Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Toyokazu YOSHIDA, Kohei FUJITA & Toru NAGASAWA (2002) Novel Reversible Indole-3-carboxylate Decarboxylase Catalyzing Nonoxidative Decarboxylation, Bioscience, Biotechnology, and Biochemistry, 66:11, 2388-2394, DOI: 10.1271/bbb.66.2388

To link to this article: http://dx.doi.org/10.1271/bbb.66.2388

Biosci. Biotechnol. Biochem., **66** (11), 2388–2394, 2002

Novel Reversible Indole-3-carboxylate Decarboxylase Catalyzing Nonoxidative Decarboxylation

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Received May 21, 2002; Accepted July 15, 2002

After enrichment culture with indole-3-carboxylate in static culture, a novel reversible decarboxylase, indole-3-carboxylate decarboxylase, was found in *Arthrobacter nicotianae* FI1612 and several molds. The enzyme reaction was examined in resting-cell reactions with *A. nicotianae* FI1612. The enzyme activity was induced specifically by indole-3-carboxylate, but not by indole. The indole-3-carboxylate decarboxylase of *A. nicotianae* FI1612 catalyzed the nonoxidative decarboxylation of indole-3-carboxylate into indole, and efficiently carboxylated indole and 2-methylindole by the reverse reaction. In the presence of 1 mM dithiothreitol, 50 mM $Na_2S_2O_3$, and 20% (v/v) glycerol, indole-3-carboxylate decarboxylase was partially purified from *A. nicotianae* FI1612. The purified enzyme had a molecular mass of approximately 258 kDa. The enzyme did not need any cofactor for the decarboxylating and carboxylating reactions.

Key words: decarboxylase; nonoxidative decarboxylation; carboxylation; indole-3-carboxylate; indole

In the microbial degradation of various carboxylic acids, enzymatic decarboxylation reactions are common. The decarboxylation activities of the decarboxylases involved in the metabolism of amino acids and 2-oxo-acids depend on pyridoxal 5′-phosphate and thiamine pyrophosphate, respectively, and their characterization, structures, and reaction mechanisms have been reported.[1–3] In decarboxylases such as ferulic acid decarboxylase,[4,5] *p*-coumaric acid decarboxylase,[5] gallic acid decarboxylase,[6,7] 4-hydroxybenzoate decarboxylase,[8] 2,3-dihydroxybenzoic acid decarboxylase,[9] and orotidine-5′-monophosphate decarboxylase,[10] no prothetic group is needed for the catalytic reaction.

We recently purified and characterized a pyrrole-2-carboxylate decarboxylase from *Bacillus megaterium* PYR2910; the enzyme catalyzes the nonoxidative decarboxylation of pyrrole-2-carboxylate to yield pyrrole and CO_2.[11] This enzyme also catalyzes the carboxylation of pyrrole at an appreciable rate,[12] and we used the carboxylation reaction for the synthesis of pyrrole-2-carboxylic acid.[13] The reverse reaction of pyrrole-2-carboxylate decarboxylase occurs when there is reaction equilibrium, and equilibrium was shifted towards pyrrole-2-carboxylic acid by optimization of the reaction conditions. 4-Hydroxybenzoate decarboxylase and 3,4-dihydroxybenzoate decarboxylase, which are involved in the anaerobic metabolism of phenolic compounds, also are reversible decarboxylases catalyzing nonoxidative decarboxylation *in vitro*.[8,14] Although the carboxylation activities of purified 4-hydroxybenzoate decarboxylase and 3,4-dihydroxybenzoate decarboxylase are weak, their physiological functions are the carboxylation of phenol and catechol, respectively, under anaerobic conditions.[8,14] Zhang and Young investigated the anaerobic biodegradation of naphthalene and phenanthrene, and showed that the initial key reaction for the use of both polycyclic aromatic hydrocarbons in the absence of oxygen is carboxylation.[15] Their results suggest the existence of an enzyme group reversibly catalyzing nonoxidative decarboxylation. However, there has been no report on decarboxylases efficiently catalyzing reverse carboxylation other than the pyrrole-2-carboxylate decarboxylase of *B. megaterium* PYR2910. In this study, we found an indole-3-carboxylate decarboxylase to be a novel reversible decarboxylase in *Arthrobacter nicotianae* FI1612 and several molds isolated by static enrichment culture. The enzyme reaction was characterized with resting cells and the partially purified enzyme of *A. nicotianae* FI1612.

