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Polyamine synthesis in plants. Purification and properties of amidinotransferase from soybean (*Glycine max*) axes

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Abstract

Three-day-old soybean (*Glycine max*) seedlings were exposed to 0.4 M sorbitol solution for 4 h to induce amidinotransferase activity, with the corresponding enzyme being purified to homogeneity by chromatographic separation on DEAE-Sephacel, Sephacryl S-300 and L-arginine Sepharose 4B. The purified enzyme used L-arginine and L-glycine as the major donor/acceptor of the amidino group, respectively, with formation of guanidinoacetic acid and ornithine products being confirmed by ESI-MS. The enzyme is a tetrameric protein having a molecular mass of 240,000 Da, whose thiol group is needed for enzymatic activity. The K_Ms for arginine and glycine were 3.8 and 0.89 mM, respectively, with optimal temperature and pH being 37 °C and 9.5, respectively. The soybean amidinotransferase could be indirectly involved in nitrogen metabolism, as suggested by the observation that arginine:glycine amidinotransferase in soybean axes is indirectly involved in putrescine biosynthesis and displays feedback control at high levels of an endogenous regulator, putrescine.

Keywords: Glycine max; Soybean; Leguminosae; Amidinotransferase; Guanidinoacetic acid; Polyamine

1. Introduction

Amidinotransferase (AT, EC 2.1.4.1) activity was first reported independently in mammalian kidney tissue by Borsook and Dubnoff (1941) and Bloch and Sehoenheimer (1941). It catalyzes transfer of the amidine group from donors such as L-arginine, guanidinoacetic acid (GAA), L-canavan (Walker, 1956) and homoarginine (Ratner and Rochovansky, 1956) to acceptors such as glycine, L-ornithine, L-canaline, γ-aminobutyric acid, β-alanine (Pisano et al., 1957), and hydroxylamine (Walker, 1958). The molecular and regulatory aspects of AT have been extensively investigated in relation to guanidine compounds, which are involved primarily in creatine metabolism (Watanabe et al., 1994; Takeda et al., 1992; Gross et al., 1986; McGuire et al., 1980; Van Pilsum et al., 1967; Ungar and Van Pilsum, 1966a,b; Ronca et al., 1966). In contrast to extensive studies on animal ATs, there has been only one paper on a plant AT (Srivenugopal and Adiga, 1980), which reported its partial purification from *Lathyrus sativus* seedlings and its possible roles in homo-arginine metabolism and amine interconversions. Detailed studies on AT from higher plants are therefore lacking.

Plants produce putrescine either directly from ornithine, by the action of ornithine decarboxylase (ODC), or indirectly from arginine through arginine decarboxylase (ADC) (Tiburcio et al., 1990). In the former biosynthetic pathway, ornithine can be derived from arginine by action of either arginase or L-arginine:glycine AT. Current biochemical studies on putrescine biosynthesis include only the evaluation of ADC and ODC activities without any reference to either arginase or L-arginine:glycine AT, both of which can affect levels of arginine and ornithine. Hence the role of AT has been overlooked.

Preliminary studies with crude soybean extracts showed that AT activity in axes of soybean seedlings cultured with sorbitol is higher than that of the control (without sorbitol), and that AT has a very high affinity for L-Arginine Sepharose 4B. Thus, the first purification and characterization of an AT from a plant origin and its possible role in putrescine biosynthesis is described.

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2. Results and discussion

2.1. Sorbitol-treatment and purification

Environmental challenge (osmotic-stress, salinity, hypoxia, etc.) causes a drastic increase in putrescine levels due to induction of ADC (Bouchereau et al., 1999). Attempts were thus made to purify AT from soybean axes exposed to sorbitol. As shown in Fig. 1, AT specific activity reached its highest levels in 3-day-old axes exposed to 0.4 M sorbitol. In soybean seedlings treated with sorbitol, to specific activity of AT increased about 40% (Fig. 2). However, since this increase in specific activity could be due not only to enzyme induction but also to low turnover, further work is thus needed to verify whether AT is induced by sorbitol or if a pre-existing inactive AT is activated. Interestingly, there was no activity in cotyledons, hooks or hypocotyls. Additionally, raising soybean seedlings either under hypoxia or in the presence of sodium chloride failed to increase activity (data not shown). Axes of soybean seedlings treated with sorbitol were therefore used to purify AT.

