Journal of Chromatography, 483 (1989) 311–318
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 936

PURIFICATION OF TRYPTOPHAN DECARBOXYLASE FROM A *CATHARANTHUS ROSEUS* CELL SUSPENSION CULTURE[a]

E. J. M. PENNINGS* and R. VERPOORTE
Center for Bio-Pharmaceutical Sciences, Division of Pharmacognosy, Leiden University, P.O. Box 9502, 2300 RA Leiden (The Netherlands)
and
O. J. M. GODDIJN and J. H. C. HOGE
Department of Plant Molecular Biology, Leiden University, P. O. Box 9505, 2300 RA Leiden (The Netherlands)

(First received March 14th, 1989; revised manuscript received August 8th, 1989)

SUMMARY

Tryptophan decarboxylase (TDC) (E.C. 4.1.1.28) was purified from a cell suspension culture of *Catharanthus roseus*. All steps were carried out in buffers of pH 7.0–7.5, as the enzyme showed the highest stability within this pH range. After ammonium sulphate precipitation (40–55% saturation) from a crude cell-free extract, the enzyme was purified by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B, anion-exchange chromatography on Q-Sepharose Fast Flow and high-performance size-exclusion chromatography (SEC) on a GF-450 column coupled to a TSK G3000SW column. The enzyme apppeared to be unstable, particularly in acidic medium. As determined by SEC, native TDC had an M_r of about 85 000. Native gel electrophoresis showed the appearance of one major band corresponding to M_r 96 000. Under denaturing gel electrophoresis conditions, the TDC band corresponded to an M_r of about 49 000. Antiserum against the M_r 49 000 protein also reacted strongly with two other proteins of M_r 33 000 and 17 000. It is concluded that the enzyme consists of two subunits of M_r 49 000 and that the two smaller proteins are degradation products of TDC.

INTRODUCTION

The aromatic amino decarboxylases (E.C. 4.1.1.28) play crucial roles in the biosynthesis of several types of alkaloids in various plants. 3,4-Dihydroxyphenylalanine (DOPA) decarboxylase and/or tyrosine decarboxylase (E.C. 4.1.1.25) are involved in isoquinoline alkaloid synthesis[1,2] and tryptophan decarboxylase (TDC) plays a key role in the formation of indole alkaloids[3,4]. TDC is also involved in the biosynthesis of indoleacetic acid, a major plant growth hormone. The few data

[a] Presented at the *3rd FPLC Symposium, Maarssen, March 14, 1989.*

0021-9673/89/$03.50

known from the plant enzymes show that different representatives of this group of enzymes have several properties in common[5–7]. Similarities also exist with enzymes of other origins as is indicated, *e.g.*, by the 39% sequence homology between TDC from *Catharanthus roseus* and DOPA decarboxylase from *Drosophila melanogaster*[8].

In our studies of indole alkaloid biosynthesis, TDC became one of the target enzymes for isolation and subsequent molecular biology studies for the encoding gene. Hence, we set out to purify the decarboxylase from a cell suspension culture of *C. roseus*. Using essentially a shortened version of the procedure published by Noë *et al.*[6], we obtained a 200-fold purified enzyme preparation[9] which, after further purification under denaturing gel electrophoresis conditions, was used to raise TDC-specific antibodies[10]. A serious problem encountered during this work was the considerable loss of activity due to instability of the enzyme. Therefore, we were urged to develop a fast procedure for the purification of TDC. In view of the above-mentioned general interest in aromatic amino acid decarboxylases, such a method may also be useful for the purification of other decarboxylases involved in scondary metabolite production. Here, we describe such a procedure, consisting of ammonium sulphate precipitation and three subsequent chromatographic steps, leading to a final enzyme preparation with a purity higher than 90%.

EXPERIMENTAL

Materials and equipment

Phenyl-Sepharose CL-4B, Q-Sepharose Fast Flow, Phastgel Silver Kit, the PhastSystem, the fast protein liquid chromatographic (FPLC) instrument and the reference proteins for size-exclusion chromatography (SEC) and for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) were all obtained from Pharmacia–LKB Biotechnology (Uppsala, Sweden). A Zorbax Bio Series GF-450 column (250 × 9.4 mm I.D.) was purchased from DuPont (Wilmington, DE, U.S.A.) and a TSK G3000SW column (600 × 7.5 mm I.D.) from Toya Soda (Yamaguchi, Japan). All chemicals were of analytical-reagent grade.