Materials and Methods

Materials. Indole-3-carboxylate and indole were obtained from Wako Pure Chemical Industiries (Osaka, Japan) and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. DEAE-Sephacel, phenyl-Sepharose CL-4B, and Sephacryl S-200 HR were from Amersham Biosciences Co. (Upsala, Sweden). Butyl-Toyopearl 650M was purchased from Tosoh (Tokyo), and marker proteins for HPLC from Orien-

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tal Yeast Co. (Tokyo). Unless otherwise stated, all other chemicals were obtained from commercial sources and were of reagent grade.

Enrichment culture. For the isolation of indole-3-carboxylate-degrading microorganisms, conventional enrichment culture was done by static culture at 28°C. Soil samples were put in 40 ml of medium I containing 2 g of indole-3-carboxylic acid, 2 g of $(NH_4)_2HPO_4$, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 5 ml of metal solution in 1 liter of tap water (pH 7.0). The metal solution consisted of 40 mg of $CaCl_2 \cdot 2H_2O$, 50 mg of HBO_3, 4 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of KI, 20 mg of $FeSO_4 \cdot 7H_2O$, 40 mg of $MnSO_4 \cdot 7H_2O$, 40 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of $H_2MoO_4 \cdot 2H_2O$, and 2 ml of conc. HCl in 1 liter of distilled water. Microorganisms were isolated on agar plates of the same medium.

Culture conditions and preparation of resting cells. The first cultivation was done aerobically at 28°C for 2 d with reciprocal shaking at 115 rpm. For bacteria, the culture medium contained 5 g of meat extract (Kyokuto Seiyaku Co., Tokyo), 5 g of Polypepton (Nihon Pharmacy Co., Tokyo), 0.5 g of yeast extract (Oriental Yeast Co.) and, 2 g of NaCl in 1 liter of tap water (pH 7.0). Molds were first cultivated in medium I containing 0.2% (w/v) yeast extract. The culture was transferred to 40 ml of medium I containing 0.2% (w/v) yeast extract, and cultured by static cultivation in a 500 ml- shaking flask at 28°C for 5 d.

Bacterial cells were harvested by centrifugation at $12{,}000 \times g$ for 30 min. Molds mycelia were obtained by the filtration of culture broth. The harvested cells or mycelia were washed once with 0.15 M NaCl and suspended in the same solution.

Assay of decarboxylation activity of indole-3-carboxylate decarboxylase by resting cells. A standard reaction mixture contained 10 μmol of indole-3-carboxylate, 100 μmol of potassium phosphate buffer (pH 7.0), and resting cells in a final volume of 2 ml. Bacterial cells harvested from 20 ml of culture broth were added to the reaction mixture. Molds mycelia from 40 ml were used for the decarboxylation reaction, which was started by the addition of indole-3-carboxylate. After static incubation of the mixture at 30°C for 1 h, the reaction was stopped with 0.2 ml of 0.2 N NaOH. The resulting mixture was centrifuged at $12{,}000 \times g$ for 5 min, and the supernatant was analyzed by HPLC (Shimadzu LC-6A and SPD 6AV system) with a Spherisorb ODS2 column (4.6 × 150 mm, Waters). A 44:55 (v/v) mixture of methanol and 0.1 M potassium phosphate buffer (pH 4.0) was used for the elution. The flow rate was 1.0 ml/min, and the absorbance of indole-3-carboxylate and indole was monitored at 230 nm. For this resting-cell reaction, total activity was defined as the amount of indole formed per minute by cells derived from 1 ml of culture broth.

