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ORIGINAL ARTICLE

Putative occurrence of lysine decarboxylase isoforms in soybean (*Glycine max*) seedlings

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Abstract The activity of lysine decarboxylase was studied in 3-day-old soybean (*Glycine max* (L.) Meer cv. Sakai) seedlings also in relation to light conditions. Lysine decarboxylase activity was mainly localized in the roots and to a lesser extent in the hypocotyls and was detectable in both the soluble and particulate fractions. The enzyme activity levels were similar during germination under light and dark conditions. With respect to lysine concentration, the initial decarboxylation rate of the soluble fraction showed a saturating curve. Conversely, the initial decarboxylation rate of the particulate fraction showed a sigmoidal curve. These results could suggest that at least two isoforms of lysine decarboxylase are present in different organs of soybean seedlings. In the root soluble fraction, the suicide inhibitor α-difluoromethyl-lysine suppressed the activity of lysine decarboxylase and of ornithine decarboxylase to the same extent, but had no effect on arginine decarboxylase activity.

Keywords Cadaverine · *Glycine max* · Lysine decarboxylase · Polyamine · Soybean

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Introduction

In plants the polyamine cadaverine (1,5-diaminopentane) is synthesized from lysine by lysine decarboxylase enzyme (LDC, EC 4.1.1.18). This compound is present in various bacteria and several higher plant families such as Gramineae, Poaceae, Solanaceae and in particular in Leguminosae (Bagni et al. 1986; Lin 1984; Rodríguez et al. 2000; Smith and Wilshire 1975). In the Leguminosae family, decarboxylation is the main pathway of cadaverine synthesis, although it can also be synthesized from homoarginine via homoagmatine, metabolites that are present during seed germination (Smith 1977). The other aliphatic polyamines, and in particular putrescine, are synthesized both from ornithine and arginine respectively via ornithine (ODC) and arginine (ADC) decarboxylase biosynthetic enzymes (Bagni and Tassoni 2001).

Up to date, no clear relationship has been found between cadaverine and other aliphatic polyamines, such as putrescine, spermidine and spermine. However, cadaverine is known to play a role in the induction of cell division in *Helianthus tuberosus* explants from the medullary parenchyma of dormant tubers (Bagni et al. 1993). Moreover, cadaverine is implicated as precursor in the biosynthesis of quinolizidine, piperidine and other types of alkaloids (Hirai et al. 2000; Leistner and Spenser 1973). In animal tumor cells exposed to α-difluoromethyl-ornithine (DFMO), a suicide inhibitor of ornithine decarboxylase, cadaverine was formed by a compensatory mechanism following the inhibition of putrescine and spermidine synthesis. Cadaverine was then rapidly converted into aminopropylcadaverine and bis(aminopropyl)cadaverine, that are spermidine and spermine analogues (Jänne et al. 1981). Similarly, some Leguminosae contain a large amount of cadaverine (Federico and Angelini 1988) which can be

converted into aminopropylcadaverine and bis (aminopropyl)cadaverine, as also suggested in *H. tuberosus* explants by Bagni et al. (1981). Although the plant distribution of cadaverine and the activity and localization of diamine oxidases cadaverine catabolic enzymes (Lin 1984; Smith and Barker 1988; Scoccianti et al. 1990; Torrigiani and Scoccianti 1995), were extensively studied in Leguminosae during seed germination and development, little attention has been given to lysine decarboxylase activity and its regulation in vitro and in vivo. The present study examines lysine decarboxylase activity in both soluble and particulate fractions of soybean (*Glycine max* (L.) Meer cv. Sakai) seedlings also by means of an innovative approach based on the use of α-difluoromethyl-lysine (DFML), a lysine decarboxylase suicide inhibitor, utilized for the first time on plant material.

Materials and methods

Chemicals

α-Difluoromethyl-DL-lysine-HCl was obtained from Peptide Institute Inc. (Osaka, Japan) and was stored between −20 and −4°C before use.

Plant material

Soybean (*Glycine max* (L.) Meer cv. Sakai) seeds, were soaked in aerated tap water for 18 h. They were then divided into two groups and grown in wet-soil pots for 3 days. One group was allowed to germinate in a greenhouse with a photoperiod of 12 h natural light/12 h darkness and a day/night temperature regime of 28/16°C, respectively. The other group was kept in darkness. After harvest, the seedling organs were separated for enzyme assay.

