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Elicitor-Induced L-Tyrosine Decarboxylase from Plant Cell Suspension Cultures[1]

II. PARTIAL CHARACTERIZATION

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ABSTRACT

Properties of purified L-tyrosine decarboxylase (EC 4.1.1.25) from elicitor-induced cell suspension cultures of *Eschscholtzia californica* Cham. and *Thalictrum rugosum* Ait. are described. L-Tyrosine decarboxylase is a dimeric enzyme with a molecular weight of 112,600 ± 600 daltons. The isoelectric point was estimated to be at pH 5.2 and pH 5.4 for the enzyme from *E. californica* and *T. rugosum*, respectively. The purified enzymes were stabilized in the presence of pyridoxal-5-phosphate. Optimum pH for the enzyme from both plants was found to be 8.4. Enzyme activity was dependent on exogeneously supplied pyridoxal-5-phosphate. The enzyme decarboxylated L-tyrosine and L-β-3,4-dihydroxyphenylalanine but was inactive toward L-phenylalanine and L-tryptophan. Apparent K_m values of *Eschscholtzia*- and *Thalictrum*-decarboxylase for L-tyrosine were 0.25 ± 0.03 and 0.27 ± 0.04 millimolar, respectively. Similar affinities were found for L-3,4-dihydroxyphenylalanine. *Eschscholtzia* L-tyrosine decarboxylase was strongly inhibited by the phenylalanine analogue L-α-aminooxy-β-phenylpropionate and largely unaffected by D,L-α-monofluoromethyl-3,4-dihydroxyphenylalanine and α-difluoromethyltyrosine.

An enhanced biosynthesis of berberine in cell suspension cultures of *Thalictrum rugosum* Ait. was achieved by treating the cells with a yeast carbohydrate elicitor (6). This increased product formation followed an increase of TDC[2] (EC 4.1.1.25) activity (8). These findings, coupled with results from feeding experiments with radioactive labeled tyrosine (8), indicate that TDC might control the branching point between primary and secondary metabolisms. Furthermore, the synthesis of dopamine (one of the direct precursors of norlaudanosoline) from tyrosine during the initial steps of alkaloid biosynthesis is not clear. Two possible routes could be considered: first, hydroxylation of tyrosine to DOPA followed by a decarboxylation of DOPA to dopamine; second, decarboxylation of tyrosine to tyramine and subsequent hydroxylation of tyramine to dopamine.

Data about the catalytic and molecular properties of plant DOPA and tyrosine decarboxylase would be valuable in establishing the function of these enzymes in alkaloid biosynthesis. However, only very limited information about these enzymes has been published (2, 7, 9, 14, 15). Possible reasons for this may be the instability of TDC during purification and its small quantities in the cells (11). We have purified TDC from elicitor-induced cell suspension cultures of *Eschscholtzia californica* Cham. and *T. rugosum*. Some properties of the purified enzyme are reported in this communication. Furthermore, a comparison of TDC from *E. californica* and *T. rugosum* has been carried out.

MATERIALS AND METHODS

Chemicals. AOPP was a kind gift from Dr. R. Chollet, Sandoz AG, Basel, Switzerland. α-Difluoromethyltyrosine (RMI 71.855) and D,L-α-fluoromethyl-3,4-dihydroxyphenylalanine (RMI 71.963) were gifts from Merrell Dow Research Institute, Strasbourg, France. L-[1-^{14}C]tyrosine (53.8 mCi/mmol) and Protosol were purchased from New England Nuclear. L-[Methylene-^{14}C]-tryptophan (59 mCi/mmol), L-3,4-dihydroxyphenyl-[1-^{14}C]alanine (5.4 mCi/mmol), and L-[U-^{14}C]phenylalanine (504 mCi/mmol) were supplied by Amersham. L-DOPA, D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49), amyloglucosidase (EC 3.2.1.3), and α-amylase from bacteria (EC 3.2.1.1) were supplied by Fluka. Polybuffer 74 was obtained from Pharmacia. Ampholines were from LKB. Other chemicals were obtained from the same sources as in the preceding paper (11).

Cell Culture and Induction. *Thalictrum rugosum* and *Eschscholtzia californica* cell suspensions were cultivated and induced for the extraction of TDC as described (11).

Purification of TDC. TDC was purified from elicitor-induced cells of *T. rugosum* and *E. californica* as described previously (11).

Enzyme Assay. Decarboxylase activity was measured as described previously (11). For the determination of pH optimum the assay contained a mixture of three different buffers (bis-Tris, Hepes, Tris) each giving a concentration of 40 mM; pH values were adjusted with HCl or NaOH.

