Plant Molecular Biology 3: 281–288 (1984).

Tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures: purification, molecular and kinetic data of the homogenous protein

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Keywords: *Catharanthus roseus*, cell culture, tryptophan decarboxylase, enzyme induction, purification

Abstract

The purification of tryptophan decarboxylase from *Catharanthus roseus* (TDC, E.C.: 4.1.1.27), to apparent homogeneity, is described. The enzyme represents a soluble protein with a molecular weight of 115 000 ± 3 000, consisting of 2 identical subunits of 54 000 ± 1 000. The pI was estimated to be 5.9 and the K_m for L-tryptophan was found to be 7.5×10^{-5} M. Phenylalanine, tyrosine and DOPA were not decarboxylated by tryptophan decarboxylase from *Catharanthus* cells. Similar to the aromatic amino acid decarboxylase from hog kidney the enzyme does not appear to be obligatorily dependent on exogenously supplied pyridoxal phosphate, as it seems to contain a certain amount of this cofactor. The average percentage of TDC in the cells was found to be 0.002% in the growth medium while the level increased up to 0.03% when indole alkaloid biosynthesis was induced. The role of the protein as a bottleneck enzyme of indole alkaloid biosynthesis is discussed.

Introduction

Tryptophan decarboxylase (TDC, E.C.: 4.1.1.27) catalyzes the conversion of L-tryptophan to tryptamine. This reaction represents a branching point from primary metabolism into a secondary pathway and seems to play an important role for the carbon flow into this pathway. Thus, it has been shown that all medium composition changes leading to increased indole alkaloid formation by suspension cultures of *Catharanthus roseus* were accompanied by increased activities of TDC (Knobloch *et al.* 1981). A similar strong correlation between enhanced TDC activity and accumulation of harman alkaloids and serotonin was found with *Peganum harmala* suspension cultures (Sasse *et al.* 1982). The conclusion that enzymes linking primary and secondary metabolism may play a decisive regulatory control for the expression of secondary pathways, can also be drawn from other systems (Heller *et al.* 1979; Selby *et al.* 1980; Berlin *et al.* 1982).

To unravel the regulatory function of these enzymes and to remove the regulatory control it is necessary to obtain more information relating to the molecular properties of such enzymes and the corresponding genes. Of the enzymes, diverting primary metabolites into secondary pathways, only phenylalanine ammonia lyase has been characterized up to the gene level in Hahlbrock's group. Although TDC activity has been measured in various higher plant systems (Gibson *et al.* 1972; Baxter & Slaytor 1972; Scott & Lee 1975; Grosse & Klapheck 1979), only partial purification has been achieved because of its instability in these systems. From our previous work it was known that TDC was inducible in suspension cultures of *Catharanthus roseus* with a maximal activity 48 h after transferring the cells into IM2-medium (Berlin *et al.* 1983). In this paper we describe the purification of TDC to apparent homogeneity and the investigation of some catalytic and molecular properties of the enzyme.

Materials and methods

Cell culture

Cell suspension cultures from *Catharanthus roseus* were propagated as described previously (Knobloch & Berlin 1980). Induction of TDC was achieved by transferring approx. 1 kg (fr. wt.) of 14-day old *Catharanthus roseus* cells from MX-medium into 20 l of IM2-medium (Berlin *et al.* 1983) in a BRAUN air lift fermenter for 2–3 days. The cells were harvested by suction and preserved in liquid nitrogen.