Lysine decarboxylase enzyme assay

All extraction procedures were carried out on ice. Samples were homogenized in three volumes of 100 mM Tris–HCl (pH 8.5) containing 50 μM pyridoxalphosphate and were centrifuged at 26,000×*g* for 30 min at 0°C. Aliquots (0.2 ml) of both supernatant (soluble fraction) and resuspended pellet (particulate fraction containing cell walls, nuclei, plastids and mitochondria) were used to assay the activities of LDC (EC 4.1.1.18), ornithine decarboxylase (ODC, EC 4.1.1.17) and arginine decarboxylase (ADC, EC 4.1.1.19) by measuring the $^{14}CO_2$ evolution from about 5 μM of L-[U-^{14}C]lysine (specific activity 12 GBq mmol^{-1}, GE Healthcare, UK), L-[1-^{14}C]ornithine (specific activity 1.93 GBq mmol^{-1}, GE Healthcare, UK) and DL-[1-^{14}C] arginine (specific activity 12.43 GBq mmol^{-1}, GE Healthcare, UK) respectively, as previously described by Altamura et al. (1993) who optimized pH, buffer conditions, pyridoxalphosphate and time of the enzyme assays. In previous studies that used similarly obtained cell fractions from a different plant system (*Arabidopsis thaliana*), detailed analysis on the pellet permitted to point out that over 80% of this fraction was represented by intact plastids and/or chloroplasts, while in the supernatant fraction, under our experimental conditions, broken nuclei and plastids were almost negligible (Bagni et al. 2006). Protein content was determined according to Lowry et al. (1951). Each experiment was repeated at least twice with 3–5 replicates for each sample.

Data fitting

Initial rates for lysine decarboxylation of supernatant or particulate fractions supplied with different lysine concentrations, were fitted directly to the Hill equation

$$V_0 = V_{\max}[S]^n/(K + [S]^n)$$

where V_0 and $V_{\max}$ are the initial and maximal initial lysine decarboxylase rates, [S] is the concentration of substrate (in this case lysine), K is the apparent dissociation constant and n is the Hill coefficient. Linear plots were obtained by replotting the data according to the logarithmic form of the Hill equation

$$\log\left(V_0/V_{\max} - V_0\right) = -\log\ K + n\ \log[S]$$

using values for $V_{\max}$ obtained from direct fitting to the Hill equation (not shown).

All experiments were repeated twice and provided similar results. Therefore, the data presented refer to a single experiment.

Results

Enzyme activities were assayed in the organs of 3-day-old soybean seedlings. The activity of LDC was mainly localized in the roots and was much lower in the hypocotyls. No appreciable activity was detected in cotyledons and shoots. The activity in the roots was localized in both the supernatant (soluble) and the pellet (particulate) fractions of plant samples, as determined by an assay using a low amount of ^{14}C-lysine (5 μM). The concentration of ^{14}C-lysine used in the assay does not interfere with the endogenous soybean lysine levels which are usually of about one order of magnitude superior (Krishnan et al.

2005). Enzyme assays were also performed with or without the addition of 10 mM of unlabelled lysine to operate in substrate saturating conditions. No significant difference was observed between the roots of plants grown under light and dark conditions. LDC activity was higher in the soluble than in particulate fraction in the presence of 5 μM ^{14}C-lysine, while particulates showed higher lysine decarboxylase activity under saturating conditions when 10 mM unlabelled lysine was added (Table 1).

Lysine decarboxylase activity was also detected in the hypocotyls, where, in contrast to the root fractions, a higher enzyme activity was found in the particulate than in the soluble fraction. In the soluble fraction of the hypocotyls, the activities of lysine decarboxylase under light and dark conditions were 9 and 19% of that of the root, respectively. Similarly, the activities of particulate LDC in the hypocotyls under light and dark conditions were 33 and 47% with respect to the roots. However, when activity was expressed as nmol per g of fresh weight, similar results were obtained for hypocotyls and roots under both light and dark condition (Table 1).

The kinetics of lysine decarboxylation by lysine decarboxylase in the soluble and particulate fractions of the roots were studied by measuring the initial decarboxylation rate over a range of substrate concentrations (0.1–3 mM of lysine). With respect to lysine concentration, in the soluble fraction, the initial decarboxylation rate showed a saturation curve. From the Hill equation, the Hill coefficient (n) and V_{max} were estimated to be 1.4 and 0.99 nmol mg protein^{-1} h^{-1}, respectively (Fig. 1). Conversely, in the particulate fraction, the initial decarboxylation rate showed a sigmoidal curve and from the Hill equation, the Hill coefficient (n) and V_{max} were found to be 2.1 and 1.15 nmol mg protein^{-1}h^{-1}, respectively (Fig. 2).