Assays

TDC was assayed by the high-performance liquid chromatographic (HPLC) method described previously[11]. Protein was determined by the method of Bradford[12] with bovine γ-globulin as a standard. Native PAGE and SDS-PAGE were carried out with the PhastSystem in Phastgel Gradient medium 8-25 and in Phastgel Homogeneous medium 12.5, according to the manufacturer's instructions. For SDS-PAGE, samples were prepared in 2.5% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol and 0.01% bromophenol blue. Protein staining was done with Phastgel Silver Kit. Immunological detection of TDS was carried out, after SDS-PAGE and Western blotting, with antibodies raised previously against the M_r 49 000 protein[10] obtained after SDS-PAGE of the 200-fold purified enzyme[9].

Cell material

Cell suspension cultures of *C. roseus* L. (G. Don) were grown as described previously[11]. Subculturing was done every 10–12 days by 5-fold dilution of the cells. for induction of TDC, 14-day-old cells were transferred to the induction medium

described by Berlin *et al.*[13], also by 5-fold dilution of the cells. After 5 days of TDC induction, the cells were harvested.

Preparation of crude cell-free extract

Cells were harvested by suction, washed once with water and immediately frozen in liquid nitrogen. The frozen cells were homogenized for 1 min at maximum speed in a Waring blender type 8010 (New Hartfort, CT, U.S.A.) equipped with an MC-3 metal bucket. To the frozen powder (per gram) were added 0.05 g of polyvinylpyrrolidone and 1 ml of 0.1 *M* Tris–HCl buffer (pH 8.0) containing 3 m*M* dithiothreitol and 1 m*M* EDTA. The material was allowed to thaw and cell debris was removed by centrifugation for 30 min at 10 000 *g*. The final pH of the extract was 7.5.

Purification of TDC

Protein extraction, ammonium sulphate precipitation and hydrophobic interaction chromatography (HIC) were carried out four separate times with four different batches of cells (2070 g, total fresh weight). Following each time, the TDC-containing fractions were frozen. After the fourth time, the active fractions were combined and processed further.

All procedures were carried out at 4–7°C with the exception of high-performance SEC, which was performed at room temperature. All buffers were filtered through 0.22-μm GSWP04700 filters (Millipore, Etten-Leur, The Netherlands). Elution of protein was monitored by measuring the absorbance of the effluent at 280 nm.

Ammonium sulfate fractionation. Protein precipitating between 40 and 55% of saturation was collected by centrifugation for 30 min at 10 000 *g*. The pellet was dissolved in a minimum volume of 0.2 *M* ammonium sulphate in buffer A (see below).

Hydrophobic interaction chromatography. Buffer A was 0.1 *M* Tris–HCl (pH 7.5) containing 1 m*M* dithiothreitol and 0.02 m*M* pyridoxal-5′-phosphate. Buffer B consisted of 0.2 *M* ammonium sulphate in buffer A. The protein sample was applied to a Phenyl-Sepharose CL-4B column (45 × 2.6 cm I.D.) equilibrated with buffer B and elution was carried out a a flow-rate of 1 ml/min with a linear gradient (1 l gradient volume) from 100% B to 100% A. Fractions of 10 ml were collected. The fractions containing TDC activity were pooled and concentrated by ultrafiltration with an Amicon YM-30 filter (W.R. Grace, Danvers, MA, U.S.A.).

Anion-exchange chromatography (AEC). The sample was loaded on a Q-Sepharose Fast Flow column (40 × 2.6 cm I.D.), equilibrated with 50 m*M* Tris-HCl buffer (pH 7.5) containing 1 m*M* dithiothreitol and 0.02 m*M* pyridoxal-5′-phosphate (buffer C). Elution was carried out with a linear gradient (1 l volume) from 0 to 0.5 *M* potassium chloride in buffer C at a flow-rate of 1 ml/min. Fractions (10 ml) with TDC activity were pooled and concentrated by ultrafiltration (YM-30 filters) to a final protein concentration of 2–3 mg/ml.

