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# EXPRESSION IN *ESCHERICHIA COLI* AND PARTIAL CHARACTERIZATION OF TWO TYROSINE/DOPA DECARBOXYLASES FROM OPIUM POPPY

PETER J. FACCHINI and VINCENZO DE LUCA*

Institut de Recherche en Biologie Végétale, 4101 rue Sherbrooke est, Département de Sciences Biologiques, Université de Montréal, Montréal, Québec, HIX 2B2 Canada

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**Key Word Index**—*Papaver somniferum*; Papaveraceae; opium poppy; heterologous expression; L-aromatic amino acid decarboxylase; tyrosine/dopa decarboxylase; isoquinoline alkaloids.

**Abstract**—Two tyrosine/dopa decarboxylases (TYDC1 and TYDC2) from opium poppy (*Papaver somniferum*) were heterologously expressed in *Escherichia coli* and partially characterized. TYDC1 and TYDC2 are representative members of the two major isoform sub-classes of genes found in opium poppy which share less than 75% amino acid identity. Although both enzymes exhibit a marginal preference *in vitro* for L-dopa over L-tyrosine, the apparent $K_m$s of both TYDC1 and TYDC2 in total protein extracts for either substrate were equal ($K_m$s = 1 mM) at pH 7.2. Both TYDC1 and TYDC2 exhibited a similar broad pH optimum in the range 7.5–8.5, and their activity was enhanced in the presence of pyridoxal phosphate co-factor. The $V_{max}$ values for TYDC1 with either tyrosine or dopa as substrate were virtually identical ($V_{max}$ = 0.59 fkat mg$^{-1}$ protein), whereas, the $V_{max}$ for TYDC2 was two-fold greater with dopa ($V_{max}$ = 0.21 fkat mg$^{-1}$ protein) than with tyrosine ($V_{max}$ = 0.12 fkat mg$^{-1}$ protein) as substrate. Bacterial cell cultures expressing the TYDC1 polypeptide accumulated up to 350 $\mu$g ml$^{-1}$ tyramine and 360 $\mu$g ml$^{-1}$ dopamine in the medium within 8 hr after the addition of exogenous tyrosine or dopa, respectively. In contrast, cultures expressing the TYDC2 polypeptide accumulated 160 $\mu$g ml$^{-1}$ tyramine and 110 $\mu$g ml$^{-1}$ dopamine 8 hr after adding tyrosine or dopa, respectively. The higher *in vivo* conversion rates by bacterial cultures expressing TYDC1 relative to bacteria expressing TYDC2 is consistent with the higher specific activity of TYDC1 measured *in vitro*. At least two TYDC isoforms, each consistent with predicted molecular weights, were detected in 7-day-old opium poppy seedlings with a polyclonal antiserum for tryptophan decarboxylase from *Catharanthus roseus* (periwinkle). A comparison of hydropathy profiles revealed extensive structural similarities between the two opium poppy isoforms and other aromatic amino acid decarboxylases with different substrate specificities.

## INTRODUCTION

Tyrosine and dopa decarboxylases (TYDC, DODC; EC 4.1.1.28) catalyse the conversion of L-tyrosine and L-dopa to tyramine and dopamine, respectively (Fig. 1). In a large number of related plant species tyramine and dopamine serve as distant precursors to the structurally diverse class of natural products known as isoquinoline alkaloids [1]. Although the biological roles of most isoquinoline alkaloids are unknown, some, such as sanguinarine, may function as antibiotic phytoalexins [2] and others, such as morphine and berberine, produce potent pharmacological effects. Although isoquinoline alkaloid biosynthesis is mostly restricted to Papaveraceae, Berberidaceae, Ranunculaceae and Menispermaceae, TYDC and DODC activities are found in plants from a wide variety of other families [3–7]. Recently, the rapid and transient transcriptional induction of TYDC has been demonstrated in parsley [3] and *Arabidopsis thaliana* [4]. Moreover, the incorporation of tyramine and its 4-coumaroyl- or feruloyl-conjugated derivatives into plant cell walls has been described [5]. The biosynthesis of conjugated aromatic amides via the oxidative polymerization of tyramine and dopamine may represent a common defense mechanism activated in plants that have been challenged by a pathogen.

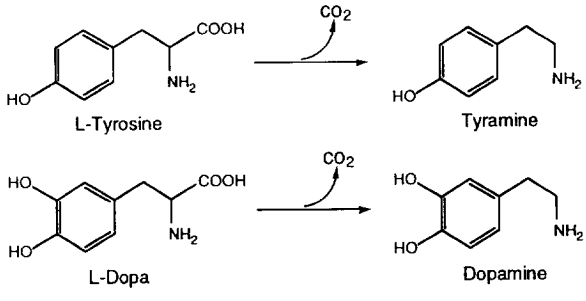


Fig. 1. Enzymatic conversions catalysed by L-tyrosine and L-dopa decarboxylases.