Identification of microorganisms. The identification of microorganisms was done by the National Collection of Industrial and Marine Bacteria, Japan (NCIMB).

Enzyme assay. Unless otherwise stated, the enzyme activity was evaluated in terms of the decarboxylating reaction of indole-3-carboxylic acid. A standard reaction mixture contained 20 μmol of indole-3-carboxylate, 100 μmol of potassium phosphate buffer (pH 7.0), 10 μmol of dithiothreitol, and enzyme solution in a final volume of 1 ml. The reaction was started by the addition of indole-3-carboxylate, continued at 30°C for 10 min, and then stopped with 0.05 ml of 2 N NaOH. The solution was centrifuged, and the resultant supernatant was analyzed by HPLC as described above. One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of indole per minute.

Carboxylation reaction. The carboxylation reaction of indole was done in a tightly closed reaction vessel. The reaction mixture contained 40 μmol of indole, 6 mmol of $KHCO_3$, 200 μmol of potassium phosphate buffer (pH 6.0), 20 μmol of dithiothreitol, and resting cells, in a final volume of 2 ml. The cells harvested from 20 ml of culture broth were used for the reaction. To increase the solubility of indole, 8% (v/v) methanol was added to the reaction mixture. The reaction was started by the addition of indole, continued at 20°C for 10 min, and stopped with 0.1 ml of 2 N NaOH. After centrifugation at 12,000 $\times g$ for 5 min, the supernatant was analyzed by HPLC.

Enzyme purification. All purification steps were at 4°C and in potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol, 50 mM $Na_2S_2O_3$, and 20% (v/v) glycerol was used, unless otherwise specified. Harvested cells from 6.5 liters of culture broth were suspended in 130 ml of 50 mM buffer and then disrupted with an ultrasonic oscillator (Insonator 201 M, Kubota, Japan) at 120 W for 30 min. The cell debris was removed by centrifugation at $15{,}000 \times g$ for 20 min. The supernatant solution was used as a cell extract. The cell extract was put on a DEAE-Sephacel column (3.5 × 14.3 cm) equilibrated with 10 mM buffer. After the column was washed with 100 mM buffer, the enzyme was eluted with 100 mM buffer containing 0.1 M KCl. The active fractions were combined and solid ammonium sulfate was added to 30% saturation. Then the enzyme solution was placed on a column (1.9 × 6.2 cm) of phenyl-Sepharose CL-4B equilibrated with 10 mM buffer containing ammonium sulfate at 30% saturation.

After the column was washed with 10 mM buffer containing ammonium sulfate at 5% saturation, the enzyme was eluted with 10 mM buffer. The active fractions were collected, and solid ammonium sulfate was added to 30% saturation. The enzyme solution was put on a butyl-Toyopearl 650 M column (1.9 × 1.1 cm) equilibrated with 10 mM buffer containing ammonium sulfate at 30% saturation. The column was washed with 10 mM buffer containing ammonium sulfate at 20% saturation, and the enzyme activity was eluted with 10 mM buffer containing ammonium sulfate at 15% saturation. The active fractions were combined. Solid ammonium sulfate was added to the enzyme solution to 90% saturation. After centrifugation of the mixture at 15,000 × *g* for 30 min, the precipitate was dissolved in 10 mM buffer containing 0.2 M KCl, and the resulting solution was placed on a column (1.7 × 93 cm) of Sephacryl S-200 HR equilibrated with 10 mM buffer containing 0.2 M KCl. The rates of sample loading and elution were maintained at 30 ml/h. Indole-3-carboxylate decarboxylase activity was eluted with the same buffer.