The inhibitory effect of increasing concentrations of α-difluoromethyl-lysine (DFML), a LDC suicide inhibitor, was tested on the decarboxylating enzyme activities of root

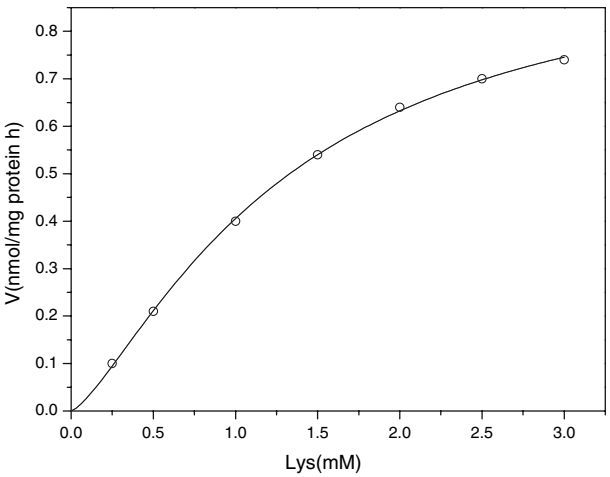

Fig. 1 Lysine decarboxylase activity of the soluble fraction measured at different lysine concentrations in roots of 3-day-old soybean seedlings

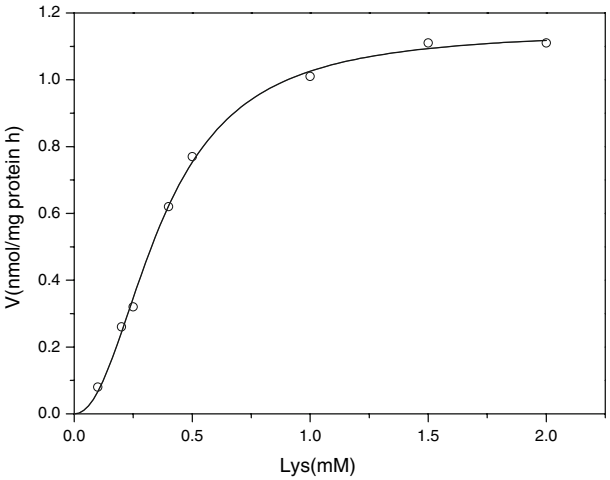

Fig. 2 Lysine decarboxylase activity of the particulate fraction measured at different lysine concentrations in roots of 3-day-old soybean seedlings

Table 1 Lysine decarboxylase activity (nmol [$^{14}CO_2$] mg protein^{-1} h^{-1}) in different organs of 3-day-old seedlings of soybean grown under light and dark conditions

Lysine concentration		Light		Dark	
		5 μM	10 mM	5 μM	10 mM
Roots	Supernatant	0.09 ± 0.01 (0.68)[a]	1.01 ± 0.20	0.10 ± 0.01 (0.48)[a]	0.92 ± 0.09
	Pellet	0.07 ± 0.01 (0.21)[a]	1.15 ± 0.31	0.06 ± 0.01 (0.17)[a]	1.33 ± 0.11
Hypocotyls	Supernatant	0.008 ± 0.001 (0.16)[a]	ND	0.019 ± 0.007 (0.44)[a]	ND
	Pellet	0.023 ± 0.006 (0.20)[a]	ND	0.028 ± 0.009 (0.23)[a]	ND

The enzyme activity was determined by using 5 μM ^{14}C-lysine with or without the addition of 10 mM unlabelled lysine. Data are the mean ± SE of three samples

ND not determined

( )[a] Lysine decarboxylase activity expressed as nmol [$^{14}CO_2$] gFW^{-1} h^{-1}

supernatant fraction (Table 2). The observed LDC activity was markedly inhibited by DFML in a dose-dependant manner. Inhibition levels ranged from 33% in the presence of 0.01 mM DFML up to 74% with 1 mM DFML.

The effects of DFML on the activity of ornithine (ODC) and arginine decarboxylase (ADC), were determined under identical experimental conditions. DFML had little effect on arginine decarboxylase and was not dose-dependent, while both lysine decarboxylase and ornithine decarboxylase were inhibited to the same extent (Table 2).

Discussion

The distribution of LDC activity in various soybean organs was similar to that reported in *Nicotiana glauca* plants by Bagni et al. (1986). Lin (1984) reported that cadaverine synthesis preceded the onset of axis growth, and its accumulation continued as growth progressed in germinating soybean seeds. Gamarnik and Frydman (1991) showed that depleted cadaverine levels were associated with morphological changes in the rooting of germinating soybean seeds. High levels of hydrogen peroxide resulting from the oxidation of cadaverine could also be needed in peroxidase-mediated reactions involved in cell wall stiffening, as suggested by Federico and Angelini (1991).

Our results have demonstrated that LDC activity in the soybean is detectable in both soluble and particulate fractions of roots and hypocotyls. In particular, in the soluble fraction of the roots LDC activity was about 10-times higher at 10 mM lysine than at 5 μM, under both light and dark conditions. The enzyme activity in the particulate fraction measured at 10 mM was about 20-times higher than at 5 μM in the light and dark conditions. These results suggest that lysine decarboxylase in the soluble fraction has different kinetic parameters than in particulate fraction.