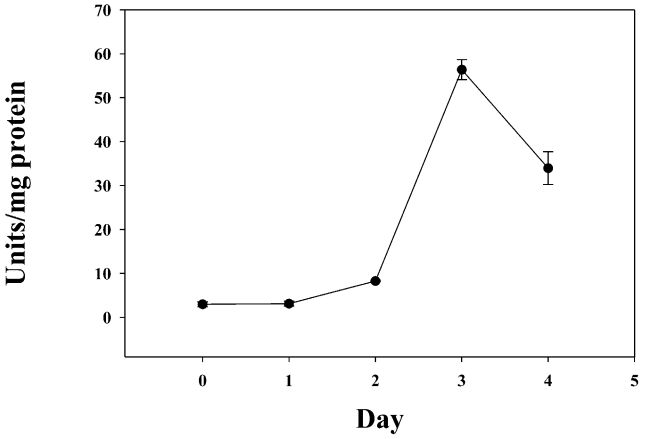

Fig. 1. Changes in amidinotransferase specific activity in soybean axes during seed germination. Soybean axes were collected on successive days and homogenized. Activities were assayed at 37 °C by measuring the formation of ornithine. Specific activities were expressed as kat/mg protein. Vertical bars represent SEs ($n=4$). Only those SEs larger than the symbols are shown.

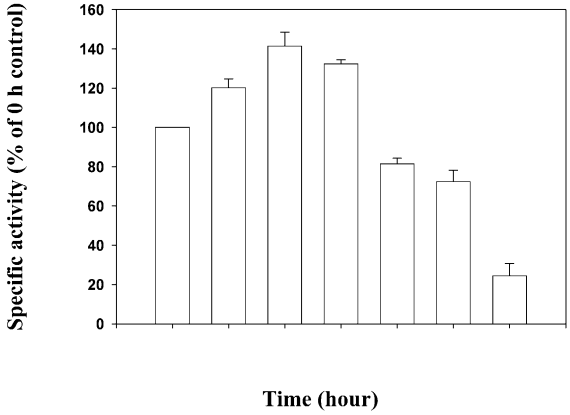

Fig. 2. Effect of sorbitol on amidinotransferase activity. Three day old soybean seedlings (2 g) were exposed to 0.4 M sorbitol for 16 h. Soybean axes were collected on successive hours and homogenized. Activities were assayed at 37 °C by measuring the formation of ornithine. Vertical bars represent SEs ($n=4$).

The method described for AT purification from *Lathyrus sativus* seedling (Srivenugopal and Adiga, 1980) was found to be unsuitable for purifying AT from soybean seedlings to homogeneity (data not shown). Since we have previously succeeded in purifying AT from rat placenta with the aid of L-arginine Sepharose 4B (Lee and Cho, 1993), we have used the same strategy to purify AT from soybean seedling, which has not been attempted before (Gross et al., 1986; Srivenugopal and Adiga, 1980; Conconi and Grazi, 1965). Precipitation by ammonium sulfate, and chromatographic steps using DEAE-Sephacel, Sephacryl S-300 and L-Arginine Sepharose 4B thus were successfully combined to achieve a 450-fold purification (Table 1). Among columns, application of an L-Arginine Sepharose 4B column resulted in a substantial increase in specific activity. This is the first purification to homogeneity and characterization of AT from a plant source, although purification and characterization of ATs from animals have been reported (Conconi and Grazi, 1965; McGuire et al., 1980; Gross et al., 1986; Lee and Cho, 1993).

2.2. Substrate specificity of the purified enzyme

In view of the wide substrate specificity of animal (Conconi and Grazi, 1965) and *Lathyrus sativus* seedlings amidinotransferases (Srivenugopal and Adiga, 1980), a series of radioisotope experiments were carried out to identify the best guanido donors/acceptors among those reported so far: Amidino group donors were arginine and its analogues such as homo-arginine and homo-agmatine. Amidino group acceptors were L-lysine, L-ornithine, L-glycine, cadaverine, putrescine and agmatine. Arginine was the best guanido group donor (Table 3) whereas its analogues, homo-arginine and homo-agmatine showed far less radioactivity ranging toward known guanidine acceptors (data not

Table 1
Purification of amidinotransferase from soybean (*Glycine max*) axes

Step	Total protein (mg)	Total activity (nkatal)	Specific activity (nkatal/mg)	Purification (fold)	Yield (%)
Crude extract	6594	11.011	1.67	1	100
35–60% $(NH_4)_2SO_4$	895	4.78	5.341	3.19	43.42
DEAE-Sephacel	117	1.23	10.54	6.31	11.19
Sephacryl S-300	15	0.471	31.42	18.82	4.28
L-Arginine Sepharose 4B	0.1	0.075	752.78	450.9	0.68

Prepared from 100 g of 3-day-old soybean axes treated with 0.4 M sorbitol for 4 h.

shown). Among acceptors of guanido group from arginine, glycine is the most efficient (data not shown), which suggests that soybean AT is involved in the synthesis of ornithine and guanido acetic acid (GAA), similar to an animal AT (Gross et al., 1986). The products, ornithine and GAA, were identified by ESI-MS (Eichhorn and Knepper, 2001).