Analytical methods. SDS-PAGE was done in 10% polyacrylamide slab gels,[16] and the gels were stained with Coomassie brilliant blue R-250. Protein concentrations were measured by the method of Bradford[17] with bovine serum albumin as the standard. The molecular mass of the native enzyme were estimated by gel-permeation HPLC with a TSK G-3000 SW column (0.75 × 60 cm, Tosoh) at 0.7 ml/min with 0.1 M potassium phosphate (pH 7.0) containing 0.2 M NaCl as the eluent. The molecular mass of the purified enzyme was calculated from line of regression obtained from the mobilities of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa). Infrared spectroscopy was done on a Perkim Elmer 1640 FTIR, and NMR spectroscopy was done on a JOEL α-400 system with dimethylsulfoxide-d_6 as the solvent.

Results

Screening of indole-3-carboxylate decarboxylase activity for microorganisms

Through enrichment culture with indole-3-carboxylate as the sole carbon source, 21 microorganisms that degraded indole-3-carboxylate were isolated. Among them, one bacterial strain and five molds formed indole by decarboxylation activities in the culture medium in static culture. In preliminary experiments on isolation, aerobic cultivation at 115 strokes/min was inadequate in screening for indole-3-carboxylate-decarboxylating microorganisms; sometimes the activity disappeared during enrichment culture. Although the growth of microorganisms in static cultivation was slower than in aerobic conditions, indole-3-carboxylate-decarboxylating activity remained in all strains isolated under static cultivation. Indole-3-carboxylate decarboxylase activity of a bacterial strain, FI1612, was confirmed by resting-cell reaction, with the total activity of 5.01 nmol/ml min. Strain FI1612 was identified as *Arthrobacter nicotianae*. A fungus with the highest activity of 0.908 nmol/ml min was identified as *Fusarium subglutinans* FI31.

Distribution of indole-3-carboxylate decarboxylase in bacteria, molds, and yeasts was examined by static cultivation with medium I containing 0.2% (w/v) yeast extract. Among the tested strains from the collection in our laboratory, *F. oxysporum* IAM5009 and *Gibberalla fujikuroi* IFO 6605 formed a small amount of indole from indole-3-carboxylate during cultivation and in the resting-cell reaction. In the following investigations, *A. nicotianae* FI1612 was used because of its high activity.

Optimization of culture conditions

To increase the indole-3-carboxylate decarboxylase activity of *A. nicotianae* FI1612, culture conditions of the bacterium were optimized. The decarboxylase activity was measured with the resting-cell reaction. The effects of carbon and nitrogen sources were examined with medium I containing 0.05% (w/v) yeast extract as the base. The following carbon sources were used at the concentration of 1% (w/v): glucose, fructose, galactose, maltose, sucrose, lactose, sorbitol, glycerol, succinic acid, sodium citrate, sodium fumarate, sodium L-glutamate, and Polypepton. Maltose was the most effective carbon source, and the optimum concentration was 1% (w/v). The following nitrogen sources were tested at the concentration of 0.2% (w/v): Polypepton, meat extract, yeast extract, casamino acids, corn steep liquor (Wako) NZ amine (Wako), malt extract (Difco, Franklin Lakes, USA), NH_4Cl, $NaNO_3$, and, $(NH_4)_2HPO_4$. The addition of meat extract at 0.5% (w/v) increased the indole-3-carboxylate decarboxylase activity of *A. nicotianae* FI1612.

Enzyme induction by indole-3-carboxylate and its analogues was examined (Table 1). The enzyme activity was strongly induced by the addition of indole-3-carboxylic acid to the culture medium. No activity was found without the addition, and the optimum concentration was 0.05% (w/v). 3-Cyanoindole and L-tryptophan also induced the enzyme activity. Other compounds tested did not induce enzyme activity.

