CONSTITUTIVE AROMATIC L-AMINO ACID DECARBOXYLASE FROM *MICROCOCCUS PERCITREUS*

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Summary

The preparation of crystalline aromatic L-amino acid decarboxylase from the cell extract of *Micrococcus percitreus* is described. The crystalline enzyme is homogeneous upon ultracentrifugation and SDS-polyacrylamide gel electrophoresis. The molecular weight is approximately 101,000. The enzyme exhibits absorption peaks at 340 nm and 415 nm, and requires pyridoxal phosphate as a cofactor. The enzyme catalyzes the stoichiometric conversion of L-tryptophan into tryptamine and CO_2. The optimal pH is 9.0 for L-tryptophan. The Km value and the maximum velocity of L-tryptophan decarboxylation were 2.4×10^{-3} M and 22 μmoles/min/mg of protein, respectively. The enzyme also catalyzes decarboxylation of L-tyrosine and L-phenylalanine.

Bacterial decarboxylation of aromatic L-amino acids to the corresponding amines has been described in a number of different strains (1, 2). In spite of a considerable amount of literature on decarboxylases of L-tyrosine, L-histidine and their derivatives (3, 4), the enzyme which catalyzes decarboxylation of L-tryptophan has never been investigated extensively.

Though Perley and Stowe (4) have demonstrated that cell free extract from *Bacillus cereus* catalyzes the formation of tryptamine from L-tryptophan, the properties of bacterial tryptophan decarboxylase still remain unknown.

In the course of investigations in our laboratory on the metabolism of L-tryptophan by bacteria, we found that a bacterial strain *Micrococcus percitreus* produced constitutively an enzyme which showed the high decarboxylation activity toward L-tryptophan. This communication describes the purification and crystallization of constitutive aromatic L-amino acid decarboxylase from *M. percitreus* and some properties of the enzyme.

Materials and Methods

Cultivation.

Micrococcus percitreus (AJ 1065) was cultured in a 50-liter fermenter jar containing 45 liters of a medium containing 0.5% of yeast extract, 3% of hydrolyzate of soybean, 0.5% of polypepton, 0.5% of glycerol, 0.1% of KH_2PO_4, 0.1% of $MgSO_4 \cdot 7H_2O$, 0.001% of $FeSO_4 \cdot 7H_2O$, 0.001% of $MnSO_4 \cdot 5H_2O$ and 0.01% of antifoam AF emulsion in tap water. The pH of the medium was adjusted to 8.0 by the addition of KOH solution. The culture was carried out at 30°C for 20 hr with aeration.

Enzyme assay.

Aromatic L-amino acid decarboxylase was assayed by measuring the amount of tryptamine formed from L-tryptophan according to the method of Kapfer and Atkinson (5) with a slight modification. Reaction mixture contained the enzyme, 20 μmoles of L-tryptophan, 1 μmole of pyridoxal phosphate and 500 μmoles of NH_4OH-NH_4Cl buffer, pH 9.0, in a total volume of 2 ml. The reaction was carried out at 30°C for 30 min and stopped by the addition of 0.5 ml of 2 N HCl. To 0.5 ml of deproteinized filtrate, 3.0 ml of 2% *p*-dimethylaminobenzaldehyde solution in a mixture of conc HCl and ethanol (25 : 75) was added, and this mixture was heated at 50°C for 40 min in a water bath. The intensity of the pink color was estimated at 30°C by measuring the absorbance at 580 nm. Though 20 μmoles of L-tryptophan reacts with the reagent slightly under these conditions, the absorbance at 580 nm is less than 0.02 and could be excluded by taking the control mixture without enzyme. The standard calibration curve had shown linearlity in the range of tryptamine concentration from 0.5 μmole to 10 μmoles in the presence of 20 μmoles of L-tryptophan. The absorbance at 580 nm is changeable with temperature, so it is necessary to estimate it at the constant temperature. Under these conditions, the color had been stable at least for 4 hr. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmole of tryptamine per minute under the assay conditions described. Specific activity was expressed as units per mg of protein.

Protein determination

Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm. An E value of 1.18 for 1 mg per ml and for 1 cm light path was used throughout. The protein in purified preparations of the enzyme was measured according to the method of Lowry *et al.* (6), with crystalline bovine serum albumin as a standard.

Spectrophotometric measurements.

Spectrophotometric measurements were done with a Beckman model DB-G recording, double beam spectrophotometer.

Results and Discussion

Purification.