To check if ornithine and GAA were formed from arginine and glycine by the action of soybean AT, the reaction mixture after enzymatic reaction was analyzed by ESI-MS (Fig. 3). The protonated molecular ions $[M+H]^+$ of arginine, glycine, ornithine and GAA were observed as base peaks with m/z 175 (Fig. 3A) for arginine, m/z 76 (Fig. 3B) for glycine, m/z 133 (Fig. 3C) for ornithine and m/z 118 (Fig. 3D) for GAA, respectively. The peak of $[M+H]^+$ ions of ornithine and GAA from the assay mixture (including substrates and products) were observed (Fig. 3E), suggesting the formation of ornithine and GAA from arginine and glycine. However, the peak of $[M+H]^+$ of glycine was not detectable: it is likely that too small an amount of glycine was used, considering its low K_M value (K_M for arginine, 3.8; K_M for glycine, 0.89). Analysis for ornithine (m/z 133) and guanidinoacetic acid (m/z 118) from ESI-MS by high resolution MS revealed formulas for ornithine, $C_5H_{13}N_2O_2$ and for GAA, $C_3H_8N_3O_2$, respectively (data not shown), indicating ornithine and GAA as AT products.

Contrary to our results, plant ATs reportedly did not transfer guanidine group from arginine to glycine (Chinard, 1952). However, it is likely that Chinard used a crude AT preparation containing many enzymes such as ornithine decarboxylase, which removed ornithine formed from glycine and arginine. On the other hand, the partially purified AT from *Lathyrus sativus* seedling was reported to catalyze arginine hydrolysis, but only with a rate as low as 1% that of the AT activity (Srivenugopal and Adiga, 1980). Stimulation of hydrolysis activity by the addition of exogenous Mn^{2+} was also reported (Srivenugopal and Adiga, 1980). In contrast, we could not detect any arginine hydrolysis activity in the purified AT from soybean even in the presence of exogenous Mn^{2+}.

2.3. Effects of group specific reagents

Among several group-specific reagents (Park and Cho, 2000), e.g. 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) for sulfhydryl, 1-ethyl-3-(3-dimethyl) aminopropylcarbodiimide for carboxyl group, pyridoxal-5-phosphate for amino group and phenylglyoxal for guanido group, the modification of a cysteine residue by DTNB resulted in total loss of soybean AT activity (data not shown). Protection by arginine against modification of cysteinyl residues was also observed, suggesting that DTNB