*Author to whom correspondence should be addressed.

Recently, we have described the isolation and characterization of a gene family for TYDC and DODC in opium poppy (*Papaver somniferum*) and demonstrated the differential and tissue-specific regulation of its members [8]. Based on amino acid homology, isolated members of the opium poppy TYDC gene family were categorized into two groups which share less than 75% sequence identity. One representative clone from each group was expressed as a β-galactosidase fusion protein in *Escherichia coli* and the two catalytically active polypeptides were used to determine substrate specificity. Although both enzymes showed marginally higher preference for L-dopa over L-tyrosine, neither would accept L-phenylalanine or L-tryptophan as substrates. Other aromatic amino acid decarboxylases such as tryptophan decarboxylase (TDC) from *Catharanthus roseus* [9], TYDC from parsley [3] and *Thalictrum rugosum* [10], DODC from *Drosophila melanogaster* [11, 12], and histidine decarboxylase (HDC) from humans [13] also share extensive homology but typically exhibit different substrate affinities.

To assess the possible regulatory function of TYDC/DODC in isoquinoline alkaloid biosynthesis in opium poppy, the catalytic properties of the major isoforms must be determined. In this paper the enzymatic properties of two heterologously expressed opium poppy TYDC isoforms [8] are partially characterized and their detection in opium poppy plants is verified. The *in vivo* activity and substrate specificity of the plant enzymes in cultured *E. coli* cells is demonstrated. Finally, the TYDC/DODC isoforms from opium poppy are compared to aromatic amino acid decarboxylases from other organisms to identify features which may confer substrate acceptance and specificity.

## RESULTS AND DISCUSSION

### *Partial characterization of enzyme activity and structure*

The substrate specificity of numerous plant and animal L-aromatic amino acid decarboxylases has been described. Animal aromatic amino acid decarboxylases typically exhibit a preference for dopa, but will also accept to a lesser extent a range of other substrates including tyrosine, phenylalanine, tryptophan, and numerous derivatives thereof [14]. In contrast, plant aromatic amino acid decarboxylases show a distinct preference for specific substrates. DODC in *Cytisus scoparius* [7] is virtually inactive toward any substrate other than dopa, whereas TDC from *C. roseus* accepts only tryptophan [15]. TYDC from parsley will accept tyrosine and, to a much lesser extent, dopa but not tryptophan or phenylalanine [3]. Tyrosine is also preferred over dopa as a substrate for TYDCs from *T. rugosum* and *Eschscholtzia californica* [16] and TYDC from *Syringa vulgaris* [17], however, the proportional acceptance of dopa relative to tyrosine is at least two-fold greater than that in parsley. The marginal preference of TYDC isoforms in opium poppy for dopa over tyrosine [8] further demonstrates the diversity in substrate specificity among plant aromatic amino acid decarboxylases (Table 1).

Double-reciprocal plots with different concentrations of either L-tyrosine or L-dopa as substrates at a fixed concentration of total soluble enzyme extract were used to determine apparent $K_m$ and $V_{max}$ values for opium poppy TYDC1 and TYDC2 expressed as β-galactosidase fusion proteins in *Escherichia coli* (Fig. 2). The apparent $K_m$s at pH 7.2 of both TYDC1 and TYDC2 for either L-tyrosine or L-dopa were 1 mM. The apparent $V_{max}$ values for the conversion of both L-tyrosine and L-dopa to tyramine and dopamine, respectively, by TYDC1 were virtually identical at 0.59 fkat $mg^{-1}$ protein. However, the apparent $V_{max}$ for the conversion of L-dopa to dopamine by TYDC2 was 0.21 fkat $mg^{-1}$ protein, whereas the apparent $V_{max}$ for the conversion of L-tyrosine to tyramine was 0.12 fkat $mg^{-1}$ protein. The TYDC1 and TYDC2 activities which catalyse the conversion of tyrosine to tyramine in transformed *E. coli* total protein extracts both showed a broad pH optimum in the range of 7.5–8.5 using a combination of Bis–Tris, Tris and glycine buffers (Fig. 3A). Standard assays for determination of reaction constants were performed in Bis–Tris buffer at pH 7.2 because of (a) the improved stability of dopa in this buffer relative to its stability in Tris, (b) the instability of dopa in alkaline conditions, and (c) the broad pH optimum range. TYDC activity was also enhanced with pyridoxal phosphate although a high level