High-performance SEC. The GF-450 column was connected in series to the TSK G3000SW column for maximum resolution. The columns were equilibrated with buffer C. The flow-rate was 0.25 ml/min and the injection volume was 0.4 ml. Fractions of 0.5 ml were collected.

Stability of TDC at different pH values

The stability of TDC was studied at 4°C, from pH 4.0 to 6.4 in 0.1 *M* citric acid–sodium citrate buffer, at pH 6.8 in 0.1 *M* KH_2PO_4–KOH buffer and from pH 7.4 to 8.0 in 0.1 *M* Tris-HCl buffer. Samples were prepared by adding 0.05 ml of partially purified TDC (after HIC) to 0.1 ml of buffer. The activity of TDC was measured after 0.1, 18 and 42 h. For the assay, 0.01 ml of each sample was diluted 10-fold in the incubation mixture and the activity was assayed at pH 7.5 as described previously[11].

Molecular-weight determination of native TDC

The M_r of native TDC was determined by PAGE in Phastgel Gradient medium 8-25 and by SEC on the GF-450 column connected to the TSK G3000SW column. The columns, equilibrated with 0.1 *M* Bis–Tris (pH 7.0), were calibrated with ovalbumin (M_r = 43 000), albumin (M_r = 67 000), aldolase (M_r = 158 000) and ferritin (M_r = 440 000).

RESULTS AND DISCUSSION

Purification of TDC

The advantage of HIC as the first chromatographic step is that the desalting step after ammonium sulphate fractionation can be omitted. The elution profile (Fig. 1) shows that TDC was eluted at about 0.1 *M* ammonium sulphate. This value is corrected for the gradient delay volume. The recovery of TDC activity was 94%. After this step, TDC had increased stability. When kept at 4°C, a 10% decrease in activity was found after 4 days. For comparison, half of the activity was lost in the crude cell-free extract when kept at 4°C for 4 days[9]. This increased stability after the

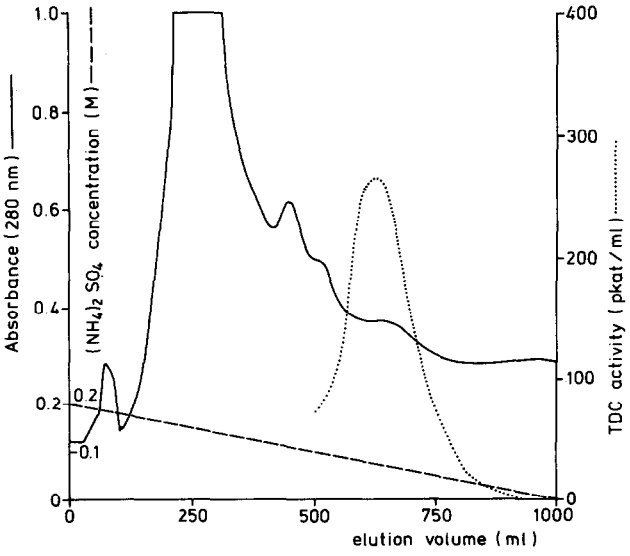

Fig. 1. Purification of *Catharanthus roseus* TDC by HIC on Phenyl-Sepharose CL-4B. Elution was carried out with a linear ammonium sulphate gradient from 0.2 to 0 *M* in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 1 m*M* dithiothreitol and 0.02 m*M* pyridoxal-5′-phosphate.