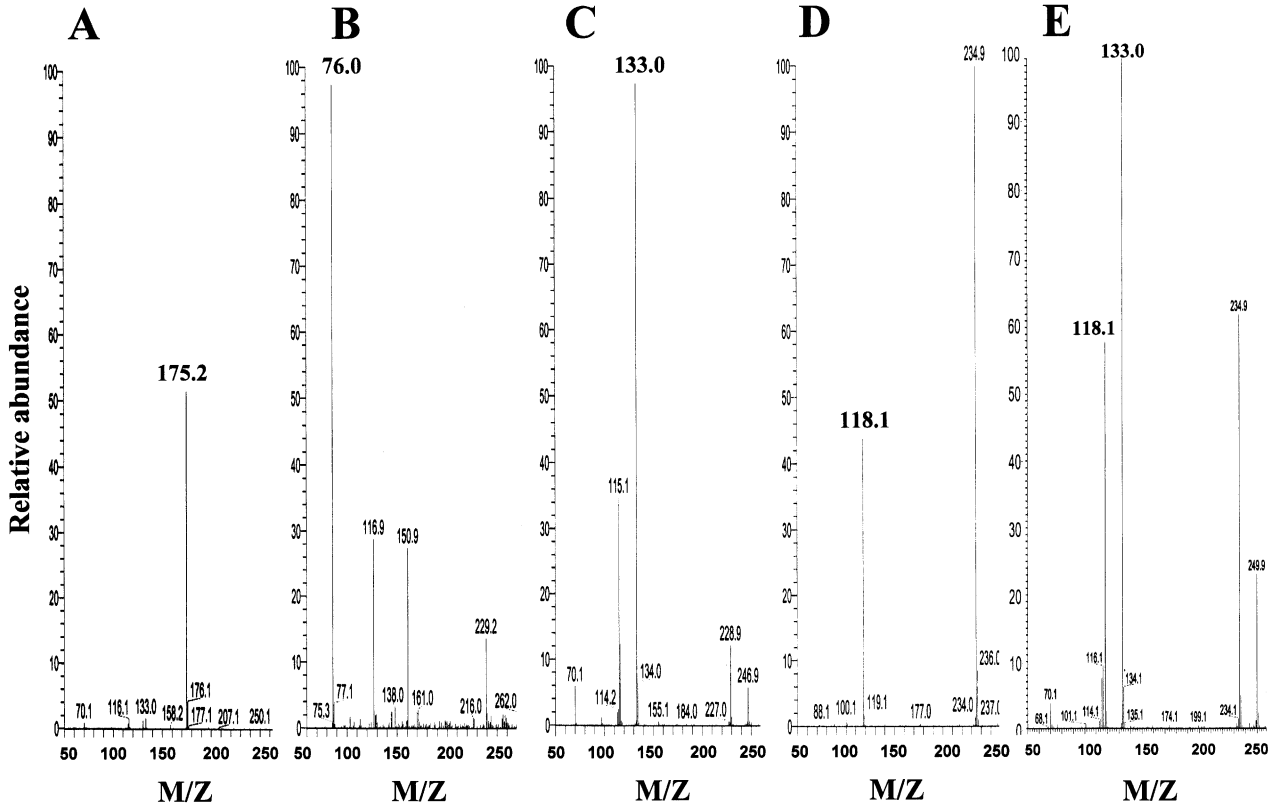

Fig. 3. ESI-MS spectra of arginine (A), glycine (B), ornithine (C), GAA (D) and the reaction mixture (E). A mobile phase of 50% acetonitrile containing 0.1% acetic acid at a flow rate of 20 ul/min.

inactivation of AT can be attributed to modification of an active site Cys residue. Involvement of a cysteine residue in the active site of AT was also observed in AT of *Lathyrus sativus* seedlings. To some extent, glycine protected the amidinotransferase from DTNB inactivation (data not shown). Other group-specific reagents had no effect on AT activity.

2.4. Molecular weight of the purified enzyme

Analysis of AT by SDS-PAGE showed one band of approximately 60,000 Da (Fig. 4B). In addition, silver staining revealed no other polypeptide except AT (chromatogram not shown). Gel filtration through Sephacryl S-300 resulted in the elution of AT activity as a symmetrical peak corresponding to a molecular mass of 240,000 Da (Fig. 4A). These results, taken together with that of SDS-PAGE, indicate that soybean AT is tetrameric. Equilibrium sedimentation analysis has previously indicated that rat (McGuire et al., 1980) and human kidney (Gross et al., 1986) ATs have native molecular masses of 83,000 Da and 89,000 Da, whereas SDS-PAGE gave values of 42,000 and 44,000 Da, respectively. Thus, subunits of animal AT appear to be dimeric with 40 kDa subunits, while soybean and the putative *L. sativus* seedlings AT is a tetramer of 4–60 kDa subunits. To date, there has been no report on higher molecular weights of AT from a plant source other than soybean.

2.5. General properties

To determine the optimal pH for AT, the activity in buffers from pH 6 to 11 was assayed using each buffer system in its effective range. The activity of AT was highest at pH 9.5, which differs from ATs from rat placenta (pH 8.3), human kidney (pH 7.5) and *L. sativus* seedlings (pH 7.5). The optimal temperature for AT activity was 37 °C, which was the same as that of *L. sativus* seedlings. AT is not thermostable, and its Treatment in Buffer B (pH 8.0) at 60 °C for 30 min resulted in complete loss of activity. Freezing and thawing also caused a 25% loss of AT activity in Buffer B, whereas its storage in Buffer B at 0–4 °C for 24 h resulted in a 20% loss of activity. Glycerol, DMSO and DMF in Buffer B were not effective in stabilizing AT. Thus, it is important that AT be used freshly, immediately after purification. The K_M value for L-arginine was determined by Lineweaver-Burk (Fig. 5) and Hanes-Woolf and Eadie-Hofstee plots (data not shown) to be 3.8 mM, which was larger than those reported for rat placenta (1.3 mM), human kidney (2.3 mM) and *L. sativus* seedlings (3.0 mM). The K_M value for glycine was also determined by Lineweaver-Burk plots to be 0.89 mM, which is larger than that of rat placenta (0.19 mM).