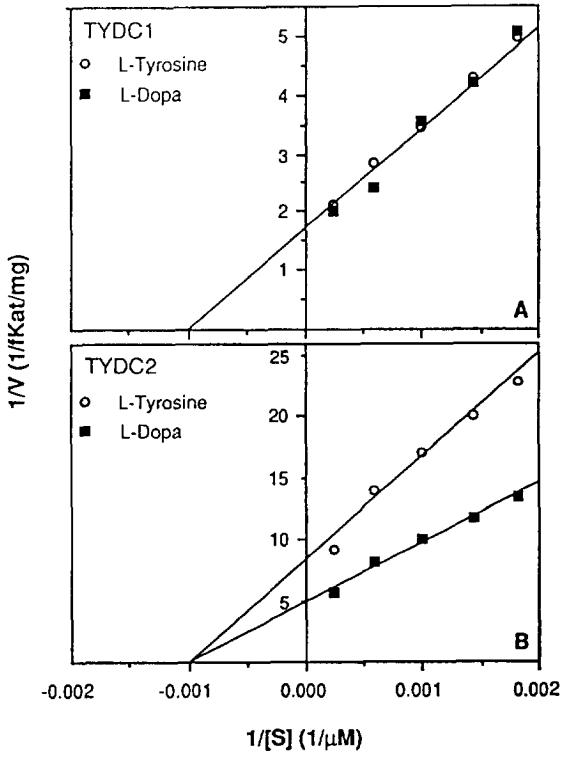


Fig. 2. Double-reciprocal plots of substrate concentration ($S$) vs specific enzyme activity ($V$) for L-tyrosine (○) and L-dopa (■) saturation of (A) TYDC1 and (B) TYDC2 expressed in *E. coli*.

Table 1. Comparison of relative substrate specificity and $K_m$ values for various plant aromatic amino acid decarboxylases

| Species | Relative enzyme activity (%) | | | $K_m$ (mM) | | Reference |
|---|---|---|---|---|---|---|
| | Tyr | Dopa | Tryp | Tyr | Dopa | |
| *Papaver somniferum* | 90 | 100 | 0 | 1.0 (7.2) | 1.0 (7.2) | [8] |
| *P. somniferum* | 65 | 100 | 0 | 1.0 (7.2) | 1.0 (7.2) | [8] |
| *Petroselinum crispum* | 100 | 28 | 0 | nd | nd | [3] |
| *Thalictrum rugosum* | 100 | 74 | 0 | 0.27 (8.4) | 0.25 (8.4) | [16] |
| *Eschscholtzia californica* | 100 | nd | 0 | 1.0 (7.0)<br>0.24 (8.4) | 1.1 (7.0) | [16] |
| *Syringa vulgaris* | 100 | 63 | 0 | 0.33 (7.5) | nd | [17] |
| *Sanguinaria canadensis* | 3 | 100 | 0 | nd | nd | [17] |
| *Hordeum vulgare* | 33 | 100 | 0 | nd | nd | [17] |
| *Cytisus scoparius* | 0 | 100 | 0 | nd | nd | [7] |
| *Catharanthus roseus* | 0 | 0 | 100 | tryp = 0.075 (7.5) | | [15] |

Numbers in parentheses refer to pH at which $K_m$ was determined.
tyr, Tyrosine; dopa, dihydroxyphenylalanine; tryp, tryptophan; nd, not determined.

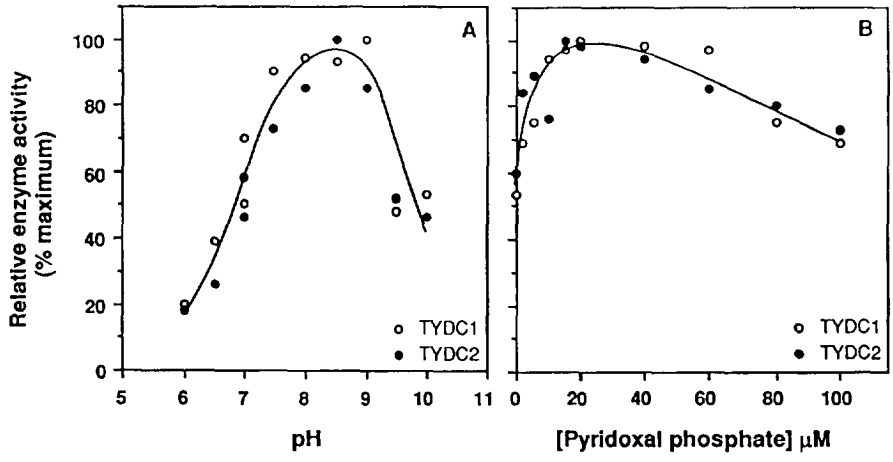


Fig. 3. (A) pH profile and (B) pyridoxal phosphate dependence of the catalytic activity of TYDC1 and TYDC2 expressed in *E. coli*.

of activity was detected even in the absence of the co-factor (Fig. 3B). The absolute dependence of plant aromatic amino acid decarboxylases on pyridoxal phosphate appears to be variable depending on protein source and assay conditions [15–17]. Lower dependence is typically observed in crude extracts probably due to the presence of endogenous co-factor.

