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Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine

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Abstract

Tyrosine decarboxylase (EC 4.1.1.25) (TDC) from the wine *Lactobacillus brevis* IOEB 9809 was purified by a rapid procedure involving anion exchange chromatography, ultrafiltration and hydrophobic interaction chromatography. The protein comprised two subunits of identical molecular mass (approximately 70 000 Da). Enzyme activity was dependent on exogenously supplied pyridoxal 5′-phosphate and the enzyme was stable at 4°C in the presence of the coenzyme. Optimum pH for the pure enzyme was 5.0. At this pH, TDC exhibited Michaelis–Menten kinetics (K_m 0.63 mM, V_{max} 998 units) and was highly substrate-specific for L-tyrosine. Other amino acids and L-DOPA are not converted by the protein. Tyramine acted as a mixed non-competitive inhibitor. Significant similarities in some biochemical properties were observed with the corresponding decarboxylase enzyme of *Streptococcus faecalis*, the sole bacterial TDC described to date.

Keywords: Tyrosine decarboxylase; Tyramine; Wine; Lactic acid bacterium; *Lactobacillus brevis*

1. Introduction

Some lactic acid bacteria involved in food fermentation may produce biogenic amines by decarboxylation of the precursor amino acids through the action of substrate-specific enzymes. The ingestion of foods containing high levels of such amines, particularly histamine and tyramine, can lead to several toxicological disturbances [1,2]. It is known that other diamines, such as diaminobutane (putrescine) and diaminopentane (cadaverine), and ethanol can potentiate the toxicity of the above-mentioned amines by inhibiting the detoxification systems [3,4]. In wines, histidine decarboxylase (HDC) activities have been detected in some *Oenococcus oeni* strains [5] and tyrosine decarboxylase (TDC) has been associated with *Lactobacillus* spp., particularly *Lb. brevis* strains [6]. More information about these enzymes could contribute to the prevention of histamine and tyramine formation during wine production and storage.

HDC has been purified and characterized from a number of different bacteria, including *Lactobacillus* 30a [7], *Lactobacillus buchneri*, *Clostridium perfringens* [8], *Micrococcus* sp. [9], *Photobacterium histaminum* [10], and *Oenococcus oeni* [11]. However, to date, very little work has been done on TDC in prokaryotes and little is known about its structure and functional properties. So far, TDC purification and characterization has only been reported for *Streptococcus faecalis* [12–14]. Recently, the TDC activity from the wine tyramine-producing *Lb. brevis* IOEB 9809 has been studied in detail [15]. Here, we describe a method that allows the purification of this enzyme to near homogeneity in one working day. Some of the biochemical properties of this *Lb. brevis* TDC are also reported.

2. Materials and methods

2.1. Chemicals

L-Tyrosine and the other amino acids, pyridoxal 5′-phosphate (PLP), L-DOPA, and EDTA were from Sigma chemicals (St. Louis, MO, USA). Prepacked fast protein liquid chromatography (FPLC) columns of Mono-Q UNO and Bio-Prep SE-1000/17, and protein molecular mass standard were obtained from Bio-Rad (Hercules, CA,

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USA). Phenyl-Superose HR 5/5 column was obtained from Pharmacia Biotech (Uppsala, Sweden). Unless otherwise mentioned, all other reagents used in this study were of analytical grade and were obtained from Sigma.

2.2. Organisms and growth conditions

The tyramine-producing strain *Lb. brevis* IOEB 9809 used in this study belongs to the bacteria collection of the Faculté d'Oenologie de Bordeaux (IOEB). It was originally isolated from Bordeaux wine [15]. 10 ml of a preculture in the exponential growth phase was grown overnight at 25°C in 1 l of MRS supplemented with L-tyrosine (1 g l^{-1}) and glucose (1 g l^{-1}) as described previously [6].

2.3. Purification procedure

Cells from an overnight 10-l culture of *Lb. brevis* IOEB 9809 were harvested by centrifugation (10 000 × *g* for 15 min at 4°C), washed twice and resuspended in 0.2 M sodium acetate buffer (pH 5.0) containing 0.1 mM EDTA, 0.2 mM PLP, and 3.6 mM L-tyrosine [6]. The cells were disrupted with ultrasonic waves (Ultrasonic MSE Scientific Instruments, Crawley, UK) at 150 W, for 10 cycles of 30 s followed by 30 s of cooling on ice each time. The suspension was centrifuged (14 000 × *g* for 20 min at 4°C) to remove cell debris, and the supernatant (crude extract) was stored at −20°C.