2.6. Arginine fate during germination

Both free and protein bound in arginine constitute a large part of the nitrogen reserves in storage tissues of

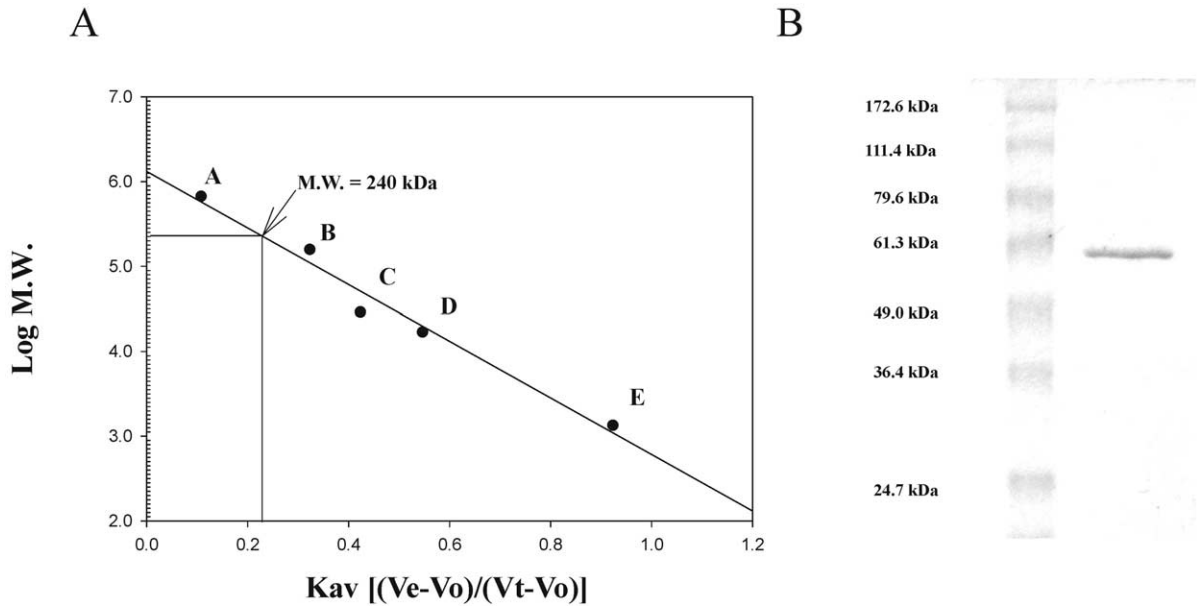

Fig. 4. Molecular weight of purified soybean amidinotransferase by Sephacryl S-300 gel filtration (A) and SDS-polyacrylamide gel electrophoresis (B). (A) Molecular mass standards were filtered individually over a Sephacryl S-300 column, elutions were monitored by absorbance at 280 nm. The elution volume of each standard was used to calculate Kav and a standard molecular weight curve was plotted. Standard proteins are A, thyroglobulin (670,000 Da); B, γ-globulin (158,000 Da); C, ovalbumin (44,000 Da); D, myoglobin (17,000 Da); and E, vitamin B_{12} (1350 Da). (B) Samples of standard solutions and an aliquot of 50 μg of purified amidinotransferase were electrophoresed on 10% SDS-polyacrylamide and stained using Coomassie Blue. The protein markers were in the Gibco BRL Bench marker. Vertical bars represent SEs ($n = 3$). Only those SEs larger than the symbols are shown.

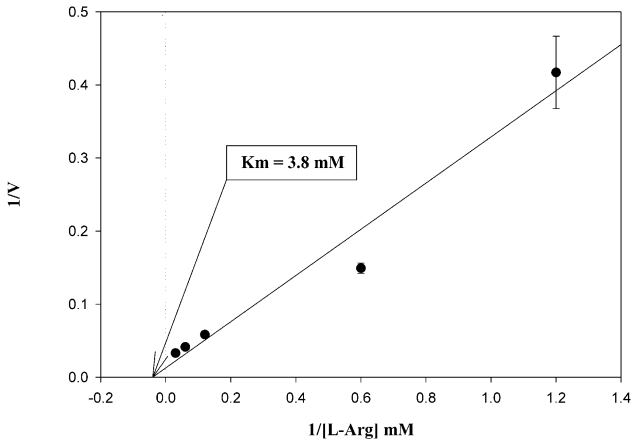

Fig. 5. Double-reciprocal Lineweaver-Burk plots of initial velocities for soybean amidinotransferase reaction against various concentrations of arginine. Vertical bars represent SEs ($n=4$). Only those SEs larger than the symbols are shown.

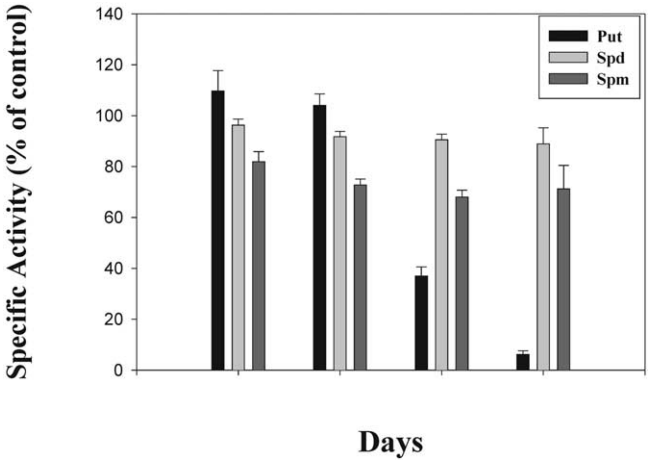

Fig. 6. Effect of polyamines on soybean amidinotransferase in vitro. Purified enzyme was preincubated with polyamines at 37 °C for 10 min and incubated at 37 °C with substrates for 1 h. Activities were assayed at 37 °C by measuring the formation of ornithine. Vertical bars represent SEs ($n=4$). Put = putrescine; Spd, spermidine; Spm, spermine.

