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LYSINE DECARBOXYLASE ACTIVITY AND ALKALOID PRODUCTION IN *HEIMIA SALICIFOLIA* CULTURES

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Key Word Index—*Heimia salicifolia*; Lythraceae; cell culture; shoot culture; lysine decarboxylase; chlorophyll; alkaloid production.

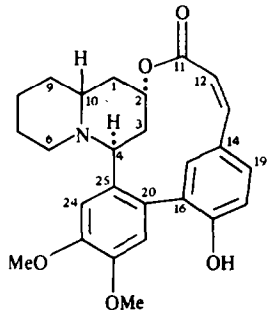
Abstract—Levels of lysine decarboxylase, thought to exercise control over the biosynthesis of alkaloids derived from the amino acid, have been determined in alkaloid-producing and non-producing cell and organ cultures of *Heimia salicifolia*. The level of this enzyme has also been measured in cultures grown in the presence and absence of light. In chlorophyllous cell cultures enzyme activity correlates positively with chlorophyll; in shoot cultures the activity also parallels alkaloid production.

INTRODUCTION

We have recently reported on the alkaloid expression of *in vitro*-grown *Heimia salicifolia* Link & Otto. Cultured shoots accumulate both phenylquinolizidines and biphenylquinolizidine lactones but none of the callus and cell suspensions yields any detectable amount of alkaloids [1, 2]. We have now initiated studies to determine some of the factors which might be limiting the capacity of the unorganized culture to produce alkaloids.

The notion is generally accepted that enzymes connecting primary and secondary metabolic pathways are at a point of regulation of secondary product biosynthesis [3–5]. The biphenylquinolizidine lactones of the Lythraceae (as vertine. 1) have been shown to be derived from lysine (C-6–10), acetate (C-1) and phenylalanine (C-2–4, 11–25) [6, 7]. Thus lysine decarboxylase (LDC) (EC 4.1.1.18) as well as phenylalanine ammonia lyase (PAL) may be thought to have a regulatory function in the biosynthesis of the alkaloids. We have at first directed our attention to lysine decarboxylase.

In this communication we report on the LDC activity in callus (chlorophyllous), cell suspensions (chlorophyllous as well as non-chlorophyllous) and shoot cultures (grown both in presence and absence of light) of *H. salicifolia*.



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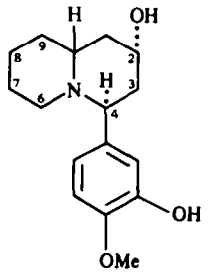

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RESULTS

Lysine decarboxylase of in vitro*-grown shoots*

The assays used for LDC activity [8, 9] led to an apparent K_m for lysine of 1.25 mM for the enzyme prepared from light-grown shoots (Lineweaver–Burk plot). This value is of the same order as those reported for LDC from *Lathyrus sativus* and *Lupinus polyphyllus* [10, 11]. Activity was found to be maximal at pH 8.3. This value is also comparable to those of 8.4 and 8.0 found for LDC from *L. sativus* and *L. polyphyllus*, respectively [10, 11].

When dialyzed preparations were assayed in the absence of pyridoxal-5′-phosphate (PLP), activity was reduced to 58–68% of a non-dialyzed control. When the dialyzed preparation was assayed under standard conditions (with PLP), activity was partially restored to 82% of control.

The effect of some potential inhibitors and protective agents is summarized in Table 1, where the standard error of each determination is 5%. It is noteworthy that when a series of metal ions (ferrous, ferric, magnesium and calcium) were each tested at 2.5 mM concentrations, Fe^{2+}, Fe^{3+} and Ca^{2+} led to a decrease in the activity of the enzyme. This effect is contrary to that observed by Hartmann *et al.* [9], who found maximal LDC activity in the presence of 2.5 mM $FeSO_4$.