In screening for indole-3-carboxylate decarboxylase, its weak activity was frequently lost during aerobic cultivation. However, after the optimization of culture conditions of *A. nicotianae* FI1612, increased activity of resting cells was found even under aerobic conditions. The course of the enzyme activity during aerobic cultivation is shown in Fig. 1. The highest total activity of about 500 nmol/ml min was ob-

Table 1. Induction of Indole-3-carboxylate Decarboxylase Activity

Compound	Growth (OD_{610})	Total activity (nmol/ml min)
None	7.15	0
Indole-3-carboxylic acid	6.79	23.8
Indole-2-carboxylic acid	6.77	0
Indole-3-carboxylic acid methyl ester	7.43	0
Indole-3-aldehyde	5.96	0
3-Indoleacetic acid	5.76	0
3-Indoleacetamide	6.83	0
3-Indolepropionic acid	7.76	0
3-Cyanoindole	3.93	0.87
L-Tryptophan	5.49	0.35
D-Tryptophan	6.27	0
Indole	1.86	0
Pyrrole-2-carboxylic acid	8.23	0
Nicotinic acid	7.00	0
3-Quinolinecarboxylic acid	8.45	0

Each compound was added at the concentration of 0.1% (w/v) in medium I containing 0.2% (w/v) yeast extract. Decarboxylation activity of cultivated cells was evaluated by a resting-cell reaction.

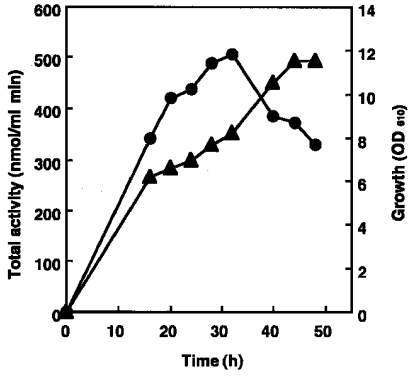

Fig. 1. Changes with Time in Indole-3-carboxylate Decarboxylase Activity of *A. nicotianae* FI1612 Cells under Optimized Aerobic Culture Conditions.

The optimized medium consisted of 0.5 g of indole-3-carboxylic acid, 10 g of maltose, 5 g of meat extract, 0.5 g of yeast extract, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 5 ml of metal solution in 1 liter of tap water, pH 7.0. The cultivation was done at 28°C in a 500-ml shaking flask containing 40 ml of medium, with reciprocal shaking at 115 strokes/min. Circles, total activity; triangles, cell growth.

served after 32 h. When the harvested cells were stored at −20°C in 0.15 M NaCl for 2 weeks, decarboxylase activity did not decrease.

Reversibility of catalytic reaction

Throughout the decarboxylation reaction for indole-3-carboxylic acid, a stoichiometric amount of indole was formed from indole-3-carboxylic acid (Fig. 2A). Indole-3-carboxylate decarboxylase reached an equilibrium, suggesting that the enzyme catalyzed the reverse carboxylation reaction. We used the conditions for the carboxylation reaction of pyrrole-2-carboxylate decarboxylase[12] in optimizing the reverse reaction of indole-3-carboxylate decarboxylase at 20°C in a tightly closed reaction vessel. Resting cells of *A. nicotianae* FI1612 catalyzed the carboxylation of indole (Fig. 2B). By 6 h, 6.81 mM indole-3-carboxylic acid had accumulated in the reaction mixture, with a molar conversion yield of 34% (mol/mol). The reaction product of the reverse carboxylation was isolated by a Dowex-1×2 anion-exchange column, and identified by ^{1}H-NMR, ^{13}C-NMR, and infrared spectroscopy with the authentic compound as a reference: NMR δ_H (Me_2SO-d_6) 7.15 (2H, m), 7.46 (2H, dd, J=1.5, 6.3 Hz), 8.02 (1H, s), 11.81 (2H, s); NMR δ_C (Me_2SO-d_6) 107.4, 112.2, 120.6, 121.0, 122.1, 126.0, 132.3, 136.4, 166.0; IR ν_{max} (KBr) cm^{-1}: 3305, 3069, 2861, 1651, 1201.