various plant species, including seeds of Leguminosae (Jones and Boulter, 1968), Pinaceae (Guitton, 1964), Fagaceae (Tixier and Desmaison, 1980), *Cucurbita moschata* (Splittstoesser, 1969) and angiospermous plant species (Van Etten et al., 1963). On seed germination, a significant quantity of L-arginine is also available (Polacco and Holland, 1993), which could be converted by several enzymes to polyamines. A large number of papers describe stimulation of protein synthesis by polyamines in vitro and in vivo (Tabor and Tabor, 1976, 1984; Smith, 1985). Polyamines influence metabolism of nitrogen-containing compounds. However, reports on the distribution and amount of arginase, arginine decarboxylase and AT in the same species, organs and suborganelles are not available. Nevertheless, assuming all three enzymes are present in the same cell and/or subcellular organelle, they could compete with each other for arginine (Table 2), such that at a high concentration of arginine (about 200 mM), all three enzymes in soybean, arginase ($K_M=83$ mM), AT ($K_M=3.8$ mM) and ADC ($K_M=60$ μM), are fully saturated with substrates and are fully operative. When the concentration of arginine decreases to about 10 mM, arginase is not saturated with arginine and there is a decrease in conversion of arginine into ornithine that is utilized to produce putrescine by action of ODC. At the same concentration of arginine, ADC and AT are, however, fully saturated and fully operative: the former converts arginine into agmatine, which is utilized to produce putrescine. The latter converts arginine into ornithine. At a very low concentration of arginine (about 150 μM), only ADC is saturated with arginine and fully operative. Therefore it would appear that AT plays an important role in soybean, being able to produce more ornithine from arginine than arginase when the concentration of arginine is below the K_M of arginase.

Table 2
K_M values of metabolically related enzymes involved in putrescine biosynthesis in soybean (*Glycine max*) axes

Enzyme	K_M value
Arginase[a]	83 mM[b]
Arginine decarboxylase[c]	60 μM[b]
Amidinotransferase[d]	3.8 mM[b]
Agmatine iminohydrolase[e]	2.5 mM[f]
Ornithine decarboxylase[g]	0.14 mM[h]

[a] Kang and Cho (1990).
[b] Arginine as a substrate.
[c] Park and Cho (1992).
[d] See Fig. 4.
[e] Park and Cho (1991).
[f] Agmatine as a substrate.
[g] Kim and Cho (1993), respectively.
[h] Ornithine as a substrate.

2.7. Effects of various compounds on AT activity

Various divalent and monovalent metal ions did not affect purified AT at any concentrations ranging from 0.1 to 10 mM (data not shown). Putrescine and spermine inhibited soybean AT, with putrescine being a potent inhibitor and spermine a less potent inhibitor (Fig. 6). These results suggest that L-arginine:glycine AT activity in soybean axes is subject to negative feedback regulation, in which putrescine and spermine are endogenous regulators of AT. Accumulation of putrescine was reportedly induced by ammonium nutrition, exposure to low pH, osmotic stress, cadmium and sulfur dioxide toxicity, anaerobiosis, UV radiation (Galston and Sawhney, 1990; Flores, 1991), atrazine treatments (Zheleva et al., 1994), ozone fumigation (Langebartels et al., 1991) and pathogen infections (Langebartels et

al., 1991). Another possible factor affecting putrescine levels is the ADC product, agmatine, which inhibits soybean *S*-adenosyl-methionine decarboxylase (SAMDC) responsible for formation of decarboxylated methionine (dcSAM) from *S*-adenosylmethionine, the aminopropyl group of which is a donor for production of spermidine from putrescine. Thus, agmatine inhibition of SAMDC indirectly increases putrescine levels (Choi and Cho, 1994). Methylthioadenosine (MTA), a product of spermidine synthase from dsSAM, was reported to inhibit soybean spermidine synthase, raising putrescine levels (Yoon et al., 2000). Therefore, it is quite possible that accumulation of putrescine could occur and prevent AT from producing ornithine from arginine. Results herein including previous observations lead us to propose the scheme in Fig. 7. Manipulation of AT in plants by stress, putrescine, MTA and agmatine could shed light on the role of AT.