Purification of TDC

Frozen cells (up to 1.5 kg) were thawed under stirring in 2 l buffer A containing 50 g DOWEX 1×2 Cl^-) and homogenized in a DYNO-MILL with glass beads. After centrifugation (30', 13 000 **g**) the supernatant was subjected to fractionated ammoiun sulfate precipitation. The resulting pellet from 42–55% saturation (4 °C), containing all TDC activity, was resuspended in 200 ml buffer A and desalted on a SEPHADEX-G 25 column (100×5 cm). The desalted protein solution was applied first to a DEAE-SEPHACEL column (40×5 cm) and the enzyme was eluted with a linear gradient of KCl in buffer A (0–0.1 M, 0.02 M/h, 3 ml/min). The fractions, containing more than 80% of TDC activity were concentrated by ultrafiltration (Amicon) and subjected without dialysis to a hydroxylapatite column (40×5 cm) which had been equilibrated with buffer C. The proteins were eluted with a linear gradient of K_2HPO_4 in buffer C (0.01–0.05 M, 0.01 M/h, 3 ml/min. The TDC-active fractions were combined, concentrated to about 10 ml by ultrafiltration and applied to a ACA-34 column (100×5 cm, LKB) and eluted with degassed buffer C (1 ml/min). TDC-active fractions were then chromatographed on a TSK-4 000 column (600×21.5 cm, LKB) with degassed buffer C (1 ml/min). The combined TDC fractions were further purified with the MONO-Q column (5×0.5 cm), Pharmacia) with a linear gradient of KCl (0–0.5 M, 1 M/h, 0.5 ml/min) in buffer D. Finally, the enzyme was chromatographed on a TSK 3 000 column (600×7.5 cm, LKB) with degassed buffer C (0.2 ml/min). After concentration by ultrafiltration, TDC was tested for homogeneity by analytical disc gel electrophoresis. All dat listed in Table 1 represent volumes and concentration after the purification steps. The purification procedure was carried out at 4 °C.

Slab gel electrophoresis

Slab gel electrophoresis (7.5% polyacrylamide in the resolving gel) was performed in the system described by Laemmli (1970) and Maizel (1971) under native and denaturing conditions. To determine the MW of the TDC and TDC subunits, electrophoresis was carried out with the enzyme, eluted from TSK 3 000 (Fig. 1c). The conditions for electrophoresis were the same as described above, except that under denaturing conditions running buffer as well as the gel contained 0.1% SDS. The molecular weight of TDC and TDC subunits was estimated after staining with the silver method (Merril *et al.* 1981) or with Coomassie Brilliant Blue R 250 using marker proteins for calibration (HMW/LMW kit, Pharmacia).

Table 1. Purification scheme for tryptophan decarboxylase (TDC). Starting conditions: 1.5 kg of 'induced' *Catharanthus roseus* cells yielding 3.5 l crude extract, approximately. The results in Table 1 represent data received from concentrated solutions after the individual purification steps. Table 1 represents mean values obtained from 3 different purifications of TDC.

Enzyme fraction	Volume (ml)	Total protein (mg)	Enzyme activity (katal)	Specific activity (katal/mg)	Purification (fold)		Yield (%)
					(mg)	(sp. act)	
Crude extract	3 500	35 000	5.2×10^{-7}	1.48×10^{-11}	–	–	100
$(NH_4)_2SO_4$-precipitation (45–55%)	165	3 210	4.5×10^{-7}	1.40×10^{-10}	11	10	90
DEAE-Sephacel	18	1 020	3.8×10^{-7}	3.72×10^{-10}	35	26	74
Hydroxylapatite	4	454	3.0×10^{-7}	6.6×10^{-10}	78	46	60
AcA-34	2	165	1.5×10^{-7}	9.1×10^{-10}	214	64	30
TSK-4 000	1.3	18	0.8×10^{-7}	4.4×10^{-9}	1 964	342	16
MONO-Q	0.6	3.7	0.3×10^{-7}	8.1×10^{-9}	9 550	642	7
TSK-3 000	0.4	1.1	0.2×10^{-7}	1.8×10^{-8}	32 141	1 565	5

