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CHARACTERIZATION OF BACTERIAL L-(—)-TYROSINE DECARBOXYLASE BY ISOELECTRIC FOCUSING AND GEL CHROMATOGRAPHY

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SUMMARY

The purification of L-(—)-tyrosine apodecarboxylase (TAD) (E.C. 4.1.1.25), obtained from extracts of cells of *Streptococcus faecalis*, has been investigated by means of preparative isoelectric focusing, molecular sieve chromatography and hydrophobic interaction chromatography. Isoelectric focusing demonstrated two separate fractions possessing enzyme activity that had p*I* values of 4.5 and *ca.* 3.2. In the chromatographic methods, however, the activity was obtained in a single peak. It was found that hydrophobic interaction chromatography on phenyl-Sepharose was particularly suitable for purification purposes. The enzyme is very firmly bound to octyl-Sepharose CL-4B but retains most of its activity even in the bound state.

INTRODUCTION

One of the most reliable methods for the determination of pyridoxal-5′-phosphate (PLP) in biological fluids is based upon initial reaction rate measurements of the enzymatic decarboxylation of L-(—)-tyrosine, a reaction that shows a very pronounced PLP dependence[1,2]. In connection with our kinetic studies on this reaction, we required a more highly purified enzyme preparation and we describe here the use of some preparative separation methods for the purification and characterization of the enzyme.

Since the work of Epps[3], Baddeley and Gale[4] and others[5,6] on crude bacterial tyrosine decarboxylase, very few attempts appear to have been made in order to purify further and to characterize chemically this enzyme. In 1970, Chabner and Livingston[7] described the elution behaviour of the enzyme in molecular sieve chromatography employing Sephadex G-200. They concluded that the molecular weight of the enzyme is greater than 200,000 and that it possibly resembles the bacterial arginine, histidine and glutamic acid decarboxylases and accordingly should be composed of sub-units.

In order to construct a suitable scheme for the purification of the enzyme and to obtain more information with respect to its electrophoretic and chromatographic behaviour and general chemical properties, we employed preparative isoelectric focusing, molecular sieving on agarose gels and hydrophobic interaction chromatography on octyl-, phenyl- and butyl-substituted Sepharose.

EXPERIMENTAL

Preparation of the cell-free material

Freeze-dried cells of specially grown cultures of *Streptococcus faecalis* (Sigma, St. Louis, Mo., U.S.A., No. T 4629) were used as the source of the tyrosine apodecarboxylase (TAD) investigated. The treatment of the cell material was essentially as described by Sundaresan and Coursin[6], with the exception that cell disrupture was effected by means of a freeze–press technique instead of sonication. The cell debris was removed by centrifugation at 20,000 *g* for 1 h, which yielded a clear supernatant. With 2 g of starting material the supernatant obtained (40 ml) was diluted to 160 ml with 0.1 *M* acetate buffer of pH 5.5.

Enzyme activity determinations

The screening of decarboxylase activity was performed with the use of PLP in excess in order to ensure complete generation of holoenzyme. The decarboxylation reaction was carried out in 25-ml erlenmeyer flasks, provided with an inner compartment in the bottom, concentrically dividing the bottom area into two separate parts[8]. The flasks were closed with rubber septa and the reaction was controlled by injection via a hypodermic needle through the septum. All reactions were carried out at 27.0°.

A 2.0-ml volume of a *ca.* 0.1 μM solution of PLP in 1 *M* sodium acetate buffer (pH 6.0) was first added to each flask, followed by 0.5 or 1.0 ml of the fraction to be analysed for decarboxylase activity. A small plastic vial, fitting into the inner compartment of the flask, was then supplied with 100 μl of the carbon dioxide trapping agent [hydroxide of Hyamine 10-X, 1 *M* in methanol (Packard, Downers Grove, Ill., U.S.A.)] and placed into the flask, which was immediately sealed and thermostated. After a pre-incubation time of *ca.* 1 h, 400 μl of a 4.1 m*M* buffer solution of L-[1-^{14}C]tyrosine (activity 250 nCi/ml) was injected, the reaction was allowed to proceed for 30 min and then stopped by injection of 0.5 ml of 20% perchloric acid. The flasks were opened after *ca.* 1 h, the plastic vials transferred to scintillator vials, each containing 10 ml of a toluene-based scintillation liquid, and finally analysed for ^{14}C activity using an Intertechnique SL-4000 liquid scintillation counter.