Relatively few $K_m$ values have been determined for plant aromatic amino acid decarboxylases. However, the TYDC1 and TYDC2 apparent $K_m$ values of 1 mM for both tyrosine and dopa (Fig. 2) at pH 7.2 are consistent with those of 1.0 and 1.1 mM obtained for tyrosine and dopa, respectively, at pH 7.0 for *E. californica* TYDC which was purified to homogeneity [16]. At the determined pH optimum of 8.4 the apparent $K_m$s for TYDC from *E. californica* and *T. rugosum* were determined to be in the range of 0.24–0.27 mM for both tyrosine and dopa. Similarly, the apparent $K_m$ for TYDC from *S. vulgaris* at pH 7.5 was found to be approximately 0.33 mM for tyrosine [18]. However, both TYDC1 and TYDC2 exhibit substrate affinities at sub-optimal pH that are consistent with those determined for other plant TYDCs and DODCs under similar conditions.

The heterologously expressed opium poppy TYDCs exhibit many characteristics typical of native tyrosine and dopa decarboxylases from plants. The additional 28 amino acids fused to the *N*-terminus of the heterologously expressed proteins do not appear to significantly alter the function of the enzymes. Addition of protein extract from untransformed *E. coli* to total protein extracts from

opium poppy had no effect on the TYDC activity in the plant extracts (data not shown). Thus, there do not appear to be factors in the *E. coli* extracts that inhibit enzyme activity. The significance of the observed difference between TYDC1 and TYDC2 enzyme function *in vitro* is unknown. However, it may be relevant to the possible function of specific TYDC isoforms in the regulatory networks of different secondary metabolic pathways. In addition, the possibility cannot be excluded that each isoform is exposed to only one substrate *in vivo* or that specific *in vivo* functions are not accurately represented *in vitro*.