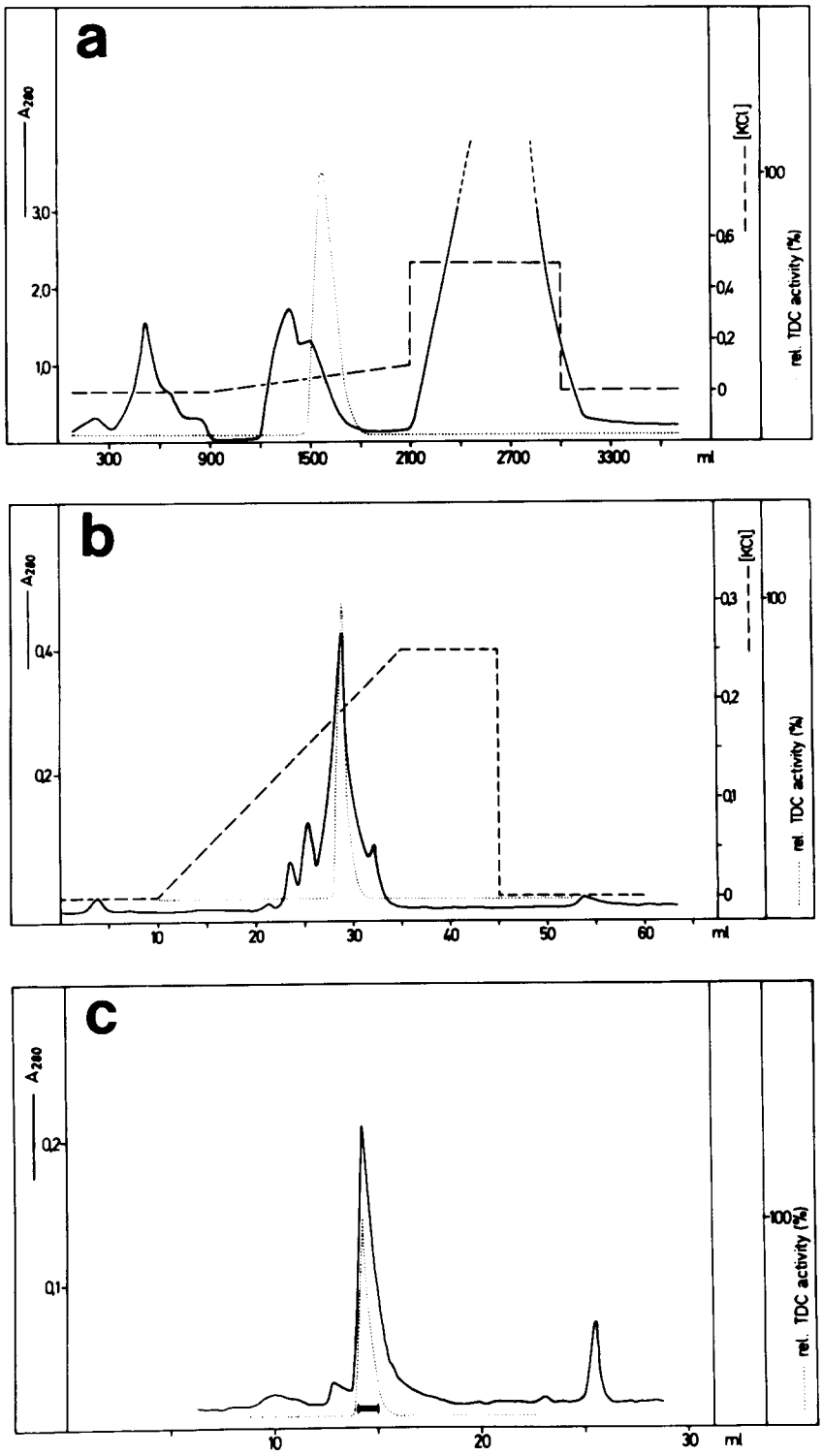

Fig. 1. a) Elution profile of tryptophan decarboxylase activity from DEAE-Sephacel (40 × 5 cm: 0–0.1 M KCl, 0.02 M/h, 3 ml/min).
b) Elution profile of TDC activity from an MONO-Q column (5 × 0.5 cm: 0–0.5 M, 1 M/h, 0.5 ml/min).
c) Elution profile of TDC from the TSK-3 000 column (LKB, 60 × 0.75 cm: 0.5 ml/min, buffer C). The bar is indicating the fractions used for electrophoretic analysis and for immunization of rabbits.