Purification methods

The preparative isoelectric focusing experiments were performed in an LKB Model 8102 (440-ml) Ampholine column. The sucrose density gradient containing the ampholyte was formed with the use of a Model 8122 gradient mixer and the pH gradient was formed prior to the introduction of the sample. The column was thermostated at 0.5° in all runs. A constant power of 10 W was maintained during the electrofocusing by means of an LKB Model 2103 power supply. On emptying the column, 5-ml fractions were automatically collected with the use of an LKB Model 7000 fraction collector.

A volume of 1.0–3.0 ml of the cell-free extract, prepared as described above and carefully introduced into the pre-formed pH gradient, was used throughout the runs, which were continued for 10–20 h after no change in the current or voltage could be observed, which indicated that steady-state conditions had been attained. A 2% Ampholine concentration was used in all experiments. The fractions collected were analysed for enzyme activity immediately without any preceding dialysis for

sucrose removal. The pH values of the samples were recorded at the electrofocusing temperature by means of a Radiometer Model PHM 61 pH meter connected to a glass–calomel 2401 C electrode.

All chromatographic separations were performed at room temperature. The equipment consisted of a peristaltic pump, the separation column, an LKB Uvicord III UV monitor, operating simultaneously at 254 and 280 nm, a multi-channel recorder (LKB Model 17000) and, when gradient elution was performed, an LKB Model 11300 gradient mixer.

The agarose gels were carefully washed before use in order to remove bacteriostatic agents. After packing, the columns were equilibrated with the first buffer system to be used and, after connection to the UV recorder system, baseline stability was rigorously checked. Application of the sample (100–300 μl) was performed manually by layering on top of the gel bed by means of a Carlsberg pipette. Columns of 9 mm × 13 cm I.D. and a flow-rate of *ca.* 17.5 cm/h were used in all experiments unless otherwise stated. To compensate for the change in the UV absorbance of the solvent during gradient elution in the hydrophobic interaction chromatographic runs, the solvent was allowed to enter the reference cell of the UV monitor just prior to its entry into the column.

Chemicals and solvents

All chemicals and solvents used were of analytical-reagent grade. The PLP and TAD solutions were stored frozen at −60° and their concentrations checked by measuring A_{390} and A_{280}, respectively, before use. The scintillation fluid used consisted of 6 g of PPO, 200 mg of POPOP, 600 ml of methanol and 1400 ml of toluene, a mixture which rapidly and completely dissolved the base used for adsorption of the carbon dioxide.

All agarose gels were of the commercial grade supplied by Pharmacia (Uppsala, Sweden), except for butyl-Sepharose 4B, which was obtained from the Institute of Biochemistry, Biomedical Centre, Uppsala University. The latter gel, likewise prepared by the glycidyl ether technique[9,10], was not crosslinked and possessed a degree of substitution of 84 mmole per mole of galactose as determined by hydrolysis and subsequent NMR spectroscopic analysis[11].

RESULTS AND DISCUSSION

Isoelectric focusing

Repeated isoelectric focusing experiments, carried out in sucrose density gradients and at different pH ranges, produced a multiple peak pattern upon subsequent screening of the eluate for enzyme activity. All activity was obtained within the pH range 2.5–6.0 and an enzyme fraction with pI = 4.5 was readily recognized in all instances. Another fraction was obtained at a lower pH, but its position was less reproducible throughout the experiments, p$I \approx$ 3.2 being obtained twice. Visual inspection of the column during the electrofocusing did not reveal any sign of turbidity due to protein precipitation, and therefore artefacts caused by sedimentation might be excluded. Heterogeneity of other decarboxylases upon electrofocusing, *e.g.*, of mammalian histidine decarboxylase[12], has been reported earlier, but its cause is not yet fully understood.