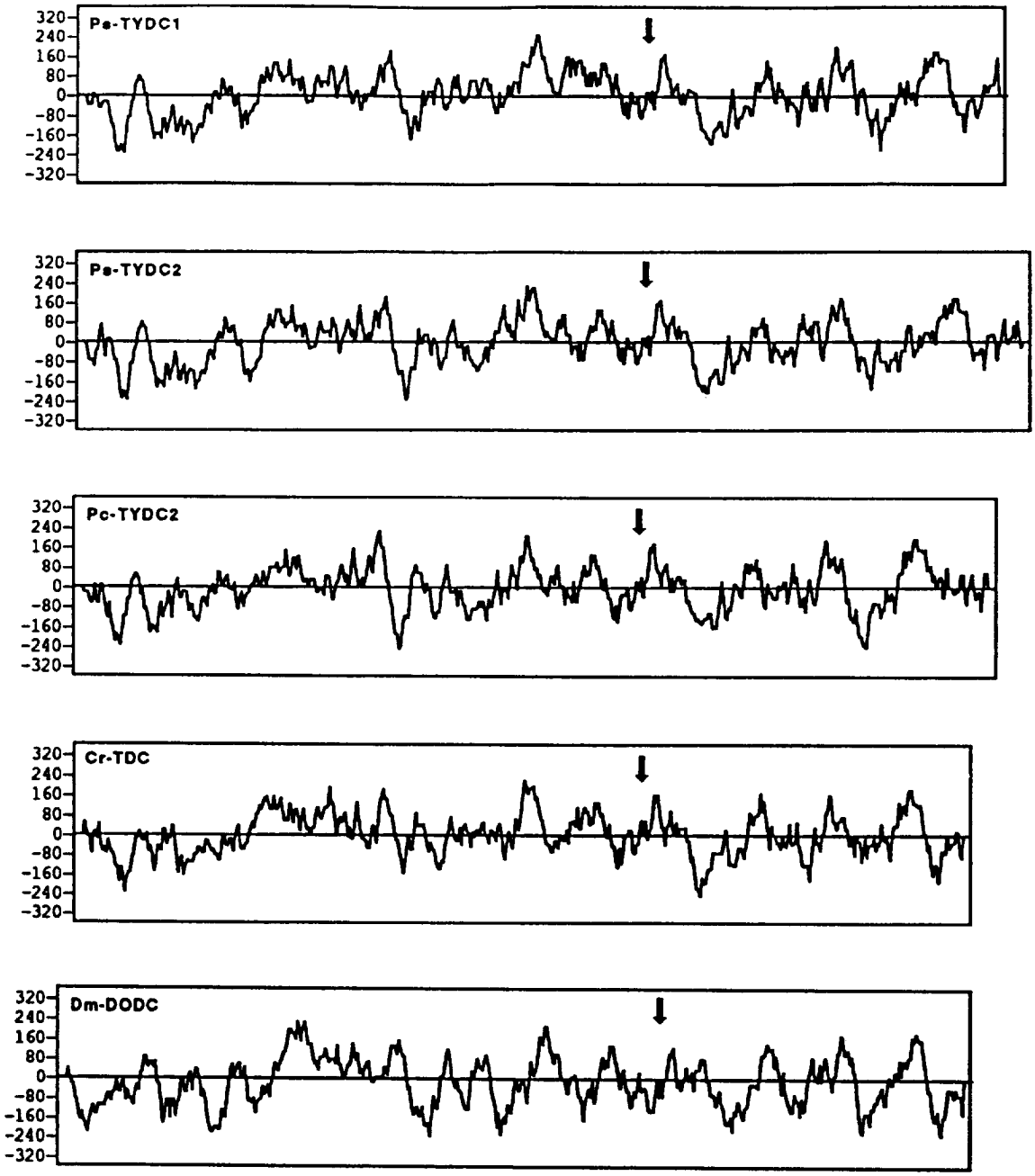


Fig. 4. Hydropathy profiles for Ps-TYDC1 and Ps-TYDC2, two tyrosine/dopa decarboxylases from opium poppy; Pc-TYDC2, a tyrosine decarboxylase from parsley [3]; Cr-TDC, a tryptophan decarboxylase from *Catharanthus roseus* [9]; and Dm-DODC, a dopa decarboxylase from *Drosophila melanogaster* [11, 12]. Each value was calculated as the average hydrophobic index of a sequence of nine amino acids and plotted to the middle residue of each sequence. Positive and negative values indicate hydrophobic and hydrophilic regions of the proteins, respectively [19]. The location of the putative pyridoxal phosphate binding site is indicated with an arrow on each protein.

The extensive structural fidelity of all aromatic amino acid decarboxylases, regardless of substrate specificity, is underlined by their remarkably similar hydropathy profiles (Fig. 4). For example, DODC from *D. melanogaster* [11, 12] which accepts a broad range of aromatic amino acid substrates shares only about 40% amino acid homology with plant aromatic amino acid decarboxylases that exhibit a high degree of substrate specificity. However, few regions that are clearly distinct in hydrophobic profile can be found in the *D. melanogaster* DODC when it is compared with plant TYDCs, DODCs, and TDC (Fig. 4). In addition, the extensive similarity in hydropathic character between TDC from *C. roseus* [9] and TYDCs from opium poppy [8] and parsley [3] is in contrast to their unique substrate specificities. Moreover, despite a divergence in amino acid sequence of more than 25%, opium poppy TYDC1 and TYDC2 have very similar catalytic functions. These data suggest that the substrate specificity observed for various aromatic amino acid decarboxylases is the result of relatively minor amino acid substitutions rather than alterations to major protein domains.

*Detection of TYDC isoforms in opium poppy protein extracts*

Polyclonal antibodies raised against tryptophan decarboxylase (TDC) from *Catharanthus roseus* [20] have been shown to cross-react with heterologously expressed TYDC proteins from opium poppy [8]. Total soluble proteins from 7-day-old light-grown opium poppy seedlings were extracted and, following fractionation by SDS–PAGE, were transferred to a nitrocellulose membrane. Proteins of various sizes showed cross-reactivity with TDC-specific polyclonal antibodies (Fig. 5). At least two prominent protein bands were detected in the $M_r$ 55 000–60 000 range. The deduced amino acid sequences of TYDC1 and TYDC2 predict proteins of 56 900 and 59 300 [8]. Other minor immunoreactive proteins are also observed in Fig. 5 but their relationship, if any, to the TYDC proteins is unknown. However, the sizes and pattern of the immunoreactive proteins detected in Fig. 5 are similar to those detected with the same polyclonal antiserum in protein extracts from *C. roseus* [20]. The synthesis and turnover of TDC in *C. roseus* appears to be highly regulated and recent evidence suggests that ubiquitination of TDC precedes degradation [21]. Considering their extensive similarities, TYDCs in opium poppy, like TDC from *C. roseus*, may also be ubiquitinated prior to proteolysis. Thus, the other immunoreactive polypeptides in Fig. 5 may represent ubiquitinated TYDC and its subsequent proteolytic products.