Enzyme assay

The activity of tryptophan decarboxylase was determined as described previously (Knobloch *et al.* 1981). The identity of the product was checked by GC/MS:

GC-conditions: Derivatization of (^{14}C-labelled) ethyl acetate extracts from standard enzyme assays was performed as described previously (Berlin & Witte 1982). Column: DB 1–15 N (methylsilicone phase), carrier gas helium 0.75 bar; split ratio 1:30; temperature program 100–300 °C, 6 °C/min; injector temperature 250 °C; detector temperature 300 °C, detection by flame ionization detection (FID) and phosphorus nitrogen detection (PND). Retention time of the N-trifluoro ethyl ester of tryptamine was 1 775 (retention index, Wehrli & Kovats 1959).

M/S-conditions: The trifluoro acetic acid conjugates of tryptamine were subjected to mass spectrophotometry as described by Berlin & Witte (1982): MS, m/z (%): M^+ (0,1), 319 (5), 239 (100), 226 (57), 148 (15), 138 (12), 125 (11), 111 (10), 69 (9).

Protein

Protein content of the probes was determined according to Bradford (1976) using gamma globulin for reference.

Calculation of the isoelectric point

The isoelectric point of TDC was determined with the materials and methods described in the PHARMACIA manual: PAGE, section 4. Tube disc gel electrophoresis (7.5%) was performed with gel system No. 1 (Maurer 1968). A constant current of 2.5 mA/rod was applied. For enzyme recovery the gels were sliced after the run with an automatic gel slicer (Gilson). The enzyme was eluted from the 80 mesh particles with buffer B and assayed for TDC activity. Recoveries of up to 30% were achieved. The pI was calculated by reference to calibration proteins (serva, pH 3–10) on reference rods which were stained with Fast Green.

Determination of bound pyridoxal phosphate

The pyridoxal phosphate (PLP) content of TDC was estimated according to the fluorometric method of Adams (1969).

Buffers

A) 0.1 M Tris-HCl pH 7.5; B) 0.05 M Tris-HCl pH 7.5; C) 0.02 M Tris-HCl pH 7.5; D) 0.02 M Bis-Tris pH 6.9. All buffers contained 0.014 M β-mercaptoethanol and 0.02% Na-azide.

Results

Purification and properties of TDC

The purification procedure was carried out on a preparative scale with 'induced' *Catharanthus roseus* cells only, to yield sufficient amounts of TDC for antibody preparation. As outlined Table 1 (track: sp. act.) ammonium sulfate precipitation, DEAE-SEPHACEL, TSK-4 000 and TSK-3 000 represented the most effective purification steps. It is evident, that the high performance columns (TSK 4 000, TSK 3 000, MONO-Q) helped greatly in shortening the purification procedure which was most important because of the instability of TDC (see below).

TDC has a molecular weight of 115 000 ± 3 000, as estimated on PAGE and TSK 3 000 and has a molecular weight similar to the aromatic amino acid decarboxylase from hog kidney (Christensen *et al.* 1970) and the DOPA decarboxylase from *Drosophila melanogaster* (Clark *et al.* 1978). Under denaturing conditions only one band with a molecular weight of 54 000 ± 1 000 was detected on slab gels, similar to the DOPA decarboxylase (Clark *et al.* 1978). The purified aromatic amino acid decarboxylase from hog kidney gives several bands of different molecular weight under these conditions. The TDC of *Catharanthus roseus* cell suspension cultures seems to be composed of two identical subunits (Fig. 2b).