In Fig. 1 the results from a typical experiment are shown. Dialysis of the two main fractions against 0.1 *M* acetate buffer (pH 6.0) followed by concentration by centrifugation via an Amicon CF 50 A membrane cone and molecular sieve chromatography on Sepharose 6B resulted in readily comparable chromatograms as judged from continuous UV monitoring of the eluates and screening of decarboxylase activity. Both fractions (from isoelectric focusing) gave only one activity maximum, each corresponding to the same value of the retention volume.

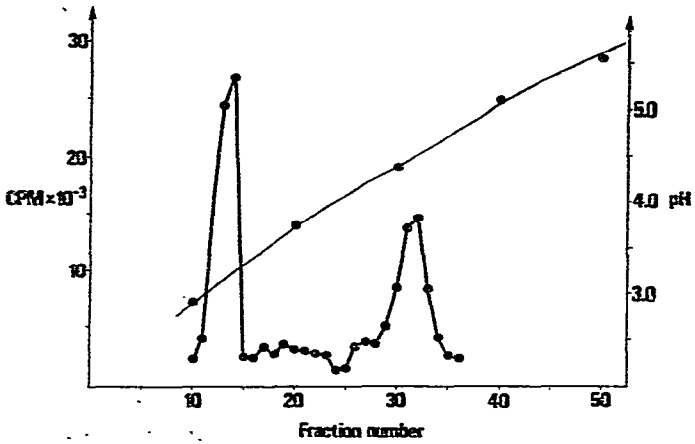

Fig. 1. Enzyme activity pattern in eluate from preparative isoelectric focusing of crude TAD.

Molecular sieve chromatography

The protein elution pattern obtained from the chromatography of a crude enzyme extract on Sepharose 6B is shown in Fig. 2, together with the decarboxylase activity profile. The activity maximum corresponded to a relative elution volume (V_e/V_0) of 2.3. In comparison, apoferritin (mol. wt. 480,000) applied to the same column and eluted under identical conditions gave $V_e/V_0 = 1.8$. As was concluded from the experiments with the substituted agarose gels, most of the protein UV absorbtion under the activity maximum originates from proteins with no decarboxylase activity.

Hydrophobic interaction chromatography

The elution behaviour of the enzyme on octyl- and phenyl-Sepharose CL-4B and on butyl-Sepharose 4B was studied under different conditions. In all instances 0.1, 0.5 and 1.0 *M* sodium acetate buffers (pH 6.0) were tried as starting buffers for an attempted adsorption of the enzymic material. After the elution of non-adsorbed proteins, a linear gradient was formed by increasing addition of 0.005 *M* sodium acetate in 50% ethylene glycol (pH 6.0). Under these conditions, no adsorption of the enzyme was obtained on butyl-Sepharose 4B, all activity being eluted together with the main part of the proteins. On the other hand, with octyl-Sepharose CL-4B, no activity could be eluted from the column under any of these conditions during 8–9 h. However, the gel showed decarboxylase activity after this time, showing that complete destruction of the enzyme had not occurred. On the phenyl-Sepharose CL-4B gels the typical elution behaviour generally associated with mechanisms of hydrophobic interaction between a protein and a gel matrix bearing hydrophobic substit-

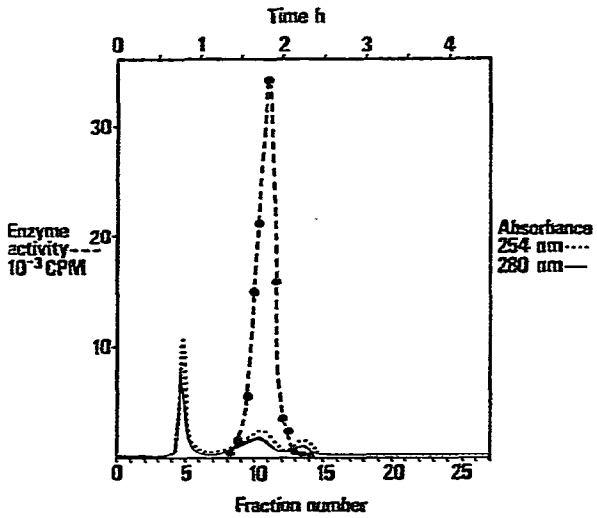

Fig. 2. Elution diagram from molecular sieve chromatography of crude TAD on Sepharose 6B. Eluent: 0.1 *M* sodium acetate solution (pH 6.0). Flow-rate: 8.9 cm/h. Bed height: 25 cm.

uents was exhibited by the enzyme. Fig. 3 shows the elution characteristics obtained when using three different conditions of adsorption. Fig. 3c demonstrates very clearly the symmetry of the enzyme peak and the high degree of resolution.

