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Purification and Characterization of Soybean Cotyledonary Spermidine Dehydrogenase

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Abstract: Decrease in the amount of cotyledonary spermidine in *Glycine max* under anaerobic conditions related to an increase in spermidine dehydrogenase. Under the same conditions, no enzymatic activity of diamine oxidase was observed. Exposure of *Glycine max* both to spermidine and 1,3-diaminopropane under anaerobic conditions resulted in a decrease in spermidine contents. Correlated with the decrease in spermidine contents, there was a drastic increase in spermidine dehydrogenase. The molecular weight of the purified enzyme estimated by Sephacryl S-300 gel column and SDS gel electrophoresis were 130,000 dalton and 65,000 dalton, respectively, indicating that the enzyme is a dimer. The optimal pH for activity was 9.3. The K_m value for spermidine was 0.61 mM. Neither metal ions nor polyamine and derivatives affected enzymatic activity, but the enzyme was inhibited by DTNB, NEM and PCMB, suggesting that a cysteine residue of the enzyme is associated with or involved in enzyme activity. To our knowledge, this is the first report describing properties of the enzyme from plants. Considered together, the data in this paper indicate that both spermidine and 1,3-diaminopropane, novel activators, enhance the spermidine dehydrogenase activity and control the intracellular spermidine contents.

Key words: anoxia, 1,3-diaminopropane, *Glycine max*, spermidine, spermidine dehydrogenase.

Spermidine, one of the polyamines, can be degraded by the action of diamine oxidase (EC 1.4.3.6) to Δ-pyrroline and 1,3-diaminopropane under aerobic conditions. It can also be catabolized by spermidine dehyrogenase to Δ-pyrroline and 1,3-diaminopropane under aerobic conditions. One of the products, 1,3-diaminopropane, has been known to act as a potent inhibitor of many enzymes, such as spermidine synthase, spermine synthase and ornithine decarboxylase (Kim *et al.*, 1987), which are responsible for the biosynthesis of polyamines. Besides such functions, 1,3-diaminopropane could be a precursor of norspermidine and norspermine which are known to be critically needed for the survival of halophilic and thermophilic bacteria (Benzamin *et al.*, 1988).

Both enzymatic activities are critical in spermidine metabolism, since enzymes can reduce the level of spermidine, which in turn may affect the concentration of spermine. Spermidine dehydrogenase has been purified and characterized from microorganisms (Hisano *et al.*, 1990). To date, the purified preparation of spermidine dehydrogenase from *Serratia marcescens* (Tabor and Kellogg, 1970) and *Pseudomonas aeruginosa* (Hisano *et al.*, 1990) are the best described. However, little information is available concerning the regulation of the enzyme by endogenous metabolites, which we have reported in the case of arginine decarboxylase (Park and Cho, 1992), SAM decarboxylase I and II (Yang and Cho, 1991; Choi and Cho, 1994). To the best of our knowledge, the regulation of spermidine dehydrogenase by anaerobic conditions has not been reported. However, we have concrete evidence of the presence of spermidine dehydrogenase and the absence of diamine oxidase under anaerobic conditions. Such preliminary findings prompted us to study the enzymes and endogenous metabolites related to enzymatic activities. In this study we report purification and characterization of spermidine dehyrogenase from soybean, which has not been previously reported. Most importantly, the data presented here also show that spermidine, 1,3-diaminepropane can elevate spermidine dehydrogenase activity significantly under anaerobic conditions, suggesting that it could be a novel mechanism by which polyamine biosynthesis is regulated intracellularly.

Materials and Methods

Materials and environmental conditions

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Soybean (*Glycine max*) seeds were imbibed as described by Kang and Cho (1990). This stage was taken as day 0 of germination. The seeds were then spread on a net, hung 0.5 cm above the surface of water in a jar, and allowed to germinate in the dark at room temperature. All chemicals were of analytical grade and were purchased from Sigma Chemical Co (St. Louis, USA). or from Bio-Rad (Richmond, USA). Anaerobic conditions were obtained by flushing nitrogen gas (99.999% nitrogen) through the jar (Reggiani *et al.*, 1988).