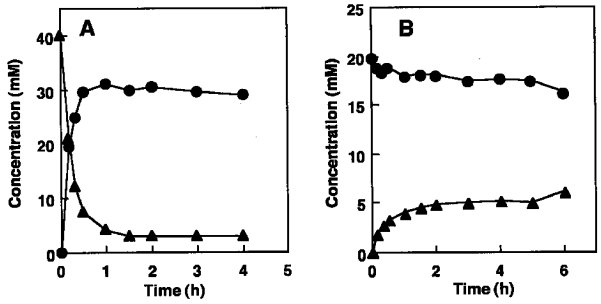

Fig. 2. Decarboxylation of Indole-3-carboxylic Acid (A) and Carboxylation of Indole (B) by Resting Cells of *A. nicotianae* FI1612.

The decarboxylation reaction was done under the standard reaction conditions with resting cells. The reaction conditions for carboxylation of indole are described in Materials and Methods. ●, Indole; ▲, indole-3-carboxylic acid.

Substrate specificity

The ability to catalyze decarboxylation of the following compounds was examined by resting cell reactions: indole-2-carboxylic acid, indole-5-carboxylic acid, thiophene-2-carboxylic acid, furan-2-carboxylic acid, 3-quinolinecarboxylic acid, 2-quinoxalinecarboxylic acid, nicotinic acid, isonicotinic acid, picolinic acid, 2-hydroxynicotinic acid, 6-hydroxynicotinic acid, 2-chloronicotinic acid, 6-chloronicotinic acid, 2,3-pyridinedicarboxylic acid, 2,5-pyridinedicarboxylic acid, pyrazine-2-carboxylic acid, and 2,3-pyrazinedicarboxylic acid. Resting cells of *A. nicotianae* FI1612 catalyzed the decarboxylation of 2-quinoxalinecarboxylic acid. The formation of quinoxaline from 2-quinoxalinecarboxylic acid was detected by HPLC, although the activity was not measured because only a small amount or quinoxaline formed.

Substrate specificity of the carboxylation reaction for the following compounds was examined: 2-methylindole, 5-methylindole, 2-hydroxyindole, 4-hydroxyindole, 5-hydroxyindole, 4-chloroindole, 5-chloroindole, 6-chloroindole, 5-aminoindole, and quinoxaline. 2-Methylindole and quinoxaline were carboxylated by resting cells of *A. nicotianae* FI1612.

Table 2. Partial Purification of Indole-3-carboxylate Decarboxylase from *A. nicotianae* FI1612

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cell extract	2940	4490	1.53	100
DEAE-Sephacel	155	19.4	0.125	0.43
Phenyl-Sepharose CL-4B	11.9	2.10	0.176	0.047
Butyl-Toyopearl 650M	4.2	1.68	0.400	0.037
Sephacryl S-200 HR	1.7	0.38	0.224	0.008

The activities toward 2-methylindole and quinoxaline were 37% and $<1\%$ of the activity toward indole, respectively. The reaction product from 2-methylindole was purified and analyzed by ^{1}H-NMR, ^{13}C-NMR, and infrared spectroscopy. The spectra of reaction product were analogous to those of indole-3-carboxylate except the signals for a methyl group at C-2, and the presence of a carboxyl group at C-3 was suggested: NMR δ_H (Me_2SO-d_6) 2.63 (3H, s), 7.08 (2H, m) 7.32 (1H, dd, $J=2.9$, 5.9 Hz), 7.92 (1H, dd, $J=3.4$, 5.9 Hz), 11.68 (1H, s), 11.84 (1H, s); NMR δ_C (Me_2SO-d_6) 13.7, 103.3, 111.0, 120.4, 120.6, 121.4, 127.3, 134.8, 144.3, 166.7; IR ν_{max} (KBr) cm^{-1}: 3377, 2910, 1660, 1213.