Analysis of the gel after the experiment corresponding to Fig. 3c showed that a small but significant decarboxylase activity was still present, the reason for which is unclear.

Estimations of relative enzyme activities

The observation of decarboxylase activity of the octyl-Sepharose gel-bound enzyme material prompted us to make a detailed comparison between free and bound enzyme activities. In a typical experiment, 100 μl of crude TAD were added to 0.5 g of octyl-Sepharose, the mixture was allowed to penetrate into the gel for 15 min and then its activity was determined and compared with that of 100 μl of free TAD analysed simultaneously under identical conditions. In this way, the bound form was shown to possess 45% of its activity in the free state, if dilution effects were neglected.

In order to estimate the degree of purification of the enzyme from the single chromatographic run shown in Fig. 3c, the pooled fractions belonging to the activity peak (26–37) were first dialysed against 0.1 *M* acetate buffer (pH 6.0) and the $A_{280}^{1\,cm}$ value was measured using the buffer as reference, which yielded $A_{280}^{1\,cm} \leqslant 0.006$ (I). Crude TAD ($A_{280}^{1\,cm} = 2.540$) was diluted with an aliquot of the same buffer to give $A_{280}^{1\,cm} = 0.056$ (II) and analysis of these samples under identical reaction conditions gave an initial rate ratio of I/II = 0.82, which roughly indicates a purification of $\geqslant 7.5\times$. A more exact value could not be obtained owing to the very low UV absorbance of I.

As shown in Fig. 4, even at rather high decarboxylation rates no significant decline in carbon dioxide formation per time unit could be observed under the conditions used for the enzyme activity determinations.