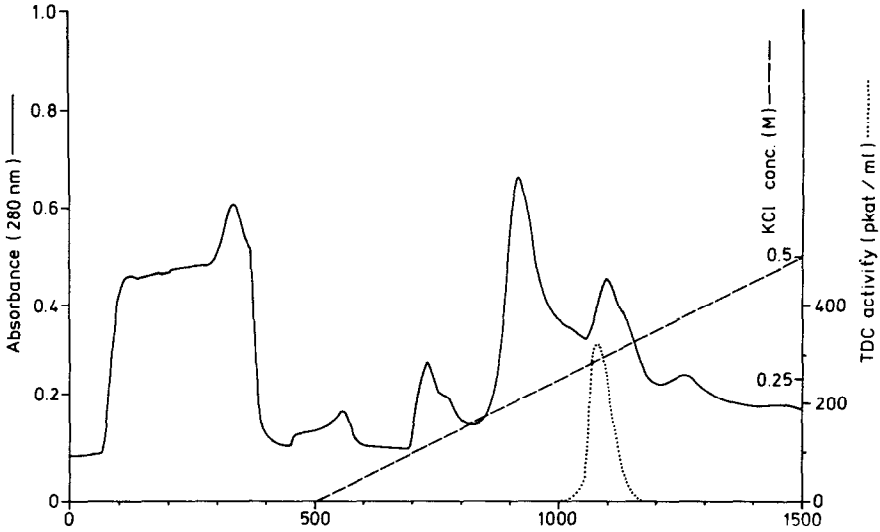

Fig. 2. Purification of *Catharanthus roseus* TDC by AEC on Q-Sepharose Fast Flow. Elution was with a linear potassium chloride gradient from 0 to 0.5 *M* in 50 m*M* Tris–HCl buffer (pH 7.5) containing 1 m*M* dithiothreitol and 0.02 m*M* pyridoxal-5'-phosphate.

first chromatographic step was also found when AEC on DEAE-Sephacel was used as the first chromatographic step[9]. After the HIC step, TDC can be stored for at least 2 months at −80°C without appreciable loss of activity.

The pooled fractions were concentrated and ammonium sulphate was removed by ultrafiltration (YM-30 filters). During ultrafiltration, about 20% of the activity was lost, resulting in an overall recovery of 74% after HIC plus ultrafiltration.

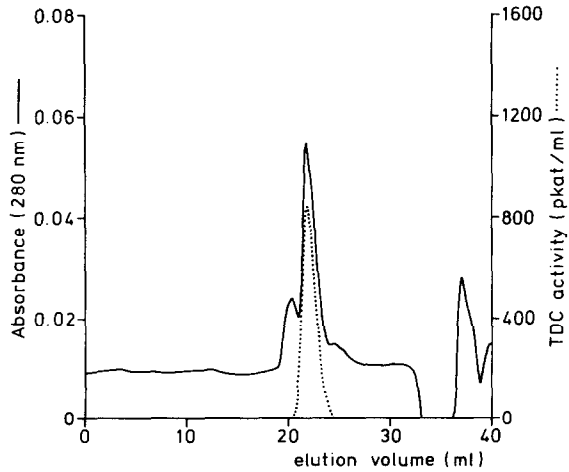

Fig. 3. Purification of *Catharanthus roseus* TDC by SEC on a GF-450 column connected to a TSK G3000SW column. Elution was with 50 m*M* Tris–HCl buffer (pH 7.5) containing 1 m*M* dithiothreitol and 0.02 m*M* pyridoxal-5'-phosphate at a flow-rate of 0.25 ml/min.

TABLE I

PURIFICATION OF TDC FROM 2070 g (FRESH WEIGHT) OF *CATHARANTHUS ROSEUS* CELLS

Fraction	*Volume (ml)*	*Total protein (mg)*	*Total activity (pkat)*	*Specific activity (pkat/mg)*	*Purification (fold)*	*Yield (%)*
42–55% saturated $(NH_4)_2SO_4$ precipitate	100	2630	118 657	45	–	100
Phenyl-Sepharose	920	179	111 340	622	14	94
YM-30 ultrafiltration	300	116	87 324	750	17	74
Q-Sepharose Fast Flow	70	20.4	31 012	1520	34	26
YM-30 ultrafiltration	6.8	15.9	15 116	948	21	13
GF-450 + TSK G3000SW	51	7.7	20 434	2653	59	17

In the next step, the enzyme was further purified by AEC on Q-Sepharose Fast Flow. TDC was eluted at a concentration of about 0.21 *M* potassium chloride (Fig. 2). This value is corrected for the gradient delay volume. The recovery of TDC activity in this step was 36% (51% when corrected for potassium chloride inhibition of TDC activity; see below). TDC-containing fractions, comprising 92% of the total amount of activity, were combined and concentrated by ultrafiltration (YM-30 filters). This led to an additional 51% decrease in activity.