Enzyme purification

All purification steps were conducted at 4℃. About 200 g of soybean cotyledons treated under anaerobic conditions in the presence of 1 μM spermidine were homogenized in 2 vol. of 50 mM Tris buffer (pH 8.5), containing 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. The homogenate was centrifuged at 10,000×*g* for 30 min, and a clear supernatant was obtained. All procedures were carried out at 0~4℃. Buffers used for enzyme purification were as follows. Buffer A: 20 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Buffer B: 5 mM potassium phosphate buffer (pH 8.0) containing 0.5 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Buffer C: 20 mM Tris buffer (pH 8.5) containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. Crude enzyme was fractionated with 20~30% $(NH_4)_2SO_4$. The suspension was centrifuged at 10,000×*g* for 30 min, the pellets were dissolved in buffer A and the solution was dialyzed against buffer A. The enzyme solution was fractionated with 20~30% acetone. The suspension was centrifuged at 10,000×*g* for 30 min, the pellets were dissolved in buffer A and the solution was flushed with nitrogen gas. The enzyme solution was loaded on a DEAE-Sepharose ion exchange column (4×15 cm) preequilbrated with buffer A. The column was washed with the same buffer. spermidine dehydrogenase was eluted with a linear gradient of 0~0.5 M KCl and the fractions containing spermidine dehydrogenase activity were pooled together, and the pooled fractions were dialyzed against buffer B. The dialysed sample was applied on a Hydroxyapatite column (4×15 cm) preequilbrated with buffer B and washed with the same buffer. Elution of the enzyme was achieved with a linear gradient of 5 mM to 1 M potassium phosphate, and the active fractions were pooled and then dialyzed against buffer C. The dialyzed enzyme was applied to an 1,8-diaminooctyl Agarose column (2×8 cm) preequilbrated with buffer C and washed with the same buffer. The bound enzyme was eluted with a linear gradient of 0~0.5 M NaCl. Active fractions were pooled and then concentrated. The purified enzyme solution was collected and used for all other studies.

Enzyme assays

During purification, spermidine dehydrogenase activity was assayed in a solution containing a 0.1 M Tris buffer (pH 8.5), 1 mM potassium ferricyanide (KFeCN), 0.5 mM spermidine (Tabor and Kellogg, 1970). All assays were performed spectrophotometrically at 400 nm. Alcohol dehydrogenase activity was determined spectrophotometrically at 340 nm (Sachs *et al.*, 1990). The reaction mixture was 0.15 M Tris buffer (pH 8.0), 0.4 mg NAD^+, 40 μl of 50% ethanol and enzyme solution (Russel *et al.*, 1990). Diamine oxidase activity was assayed as previously described (Kang and Cho, 1990). All values of enzyme activity are the means of five replicates.

Electrophoresis and molecular mass determination

The molecular weights of native enzymes were determined by the Sephacryl S-300 gel filtration. The molecular weight of subunits was obtained by the procedure of Laemmli (1970).

Miscellaneous techniques

Protein concentration was determined as described by Lowry with bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951), or spectrophotometrically at 280 nm. Polyamine concentration was determined as described by Galston and Flores (1982). All values of protein and polyamine contents are the means of five replicates. Chemical modifications of the spermidine dehydrogenase by modification reagents such as p-chloromercurybenzoate (PMSF), 5,5-dithiobis-(2-nitrobenzoate) (DTNB), N-ethylmaleimide (NEM), p-chloromercurybenzoate (PCMB), pyridoxal-5-phosphate (PLP), and phenylglyoxal (PhxG) were carried out as described (Lee and Cho, 1993). Substrate protection was also carried out (Lee and Cho, 1993). Effect of putrescine, agmatine, cadaverine, diaminopropane, and spermidine on the spermidine dehydrogenase were carried out as previously described (Kang and Cho, 1990; Yang and Cho, 1991).