Enzyme characterization

When a cell extract prepared from *A. nicotianae* FI1612 was stored without the addition of sulfhydryl-protecting reagents, 80% of the initial activity was lost after storage at 4°C for 4 d. The enzyme activity was stabilized by the addition of a mixture of 1 mM dithiothreitol, 50 mM $Na_2S_2O_3$, and 20% (v/v) glycerol to the purification buffer, with the residual activity of 80% after storage for 4 d. However, much loss of activity seemed inevitable during enzyme purification (Table 2). Partially purified indole-3-carboxylate decarboxylase, which gave three bands on SDS-PAGE, was obtained from *A. nicotianae* FI1612. The molecular mass of the enzyme was estimated to be 258 kDa by gel-permeation HPLC.

The effects of temperature and pH on the enzyme were investigated. When the enzyme had been incubated at temperatures up to 20°C for 30 min, activity was not decreased. Treatment at 30, 40, 50, and 60°C caused 13, 35, 65, and 100% losses of the initial activity, respectively. The enzyme was stable on incubation at 20°C for 1 h in the pH range of 6.0–8.0. When the decarboxylation reaction was done for 30 min at various temperatures and under various buffer conditions at the concentration of 75 mM, the activity was maximal at 50°C and pH 7.0 (potassium phosphate buffer). For the carboxylation reaction, temperatures over 30°C were not appropriate. The activities at 10, 20, and 30°C were about the same. The activity was maximal at pH 8.0 (Tris-HCl buffer, 100 mM).

The effects of various compounds on the enzyme activity were investigated under the standard enzyme assay conditions (Table 3). *N*-Ethylmaleimide, phenylhydrazine, *o*-phenanthroline, and Cu^{2+} inhibited the activity. The enzyme activity was increased by the addition of several metal ions such as Zn^{2+} and Mn^{2+}. However, the addition of these metal ions to the purification buffer did not protect against loss of enzyme activity. The purified pyrrole-2-carboxylate decarboxylase from *B. megaterium* PYR2910 absolutely required an organic acid as a co-factor for its decarboxylation and carboxylation activities.[11] In the absence of an organic acid such as acetate, propionate, butyrate, or pimelate, the decarboxylation of pyrrole-2-carboxylate and the carboxylation of pyrrole were not observed.[11] With this indole-3-carboxylate decarboxylase, the decarboxylation and carboxylation activities were independent of the addition of organic acids. The K_m values for indole-3-carboxylic acid and indole were calculated to be 0.631 and 0.719 mM from double reciprocal plots, respectively.

Table 3. Effects of Various Compounds on the Decarboxylation Activity of Indole-3-carboxylate Decarboxylase

Compound	Relative activity (%)	Compound	Relative activity (%)
None	100		
N-Ethylmaleimide	3	$ZnSO_4$	215
Iodoacetate	100	$MnSO_4$	215
p-Chloromercuribenzoate	110	$MgSO_4$	188
Hydroxylamine	65	$CaCl_2$	127
Phenylhydrazine	13	$CoCl_2$	106
Phenylmethylsulfonyl fluoride	90	$Fe_2(SO_4)_3$	94
EDTA	96	$AgNO_3$	50
2,2′-Bipyridyl	28	$HgCl_2$	48
o-Phenanthroline	15	$NiCl_2$	31
Tiron	119	$CuCl_2$	0
8-Hydroxyquinoline	51		
NaN_3	86		

The enzyme reaction was done in the standard reaction mixture containing the tested compound at 1 mM except for *p*-chloromercuribenzoate at 0.1 mM.