Diethyldithiocarbamic acid (DIECA) (5 and 10 mM) causes a significant decrease in the $^{14}CO_2$ trapped in the assays (Table 1). Since DIECA is an inhibitor of diamine oxidase (DAO) [12, 13], we verified that the $^{14}CO_2$ had not been partially generated by H_2O_2, which might have been formed by action of DAO on either [U-^{14}C]cadaverine, [U-^{14}C]lysine, or both. Such an H_2O_2-caused decarboxylation of lysine would have been diamine dependent [13]. [U-^{14}C]Cadaverine (0.1 mM; 9.7 μCi/mmole) was found to have no effect on the activity (Table 1). Thus, cadaverine, in the amount formed in our assays, does not contribute to the amount of volatile radioactivity released by the enzyme mixture, either by its

Table 1. The effects of various reagents on the activity of lysine decarboxylase isolated from cultures of *Heimia salicifolia*

Additions	Relative activity Shoots	Non-chlorophyllous suspensions
None*	100	100
1 mM DTE	104	—
3 mM DTE	113	—
5 mM DTE	56	—
10 mM DTE	18	—
2.5 mM $FeSO_4$	59	—
2.5 mM $MgSO_4$	88	107
2.5 mM $MgCl_2$	94	114
2.5 mM $CaCl_2$	59	56
2.5 mM $FeCl_3$	43	—
1 mM EDTA	34	—
10 mM EDTA	30	—
20 mM EDTA	28	—
5 mM DIECA	46	64
10 mM DIECA	28	—
0.1 mM [^{14}C]cadaverine	96	—
10 mM cadaverine	23	28
15 mM KCN	24	33
5 mM **2**	29	27
5 mM **1**	16	15

*Standard assay conditions are described in Experimental.
— = Not determined.

metabolism to a volatile product, or through its interaction with DAO, with the concomitant production of H_2O_2. When 10 mM cadaverine was added to the standard assay (Table 1), a decrease in activity to 25% of control was seen. That measured radioactivity was not enhanced further indicates that DAO has no bearing on the $^{14}CO_2$ assay for LDC. With the radiochromatographic method [9] we have established that the amount of [^{14}C]cadaverine produced in the assay corresponds directly to the amount of $^{14}CO_2$ trapped. Furthermore, [^{14}C]Δ^1-piperideine-2-carboxylic acid [12] was not detected.

LDC activity in shoot organs, callus and cell suspensions

The LDC activity of *H. salicifolia* shoots cultured under standard conditions (that is with illumination) is shown in Fig. 1c, d (open circles). Table 2 compares the LDC activity at an arbitrarily selected time after transfer (11–15 days) of cultured shoots (13 days, 27.7 pkat/mg protein), non-alkaloid-yielding green callus (11 days, 1.3 pkat/mg protein) and non-alkaloid-yielding suspension cultures, both chlorophyllous (15 days, 7.7 pkat/mg protein) and non-chlorophyllous (15 days, 86.8 pkat/mg protein).

The two types of suspension cultures studied, chlorophyllous and non-chlorophyllous, were maintained on identical media but were selected visually into two cell lines based on either their green or brown appearance. Neither cell line produced detectable alkaloids. The level of chlorophyll in the chlorophyllous suspension cultures was of the same order as that of green callus cultures. LDC activity per fresh weight was similar to that of cultured shoots, but specific activity more closely resembled that calculated for callus tissue. In the brown suspension cultures, surprisingly, we observed levels of total and specific LDC activity which were significantly higher than those found in any of the alkaloid-yielding shoot cultures. Obviously this result stands in marked contrast to the pattern of results obtained from shoot, callus and green suspension cultures. Among these chlorophyll-containing cultures, the correlation between chlorophyll content and specific activity of LDC is 87% (Fig. 2).