The column Mono-Q UNO anion exchange chromatograph connected to a Biologic HR System (Bio-Rad, Hercules, CA, USA) was previously equilibrated with 25 mM Bis–Tris propane (pH 7.0), 0.1 mM EDTA, and 5μM PLP. After loading 1 ml of crude extract, proteins were eluted with a linear NaCl gradient of 0–0.7 M at a flow rate of 1 ml min^{-1}. Fractions with enzyme activity were collected and concentrated by ultrafiltration in a Centricon-30 microconcentrator (Amicon Inc., Beverly, MA, USA). Ammonium sulfate and PLP were added to the pooled fractions to final concentrations of 1.2 M and 0.2 mM, respectively. The sample was applied to a phenyl-Superose HR 5/5 column which had been equilibrated with 50 mM sodium phosphate buffer (pH 6.0), 0.1 mM EDTA, and 5μM PLP containing 1.2 M $(NH_4)_2SO_4$. Elution was done by a reversed linear gradient of 1.2–0.5 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml min^{-1}. The enzyme-active fractions were desalted by dialysis against water containing 0.1 mM EDTA and 5 μM PLP, concentrated by ultrafiltration, brought to 0.2 mM PLP to stabilize the protein, and stored at 4°C. Between purification steps, TDC-active fractions were stored a 4°C. All chromatographic separations were performed at room temperature.

2.4. Enzyme assays

The enzyme activity was determined by measuring the CO_2 released from tyrosine with a specific CO_2 electrode (Eischweiler and Co., Kiel, Germany) by the method of Lonvaud and Ribéreau-Gayon [16]. The reaction mixture contained tyrosine (3.6 mM) and PLP (0.4 mM) in 2.0 ml of 0.2 M sodium acetate buffer (pH 5.0). The experiments were conducted at 25°C and the reaction was started by adding the enzyme extract. Release of CO_2 was monitored for 20 min and specific activity was expressed as μmol of CO_2 released per min per mg of protein. Each experiment was performed twice. Data shown correspond to the average values.

To check the substrate specificity of the purified enzyme, L-DOPA and other amino acids were used as substrate under similar conditions.

To determine K_m and V_{max} constants, the enzyme was incubated with increasing amounts of substrate and the measured activities from duplicate experiments were plotted in a Lineweaver–Burk diagram.

2.5. Electrophoresis analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and native PAGE were performed with a Mini-Protean II cell system and 4–15% (w/v) polyacrylamide gradient Mini-Protean II Tris–glycine Ready Gels as specified by the manufacturer (Bio-Rad, Hercules, CA, USA). Proteins were visualized with Coomassie brilliant blue or by silver staining with the silver staining kit (Pharmacia Biotech).

2.6. Molecular mass estimation

The relative molecular mass of the purified enzyme was estimated both by FPLC gel filtration on a Bio-Prep SE-1000/17 column (0.1 M sodium phosphate elution buffer, containing 0.1 M NaCl (pH 6.0)) and by SDS–PAGE as described above.

Table 1
Purification scheme for TDC of *Lb. brevis* IOEB 9809

Purification step	Amount of protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Activity yield (%)
Cell extract	26.5	424	16	1	100
Mono-Q	1.6	269	168	10.5	63
Ultrafiltration	0.9	183	203	12.7	43
Phenyl-Superose	0.1	106	1058	66	24

U = μmol min^{-1}.