Discussion

In studies of various kinds of decarboxylases, the interest in nonoxidative decarboxylases is focused on their reaction mechanisms because they do not use any co-factors. With orotidine-5′-monophosphate decarboxylases, reaction mechanisms have been suggested that explain how the enzyme might catalyze nonoxidative decarboxylation, and the crystal structures of the enzymes have been analyzed.[18,19] On the other hand, in studies of the microbial anaerobic degradation of phenolic compounds, two kinds of reversible decarboxylases catalyzing nonoxidative decarboxylation were found in *Clostridium hydroxybenzoicum*.[8,14] The primary structure of the 4-

hydroxybenzoate decarboxylase of the bacterium has been published,[20] and is similar to many hypothetical proteins deduced from open reading frames in genomes from bacteria and archaea; a novel gene family has been proposed. However, the catalytic residues involved in the reversible decarboxylation have not been verified yet. We also found a reversible pyrrole-2-carboxylate decarboxylase in *Bacillus megaterium* PYR2910,[11] and the pyrrole-2-carboxylate decarboxylase efficiently catalyzes the carboxylation of pyrrole.[12] In our study, to find novel reversible decarboxylases catalyzing the nonoxidative decarboxylation of aromatic compounds and then to analyze their reaction mechanisms, microorganisms with indole-3-carboxylate decarboxylase activity were isolated from soils, and the indole-3-carboxylate decarboxylase of *A. nicotianae* FI1612 was characterized.

An oxygen-sensitive 4-hydroxybenzoate decarboxylase and 3,4-dihydroxybenzoate decarboxylase are induced by 4-hydroxybenzoate and 3,4-dihydroxybenzoate, respectively, in the anaerobic cultivation of *C. hydroxybenzoicum*.[8,14] The pyrrole-2-carboxylate decarboxylase of *B. megaterium* PYR2910 is strongly induced by pyrrole-2-carboxylate and its analogues with a carboxyl group, but not by pyrrole.[11] In the presence of dithiothreitol, a pyrrole-2-carboxylate decarboxylase with high stability has been purified from *B. megaterium* PYR2910.[11] We did enrichment culture using indole-3-carboxylic acid as the sole carbon source in static culture. Indole-3-carboxylate decarboxylase activities found in the isolated bacteria and molds were quickly lost by aerobic cultivation in all strains. Similarly, some enzyme activity was lost during the purification of indole-3-carboxylate decarboxylase from *A. nicotianae* FI1612, and the addition of 50 mM $Na_2S_2O_3$ to the purification buffer stabilized the enzyme activity somewhat. This result indicates the importance of Cys residue(s) for the enzyme activity or the protein structure. *A. nicotianae* FI1612 grew well in aerobic culture, and the indole-3-carboxylate decarboxylase activity of resting cells of the bacterium was fairly stable. However, the enzyme activity in the cell extract was sensitive to oxygen. Thus, in the screening for such reversible decarboxylases, anaerobic or semianaerobic cultures are probably appropriate for enrichment. For indole-3-carboxylate decarboxylase activity of resting cells, after the highest activity was reached by 32 h of aerobic cultivation of *A. nicotianae* FI1612, activity was lost rapidly. The disappearance of decarboxylase activity during enrichment culture under aerobic conditions and the loss of the activity of isolated strains seem to arise during a long cultivation.

Pyrrole-2-carboxylate decarboxylase of *B. megaterium* PYR2910 catalyzed the reverse carboxylation of pyrrole to pyrrole-2-carboxylate, with a molar conversion ratio of 80% (mol/mol).[12] The carboxylation of indole into indole-3-carboxylate was observed by the purified indole-3-carboxylate decarboxylase as well as by the resting cells (not shown). The molar conversion yield of indole into indole-3-carboxylate was 34% (mol/mol). The low value might derive from the solubility of indole in the reaction mixture. Although the pyrrole-2-carboxylate decarboxylae activity of *B. megaterium* PYR2910 was completely dependent on an organic acid such as acetic acid,[11] indole-3-carboxylate decarboxylase did not require any cofactor for its activity. It is difficult to identify the causes of this difference because of the scarcity of knowledge on catalytic mechanisms and primary structures of reversible decarboxylases.

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