Phytochemistry, Vol 22, No 1, pp 103–106, 1983
Printed in Great Britain

0031–9422/83/010103–04$03 00/0

BIOSYNTHESIS OF SPERMIDINE IN MAIZE SEEDLINGS

EIJI HIRASAWA and YONEZO SUZUKI

Department of Biology, Faculty of Science, Osaka City University, Sumiyoshi-ku, 558 Osaka, Japan

(*Received* 26 *March* 1982)

Key Word Index—*Zea mays*, Gramineae, maize, putrescine, spermidine, spermidine synthetase

Abstract—The addition of [1,4-^{14}C]putrescine into *Zea mays* seedlings yielded radioactive spermidine The rate of incorporation of [^{14}C]putrescine was depressed by pretreatment with methylglyoxal-bis-(guanylhydrazone) (MGBG) Spermidine synthetase has been purified 115-fold from etiolated maize shoots The MW determined by Sephadex G-200 filtration was *ca* 43 000 and the optimum pH was 7 2 The enzyme was inhibited by sulfhydryl reagents The purified enzyme had no spermine synthetase or polyamine oxidase activities

INTRODUCTION

The polyamines spermidine and spermine have been demonstrated in higher plants [1], as well as in microorganisms and animals [2] In animals and microorganisms [3–6], polyamines are synthesized by two separate enzymes, namely, *S*-adenosylmethionine (SAM) decarboxylase (EC 4 1 1 50) and aminopropyltransferase (EC 2 5 1 16) from SAM and putrescine, respectively In animals, spermidine synthetase and spermine synthetase [5, 6] are separate aminopropyltransferases

In higher plants, SAM decarboxylase activity has been detected in extracts of mung bean [7] and *Vinca rosea* [8], and the enzyme has been partially purified from *Lathyrus sativus* [9] and maize [10] On the other hand, spermidine synthetase activity was detected in the homogenate of *Vinca* [8] and Chinese cabbage [11] This paper describes the biosynthesis of spermidine *in vivo*, and the properties of partially purified spermidine synthetase in maize seedlings

RESULTS AND DISCUSSION

In vivo *synthesis of spermidine in maize seedlings*

Seedlings grown for 5 days were allowed to absorb the solution of radioactive putrescine through the roots Spermidine was then estimated using an ion exchange column and PC The amount of putrescine absorbed into the seedling axes was 2 3% (1 hr) and 4 6% (3 hr) of that in the solutions, and the putrescine absorbed was converted to spermidine in the ratio of 0 33% (1 hr) and 1 98% (3 hr), respectively (Table 1) No radioactivity in spermine was detected in the 1 8–4 0 M HCl fraction which should contain this polyamine (see Experimental) The radioactivity of the 1 8 M hydrochloric acid fraction was mainly distributed in putrescine and in an unidentified compound that was found in small amounts An unidentified compound in our tracer experiment might be the product to which spermidine is converted by polyamine oxidase [12]

Therefore, spermidine may be synthesized from SAM and putrescine in maize seedlings as it is in *E coli* [13] and rat [14] This route is supported by tracer experiments with other plants Spermidine and spermine was formed from [^{14}C]putrescine in barley leaf [15] and from [^{14}C]methionine in leaf protoplasts of Chinese cabbage [11] SAM decarboxylase has been partially purified from *Lathyrus sativus* [9] and maize [10] Spermidine synthetase has been demonstrated in crude extracts from *Vinca rosea* [8] and Chinese cabbage [11], but has not hitherto been purified

Table 1 Effect of MGBG on *in vivo* spermidine synthesis from [^{14}C]putrescine in maize seedlings

Culture medium	Time after feeding (hr)	Fraction: Total activity ($\times 10^3$ dpm)	Fraction: Spermidine dpm (%)
H_2O	1	201	669 (0 33)
	3	406	8040 (1 98)
MGBG (10^{-4} M)	1	153	611 (0 40)
	3	265	1060 (0 40)

In order to examine the effect of MGBG on spermidine synthesis, the seedlings (5 days old) were pretreated with 10^{-4} M MGBG for 16 hr and then transferred to the solution containing radioactive putrescine with 10^{-4} M MGBG The absorption ratios of putrescine from the solution were 1 8% (1 hr) and 3 0% (3 hr) Spermidine which formed in the MGBG-treated axes was 0 4% of putrescine absorbed for 1 hr After 3 hr, the spermidine synthesized was only 12% of that found in the untreated roots In a previous paper we showed that maize SAM decarboxylase is inhibited by MGBG [10] The extract of maize seedlings grown in the culture solution containing MGBG had no SAM decarboxylase activity [unpublished data]