High-performance SEC was used as the last step of the purification procedure (Fig. 3). The recoveries of TDC activity, measured in two runs, were 137% and 139%. This indicated that 0.21 *M* potassium chloride, present in the sample before injection, had an inhibiting effect on enzyme activity.

The results are summarized in Table I. Based on the amount of protein recovered, the purification factor (final preparation relative to the ammonium sulphate precipitate) is about 340, which is higher than the value of 180 calculated from the data published previously[9]. The specific activity of the final preparation was not higher than the value obtained before[9], but this can be ascribed to the decrease in enzyme activity occurring during the purification procedure. Similar results were reported by Noé *et al.*[6], who found a 90% decrease in activity during a purification procedure lasting about 3 weeks and taking six chromatographic steps.

The decrease in activity in a TDC sample (after SEC) was 30% after 1 day at 4°C. Freezing of a sample at −20°C immediately after collection and subsequent thawing led to the same 30% decrease in activity, but three other cycles of freezing and thawing did not give a further decrease in activity. Similar results were obtained when the TDC sample was rapidly frozen in liquid nitrogen and stored at −80°C. After this first decrease in activity, TDC appeared to be stable for at least 2 months at −20°C. For longer periods of time the enzyme is best stored at −80°C.

Stability of TDC at various pH values

The effect of storage of TDC at different pH values was studied as we planned to use cation-exchange chromatography in the purification procedure. The results (Fig. 4) show that TDC is unstable at acidic pH values. At pH 4.0, the activity is lost instantaneously and at pH 6.0, which is close to the isoelectric point of 5.9 for TDC[6], the activity is almost completely lost overnight. Cation-exchange chromatography and chromatofocusing were therefore rejected as possible purification procedures. A

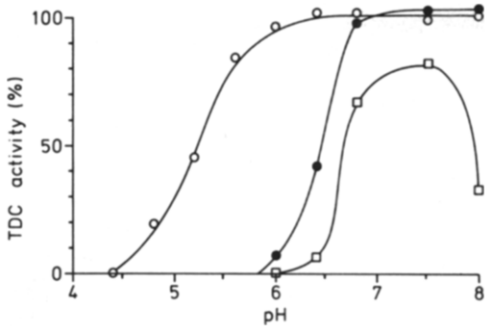

Fig. 4. Stability of TDC at different pH values. Stability was studied with TDC after HIC. Samples were prepared as described in the text and assayed for activity after (○) 0.1, (●) 18 and (□) 42 h. Values are given as percentages of the activity of the original sample after HIC.

similar instability under acidic conditions has been found for TDC from tomato shoots[5].

Molecular-weight determination

The M_r of native TDC, calculated from its retention time on the coupled SEC columns, was about 85 000. With native PAGE in an 8–25% gradient gel we calculated an M_r of about 96 000 for TDC. These values are lower than that of 115 000 found by others[6]. A possible explanation might be that in the cited study, the retention of TDC on the SEC column was not only governed by size exclusion, but also slightly by ion exclusion. The buffer concentration may be critical in this respect[14,15]. The concentration used by Noé *et al.*[6] (0.02 *M*) was lower than that used here (0.1 *M*). As a consequence, their estimate might have been too high.

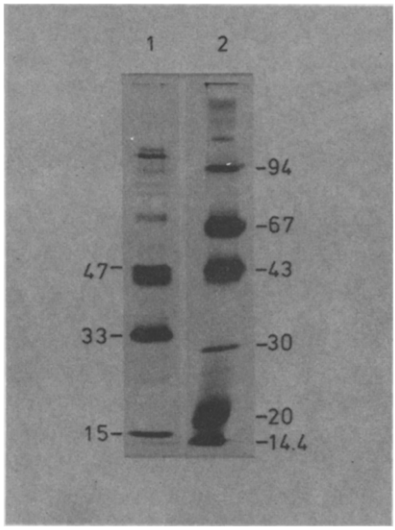

Fig. 5. SDS-PAGE of TDC peak fraction after SEC in Phastgel Homogeneous 12.5 medium. Lane 1, peak fraction (830 pkat/ml; 0.3 mg/ml); lane 2, reference proteins. Molecular weights are indicated in kilodaltons. Protein staining occurred with silver.