Stability of the enzyme

The purified TDC is a relatively unstable enzyme. When stored at 0 °C, pH 7.5, the half-life time of about 4 days can be calculated from the loss of activity monitored in 2 day intervals (data not shown). Partially purified TDC preparations (after hydroxylapatite column) revealed a slightly enhanced stability. Though, for the purification procedure lasting about 3 weeks (4–5 half-lives) a 90% loss in enzyme activity can be calculated. For long term storage, 20% glycerol was added to TDC, dissolved in buffer C. Best results of recovery during purification were achieved with buffer conditions (A–C), storing at 0 °C, without freezing the

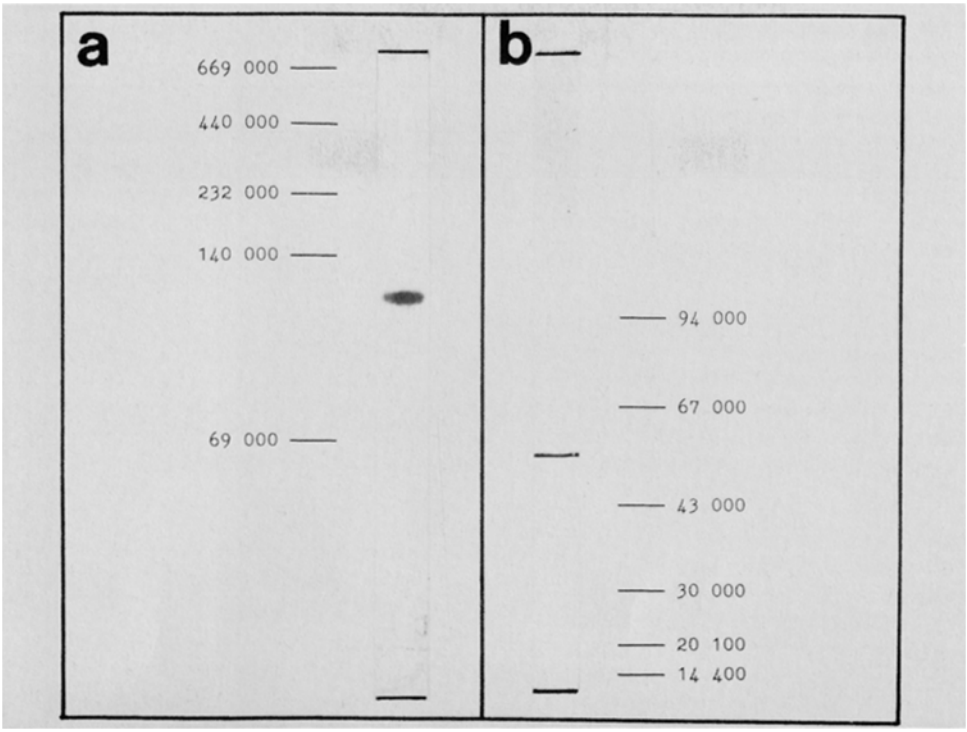

Fig. 2. a) Analytical polyacrylamide disc gel electrophoresis of appr. 1 μg purified TDC (see Table 1 and Fig. 1c) on slab gels. Protein was visualized with the silver stain method. The position of reference proteins is indicated (Pharmacia HMW-kit).

b) SDS disc gel electrophoresis of purified TDC (appr. 1 μg) from TSK-3 000, as indicated in Fig. 1c, on slab gels. The protein was visualized with Coomassie Brilliant Blue. The position of marker proteins is indicated (Pharmacia LMW-kit). Bars at the end of the tracks represent the position of the marker dye.

protein solution. TDC, dissolved in phosphate buffer (0.1 M, pH 7.5) showed only 40% of the enzyme activity in the Tris-buffer. Neither added pyridoxal phosphate nor added L-tryptophan or tryptamine, nor the use of phenylmethyl sylfonyl fluoride (PMSF) as a proteinase inhibitor increased the stability of TDC.