Purification of maize spermidine synthetase

A 115-fold purification was achieved using the procedures given in the Experimental (Table 2) Enzyme activity in the crude extract fraction could not be detected

Table 2 Purification of maize spermidine synthetase

Step Fraction	Total vol (ml)	Total activity (units)	Protein (mg/ml)	Sp act (units/mg)	Recovery (%)
1 Crude extract*	700	305	2 80	0 156	100
2 Ultracentrifugation	117	—	7 75	—	—
3 DEAE-Sephacel	7 5	447	3 35	17 8	147

*The enzyme activity in this fraction was estimated after passing through a CM-Sephadex column

due to the presence of polyamine oxidase When the crude extract was passed through a CM-Sephadex column, the enzyme activity was detectable, but spermidine synthetase could be completely separated from polyamine oxidase by a DEAE-Sephacel column (Fig 1)

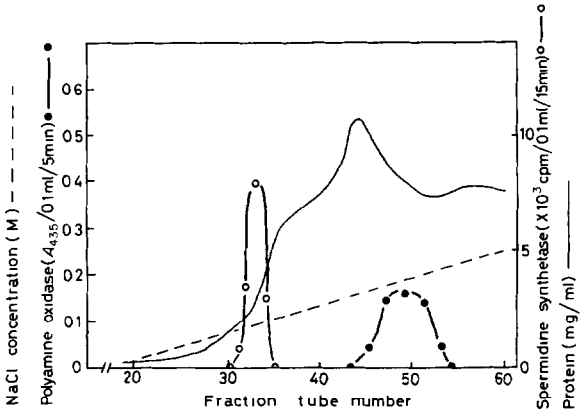

Fig 1 DEAE-Sephacel column chromatography of maize spermidine synthetase and polyamine oxidase

Substrate specificity

No detectable activity for the spermine synthetase was found in the crude extract passed through the CM-Sephadex column or the DEAE-Sephacel fraction, using [^{14}C]spermidine in the assay system (see Experimental) When SAM was used as aminopropyl donor, spermidine synthetase activity was less than 20% of that using decarboxylated SAM Aminopropyltransferase from *E coli* could use putrescine, spermidine and cadaverine as aminopropyl acceptor [4] It seems likely that spermidine synthetase in maize seedlings is similar to that of the animal enzyme which is specific for putrescine Although spermine synthetase could not be detected in the maize extracts, this may be caused by the presence of minor contamination by polyamine oxidase [16]

Other properties

The enzyme had a pH optimum of 7 2, using Tris–malate buffer (pH 6 0–8 5) The enzyme was stable near pH 7 5–8 0 (Tris–malate buffer), and this value was near that of rat (*ca* 8) [17] rather than that of *E coli* [4] However, the enzyme completely lost the activity in 16 hr at 2° in the same pH range

The apparent MW of spermidine synthetase from maize seedlings, estimated by Sephadex G-200 gel filtration, was 43 000 This value is closer to that of rat liver (*ca* 50 000) [5] rather than that of *E coli* (73 000) [4] The enzyme was inhibited by *p*-hydroxymercuriphenylsulfonate and *N*-ethylmaleimide (Table 3) in a manner similar to that of the enzyme from *E coli* [4]

Table 3 Effects of thiol reagents on maize spermidine synthetase

Compounds	Concn (mM)	Relative activity
Control	—	100
p-Hydroxymercuriphenylsulfonate	0 1	10
	1 0	0
N-Ethylmaleimide	0 1	70
	1 0	35

EXPERIMENTAL

Plant material Seeds of maize (*Zea mays* L cv Goldencross Bantam) were germinated in moist vermiculite at 25° for 2 days in the dark The seedlings were then transferred to glass trays containing H_2O They were grown at 25° for 3 days in the dark and used for tracer expts while the etiolated seedlings were grown in moist vermiculite at 25° for 7 days in the dark The shoots were used for purification of spermidine synthetase

Tracer experiments 5-Day-old seedlings were transferred to a glass tray containing 43 nM radioactive putrescine ([1, 4-^{14}C], sp act 102 mCi/mmol) After 1 or 3 hr, the axes (8 g fr wt) free from endosperm were washed with 0 01 M HCl and ground in a mortar with 5% TCA (80 ml) The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10 000 *g* for 15 min The supernatant was applied to a column (0 7 × 4 cm) of Dowex 50 W-H$^+$ (mesh 100–200) and the column was washed with deionized H_2O (50 ml) The fractions eluted with 1 8 M HCl (8 ml) and then with 4 M HCl (8 ml) from the column were evaporated to dryness at 50° The residues were dissolved in 0 01 M HCl and separated by PC