Results and Discussion

Change in spermidine dehydrogenase activities by spermidine and 1,3-diaminopropane

Glycine max seeds were imbibed for 24 h and then exposed to anaerobic conditions for 6, 12 and 24 h as described (Reggiani *et al.*, 1988). Under aerobic conditions, cotyledonary alcohol dehydrogenase activity tended to decrease steadily for 24 h and reach a pla-

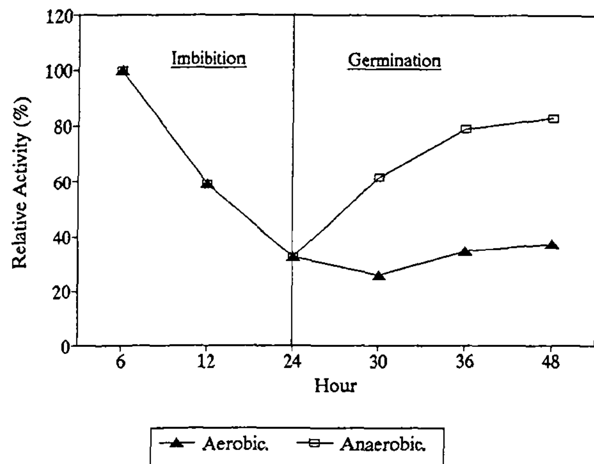

Fig. 1. Induction of cotyledonary alcohol dehydrogenase in *Glycine max* imbibed for 24 h in distilled water and then exposed to anaerobic state for 6, 12, and 24 h.

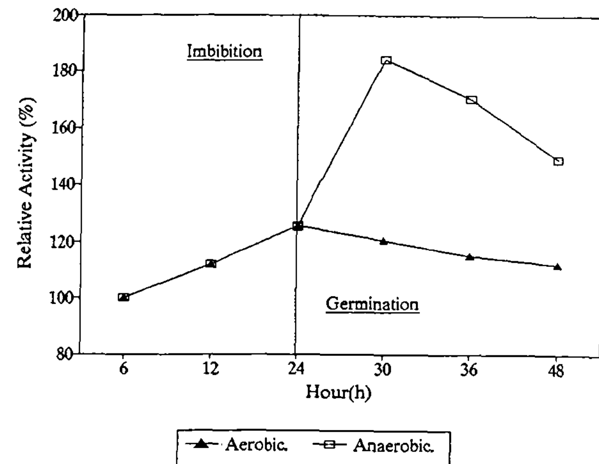

Fig. 3. The anoxic induction of cotyledonary spermidine dehydrogenase in *Glycine max* seed imbibed for 24 h in distilled water and then exposed to anaerobic state for 6, 12, and 24 h.

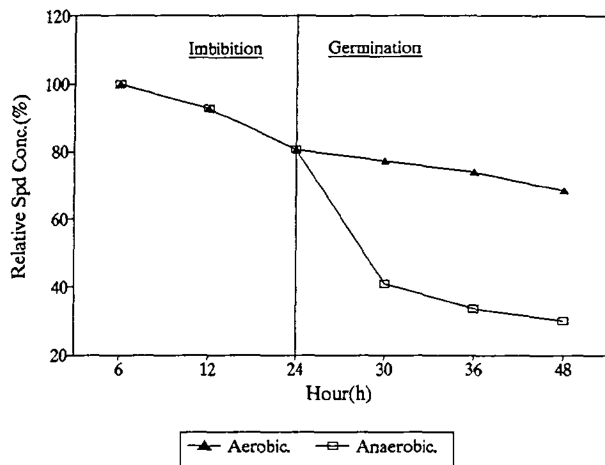

Fig. 2. Decrease in cotyledonary spermidine contents in *Glycine max* imbibed for 24 h in distilled water and then exposed to anaerobic state for 6, 12 and 24 h.