In vivo *activity of tyrosine/dopa decarboxylase in* E. coli

Cultures of *E. coli* harboring the pBluescript-TYDC1 and pBluescript-TYDC2 constructs were induced with IPTG and fed exogenous L-tyrosine, L-dopa, or L-tryptophan to a final concentration of 1 mM. Eight hours subsequent to the addition of substrates the corresponding reaction products were detected in the culture medium by direct HPLC analysis (Fig. 6). Exogenously added tyrosine was almost completely converted to tyramine by bacteria expressing the TYDC1 fusion, whereas only about 25% was converted to tyramine by cultures expressing the TYDC2 fusion. Similarly, more than 80% of the exogenously added dopa was converted to dopamine by the TYDC1 fusion, but less than 20% was converted to dopamine by the TYDC2 fusion. Tyramine also accumulated in the dopa- and tryptophan-supplemented culture medium of cells harbouring both the pBluescript-TYDC1 and pBluescript-TYDC2 constructs, although the amounts produced by bacteria expressing TYDC2 were only about 50% of those produced by cells expressing TYDC1. Tyramine accumulation in dopa- and tryptophan-supplemented cultures was representative of its accumulation in control cultures with no exogenous amino acids in the media (data not shown). Thus, cells expressing TYDC1 and TYDC2 produced large amounts of tyramine either from their endogenous tyrosine pools or from small amounts of tyrosine available in the Luria-Bertani culture medium. Neither TYDC1- nor TYDC2-expressing cultures were able to

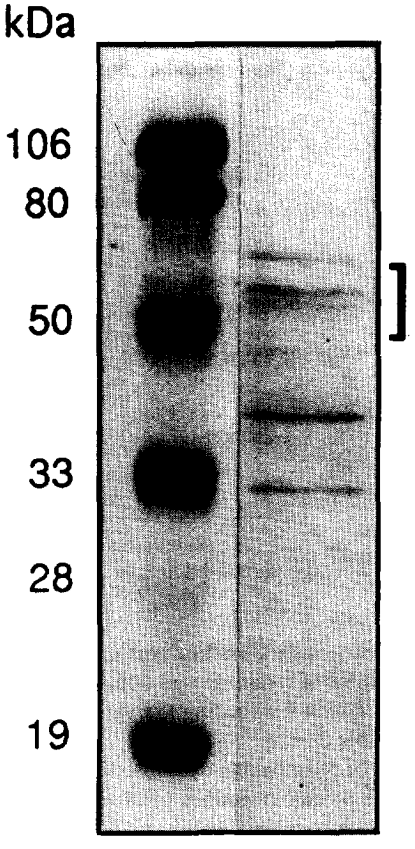


Fig. 5. Immunoblot detection of tyrosine/dopa decarboxylase isoforms in total soluble protein extracts from 7-day-old light-grown opium poppy seedlings. Immunoreactive proteins in the expected range of 55 000–60 000 are indicated. Bands that appear in the lane on the left are $M_r$ standards stained with Coomassie Brilliant Blue.

