained after DEAE-cellulose chromatography, 3.6 mg protein) was dialysed overnight against solution E (see text) and applied to a spermine–Sepharose column (1 × 5 cm) pre-equilibrated with solution E. The column was washed with 6 ml solution E, followed by elution with 10 ml solution E containing 0.3 M NaCl (A), 6 ml solution E containing 0.3 M NaCl and 1 mM putrescine (B), 8 ml solution E containing 0.3 M NaCl and 1 mM spermidine (C) and finally with solution E. Fractions, 2 ml each, were collected. White columns, protein concentration; hatched columns, spermine synthase activity.

approx. 6300-fold overall purification of the enzyme was achieved. The final preparation had a specific activity about 55-times higher than that reported for the partially purified enzyme from rat brain [3].

The purified enzyme was free of any *S*-adenosylmethionine decarboxylase or spermidine synthase activity. Preliminary analysis on SDS–gel electrophoresis indicates that it was at least 30–50% pure. Large-scale purifications for closer characterization of the enzyme are now in progress in our laboratory.

There is ample evidence to suggest that polyamines are essential elements in macromolecular synthesis especially in rapidly proliferating tissues [9,10]. Inhibitors of polyamine synthesis have been shown to arrest or delay cell proliferation [10]. As described here, spermine–Sepharose affinity chromatography is a powerful tool for the isolation of spermine synthase from tissues. Application of this method would facilitate the search of new inhibitors of polyamine synthesis, i.e., inhibitors of aminopropyltransferases.

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References

[1] Raina, A. and Hannonen, P. (1970) Acta Chem. Scand. 24, 3061–3064.
[2] Raina, A. and Hannonen, P. (1971) FEBS Lett. 16, 1–4.
[3] Hannonen, P., Jänne, J. and Raina, A. (1972) Biochim. Biophys. Acta 289, 225–231.

Table 1
Purification of spermine synthase

Step	Total protein (mg)	Total units	Recovery (%)	Spec. act. (units/mg protein)	Purification (-fold)
Crude supernatant	10 820	995	100	0.092	1
Ammonium sulphate fraction (60–75%)	1207	309	31	0.256	2.8
DEAE cellulose	182	159	16	0.875	9.5
Ammonium sulphate fraction (0–80%)	129	115	11.5	0.890	9.7
Spermine–Sepharose	0.16	93	9.3	579	6290

[4] Raina, A., Pajula, R-L. and Eloranta, T. (1976) FEBS Lett. 67, 252–255.
[5] Pegg, A. E. and Williams-Ashman, H. G. (1969) J. Biol. Chem. 244, 682–693.
[6] Wickner, R. B., Tabor, C. W. and Tabor, H. (1970) J. Biol. Chem. 245, 2132–2139.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[8] Kalckar, H. M. (1947) J. Biol. Chem. 167, 461–475.
[9] Raina, A. and Jänne, J. (1975) Med. Biol. 53, 121–147.
[10] Jänne, J., Pösö, H. and Raina, A. (1978) Biochim. Biophys. Acta, in press.