teau (Fig. 1). But the enzymatic activity was observed to increase steadily under anaerobic conditions. Such increases in enzymatic activities under anaerobic conditions were also reported in roots, and shoots of seedlings of *Zea mays* (Andrew *et al.*, 1993). Under the same conditions in the case of alcohol dehydrogenase of soybean, the spermidine content was slightly decreased under aerobic conditions whereas under anaerobic conditions it was significantly reduced (Fig. 2). Such drastic decrease in spermidine contents could be due either to the conversion of spermidine to spermine or by the action of diamine oxidase and spermidine dehydrogenase. The decrease in spermidine contents, however, seems to be not linked to conversion from spermidine to spermine since the spermine contents were almost constant. Diamine oxidase has been purified and characterized from soybean by the authors (Kang and Cho, 1990). Putrescine, spermidine and spermine were observed to be attacked by diamine oxidase (Kang and Cho, 1990). In addition, the amount of the enzyme on the basis of protein was relatively larger than that the enzyme responsible for polyamine metabolism under aerobic conditions (Kang and Cho, 1990). But in the case of anaerobic conditions, we could not detect any enzymatic actitivity at all. Instead, spermidine dehydrogenase activity, responsible for catabolizing spermidine, was observed to increase drastically under anaerobic conditions compared to that under aerobic conditions (Fig. 3). These cumulative results as the lack of relationship between the amounts of spermidine and spermine, the absence of diamine oxidase, and the increase in spermidine dehydrogenase activity strongly suggests that the drastic decrease in spermidine contents might be due to the elevation of the spermidine dehyrogenase activity under anaerobic conditions. For years we have tried to find out which endogenous regulatory substances can elevate or reduce the activity of enzymes responsible for polyamine biosynthesis and degradation. One of them is agmatine, which inhibits SAMDC I (Yang and Cho, 1991), SAMDC II (Choi and Cho, 1994), and ADC (Park and Cho, 1992). In connection with such studies, we have made attempts to find out which intracellular substances can have an effect on spermidine dehydrogenase. Spermidine, the substrate for spermidine dehydrogenase, turned out to elevate enzymatic activity significantly under anaerobic conditions. Also, it could elevate enzymatic activity under aerobic conditions but less than under anaerobic conditions, which showed activity slightly higher than that of the control. Such increase in enzymatic activity is related to the decrease in the spermidine applied

Table 1. Effects of spermidine and 1,3-diaminopropane on cotyledonary spermidine dehydrogenase activity and spermidine contents

		Relative[a] spermidine dehydrogenase activity (%)						Relative[b] spermidine contents (%)					
		Imbibition			Germination			Imbibition			Germination		
Time (h)		6	12	24	30	36	48	6	12	24	30	36	48
Spermidine[c]	aerobic	100	106	110	119	120	124	100	93	90	84	82	79
	anaerobic	100	106	110	180	193	202	100	93	90	47	44	42
1,3-diaminopropane[d]	aerobic	100	97	97	90	83	73	100	101	102	107	117	126
	anaerobic	100	97	97	96	91	87	100	101	102	103	106	113

[a]Relative spermidine dehydrogenase activity at various times were calculated by taking spermidine dehydrogenase activity of 6 h imbibition as 100%.
[b]Relative spermidine contents at various times were calculated by taking spermidine contents of 6 h imbibition as 100%.
[c]Same conditions as Fig. 1 except for distilled water containing 1 μM spermidine.
[d]Same conditions as Fig. 1 except for distilled water containing 1 μM 1,3-diaminopropane.

Table 2. Purification of spermidine dehydrogenase from *Glycine max* cotyledons under anoxia in the presence of spermidine

Fraction	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude	3647	28800	7.9	100	1
$(NH_4)_2SO_4$ (20~30%)	248	5000	20	17	2.5
Acetone	12	1400	112	5	14.2
DEAE-Sepharose	6.3	1200	190	4	24.1
Hydroxylapatite	5	1000	200	3	25.3
1,8-diaminooctyl Agarose	0.8	800	1000	2.7	127

[a]1 U is defined as 0.01 increase in absorbance density at 400 nm following 10 min incubation at 50℃.