3. Experimental

3.1. General

Putrescine, bovine serum albumin, L-arginine, L-ornithine, acrylamide, ammonium persulfate, *N*,*N*-methylene-bisacrylamide, bromophenol blue, Coomassie brilliant blue R-250 and dithiothreitol (DTT) were purchased from Sigma Chemical Co (St. Louis, MO, USA), whereas ninhydrin was obtained from Merck (Darmstadt, Germany). L-[^{14}C(U)] Arginine, DEAE-Sephacel, Sephacryl S-300 and L-Arginine Sepharose 4B were purchased from Pharmacia Biotech (San Francisco, CA, USA). All other chemicals used were reagent grade materials from various commercial sources.

For all measurements, each treatment was repeated four times. All experiments described here were repeated three times. Similar results and identical trends were obtained each time.

3.2. Plant materials

Soybean seeds (*Glycine max* [L.] Merrill cv. Baek Tae) were sterilized with 1% sodium hypochlorite and 0.02% Tween for 20 to 30 min and then washed extensively with distilled water. Sterilized seeds were germinated on gauze in plastic trays at 25 °C in the dark and grown for 4 days. All plants were watered daily as described (Kang and Cho, 1990). Three day old soybean roots were exposed to 0.4 M sorbitol for 16 h (Flores and Galston, 1982). Soybean seedlings of each group were harvested at indicated intervals and activities were assayed as described in the Enzyme Assay section.

3.3. Purification of amidinotransferase

All purification procedures were performed at 0–4 °C, unless otherwise stated. Three-day-old soybean roots

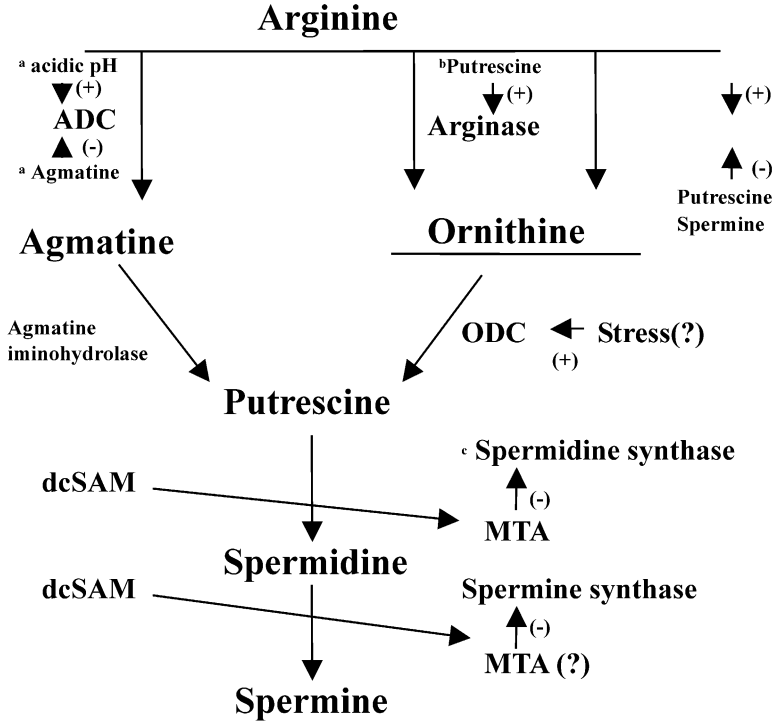

Fig. 7. Scheme of possible regulation of putrescine biosynthesis by stresses and amines in soybean axes. (+), induction or activation ; (−), inhibition. a, Data from Park and Cho (1992); b, data from Kang and Cho (1990); c, data from Yoon et al. (2000). SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; dcSAM, decarboxylated S-adenosylmethionine; MTA, methylthioadenosine.

Table 3
Substrate combinations and the products of purified soybean seedling amidinotransferase

Donor	Acceptors	Concentrations of acceptors (mM)	Products	Relative activity (% of control)
[^{14}C]Arginine	L-Glycine	100	Ornithine[a] + Guanido acetic acid	376.0±60.4
		10		196.5±15.7
		1		100[b]
	L-Lysine	100	Ornithine[a] + Homoarginine	70.1±22.7
		10		65.2±3.0
		1		64.4±6.5
	Agmatine	100	Ornithine[a] + Arcain	111.0±6.0
		10		91.8±5.1
		1		56.1±8.3
	Putrescine	100	Ornithine[a] + Agmatine	47.6±5.6
		10		31.8±2.1
		1		24.2±4.5

Labeled arginine (0.1 μCi) was employed. The different acceptor concentrations were incubated in the presence of the purified enzyme (5 μg) under assay conditions (see Section 3).