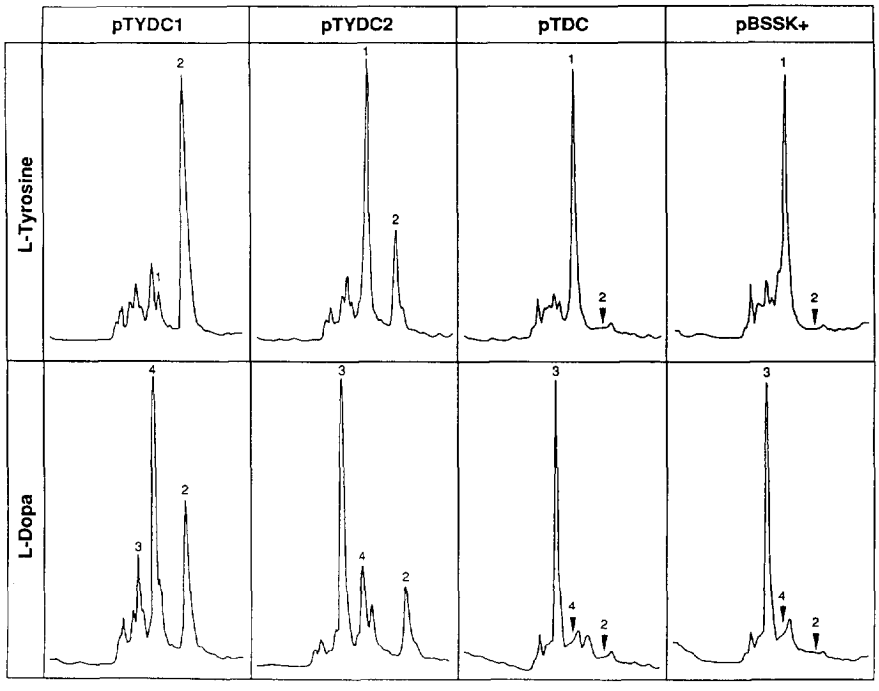


Fig. 6. HPLC analysis of L-tyrosine- or L-dopa-supplemented nutrient medium from *E. coli* cell cultures transformed with pBluescript-TYDC1 (pTYDC1), pBluescript-TYDC2 (pTYDC2), pTDC5 (pTDC), and untransformed pBluescript ($SK^+$) (pBSSK$^+$). Identification of peaks: 1, L-tyrosine; 2, tyramine; 3, L-dopa; 4, dopamine. The expected location of tyramine and dopamine peaks, when absent, are indicated with arrows.

Table 2. Aromatic amine concentrations in the culture medium of *Escherichia coli* cells expressing TYDC1 and TYDC2 polypeptides

| Heterologous expressed enzyme | Exogenous added substrate | Amount of product in medium ($\mu g\,ml^{-1}$) | | |
|---|---|---|---|---|
| | | Tyramine | Dopamine | Tryptamine |
| TYDC1 | L-Tyrosine | 350 | 0 | 0 |
| | L-Dopa | 270 | 360 | 0 |
| | L-Tryptophan | 250 | 0 | 0 |
| TYDC2 | L-Tyrosine | 170 | 0 | 0 |
| | L-Dopa | 160 | 110 | 0 |
| | L-Tryptophan | 140 | 0 | 0 |

Exogenous substrates were added to a final concentration of 1 mM and the medium sampled 8 hr later.

convert exogenously added L-tryptophan to tryptamine. Concentrations of tyramine and dopamine in the culture medium of *E. coli* harbouring the various constructs are listed in Table 2.

Analysis of cell extracts demonstrated that more than 95% of the aromatic amines produced after 8 hr were exported to the culture medium. Time course studies showed that maximum levels of product accumulation in the medium were obtained within 6 hr after the addition of exogenous substrates (data not shown). No tyramine or dopamine was detected in cell extracts or in the medium of *E. coli* cultures transformed with pTDC5 or non-recombinant pBluescript ($SK^+$) and fed exogenous tyrosine or dopa (Fig. 6). The inability of heterologously expressed *C. roseus* TDC to accept tyrosine or dopa as substrates *in vivo* is consistent with its substrate specificity determined using the purified plant protein [15]. The lack of any accumulation of aromatic amines in the medium of pBluescript-transformed cultures demonstrates that *E. coli* does not possess endogenous aromatic amino acid decarboxylase activity.

Tyramine, $\beta$-hydroxytyramine (octapamine), and other substituted phenylethylamines have been reported to be toxic to tobacco cell cultures grown in the presence of auxins [22]. The toxicity of certain aromatic amines in plants may be due to their oxidation by phenoloxidases

resulting in the formation of indolequinones [23]. Tyramine and dopamine themselves do not appear to be directly toxic since some plants, including opium poppy, have been reported to accumulate large amounts of dopamine [24]. As it is also unlikely that *E. coli* contains phenoloxidases capable of catalysing the conversion of tyramine or dopamine to indolequinones, it is not surprising that the high levels of these substituted phenylethylamines in the culture medium do not appear to be toxic to the bacterial cells. In addition, despite the possible contribution of the endogenous tyrosine pool to the production of exported tyramine (Fig. 6 and Table 2), *E. coli* cells appear to be capable of compensating for this loss. No obvious difference in growth rates was observed between tyramine producing cultures (cells harbouring pBluescript-TYDC1 and pBluescript-TYDC2) and tyramine non-producing cultures [cells harbouring pTDC5 and pBluescript ($SK^+$)]. Over-expression of opium poppy TYDC genes in plants is currently in progress in our laboratory in order to further investigate effects of metabolic diversion as shown here in transformed bacteria.

## EXPERIMENTAL

*Expression vector construction and bacterial cell cultures.* The open reading frames of TYDC1 and TYDC2 were amplified by PCR with specific primers designed to incorporate flanking 5′ *Xba*I and 3′ *Sal*I restriction endonuclease sites, and were subsequently inserted into pBluescript ($SK^+$) as described previously [8]. Both constructs contain a 28 amino acid β-galactosidase peptide at the N-terminus of the expressed TYDC protein. *Escherichia coli* XL-1 Blue cells harbouring the pBluescript-TYDC1 and pBluescript-TYDC2 constructs, were grown at 30° in Luria–Bertani medium to $A_{600} = 0.5$, and expression of the fusion proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were collected 1 hr after addition of IPTG, centrifuged to remove the medium, and the pellets were washed with 50 mM Bis–Tris, pH 7.2 then frozen at − 80° until used for analysis. As controls, *E. coli* XL-1 Blue cells harbouring pTDC5 [9], a pBluescript vector containing a *C. roseus* TDC cDNA and capable of directing the expression of a catalytically active TDC, and non-recombinant pBluescript ($SK^+$) were grown, induced, and processed under identical conditions [8].