(Table 1). One product, 1,3-diaminopropane, which was produced by the action of spermidine dehyrodenase, also turned out to be a good regulator. It increased the spermidine dehydrogenase activity both under aerobic and anaerobic conditions. The effect of the 1,3-diaminopropane under anaerobic conditions is greater than that under aerobic conditions. The increase in enzymatic activity was coupled with an increase in spermidine contents. And the amounts of the spermidine in cotyledon under anaerobic is less than that under aerobic conditions and depends on the amount of 1,3-diaminopropane applied (Table 1). Especially, under anaerobic conditions, a drastic increase in spermidine dehyrogenase activity by spermidine and 1,3-diaminopropane have not been reported in plants. Such regulation of the spermidine dehydrogenase activity might be a novel mechanism by which polyamine metabolism is controlled intracellularly. It, however, is not clear how exogenously added spermidine, 1,3-diaminopropane and anoxia can effect spermidine dehydrogenase activity. Some preliminary results have failed to clarify the accessibility of antibiotics such as cycloheximide to the inside of the cotyledon, since the effects of antibiotics on enzymeric activity were not reproducible (unpublished data). Therefore, parallel experiments using different approaches to clarify the response of the enzymatic activity to anoxia, spermidine and diaminopropane are needed.

Purification of spermidine dehydrogenase

Aside from the properties of the spermidine dehyrogenase described above, other properties of the enzyme have not been known, since no one has purified and characterized the enzyme from plants. For further characterization, we made attempts to purify the enzyme from soybean. The results are summarized in Table 2. Because the method employed for *serratia* marcescens by Tabor and Kellogg (1970) was not suitable for the purification of soybean enzyme, we modified the procedure. We used 1,8-diaminooctyl agarose and DEAE-Sepharose instead of Sephadex G-150 step. Spermidine dehydrogenase was fractionated in a range of 20 to 30% ammonium sulfate. After dialysis, the enzyme solution was fractionated with 20~30% ace-

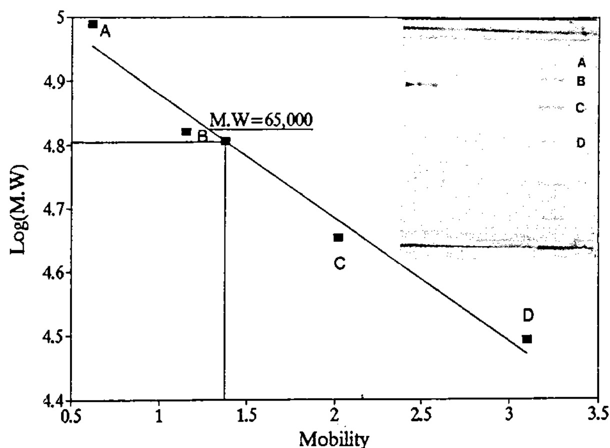

Fig. 4. Determination of subunit molecular weight of the purified spermidine dehydrogenase was estimated by SDS gel electrophoresis. A: Phosphorylase b (97.4 kDa); B: Bovine serum albumin (66.2 kDa); C: Ovabumin (45kDa); D: Carbonic anhydrase (31 kDa).

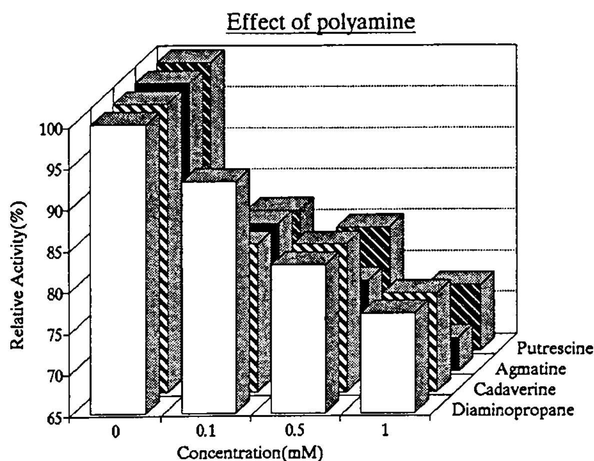

Fig. 5. Effect of polyamine on the purified spermidine dehydrogenase.

tone. The enzyme solution was applied to a DEAE-Sepharose column, and the enzyme was eluted in a range of 0 to 0.5 M KCl. The enzyme was bound to the hydroxyapatite column and eluted in a range of 0.005 to 1 M phosphate. The hydroxyapatite enzyme pool was applied to a 1,8-diaminooctyl agarose column, and the enzyme was eluted in a range of 0 to 0.5 M NaCl. During the purification, we could not find any evidence of the prescence of an isozyme. The protocol developed resulted in a 127-fold purification of spermidine dehydrogenase.