The relatively high LDC activity of the non-chlorophyllous suspensions was unexpected. We therefore initiated experiments to investigate the possibility that it might be due to an isoenzyme of the form found in chlorophyll-containing cultures. Both the apparent K_m for lysine (0.84 mM) and the pH optimum (8.3) of the LDC isolated from the non-chlorophyllous suspensions, however, were shown to be comparable to the values given by the shoot cultures. A series of differential inhibition studies were performed in which the effects of adding each of several inhibitors to the standard assay were determined (Table 1) for the two enzyme preparations. Allowing a 20% margin which incorporates errors which might be introduced because of the innate differences between the culture types, as well as experimental error, we found that the two enzymes are inhibited to similar extents by each of the compounds tested. As expected, the enzyme was strongly inhibited by the carbonyl reagent KCN. Moreover, we found that LDC is apparently sensitive to inhibition by both phenyl- (**2**) and biphenylquinolizidine (**1**) alkaloids.

Comparison of LDC activity, protein- and chlorophyll-content in light- and dark-grown shoot cultures

Light- and dark-grown shoot cultures were analyzed for LDC activity at various stages during the culture period in order to determine the effect of a reduction in chloroplast development on a potentially alkaloid-positive tissue (Figs. 1a–d). In the particular shoot cultures used for these enzymatic experiments chlorophyll concentration followed the pattern shown in Fig. 1b. While these changes appear to be random in light-grown shoots, they fall within the range established from a number of determinations made over the course of the culture period. Dark-grown shoots, without exception, show a time-dependent decrease in chlorophyll content per fresh weight.

In both light- and dark-grown shoot cultures, total protein per fresh weight increased markedly during the first week to 10 days after subculture (Fig. 1a). This increase is slightly greater in dark-grown shoots. Likewise LDC activity per fresh weight showed an initial increase, reaching a maximum 14–15 days after subculture, and again the increase is slightly greater in dark-grown shoots (Fig. 1c). In the light, the specific activity rises as protein/fresh weight falls (Fig. 1d). An apparent parallelism (81%) exists between the specific activity of LDC and the chlorophyll content of the tissues (Fig. 1b, d). In dark-grown shoots, LDC activity per unit fresh weight is maximal at day 14–15 when the chlorophyll concentration has already decreased to one half of its original value (Fig. 1b, c). Specific activity (Fig. 1d) decreases, although irregularly, with time. A correlation of 74% exists between LDC specific activity and chlorophyll in dark-grown shoots.

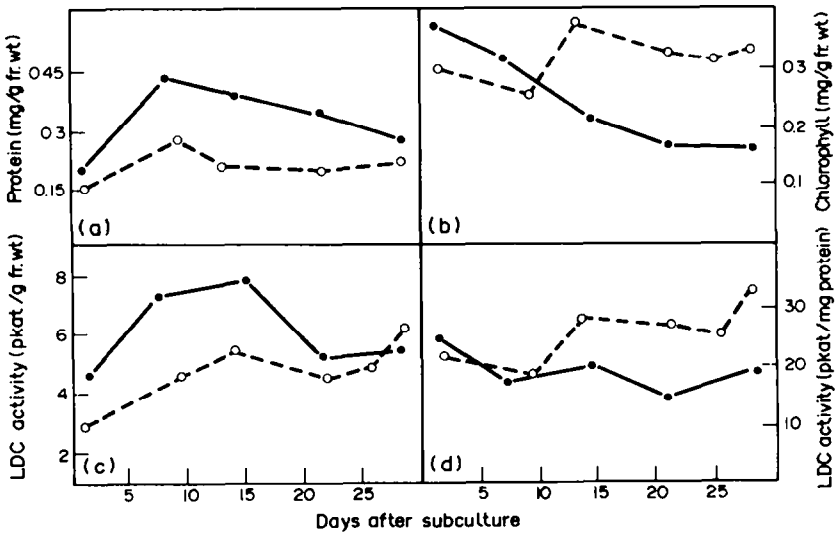

Fig. 1. Changes in (a) protein, (b) chlorophyll, (c) activity of LDC and (d) specific activity of LDC with time after subculture in light-grown (-○-○-○-) and dark-grown (-●-●-●-) shoot cultures.