[a] Radioactivities of ornithine were measured. For all measurements, each treatment was repeated four times.

[b] Amount of radioactivity in ornithine formed at 1 mM glycine is arbitrarily given at 100%.

treated with 0.4 M sorbitol for 4 h (100 g, fresh weight) were homogenized in a chilled electric blender with 150 ml Buffer A (50 mM Tris/Cl, 0.1% β-mercaptoethanol, 0.5 mM EDTA, pH 8.0). The homogenate was filtered through four layers of gauze and the filtrate was centrifuged at 13,000 *g* for 30 min. The active supernatant in Buffer A was adjusted to 35% saturation with solid $(NH_4)_2SO_4$ and stirred at 4 °C for 2 h. The solution was centrifuged at 13,000 *g* for 30 min and the pellet was discarded. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$ and treated as above, except that the pellet was retained, dissolved in Buffer B (10 mM Tris/Cl, 0.1% β-mercaptoethanol, 0.5 mM EDTA, pH 8.0), and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sepharose (20 × 2 cm) column, pre-equilibrated with Buffer B. After washing the column with equilibration buffer, proteins were eluted with a linear gradient of 0–0.4 M sodium chloride at a flow rate of 30 ml/h. Active fractions were collected, and concentrated by ultrafiltration (Amicon stirred cell, YM-10). The concentrated sample was applied to a Sephacryl-S 300 gel (135 × 0.9 cm) column, pre-equilibrated with Buffer B. Active fractions were collected, then concentrated by ultrafiltration (Amicon stirred cell, YM-10). The concentrated sample was applied to a L-Arginine Sepharose 4B (10 × 0.6 cm) column pre-equilibrated with Buffer B. After washing the column with equilibration buffer, proteins were eluted with a linear gradient of 0–0.2 M potassium chloride at a flow rate of 10 ml/h. The active fractions were concentrated by ultrafiltration and dialyzed against Buffer B, and the concentrated solution was used for all other studies.

3.4. Enzyme assay

AT activity was assayed at 37 °C by modification of the method of Van Pilsum et al. (1970). The assay mixture containing 30 mM Tris/Cl buffer (pH 9), 0.5 mM EDTA, 1 mM DTT, 8 mM L-arginine, 2 mM glycine, and enzyme solution in a total volume of 0.3 ml was placed in the microcentrifuge tube. The microcentrifuge tube was incubated with shaking for 1 h at 37 °C. At the end of this period, 0.3 ml of 10% TCA was added to the enzyme mixture. After centrifugation, 0.5 ml of the supernatant was mixed with 0.5 ml of ninhydrin solution (25 mg/EtOH, 1 ml) and 1.5 ml of acid mixture (acetic acid/H_2PO_4/H_2O, 1:1:3, V/V/V). After boiling the reaction mixture at 100 °C for 1 h, the enzyme activity was determined by measuring optical density at 515 nm. Enzymatic activities were also determined with analogs of substrates in order to find enzyme specificity. Specific activity was expressed as nkatals/mg protein. AT activity was a linear function of both incubation time and concentration under these conditions. Another method for enzyme assay was also used. L-[^{14}C(U)] Arginine was added in the enzyme mixture and the production of labeled ornithine was measured by a Beckman LS-5000 TA liquid scintillation counter. Ornithine and other compounds were resolved using TLC, with the radioactivity of ornithine being measured as described previously (Lee and Cho, 1993). For detection of products, preparative experiments were carried out in a total of 3 ml reaction mixture. After terminating the enzymatic reaction, the enzyme was removed using a Centricone. The filtrate was evaporated to dryness by lyophilization and the residue was dissolved in 1 M acetic acid (300 ul). Each aliquot of

standard compounds and the reaction mixture was analyzed by ESI-MS for detection of ornithine and GAA, respectively. The apparatus used was Mariner (Perseptive Biosystem, USA) mass spectrometer. The conditions were as follows: nozzle temperature, 140 °C; nozzle voltage, 100 eV; ionspray voltage, 3.5 kV. Authentic compounds, ornithine and GAA in products were separated with a mobile phase, 50% acetonitrile containing 0.1% acetic acid at a flow rate of 20 ul/min.

3.5. Protein determination

Protein was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

3.6. Molecular weight determination

The molecular weight of purified AT was estimated by gel filtration as described previously (Choi and Cho, 1994). SDS-PAGE was performed by the Laemmli method (Laemmli, 1970).

3.7. General methods

The K_M, optimum pH, and optimum temperature of purified enzyme were determined as described previously (Kamio and Terawaki, 1983).

Acknowledgements

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