Electrophoretic properties

The molecular weight of the purified enzyme was determined to be 130,000 by Sephacryl S-300 gel filtration. And SDS gel electrophoresis showed a single band indicating that the subunit molecular weight of

Table 3. Effect of several modification reagents on spermidine dehydrogenase

Putative modified groups	Modifiers[a]	Conc.	Relative activity (%)
Ser	DFP	100 μM	92
		1 mM	90
−OH	PMSF	500 μM	93
		1 mM	90
−SH	DTNB	100 μM	66
		1 mM	20
	NEM	100 μM	80
		1 mM	24
	PCMB	100 μM	87
		1 mM	43
Lys	PLP	100 μM	91
		1 mM	81
Arg	PhGx	100 μM	90
		1 mM	86

[a]Modification reaction were initiated by adding the reagent to the enzyme solution and then incubated for 10 min at 25℃.

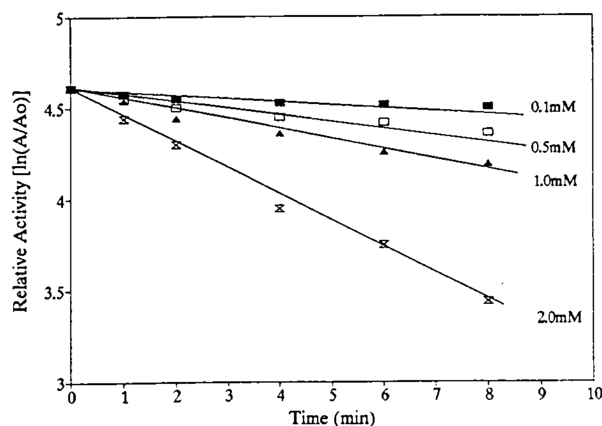

Fig. 6. Semilogarithmic plot for the inactivation of the spermidine dehydrogenase activity by NEM. The enzyme (0.2 μg) was incubated with various concentrations of the NEM in 20 mM Tris-HCl buffer (pH 7.4) at 25℃. Ao: original enzyme activity; A: enzyme activity at time indicated.

the enzyme was 65,000 (Fig. 4). These data suggest that the enzyme is a homodimeric enzyme. In the cases of both *Pseudomonas aeruginosa* and *Citrobacter freundii*, the enzyme was a monomer of mass 63,000 dalton (Hisano *et al.*, 1990).

Kinetics and chemical modification

Soybean spermidine dehydrogenase obeyed typical Michaelis-Menten kinetics, and its K_m value was 0.61 mM for spermidine, which is larger than that of observed in pseudomonas aeruginosa (0.037 mM) (Hisano *et al.*, 1990). Enzyme activity was evaluated from pH

6.5 to 10.1 using the buffer system: potassium phosphate (pH 6.5~7.7), Tris-HCl (pH 7.5~9.7), and Glycine-NaOH (pH 9.3~10.1). The optimum pH turned out to be 9.3. The effect of metal ions and polyamine analogues such as putrescine, agmatine, cadaverine, and 1,3-diaminopropane on enzyme activity were innocuous (Fig. 5). Inactivation of spermidine dehydrogenase by modification reagents are shown in Table 3. Fig. 6 shows the time course for inactivaton of the enzyme incubated with NEM. Loss of activity followed pseudo first order kinetics. But the substrate provided protection against inactivation by NEM as expected (data not show). The second order rate constant for inactivaton was 14 $M^{-1} \cdot min^{-1}$, determined from the rate of inactivation at 0.1, 0.5, 1 and 2 mM. When the data are plotted as indicated above, the slope of n=0.77 suggests that inactivation is the result of the reaction of one sulfhydryl group in active site or near active site of the enzyme.

Acknowledgement

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