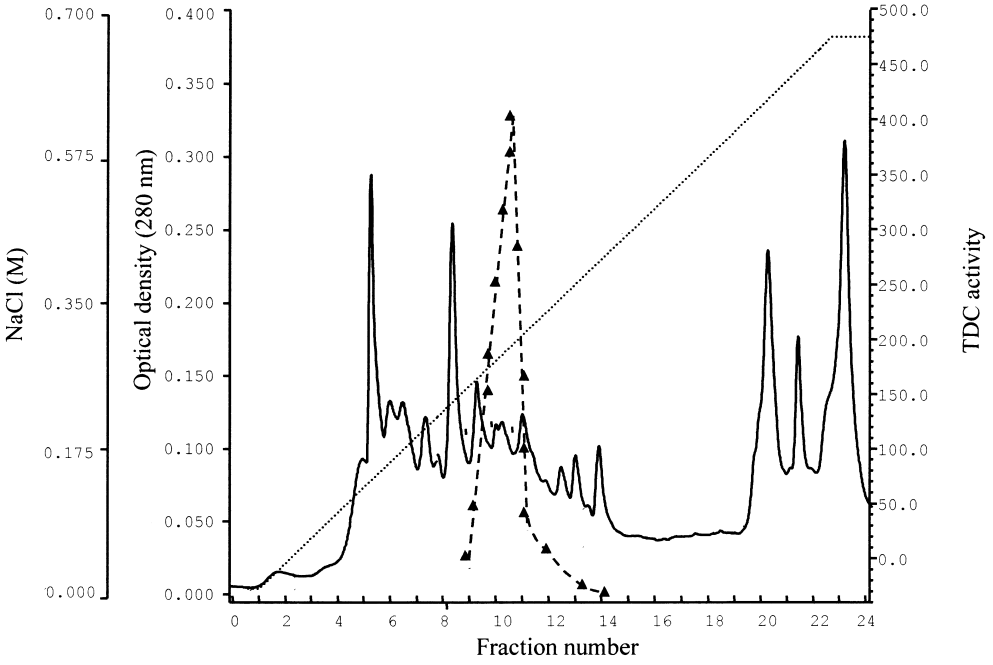

Fig. 1. Elution profile of anion exchange chromatography of the crude extract. The Mono-Q column was eluted with a 0–0.7 M NaCl gradient in 25 mM Bis–Tris propane (pH 7.0), 0.1 mM EDTA, and 5μM PLP. Absorbance at 280 nm (solid line), TDC activity (μmol min^{-1} mg^{-1} protein) (▲), and NaCl gradient (dotted line).

2.7. Protein quantification

After all the purification steps, the amount of protein was determined spectrophotometrically using a protein assay (BCA Protein Assay, Pierce) [17] with bovine serum albumin as the standard.

3. Results

A summary of the purification of TDC from *Lb. brevis* IOEB 9809 is shown in Table 1. The crude extract showed a specific TDC activity of 16 μmol CO_2 released min^{-1} mg^{-1} of protein. The enzyme was purified 66-fold to a final specific activity of 1058 μmol CO_2 released min^{-1} mg^{-1} of protein with 24% recovery. All chromatographic

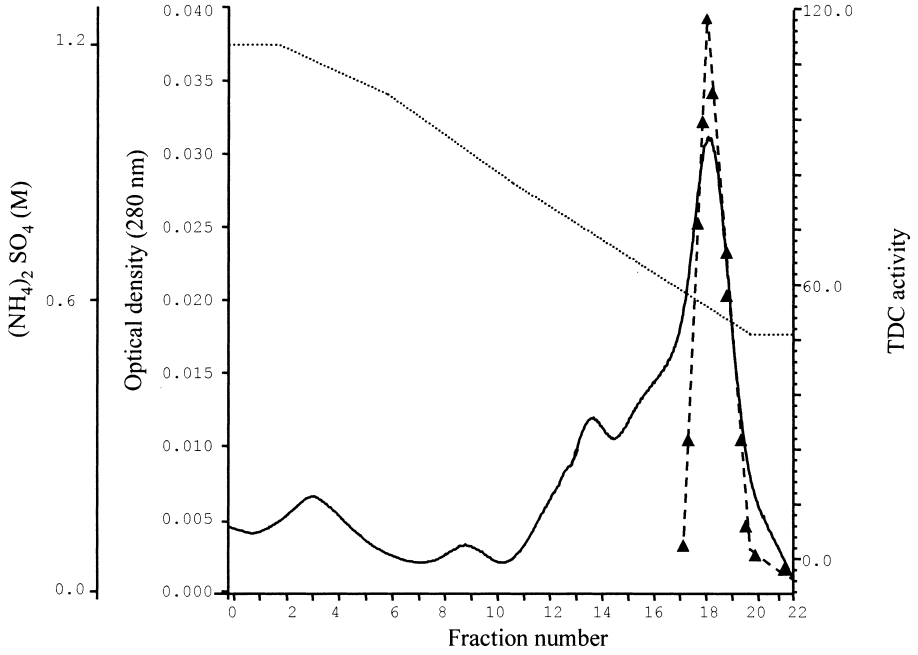

Fig. 2. Elution profile of hydrophobic interaction chromatography of the partial purified extract. The phenyl-Superose column was eluted with a reversed linear gradient of 1.2–0.5 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate buffer (pH 6.0), 0.1 mM EDTA, and 5μM PLP. Absorbance at 280 nm (solid line), TDC activity (μmol min^{-1} mg^{-1} protein) (▲), and NaCl gradient (dotted line).