Substrate specifities and kinetic properties of TDC from Catharanthus roseus

The only two natural aromatic amino acids metabolized by the enzyme are L-tryptophan and L-5-hydroxytryptophan. The K_m for L-tryptophan was 7.5×10^{-5} M, whereas L-5-hydroxytryptophan had a K_m of 1.3×10^{-3} M according to Lineweaver & Burk plots. L-phenylalanine, L-tyrosine and L-DOPA were not decarboxylated (Table 2), as monitored by TLC (data not shown). Tryptamine as the exclusive product of the decarboxylation reaction was identified by GC/MS.

The enzymatic conversion of L-tryptophan to tryptamine is linear for more than 2 h with the homogeneous protein, as well as with non-purified preparations. No cooperative effects could be detected. D-tryptophan was found to be a non-competitive inhibitor of the enzyme (data not shown) while tryptamine is a competitive inhibitor of the decarboxylation reaction (K_I: $3{,}1 \times 10^{-4}$ M). Carbon dioxide showed no inhibitory effect on the enzyme reaction.

Calculation of turnover number of TDC

As outlined in Table 2, the half-life time of TDC was between 4–7 days, depending upon the degree of purity. Therefore, during purification one has to take into account that more and more enzyme will exist in an inactive form. The possibility of degradation of the enzyme can be ruled out by the fact that in pure enzyme preparations the electrophoretic pattern remains constant, whereas the enzyme activity declines if TDC is stored at 0 °C for several days. Variation of the pyridoxal phosphate concen-

Table 2. Molecular and kinetic properties of tryptophan decarboxylase from *Catharanthus roseus* cell suspension cultures in comparison with TDC from tomato and aromatic amino acid decarboxylase from hog kidney.

Properties	TDC		Aromatic aminoacid decarboxylase f. hog kidney[b]
	C.r.	Lyc. esc.[a]	
Molecular weight[1]	115 000 ± 3 000		112 000
Subunits	2(54 000 ± 1 000)		57 000/40 000/21 000
Substrate specificities			
K_M (M)	7.5×10^{-5}	3×10^{-3}	1×10^{-2}
L-Tryp			
5-Hydr	1.3×10^{-3}	30%	1×10^{-4}
L-Phe	∞	∞	4.2×10^{-2}
L-Tyr	∞	∞	8.4×10^{-3}
L-Dopa	∞	-	7.9×10^{-4}
Cooperative effects	$R_S = 80$	-	-
(L-Tryp)	$n_H = 1.06$	-	-
Product inhibition			
[tryptamine]	$K_I = 3.1 \times 10^{-4}$	-	-
Cofactor content			
(PLP)	0.5–1 mol	-	0.7–1.2 mol
Half-life time[2]	4–7 days	1 day	6 weeks
PH-optimum			
(test)	8.5	8.0	8.5
Turnover number			
(min^{-1})	200	-	1000
TDC part of total	IM2 = 0.03%	-	0.3%
protein	MX = 0.002%		

[1] Determined by PAGE and gel filtration.
[2] For the homogenous preparation 4, for partially purified TDC (hydroxylapatite step) 7 days.
[a] Gibson *et al.* 1970.
[b] Christensen *et al.* 1970.

tration for the assay could not restore the initial activity.

Nevertheless, a turnover number of app. 200 was calculated for TDC from *Catharanthus* on the basis of the specific activity of the homogenous enzyme preparation.

Proportion of TDC in the cells

TDC is only present in rather small amounts in the cells as it represents 0.03% even in 'induced' cells of total protein, calculated on the base of Table 1. The percentage of TDC in 'growing' cells can be estimated in an order of magnitude smaller (0.002%), based on the enzyme activity of the corresponding cells in MX-medium (Knobloch *et al.* 1981).