PC Polyamines were separated by ascending PC, with methyl cellosolve–PrOH–H_2O (14 3 3) [18] R_fs spermine, 0 15, spermidine, 0 30, and putrescine, 0 50

Purification of enzyme All operations were performed at 4° The shoots (300 g) which were sterilized for 5 min in 0 1% benzalkonium chloride soln were blended with 0 05 M Tris–HCl buffer (pH 8) containing 1 mM EDTA and 10 mM 2-mercapto-

ethanol (2ME) (600 ml) The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10 000 *g* for 15 min The supernatant was pptated with solid $(NH_4)_2SO_4$ to 80% satn and the ppt was collected by centrifugation at 10 000 *g* for 15 min The pellet was dissolved in the grinding buffer (100 ml) and the resulting soln was dialysed overnight against 10 mM Tris–HCl buffer (pH 8) containing 0 1 mM EDTA and 10 mM 2ME The dialysate was ultracentrifuged for 1 hr at 100 000 *g*, and the supernatant was applied to a column (1 1 × 25 cm) of DEAE-Sephacel previously equilibrated with 10 mM Tris–HCl buffer (pH 8) containing 0 1 mM EDTA and 10 mM 2ME The column was washed with the same buffer, and then the enzyme was eluted with 150 ml of a linear gradient of from 0 to 1 M NaCl made up in the equilibration buffer (flow rate, 9 ml/hr) Active fractions were pooled (Fig 1) and used as 'spermidine synthetase'

Determination of enzyme activity The labelled spermidine was estimated The total vol of the reaction mixture was 1 ml The assay system was as follows 0 4 ml 0 5 M Tris–malate buffer (pH 7 2), 0 1 ml 1 μCi/ml [1,4-^{14}C]putrescine (sp act 102 mCi/mmol), 0 1 ml 1 6 μM decarboxylated SAM, 0 1 ml 1 mM dithiothreitol (DTT) and 0 1 ml enzyme soln The mixture was incubated at 30° for 15 min, with shaking, and the reaction was stopped by the addition of 0 1 ml 50% TCA The labelled spermidine formed in the mixture was separated by a modification of the method in ref [14] as follows After centrifugation, the supernatant was applied to a small column (0 7 × 4 cm) of Dowex 50 W–H$^+$ (100–200 mesh) The column was washed with 10 ml H_2O followed by 25 ml 1 8 M HCl to remove putrescine and then it was washed with 4 ml 2 8 M HCl for the separation of spermidine This fraction was collected and mixed with 1 ml 20% NaOH The mixture was added to 10 ml scintillation fluid (Scintisol EX-H from Wako Pure Chemicals) and counted The recovery of authentic spermidine from the column was *ca* 92% Enzyme activity in the crude extract was estimated by the following procedures The filtrate of the homogenate was ultracentrifuged for 1 hr at 100 000 *g* The supernatant was applied to a column (1 1 × 5 cm) of CM-Sephadex previously equilibrated with 50 mM Tris–malate buffer (pH 6 8) containing 0 1 mM EDTA and 10 mM 2ME The eluate with the same buffer was used as the enzyme source of the crude extract fraction 1 unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 dpm of [^{14}C]spermidine per sec under the above assay conditions Determination of polyamine oxidase was given in ref [19] using a method similar to that described in ref [20]