Gel Filtration. The mol wt of the native enzyme was estimated by gel filtration with a Superose 12 column (Pharmacia). The eluent was 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT; flow rate: 1 ml/min. Reference proteins were D-glucose-6-phosphate dehydrogenase (M_r 102,000 D), amyloglucosidase (M_r 97,000 D), and α-amylase (M_r 58,000 D).

Isoelectric Point. pI values of TDC were determined by using a polybuffer exhange column (Mono P, HR 5/20; Pharmacia). A sample (0.5 ml) of purified TDC was desalted by centrifugation through a 5 ml Sephadex G-25 column equilibrated in 25 mM bis-Tris-HCl (pH 6.7) and applied to the Mono P column, which had been equilibrated with the same buffer. Elution was performed with a decreasing pH gradient (30 ml) using a 10-fold diluted Polybuffer 74-HCl (pH 4.9). The pI values were also estimated by isoelectric focusing. IEF was carried out on tubes as described (5) using ampholine pH 4 to 6.

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[2] Abbreviations: TDC, L-tyrosine decarboxylase; DOPA, L-3,4-dihydroxyphenylalanine; PLP, pyridoxal-5-phosphate; AOPP, L-α-aminooxy-β-phenylpropionate; pI, isoelectric point.

RESULTS AND DISCUSSION

Few enzymes involved in alkaloid biosynthesis within higher plants have been purified and characterized. We have been involved in studies on the initial steps of isoquinoline alkaloid synthesis. A correlation between elicitor-induced TDC activity and enhanced berberine biosynthesis has been established (6, 8). In order to further evaluate the possible role of TDC as a key enzyme in isoquinoline alkaloid synthesis, we purified the enzyme from plant cell suspension cultures of *Thalictrum rugosum* and *Eschscholtzia californica*, both of which produce these alkaloids (11). The two enzymes were partially characterized as described below.

Molecular Weight. The mol wt of TDC as determined by gel filtration on a Superose 12 column was 95,000 D. Native PAGE indicated a M_r around 90,000 D (data not shown). Since TDC was retained by the ultrafiltration membrane YM 100 (Diaflo, Amicon), a $M_r > 100{,}000$ D can be assumed. From SDS-PAGE, a subunit M_r of 56,300 ± 300 D was determined (11). These findings suggest that TDC from *T. rugosum* or *E. californica* is a dimer composed of two identical subunits with a mol wt of 112,600 ± 600 D. Published M_rs of TDC from plant tissue do not exist. However, this mol wt is similar to that reported for the aromatic amino acid decarboxylase from hog kidney (3) and the DOPA decarboxylase from *Drosophila melanogaster* (4). Furthermore, it is interesting to note that tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures consists of two identical subunits with a M_r of 54,000 D (13). Despite the similarities between TDC and tryptophan decarboxylase, it is clear from substrate specificity studies that these enzymes are not the same. The former showed no decarboxylase activity toward L-tryptophan (see below) while the latter does not decarboxylate L-tyrosine or L-DOPA (13).

Isoelectric Point. pI Values for the purified TDCs were estimated by chromatofocusing and isoelectric focusing (data not shown). The pI values for TDC from *T. rugosum* and *E. californica* were 5.2 and 5.4, respectively. No pI values have been reported for plant TDC. For TDC from *Streptococcus faecalis*, a pI value of 4.5 has been reported (1). An pI of 5.9 was determined for tryptophan decarboxylase from *C. roseus* (13).

Enzyme Stability. The purified enzymes are very unstable, and addition of PLP to the storage buffer is essential for stability as indicated in Figure 1. The purified enzyme had a half-life of around 3 and 15 d at 2°C in the absence and presence of PLP, respectively. In contrast, tryptophan decarboxylase from *C. roseus* was not stabilized by addition of PLP to the storage buffer (13). This enzyme showed a half-life of around 4 d when stored at 0°C in 20 mM tris-HCl buffer (pH 7.5). Attempts to stabilize TDC further by the addition of $(NH_4)_2SO_4$ or sucrose have not been successful, as shown in Table I. Freezing of the purified enzyme at −70°C resulted in an immediate loss of activity. Likewise, freezing of purified tryptophan decarboxylase from *C. roseus* resulted in a rapid loss of activity (13). The loss of activity during freezing and/or thawing may be due to the relatively low protein content of the samples. Glycerol appears to stabilize the enzyme, but it also inhibits the enzyme considerably (Table I). However, during the removal of such a stabilizing agent (*e.g.* by gel filtration), a major loss of activity is observed due to the low protein content of the sample. Because of this instability, freshly

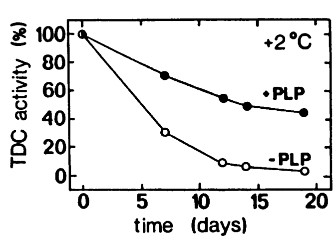

FIG. 1. Stability of purified TDC from *E. californica* as function of storage time in the absence and presence of 80 µM PLP, at 2°C. The storage buffer was 10 mM Tris-HCl (pH 8.4), containing 0.2 M $(NH_4)_2SO_4$, 1 mM DTT, and 0.1 mM EDTA.