Table 2 Effect of α-difluoromethyl-lysine (DFML) on the soluble-fraction activities of lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) from soybean seedlingsDMFL was added at the beginning of the incubation

DFML (mM)	(%) Relative activity		
	LDC	ODC	ADC
0	100	100	100
0.01	67.3	59.4	96.9
0.1	35.6	33.1	91.3
0.5	27.7	24.8	94.4
1.0	25.7	22.0	93.5

The activities of the controls, taken as 100%, were respectively 1.01 nmol [$^{14}CO_2$] mg protein^{-1} h^{-1} for LDC, 47.1 nmol [$^{14}CO_2$] mg protein^{-1} h^{-1} for ODC and 72.1 nmol [$^{14}CO_2$] mg protein^{-1} h^{-1} for ADC

Moreover, the enzyme seems to be present in both fractions in relatively abundant levels.

The LDC activities in both the soluble and particulate fractions in the hypocotyls of soybeans grown under dark conditions were greater than in those grown in the light (Table 1). This might be due to differences in the length of the hypocotyls, as those of seedlings that were germinated in darkness extended more rapidly than those grown under light conditions. Smith and Davies (1985) reported that polyamines in the hypocotyls increase during internode elongation, probably as a result of initial cell proliferation.

The present kinetic study has demonstrated that lysine decarboxylation in the soluble fraction is not cooperative with respect to substrate concentration (Fig. 1). From the Hill equation, the apparent dissociation constant is 0.70, in good agreement with the K_m value previously determined for lysine decarboxylase purified from soybean roots (Kim et al. 1998) and also similar to the K_m of lysine decarboxylase from *Selenomonas ruminantium* (Takatsuka et al. 1999). Conversely, the lysine decarboxylation in the particulate fraction is cooperative with respect to substrate (Fig. 2), with a Hill coefficient (n) of 2.1. In contrast to the LDC of the soluble fraction, which was shown to be monomeric (Kim et al. 1998), the LDC enzyme present in the particulate fraction could probably be oligomeric, as demonstrated by the Hill coefficient. Consequently, our results could lead to hypothesize for the first time in higher plants the contemporary presence of two different lysine decarboxylase isoenzymes in soybean soluble and particulate fractions. A chloroplast-localized lysine decarboxylase activity was in fact previously detected in *Lupinus polyphyllus* by Hartmann et al. (1980). Goldemberg (1980) reported two enzyme isoforms in lysine decarboxylase mutants of *Escherichia coli*, as also confirmed by the presence of two lysine decarboxylase genes in *E. coli* (Kikuchi et al. 1997; Meng and Bennet 1992).

Based on database searching, no lysine decarboxylase gene sequence from soybean (or other Leguminosae) has been up to now identified. Instead, according to the TAIR database (www.arabidopsis.org) in *A. thaliana* genome there are three putative LDC genes respectively At5g26140, At5g11950, At1g50575. Expression results from different authors (ATGenExpress Visualization Tool; http://jsp.weigelworld.org) evidenced that these genes are differently expressed depending on the plant organ, plant growth stage and stress conditions. These data, even if obtained in *A. thaliana*, could lead to hypothesize, even in the soybean plant system, the existence of two or more genes coding for lysine decarboxylase and consequently the possible presence of different LDC protein isoforms.

α-Difluoromethyl-lysine (DFML) was shown to be a selective irreversible inhibitor of lysine decarboxylase of *Mycoplasma dispar* (Pösö et al. 1984) and *S. ruminantium* in

vitro (Kamio et al. 1986). However, the *S. ruminantium* lysine decarboxylase is capable of decarboxylating both L-lysine and L-ornithine with similar K_m values (Takatsuka et al. 1999). Our results showed that lysine decarboxylase activity was much lower than ornithine decarboxylase activity, although, in the soluble fraction of the soybean roots (Table 2), both were similarly inhibited by DFML. Gamarnik and Frydman (1991) reported that increasing concentrations of α-difluoromethyl-ornithine (DFMO) affected the enzymatic activities of lysine and ornithine decarboxylase differently in the soluble fractions of 2-day-old soybean roots. Kim et al. (1998) reported that purified lysine decarboxylase from soybean roots was inhibited by cadaverine, but was less inhibited by putrescine. These results could suggest that lysine and ornithine decarboxylases show a high degree of substrate specificity, although they can be inhibited by both DFML and DFMO due to the irreversible binding between the enzymes and the inhibitors. Site-directed mutagenesis experiments of the conserved amino acids demonstrated that *N. glutinosa* ODC has decarboxylase activity toward both L-lysine and L-ornithine providing definitive evidence for both ODC and LDC activities being modulated via the same active site in the same protein (Lee and Cho, 2001). With the use of a different approach, our results show the same amount of inhibition by DFML of both LDC and ODC activities in the soluble-fraction of soybean roots are, and seem to be therefore in agreement with the findings of Lee and Cho (2001).

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