separations required for the purification of TDC from *Lb. brevis* were carried out within one working day. With anion exchange chromatography, most of the enzyme activity eluted from the column between 0.26 and 0.30 M NaCl (Fig. 1). The elution profile was constant in repeated runs. The TDC cell extract was purified approximately 11 times with a recovery of 63% in this step. With ultrafiltration, the enzyme was purified 13-fold with a recovery of 43%. Hydrophobic interaction resulted in a higher factor of purification. Fig. 2 shows the elution profile on the phenyl-Superose column. The enzymatic activity was tested in fractions 17–22, and the highest activity was found in fraction 18 (Fig. 2).

After the final hydrophobic interaction chromatography, one major band running at an apparent molecular mass of ca. 70 kDa and a few minor bands were stained by SDS–PAGE (Fig. 3). This enzyme preparation eluted from the gel filtration on Bio-Prep SE as a single peak at a position corresponding to ca. 140 kDa (data not shown). Furthermore, the fractions that eluted in hydrophobic interaction chromatography preceding the TDC-active fractions contained the same minor bands but not the major band at 70 000 by SDS–PAGE. It suggests that the major band corresponds to the TDC enzyme. These results led to the presumption that *Lb. brevis* TDC is a dimer composed of two identical subunits of approximately 70 kDa. This enzyme preparation was used for further biochemical characterization.

The effect of temperature to prevent the loss of enzyme activity during the purification procedure was investigated. The enzyme was stable up to 37°C for 10 min, whereas only 25% of the activity remained at this temperature after 20 min. Between the purification steps, the active enzyme eluates were completely inactivated by freezing (−20°C). After repeated storage at 4°C, the enzyme showed a half-life of about 1.5 days.

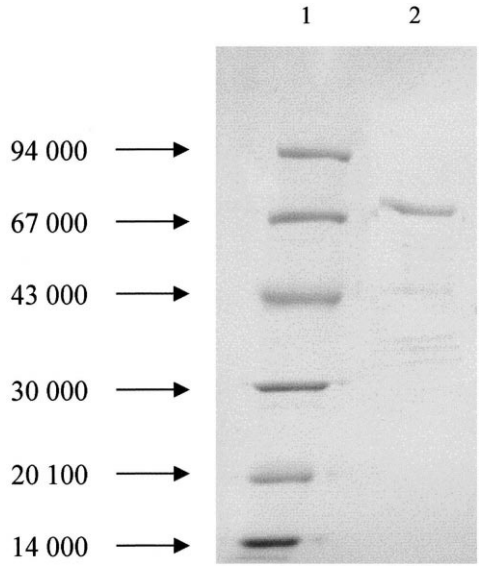

Fig. 3. SDS–PAGE of the purified TDC from *Lb. brevis* IOEB 9809. Lane 1: reference proteins; lane 2: TDC.

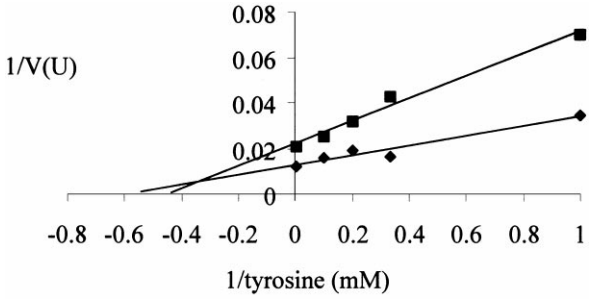

Fig. 4. Lineweaver–Burk plot of the TDC activity in the absence (●) and presence (■) of 50 mM tyramine.

The TDC enzyme was PLP-dependent. Washing the purified extract with sodium acetate buffer (pH 5.0) by ultrafiltration resulted in a complete loss of the activity toward L-tyrosine. The activity was partially restored (55% of the maximum activity) upon addition of 0.2 mM PLP to the assay buffer. On the other hand, a strong loss of TDC activity occurred during the purification procedure. If PLP (0.2 mM) was added to the active fractions after each purification step, the activity was enhanced about 10-fold and the eluates were relatively stable for 3 days at 4°C. Because of this instability, daily prepared enzyme was used in the characterization experiments.