PAGE

Native PAGE in an 8–25% gradient gel showed the presence of one major protein band (M_r 96 000). After SDS-PAGE, in 12.5% homogeneous gel and 8–25% gradient gel, TDC could be identified as an M_r 49 000 band (Fig. 5) occurring in the gel synchronously with enzyme activity in the fractions. Antibodies raised previously against the M_r 49 000 band[10] reacted strongly with the M_r 49 000 band obtained with the present method, but also with two other bands corresponding to M_r 33 000 and 17 000 This indicated that the two additional bands might represent degradation products of TDC. Determination of the amino acid sequences of the N-termini of the M_r 49 000 and 33 000 fragments showed that the latter is indeed a degradation product of the former[10].

It is concluded that TDC from *C. roseus* is a dimeric protein consisting of two subunits of M_r 49 000. This is in agreement with results of others[6,8]. From the sum of the intensities of the three bands after silver staining, it could be estimated that the purity of TDC after the high-performance SEC step was at least 90% (Fig. 5).

CONCLUSIONS

HIC is attractive as the first chromatographic step as the desalting step used previously[9] can be omitted. Based on the amount of protein, the purification factor was 340. Using only three chromatographic steps, we obtained a final enzyme preparation with an estimated purity of at least 90%.

ACKNOWLEDGEMENT

The authors thank the Toya Soda for the generous gift of the TSK G3000SW column.

REFERENCES

1 H. R. Schütte and H. W. Liebisch, in K. Mothes, H. R. Schütte and M. Luckner (Editors), *Biochemistry of Alkaloids*, VCH, Weinheim, 1985, p. 188.
2 R. B. Herbert, in J. D. Phillipson, M. F. Roberts and M. H. Zenk (Editors), *The Chemistry and Biology of Isoquinoline Alkaloids*, Springer, Berlin, 1985, p. 213.
3 D. Gröger, in K. Mothes, H. R. Schütte and M. Luckner (Editors), *Biochemistry of Alkaloids*, VCH, Weinheim, 1985, p. 272.
4 K.-H. Knobloch, B. Hansen and J. Berlin, *Z. Naturforsch., Teil C*, 36 (1981) 40.
5 R. A. Gibson, G. Barrett and F. Wightman, *J. Exp. Bot.*, 23 (1972) 775.
6 W. Noé, C. Mollenschott and J. Berlin, *Plant Mol. Biol.*, 3 (1984) 281.
7 I. A. Marques and P. E. Brodelius, *Plant Physiol.*, 88 (1988) 52.
8 V. De Luca, C. Marineau and N. Brisson, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 2582.
9 E. J. M. Pennings, R. v.d. Bosch, R. v.d. Heijden, T. v.d. Leer and R. Verpoorte, in R. J. Robins and M. J. C. Rhodes (Editors), *Manipulating Secondary Metabolism in Culture*, Cambridge University Press, Cambridge, 1988, p. 79.
10 O. J. M. Goddijn, J. H. C. Hoge, E. J. M. Pennings and R. Verpoorte, in preparation.
11 E. J. M. Pennings, I. Hegger, R. v.d. Heijden, J. A. Duine and R. Verpoorte, *Anal. Biochem.*, 165 (1987) 133.
12 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
13 J. Berlin, E. Forche, V. Wray, J. Hammer and W. Hösel, *Z. Naturforsch., Teil C*, 38 (1983) 346.
14 B. Anspach, H. U. Gierlich and K. K. Unger, *J. Chromatogr.*, 443 (1988) 45.
15 S. Mori and M. Kato, *J. Liq. Chromatogr.*, 10 (1987) 3113.