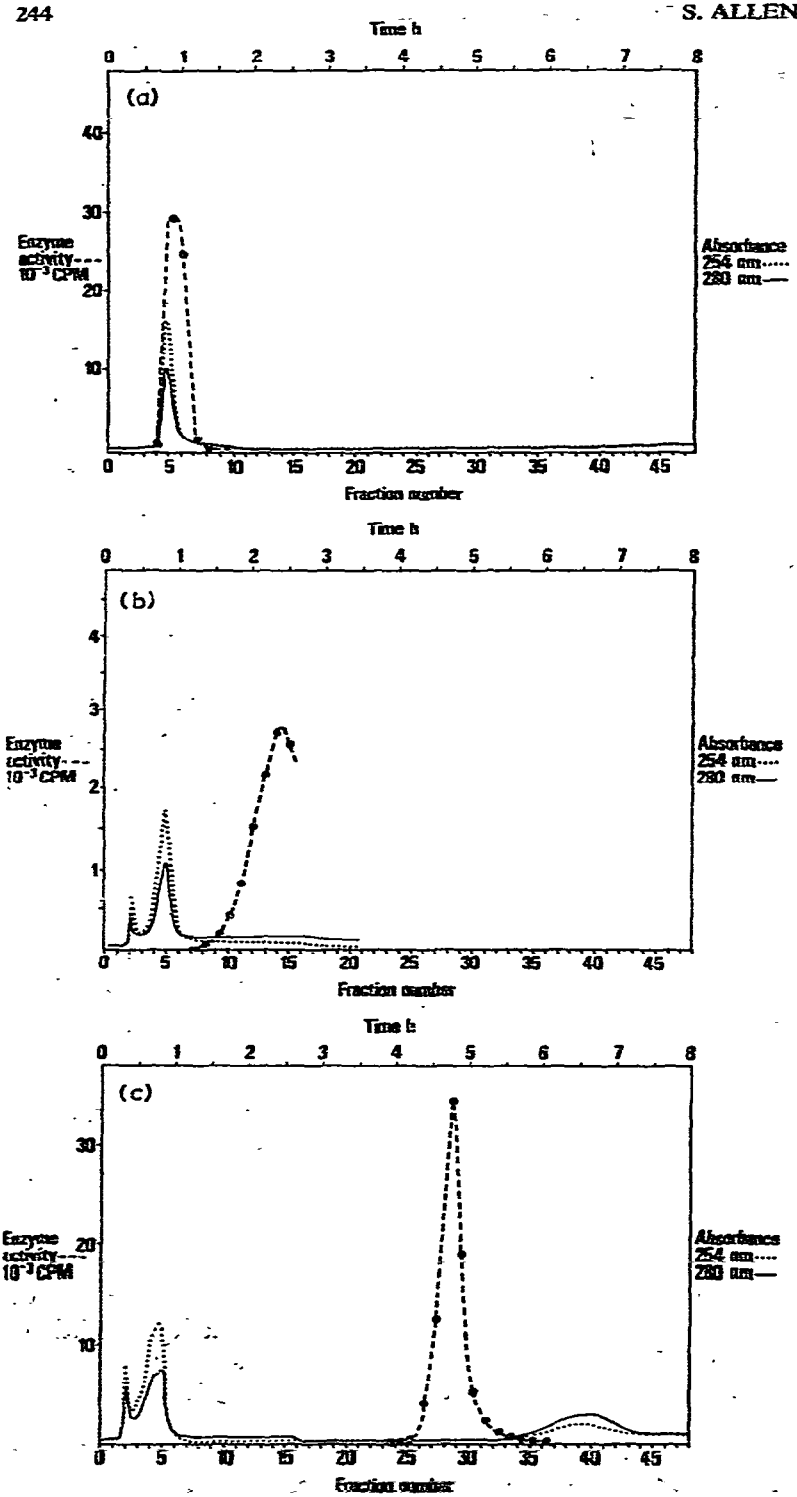

Fig. 3. Elution diagrams from hydrophobic interaction chromatography of crude TAD on phenyl-Sepharose CL-4B using different conditions for protein adsorption: (a) 0.1 *M* sodium acetate solution (pH 6.0); (b) 0.5 *M* sodium acetate solution (pH 6.0); (c) 1.0 *M* sodium acetate solution (pH 6.0). In (b) a different TAD preparation, with a much lower specific activity, was used. Flow-rate: 17.5 cm/h. The gradient was started after 1 h of isocratic elution.

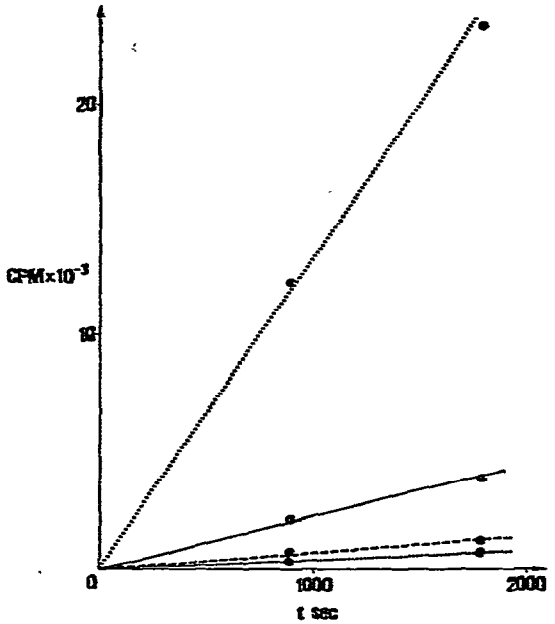

Fig. 4. Illustration of the conditions of constant decarboxylation rate (pseudo-zeroth order) used for the enzyme activity determinations. The lines correspond to different fractions obtained on elution from a phenyl-Sepharose CL-4B column.

The crude TAD used in all purification experiments contained very little PLP. The degree of resolution from cofactor can be seen from the initial rate ratio of 306 found for conditions of added and excluded PLP, respectively.

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