Table 2. Comparative study of tissue cultures of *H. salicifolia*

	Shoots*	Callus†	Non-chlorophyllous suspension‡	Chlorophyllous suspension‡
Chlorophyll (mg/g fresh wt)	0.381	0.103	0.003	0.033
Protein (mg/g fresh wt)	0.20	3.28	0.22	0.96
LDC (pkat/g fresh wt)	5.5	4.3	19.1	7.4
LDC (pkat/mg protein)	27.7	1.3	86.8	7.7

Days after subculture: *13, †11, ‡15.

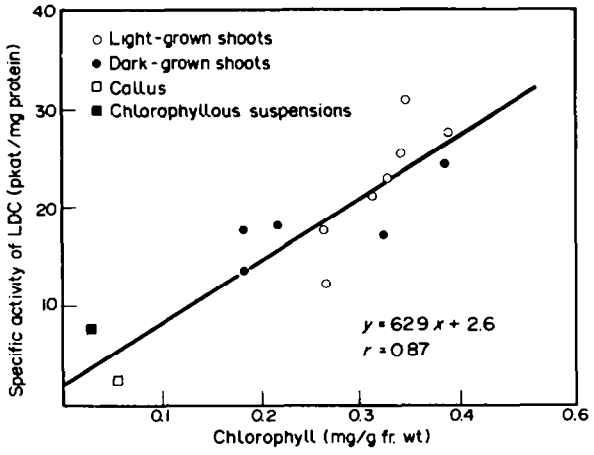

Fig. 2. The relationship between chlorophyll concentration and specific activity of LDC in light- and dark-grown shoot cultures, partially differentiated callus and chlorophyllous suspension cultures.

DISCUSSION

Lysine decarboxylase from *Heimia salicifolia* appears to resemble the enzymes from *Lathyrus sativus* [10] and *Lupinus polyphyllus* [11] with regard to both pH optima and apparent K_m values. While the plant enzymes exhibit maximal activity at pH greater than 8.0, it is interesting to note that LDC from bacterial sources show optima between pH 5 and 6.5. In the light of evidence for the localization of LDC in the chloroplasts of *L. polyphyllus* [9], it was pointed out that the light-mediated shift in the pH of the chloroplast stroma from 7 to 8 might play a role in regulating LDC activity *in vivo* [11].

The reduction we observed in LDC activity after dialysis, and its partial restoration by added PLP, suggest that the enzyme, like most other amino acid decarboxylases, requires this cofactor for optimal activity. While metal ions have been found to enhance the activity of LDC isolated from various sources [9, 14–16], we observed a reduction in activity by three of the salts tested. Since the removal of metal ions, either by the addition of EDTA or dialysis, also effects a reduction in activity, we must conclude that a critical concentration of metal ion(s)

exists which promotes optimal activity. The nature of this requirement has not been determined.

The effect of cadaverine on LDC activity (Table 1) suggests that if LDC is regulated by feed-back inhibition, the inhibition is not exerted by its immediate product, cadaverine, at 0.1 mM concentration. The inhibition seen at 10 mM concentration may not be relevant to the *in vivo* regulation of LDC, as cadaverine concentrations *in vivo* have been found to be very low [9, 17].

The findings that 5 mM concentrations of *trans*-2-hydroxy-4-(3-hydroxy-4-methoxyphenyl)quinolizidine (2) and vertine (1) inhibited activity to 28 and 16% of control, respectively, imply that LDC may be subject to an end-product feedback inhibition mechanism *in vivo*. While concentration gradients may exist *in vivo* which allow alkaloid levels to reach 5 mM in the vicinity of their biosynthesis, it is also possible that such concentrations are never reached.