Decarboxylated SAM Prepared from SAM with the aid of the enzyme from *Saccharomyces cerevisiae* SAM decarboxylase was purified by a modification of the method in ref [21] as follows The yeast cells (*ca* 200 g) suspended in 25 mM Tris–HCl buffer (pH 7 5) (100 ml) containing 0 1 mM EDTA and 1 mM DTT were disrupted with glass beads (0 3 mm) in a vibration homogenizer (Buhler) Suspensions of broken cells were centrifuged at 10 000 *g* for 15 min The supernatant was added slowly to 10% (v/v) streptomycin sulfate (40 ml) It was left for 30 min and centrifuged at 10 000 *g* for 15 min The supernatant was further fractionated with solid $(NH_4)_2SO_4$, and the fraction obtained between 40 and 60% satn was collected by centrifugation for 15 min at 10 000 *g* The ppt dissolved in the homogenizing buffer (50 ml) was dialysed for 16 hr against the same buffer The dialysate was applied to a MGBG-Sepharose 4B column (1 1 × 25 cm) previously equilibrated with homogenizing buffer The column was washed with the same buffer (200 ml) followed by 100 ml 0 5 M NaCl in the buffer, and then active fractions were eluted with the buffer containing 0 3 M NaCl and 1 mM MGBG They were concd in a collodion-bag and then dialysed against 25 mM Tris–HCl buffer (pH 7 5) containing 0 1 mM EDTA, 1 mM DTT and 0 1 mM putrescine The dialysed enzyme was used for decarboxylation of SAM The reaction mixture contained, in a total vol of 2 ml, 200 μmol NaPi buffer, pH 7 2, 2 μmol DTT, 2 μmol putrescine, 20 μmol SAM and the enzyme soln After incubation for 3 hr at 37°, the mixture was deproteinized by the addition of 0 2 ml 50% TCA followed by centrifugation The supernatant was applied to a column (0 7 × 4 cm) of Dowex 50 W-H$^+$ (100–200 mesh) and the decarboxylated SAM was eluted with 4 M HCl (8 ml) after washing with 1 8 M HCl (20 ml) to remove putrescine Decarboxylated SAM in the 4 M HCl fraction was further purified as follows according to ref [22] The remaining SAM and decarboxylated SAM were separated by paper electrophoresis (380 V, 75 min, in 0 1 M NaOAc, pH 3) After electrophoresis the paper strip was gently dried and the fractions visualized with a UV lamp The decarboxylated SAM fraction (migrating faster than SAM) was cut out and eluted with 20 ml 1 M HCl for 15 min The eluate, after filtration, was then evaporated to dryness under red pres at 50° and the residue dissolved in a small vol of 0 01 M HCl and stored at −20° The concn of decarboxylated SAM was estimated by A_{260}

The MW of the enzyme was determined with a molecular sieve according to ref [23] The column was equilibrated by the ascending method with 10 mM Tris–HCl buffer (pH 8) containing 0 1 mM EDTA and 0 1 M NaCl Cytochrome *c* (MW = 13 000), trypsin inhibitor from egg white (MW = 28 000), BSA (MW = 68 000) and γ-globulin (MW = 140 000) were used to calibrate the Sephadex G-200 column Protein content was determined according to ref [24]

Acknowledgements—This work was supported in part by a Grant-in Aid for Co-operative Research (434034) from the Ministry of Education, Science and Culture, Japan

REFERENCES

1 Smith, T A (1970) *Phytochemistry* **9**, 1479
2 Bachrach, U (1973) *Function of Naturally Occurring Polyamines* Academic Press, New York
3 Wickner, R B, Tabor, C W and Tabor, H (1970) *J Biol Chem* **245**, 2132
4 Bowman, W H, Tabor, C W and Tabor, H (1973) *J Biol Chem* **248**, 2480
5 Hannonen, P, Janne, J and Raina, A (1972) *Biochem Biophys Res Commun* **46**, 341
6 Pajula, R-L, Raina, A and Eloranta, T (1979) *Eur J Biochem* **101**, 619
7 Coppoc, G L, Kallio, P and Williams-Ashman, H G (1971) *Int J Biochem* **2**, 673
8 Baxter, C and Coscia, C J (1973) *Biochem Biophys Res Commun* **54**, 141
9 Suresh, M R and Adiga, P R (1977) *Eur J Biochem* **79**, 511
10 Suzuki, Y and Hirasawa, E (1980) *Plant Physiol* **66**, 1091
11 Cohen, S S, Balint, R and Sindhu, R K (1981) *Plant Physiol* **68**, 1150
12 Smith, T A (1970) *Biochem Biophys Res Commun* **41**, 1452
13 Tabor, H, Rosenthal, S M and Tabor, C W (1958) *J Biol Chem* **233**, 907
14 Pegg, A E and Williams-Ashman, H G (1969) *J Biol Chem* **244**, 682
15 Smith, T A (1970) *Ann N Y Acad Sci* **171**, 988
16 Suzuki, Y and Yanagisawa, H (1980) *Plant Cell Physiol* **21**, 1085

17 Janne, J, Schenone, A and Williams-Ashman, H G (1971) *Biochem Biophys Res Commun* **42**, 758
18 Herbst, E J, Keister, D L and Weaver, R H (1958) *Arch Biochem Biophys* **15**, 178
19 Suzuki, Y and Hirasawa, E (1973) *Phytochemistry* **12**, 2863
20 Holmstedt, B, Larsson, L and Tham, R (1961) *Biochim Biophys Acta* **48**, 182
21 Pöso, H, Sinervirta, R and Janne, J (1975) *Biochem J* **151**, 67
22 Janne, J, Williams-Ashman, H G and Schenone, A (1971) *Biochem Biophys Res Commun* **43**, 1362
23 Andrews, P (1970) *Methods Biochem Anal* **18**, 1
24 Lowry, O H, Rosebrough, N J, Farr, A L and Randall, R J (1951) *J Biol Chem* **193**, 265