The activity of the TDC enzyme was optimal at pH 5.0, keeping more than 90% of the maximum value at pH 6.0 (data not shown). At pH values below pH 2.0 or above 9.0, no enzyme activity could be detected. At pH 5.0, TDC exhibited simple Michaelis–Menten kinetics (Fig. 4). The biochemical constants K_m and V_{max} were calculated using L-tyrosine as the substrate, leading to a K_m value of 0.63 mM and a V_{max} value of 998.6 U mg^{-1} protein (1 U = 1 μmol substrate decarboxylated min^{-1}). The effect of tyramine, the final product of the reaction, on the purified TDC activity was investigated. It acted as a mixed non-competitive inhibitor (Fig. 4).

To determine the substrate specificity of TDC, the enzyme was incubated with L-DOPA (3.6 mM) and with different amino acids (histidine, lysine, phenylalanine, tryptophan, and ornithine, final concentration 5 mM) which may be precursors of biogenic amines in wine via decarboxylation, in 0.2 mM sodium acetate buffer, pH 5.0. CO_2-specific electrode and biogenic amine HPLC analyses revealed that none of these compounds was decarboxylated to an amine by TDC.

4. Discussion

This paper reports the purification and characterization of an enzyme responsible for tyrosine decarboxylation in lactobacilli. So far, TDC had been only isolated and characterized in the species *S. faecalis* [12–14]. Based on the specific activity, the enzyme was purified 66-fold in a three-step procedure with a recovery of 24%. Using a sim-

ilar method, Borrensen et al. [14] reported a specific activity of 115 U mg^{-1} protein after a 30-fold procedure with 3.4% recovery. In agreement with the results from Borrensen et al. [14], the major problem in the purification of TDC was its lability during the different purification steps. Therefore, the procedure needed as few steps as possible. Previous experiments had shown that after extraction, the enzyme was unstable at 4°C [6]. Freezing and storage at −20°C with addition of PLP and tyrosine to the storage buffer prevented a decrease of the TDC activity. However, in contrast, between the purification steps, TDC was inactivated by freezing, but it was relatively stable at 4°C. Although TDC activity of crude extract is freeze-stable, purified TDC quickly loses catalytic activity at this temperature. Using similar methods, Marques and Brodelius [18] also reported different stability conditions for plant tyrosine decarboxylases. In this respect, *Lb. brevis* TDC differs from the *S. faecalis* TDC, which could be stored for days at 0°C or for months at −18°C [14]. Removal of PLP from the TDC-active fractions completely inactivated the enzyme, and it could be partly reactivated by adding 0.02 mM of PLP. Purified preparations were relatively stable for 3 days at 4°C with addition of PLP. These results confirm the coenzyme dependence of the pure TDC as previously demonstrated for cell-free extract [6] and for *S. faecalis* pure TDC [14].

The molecular mass of the native form of TDC, as estimated by gel filtration chromatography, was approximately 140 kDa, consisting apparently of two subunits of approximately 70 kDa according to SDS–PAGE. This result differs slightly from those obtained for the TDC from the non-wine *S. faecalis*, which has a dimer structure but composed of two different subunits of approximately 74 500 and 76 000 Da [14].

Kinetic studies on pure enzyme confirmed a pH optimum at 5.0 for activity. Like a cell suspension and crude extract [6] at this optimal pH, the isolated TDC exhibited simple Michaelis–Menten kinetics. Tyramine, the product of the reaction, inhibited the TDC. The kinetic parameter for pure TDC ($K_m^{tyrosine} = 0.63$ mM) was almost the same as for cell-free extract ($K_m^{tyrosine} = 0.67$ mM) [6]. Both the optimal pH and the $K_m^{tyrosine}$ for pure *Lb. brevis* IOEB 9809 TDC were close to those of *S. faecalis* [12].

TDC from *Lb. brevis* IOEB 9808 was substrate-specific like the HDC from the wine *O. oeni* [11] in being only active towards the respective amino acid precursors, tyrosine and histidine. Unlike *S. faecalis* TDC, the TDC from *Lb. brevis* was inactive toward L-DOPA.

In conclusion, the rapid procedure described led to a near homogeneous enzyme preparation of TDC using only a few steps. *Lb. brevis* TDC shares some biochemical properties with the corresponding decarboxylase enzyme of *S. faecalis*, mainly with regard to the catalytic mechanism. Nevertheless, the two enzymes differ in their structure, stability, and substrate specificity.

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