The relationship which appears to exist between the activity of LDC and the amount of chlorophyll in cultured *H. salicifolia* shoots would provide circumstantial evidence for the possible localization of LDC in the chloroplast. Furthermore, we have found that the level of alkaloid accumulation in shoot tissues is also proportional to the chlorophyll content of these tissues [2]. In light- and dark-grown shoots, the apparent correlation obtained between chlorophyll and phenylquinolizidinol (2) concentration was 79 and 78% respectively; it was 99 and 98%, respectively, between chlorophyll and vertine (1) content [2]. These findings point to an integral role of LDC in the biosynthesis of the phenyl- and biphenylquinolizidine alkaloids.

The results obtained from callus and chlorophyllous suspension cultures extend the apparent relation which may exist between activity of LDC and chlorophyll concentration. In these morphologically less organized tissues, however, the correlation with alkaloid content does not hold any longer. Although it is low, the LDC activity of callus and cell suspensions would not have precluded alkaloid detection, provided that under a non-limiting supply of lysine, any cadaverine formed would have been channeled into alkaloids. A linear relationship between either chlorophyll concentration or LDC activity and alkaloid accumulation does not appear to exist.

The present study was developed to initiate investigations into the possible causes for the observed lack of alkaloid accumulation when *H. salicifolia* is grown as a callus or a cell suspension. One major problem of secondary metabolite production by plant cell cultures is that when grown *in vitro* a species may present an altered, generally simpler, secondary metabolism. The causes for this are not yet understood [3–5, 18]. In the quinolizidine alkaloid field it has been shown that cell cultures of legume species produce alkaloids, the total amount is 1–3 orders of magnitude lower in the cultures than in the respective field-grown species [19]. Alkaloid content has been shown to correlate positively with greening of cell suspensions of *Lupinus polyphyllus* [20] and *Sarothamnus scoparius* [21] as well as with regreening of *L. polyphyllus* leaves [12]. The enzyme that catalyzes the synthesis of 17-oxosparteine from cadaverine as well as the LDC, active in the formation of cadaverine from lysine, has been shown to be localized in the chloroplasts of *L. polyphyllus* [22]. LDC activity of *L. polyphyllus* was shown to correlate positively with chlorophyll content during leaf regreening. A positive correlation was also found between LDC activity and leaf alkaloid concentration in *L. polyphyllus*, *L. luteus* and *L. albus* [12].

These findings would suggest that LDC may have a regulatory function in tetracyclic quinolizidine alkaloid biosynthesis. The consistency of the apparent LDC, chloroplast and alkaloid interdependency, however, is disturbed by findings of LDC activity not only in non-alkaloid producing organs of quinolizidine yielding plants but also in species that do not produce any lysine-derived alkaloids [12]. A similar picture emerged from our work with *H. salicifolia*. Whereas in all the chlorophyllous cultures analyzed, LDC correlates positively with chlorophyll (Fig. 2), only in shoot cultures does LDC correlate also with biphenylquinolizidine lactone concentration. LDC activity, however, is not limiting in non-alkaloid producing cultures.

In view of these results we can conclude that control of alkaloid biosynthesis in *Heimia* is not mediated by LDC. Other enzymes of the lysine branch of the biosynthetic pathway [6] may exercise this control. The function may also lie in the pathway leading to the isoferulic acid moiety of the alkaloid molecules, i.e. with PAL or some subsequent enzyme.

The comparatively high LDC levels in the non-chlorophyllous suspension cultures would imply that the enzyme might be serving a function in the cells unrelated to phenyl- and biphenylquinolizidine production. At present we have no evidence to indicate that this is indeed the case, nor do we have any results that might indicate that we are dealing with isoenzymes. We would be able to answer these questions once the LDC has been purified and studied in more detail.

EXPERIMENTAL

Chemicals. L-[^{14}C(U)]Lysine, [^{14}C(U)]cadaverine and Protosol were purchased from New England Nuclear. Unlabeled lysine and cadaverine, cofactors and other biochemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Plant material. Shoot cultures were developed and maintained as in ref. [2]. Suspension cultures were grown in medium C described in ref. [1]. The greener tissue was selected and propagated separately from the more rapidly browning cell cultures. Chlorophyllous callus cultures were developed from 4- to 6-week old seedlings on medium containing Murashige and Shoog salts [23], thiamine hydrochloride (0.1 mg/ml), inositol (100 mg/ml), NAA (10^{-1} μM), 2,4-D (1 μM), BA (10 μM), kinetin (2.3×10^{-1} μM) and 3% sucrose. Growth was under continuous illumination at 23–26° with a monthly subculture schedule.