Pyridoxal phosphate content of TDC

Pyridoxal phosphate, a common cofactor for decarboxylases, enhances the activity of TDC between 2–3 fold, depending upon the degree of purity of the enzyme. Therefore, not surprisingly, the purified TDC from *Catharanthus roseus* was found to contain pyridoxal phosphate, as with the hog kidney enzyme (Christensen *et al.* 1970), according to the fluorometric method of Adams (1969). The excitation and emission spectra of the fluorescent derivative of the enzyme agreed well with those of pyridoxal phosphate standards and with the published spectra (Adams 1969). Approximately 1 mole pyridoxal phosphate seems to be bound by 1 mole of TDC.

Requirement of a free sulfydryl group

We examined the effect of p-chloromercuribenzoate (sodium salt) on the TDC from *Catharanthus roseus* and found that pretreatment of the enzyme for 30 min with this reagent resulted in almost complete inhibition (>90%) of the enzyme activity at a concentration of 1 mM. Christenson *et al.* (1970)

found 97% inhibition of the hog kidney enzyme at an unspecified concentration of the reagent.

Calculation of the isoelectric point

Electrofocusing in a pH gradient in the range 3–10 revealed that tryptophan decarboxylase of *Catharanthus roseus* has an approximate isoelectric point of 5.9. The presence of the enzyme within this pH gradient was determined by slicing and elution of the protein from the gel rods.

Discussion

Tryptophan decarboxylase diverts the primary metabolite tryptophan into secondary pathways in many plants (Waller & Dermer 1981). Despite its importance this enzyme has not been particularly characterized. Former attempts to purify TDC to apparent homogeneity failed mainly due to instability of the partially purified enzyme (Gibson *et al.* 1972; Baxter & Slaytor 1972). The enzyme from *Catharanthus roseus* cell suspension is more stable, a fact which enabled the multi-step purification outlined in Table 1. Our strategy for the purification of TDC can be divided into two sections: A) Common procedures including low pressure LC for enrichment, and B) HPLC chromatography for purification to apparent homogeneity.

The first step of the scheme involved high capacity ion exchange chromatography (DEAE, hydroxylapatite) followed by preparative gel filtration (ACA-34). This led to a coarse enrichment of the 100 000 D region, while the following HPLC steps were successful in separating the remaining impurities.

The instability of the enzyme caused a significant loss of activity during the purification. The two tracks (Table 1) for the 'purification factor' show the difficulties in calculating the effective enrichment.

The resulting protein preparation appeared to be free of impurities on nondenaturing disc gels (Fig. 2a) and had a molecular weight of 115 000 ± 3 000, as confirmed by gel filtration on TSK 3 000. For the investigations of the subunit structure the preparation (after TSK 3 000, see Table 1) was subjected to SDS gel electrophoresis which showed only one band (Fig. 2b) with a molecular weight of 54 000 ± 1 000. Thus TDC from *Catharanthus roseus* seems to be composed of two identical subunits. Experiments to test this proposal and to elucidate the number of active sites are in progress. A preliminary turnover number in the range of 200 can be predicted, based on the specific activity of the homogeneous protein.

In contrast to the aromatic amino acid decarboxylase from hog kidney (Christensen *et al.* 1970), the enzyme from *Catharanthus roseus* exhibits extreme substrate specificity for L-tryptophan and to a reduced extent for 5-hydroxytryptophan (K_m: 7.5×10^{-5}, and 1.3×10^{-3} M, respectively), the precursor of serotonin in animal cells.

As shown in Table 1, tryptophan decarboxylase is only present in rather small amounts even in 'induced' cell suspension cultures as it represents 0.03% of the soluble protein respectively. Thus the role of the TDC as a 'bottle-neck enzyme' seems reasonable: our aim is to generate cells with constitutively expressed TDC activity by the method of genetic engineering in cases where the lack of sufficient TDC activity was found to be the reason for low production of indole derived alkaloids. The basis for the construction of the corresponding cDNA has been laid and highly specific antibodies against TDC have already been prepared (Noé, unpublished).

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