Table I. *Relative Stability of Purified TDC from E. californica Stored in 35 mM Tris-HCl Buffer (pH 8.0), Containing 0.2 M $(NH_4)_2SO_4$, 80 µM PLP, 0.1 mM EDTA, and 1 mM DTT after Addition of Various Agents*

Addition	Storage Temperature	Storage Time			
		0	1	5	22
	°C		*d*		
None	2	100	95	53	34
	−70		30	14	ND[a]
30% Glycerol	2	52	52	50	52
	−70		23	9	ND
150 mM Sucrose	2	92	62	35	29
	−70		11	5	ND
3 M $(NH_4)_2SO_4$	2	85	52	21	21
	−70		48	30	ND

[a] Not determined.

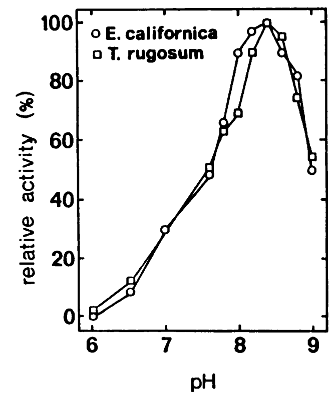

FIG. 2. Relative TDC activity as a function of pH. Buffers were composed of BIS-Tris, Hepes, and Tris each giving a concentration of 40 mM.

Table II. *K_m Values for Purified TDC from Plant Cell Cultures*

Plant Species	K_m			
	L-Tyrosine		L-DOPA	
	pH 8.4	pH 7.0	pH 8.4	pH 7.0
T. rugosum	0.27 ± 0.04[a]	ND[b]	0.24 ± 0.08[a]	ND
E. californica	0.25 ± 0.03[a]	0.97	ND	1.1

[a] SD, $n = 3$. [b] Not determined.

Table III. *Substrate Specificity of Purified TDC*

The substrate concentration was 0.8 mM in the standard assay mixture (pH 8.4).

Substrate	Relative Activity	
	T. rugosum	*E. californica*
	%	
L-Tyrosine	100	100
L-DOPA	74	ND[a]
L-Phenylalanine	<1	<1
L-Tryptophan	ND	0

[a] Not determined.

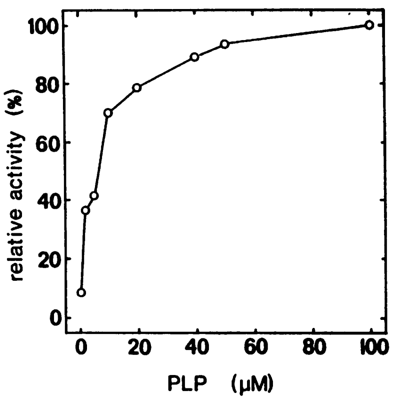

FIG. 3. Relative TDC activity as a function of PLP concentration.

Table IV. *Inhibition of Purified TDC from E. californica by Various Inhibitors*

The inhibitors were added at a concentration of 0.2 mM to the standard assay (pH 8.4). Results are the average of two independent experiments.

Inhibitor	Relative Activity	
	No preincubation	60 min preincubation[a]
	%	
None	100	89
α-Fluoromethyl-DOPA	87	86
α-Difluoromethyl-tyrosine	91	74
α-Aminooxy-β-phenylpropionate	7	4

[a] Before addition of substrate the enzyme was preincubated at 30°C with the inhibitor.

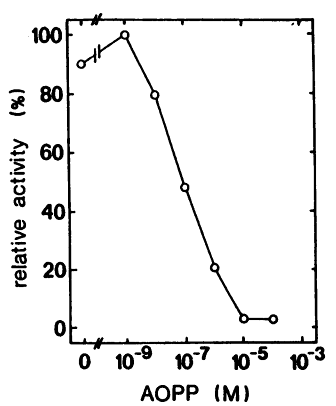

FIG. 4. Relative TDC activity as a function of AOPP concentration.

prepared enzyme (less than 3 d old) was used in the subsequent experiments.

pH Optimum. The pH optimum for the decarboxylation of L-tyrosine was at 8.4 (Fig. 2). Our measurements reflect quite well the pH dependence of the aromatic L-amino acid decarboxylase from hog kidney (3) but are in contrast to the data reported (9) for barley root TDC, which had a pH optimum at 7.3.