Harvesting and extraction of tissues. Tissues were harvested between 11 a.m. and 1 p.m. Immediately after weighing, fresh tissue (10–20 g) was homogenized with Me_2CO (2 × 100 ml) at −15 to −20° (explosion-proof Waring blender). The powder was dried under vacuum and stored desiccated at −20° until it was analyzed for total protein and LDC activity. The combined filtrates were analyzed for chlorophyll and alkaloids as in ref. [2].

Assay for lysine decarboxylase activity. Two methods [8, 9] for LDC assay were adapted to the *H. salicifolia* cultures. Both the $^{14}CO_2$ [8] and the [U-^{14}C]cadaverine [9] produced upon decarboxylation of [U-^{14}C]lysine were measured. The $^{14}CO_2$ assay, being the more convenient of the two, was used routinely. Assays were performed in 10 ml side-arm reaction flasks sealed with a rubber septum at the side-arm and a rubber stopper in the top. From the stopper, an 8 mm Whatman 3 MM filter disc satd with 20 μl of 5 M Protosol soln was suspended by a straight pin.

In a total vol. of 1.0 ml, the standard assay contained: 100 μmol KPi buffer, pH 8.0, 0.1 μmol L-lysine containing 0.5–1.0 μCi L-[U-^{14}C]lysine, 0.3 nmol pyridoxal-5'-phosphate (PLP), and 300–800 μl plant enzyme extract, containing 0.02–0.20 mg protein. This extract was prepared by suspending the acetone powder in 0.1 mM KPi buffer, pH 8.0 (50–100 mg powder), and stirring 30 min at 0–4°. The suspensions were then centrifuged 10 min at 10000 *g* and the supernatant was used in the enzyme assays. Incubation was 1 hr at 37°. The enzymatic reaction was terminated by injection of 100 μl 3 N HCl through the side-arm; flasks were then allowed to stand for an additional hour for the complete entrapment of $^{14}CO_2$ on the filter disc. Filter discs were then removed to 20 ml vials and 10 ml of scintillant was added (7 g PPO and 0.2 g POPOP per 1. toluene). Radioactivity was measured using the external standard method.

In some cases, the amount of [^{14}C]cadaverine produced was also determined by a modification of the method of Hartmann *et al.* [9]. Aliquots of 100 μl of the assay mixture applied together with lysine and cadaverine were chromatographed on 'Baker-flex' silica thin layer plates in 96% EtOH–25% NH_4OH (3:2). The cadaverine region, which was localized by radio-scanning and ninhydrin reaction, was cut out and eluted in the scintillation vial with 2 ml 0.5 N HCl in 50% MeOH. Ten milliliters of cocktail (6 g naphthalene, 0.4 g PPO, 0.02 g POPOP and 2 ml ethyleneglycol per 100 ml dioxane) were then added and radioactivity was determined.

Protein was determined by the method of Bradford [24].

Determination of pH optima. Me_2CO powders were suspended in doubly distilled water and centrifuged as described for the standard assay. The supernatant was assayed using 0.1 ml of 1 M KPi buffers, ranging in pH from 6.0 to 9.5, under conditions which were otherwise standard.

Determination of apparent K_m. The concentration of unlabelled L-lysine in the otherwise standard assay was varied between 0.1 and 12 μM.

Determination of the effects of potential inhibitors, protective agents and cofactors. When testing the effect of PLP on LDC activity, the supernatant obtained after centrifuging the suspended Me_2CO powder was dialyzed overnight against 0.1 M KPi, pH 8.0, at 4°. Other agents tested were added to the standard assay described above.

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