*Enzyme assays.* Transformed *E. coli* extracts were assayed for decarboxylase activity by measuring the release of $^{14}CO_2$ from L-*carboxyl*-$^{14}$C-labelled L-tyrosine and L-dihydroxyphenylalanine (L-dopa), as described previously [8, 25, 26]. Enzymatically liberated $^{14}CO_2$ was trapped on quaternary ammonium-saturated GF/A filter disks suspended above the reaction solution in air-tight vials. Bacterial cells were lysed by sonication in 200 mM Bis–Tris, pH 7.2, debris was removed by centrifugation and the supernatant was desalted by passage through a PD-10 column (Pharmacia). The standard assay mixt. for decarboxylase activity contained 50 mM Bis–Tris, pH 7.2, 1 mM EDTA, 25 μM pyridoxal 1-phosphate, 0.1 μCi (specific activity, 55 mCi mmol$^{-1}$ Ci = 37 GBq) radiolabelled aromatic amino acid substrate, additional cold aromatic amino acid to bring the substrate concentration to the specified value, and 250 μl of protein extract in a total volume of 1 ml. Buffers for pH optimum assays were 100 mM each of Bis–Tris (pH 6–7), Tris (pH 7–9), and glycine (pH 9.5–10). All reactions were incubated for 60 min at 35° with constant agitation to liberate the released $^{14}CO_2$ from the aq. soln. The reactions were stopped by the addition of 0.2 M HCl and agitated for an additional 1 hr before scintillation counts from the GF/A filters were determined. Total protein concn of bacterial enzyme extracts was determined by the method of ref. [27].

*Precursor additions to bacterial cell cultures.* For precursor-feeding expts, bacterial cultures were grown and induced as described for enzyme extraction. However, exogenous L-tyrosine, L-dopa, and L-tryptophan were added to the cell culture medium 1 hr after IPTG induction. Cultures were grown for 8 hr at 30° in the presence of exogenous substrates. Subsequently, medium samples were collected after removal of cells by centrifugation. Cell pellets and medium samples were frozen separately at − 80° until analysed. Bacterial cells were extracted by sonication in methanol. Cell debris was removed by centrifugation and the solvent was evapd to dryness under red. pres. The dry extracts were redissolved in methanol for analysis. Medium samples were analysed directly.

*High pressure liquid chromatography.* Substrates (L-tyrosine, L-dopa, and L-tryptophan) and *in vivo* reaction products (tyramine, dopamine and tryptamine) in transformed bacterial cultures were identified and quantitated by HPLC on a Waters 600E HPLC system and Waters 991 photodiode array detector. Compounds were sepd on a Waters Nova Pak C18 reversed phase column (3.9 × 300 mm) at 1200 psi with MeOH–$H_2O$–HOAc (20:179:1, pH 3.4) isocratic gradient and detected by their absorbance at 280 nm. Ten microlitres of bacterial cell extract in methanol or 10 μl of cell-free medium were injected onto the column. Peaks were identified from UV spectra and by comparison of $R_t$s to those of known standards.

*Detection of TYDC isoforms in opium poppy.* Seven-day-old light-grown (3 days dark followed by 4 days with a 16 hr photoperiod) poppy seedlings were ground to a fine powder under liquid $N_2$. Total soluble proteins were extracted in the presence of polyvinylpolypyrrolidone (PVPP) in 200 mM Bis–Tris, pH 7.2 and 28 mM β-mercaptoethanol. Debris was removed by centrifugation and the supernatant was desalted on a PD-10 (Pharmacia) column. Total protein concn was determined [27] and 25 μg aliquots were diluted 3:1 with acetone and pptd overnight at − 80°. Samples were centrifuged and the protein pellets were dried under red. pres. The pellets were solubilized in SDS sample buffer [0.1 M Tris–HCl, pH 6.8, 1.6% (v/v) glycerol, 0.008% Bromphenol Blue, 4 mM EDTA, 10 mM β-mercaptoethanol, 3% (w/v) SDS], sepd on a 12% SDS–polyacrylamide gel, transferred to nitrocellulose, and immunodetected as described in ref. [28].

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