Substrate Specificity. Despite the differences in specific activity (11), the apparent K_m values of *Thalictrum* TDC for L-tyrosine (0.27 mM) and DOPA (0.24 mM) were close to the K_m of *Eschscholtzia* TDC for L-tyrosine (0.25 mM) (Table II). The pH optimum for DOPA decarboxylation was also at 8.4. TDC in desalted but not further purified extracts from *Eschscholtzia* cells had an apparent K_m value of 0.26 ± 0.08 (SD) mM ($n = 7$) for L-tyrosine, indicating that the purification procedure did not change the substrate affinity of the enzyme. With *Thalictrum*

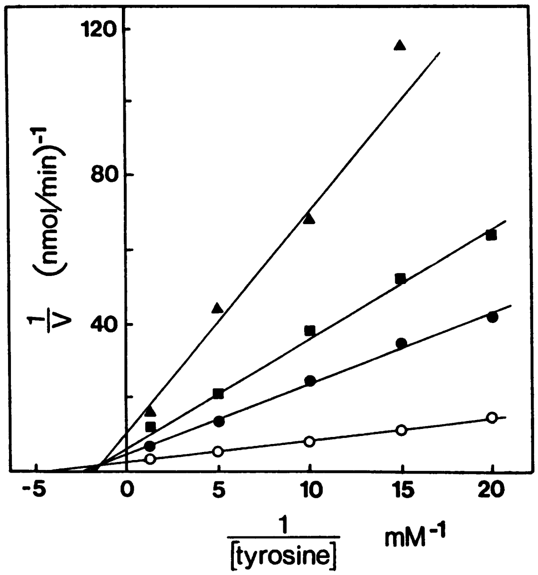

FIG. 5. Double-reciprocal plot of purified *Eschscholtzia* TDC in the presence of various concentrations of AOPP. (○), 0; (●), 0.3; (■), 0.5; and (▲), 1.0 μM AOPP.

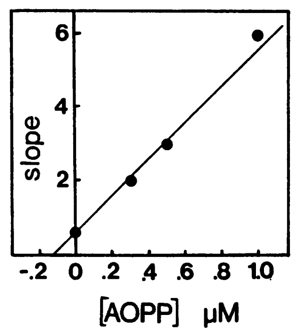

FIG. 6. Slopes of the lines in the double-reciprocal plot (Fig. 5) as a function of inhibitor concentration. The apparent K_i value for AOPP is obtained from the intercept on the base line.

TDC, the decarboxylation of DOPA as a function of incubation time was linear for at least 30 min at pH 7.0, whereas at pH 8.4 it was linear for only 10 min (data not shown), confirming an instability of DOPA at higher pH as mentioned in the literature (10). The decarboxylase activity of *Eschscholtzia* TDC toward DOPA was determined at pH 7.0. At this pH, the enzyme showed similar affinities for L-tyrosine and L-DOPA (Table II). L-Phenylalanine and L-tryptophan were virtually not decarboxylated by TDC (Table III).

Coenzyme Dependence. Addition of PLP stimulated *Eschscholtzia* TDC activity about 10-fold as illustrated in Figure 3. Barley root TDC activity was enhanced 4-fold by PLP but reached an activity plateau at 5 μM PLP (9), whereas *Eschscholtzia* TDC had to be supplied with around 50 μM PLP to reach maximum activity (Fig. 3). The activity of tryptophan decarboxylase from *C. roseus* was enhanced 2- to 3-fold by the addition of PLP (13).

Inhibitors. TDC from certain plants has been shown to be strongly inhibited by α-fluoromethyl-3,4-dihydroxyphenylalanine (a suicide inactivator [12]) and by the phenylalanine analog AOPP (2). *Eschscholtzia* TDC was largely unaffected by preincubation with α-fluoromethyl-(3,4-dihydroxyphenyl)-alanine and difluoromethyl-tyrosine (Table IV) but was inactivated by AOPP (Table IV; Fig. 4). The mode of AOPP inhibition of *Eschscholtzia* TDC is a mixed-type as illustrated in Figure 5. By plotting the slopes of the lines in Figure 5 against inhibitor concentration, an apparent K_i value of 135 nM AOPP may be calculated (Fig. 6). An apparent K_i value of 11 nM AOPP has been reported for crude TDC from *Syringa vulgaris* (2). The *Syringa* enzyme was also essentially unaffected by α-fluoromethyltyrosine or α-fluoromethyl-DOPA (2).

Concluding Remark. In comparing the properties of TDC isolated from *Eschscholtzia* or *Thalictrum* cell cultures, we found similarities for pH optimum, apparent K_m values, substrate specificity, and mol wt but differences in the induction pattern, specific activity, hydrophobic interaction during chromatography (11), and pI value.

The catalytic and molecular properties of TDC from plant sources are under further investigation in our laboratory. Furthermore, the presence of mRNA for this enzyme within the cells under various cultivation conditions is being investigated to evaluate the possible regulatory properties of TDC in isoquinoline alkaloid biosynthesis.

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