The cells (approximately 10,500 g wet weight) harvested from 700 liters of the cultured broth, were suspended in 0.05 M potassium phosphate buffer, pH 7.0, to give a suspension of about 5 g/10 ml. All subsequent procedures were performed at 0 - 5°C. Potassium phosphate buffers used in the purification procedures contained 10^{-5} M pyridoxal phosphate, 10^{-2} M β-mercaptoethanol and 10^{-4} M EDTA.

Step 1. The suspended cells were disrupted by a Dyno Mill (W. A. Bachofen, Basel, Switzerland).

Step 2. One-fifth volume of a 10% protamine sulfate solution at pH 7.0 was added to the cell extract (22.5 liters) and the precipitate formed was removed by centrifugation at 16,000 x g for 30 min.

Step 3. The supernatant solution was fractionated with ammonium sulfate (30 - 50% saturation), followed by dialysis against 0.05 M potassium phosphate buffer, pH 7.0.

Step 4. Ninety-three milliliters of 10% protamine sulfate at pH 7.0 was added to the dialyzate (1,430 ml) and the precipitate formed was removed by centrifugation at 16,000 x g for 30 min.

Step 5. The supernatant solution (1,420 ml) was applied on a DEAE-Sephadex A-50 column (13 x 20 cm) equilibrated with 0.05 M potassium phosphate

buffer, pH 7.0. After the column was washed with the same buffer, the enzyme was eluted stepwise with 0.1 M potassium phosphate buffers, pH 7.0, containing 0.2 and 0.3 M KCl. Active fractions were combined and concentrated by the addition of ammonium sulfate to 50% saturation. The precipitate was collected and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0.

Step 6. The dialyzed enzyme solution (500 ml) was again fractionated with ammonium sulfate (30 - 38% saturation), followed by dialysis against potassium phosphate buffer, pH 7.0.

Step 7. The dialyzed enzyme solution (92 ml) was applied on a DEAE-Sephadex A-50 column (4.4 x 14 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of KCl concentration from 0.1 to 0.3 M in 0.1 M potassium phosphate buffer, pH 7.0. Active fractions were combined and concentrated by the addition of ammonium sulfate to 50% saturation. The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.0.

Fig. 1. Photomicrograph of crystalline aromatic L-amino acid decarboxylase from *M. percitreus* (x 600).

Step 8. The enzyme solution (3 ml) was passed through a Sephadex G-200 column (2.4 x 90 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. Active fractions were combined and concentrated by the addition of ammonium sulfate to 45% saturation. The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.0.

Step 9. The enzyme solution (0.5 ml) was again passed through a Sephadex G-200 column (2.4 x 90 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. Active fractions were combined and concentrated with ammonium sulfate to 50% saturation. The precipitate was dissolved in a minimum amount of 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-3} M dithiothreitol.

Step 10. Fine powdered ammonium sulfate was cautiously added to the enzyme solution until it became slightly turbid, and the mixture was placed in an ice bath. Crystallization began after about 8 hours and was virtually

Table I. Purification of aromatic L-amino acid decarboxylase from *M. percitreus.*

	Step	Total protein (mg)	Total activity (units)	Specific activity	Yield (%)
1.	Cell extract	2,920,000	2089	0.00072	100
2.	1st Protamine sulfate	439,000	1734	0.0040	83
3.	1st Ammonium sulfate	46,300	1130	0.024	54
4.	2nd Protamine sulfate	19,300	1179	0.061	56
5.	1st DEAE-Sephadex	7,710	846	0.11	40
6.	2nd Ammonium sulfate	1,860	646	0.35	31
7.	2nd DEAE-Sephadex	476	787	1.65	38
8.	1st Sephadex G-200	141	690	4.89	33
9.	2nd Sephadex G-200	60	616	10.3	29
10.	Crystallization	16	268	16.5	13
	Recrystallization	5	91	18.4	4.4

complete within 10 days. Fig. 1 is a photomicrograph of the crystalline aromatic L-amino acid decarboxylase of *M. percitreus*, which appears as fine needles with a yellow color. Recrystallization was carried out by repeating the last step. A summary of typical purification procedure is presented in Table I. The aromatic amino acid decarboxylase was purified 25,000-fold with a 4.4% yield.

Homogeneity.

The recrystallized preparation of enzyme sedimented as a single symmetric peak in the ultracentrifuge performed in 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal phosphate, 10^{-2} M mercaptoethanol, 10^{-3} M dithiothreitol and 10^{-4} M EDTA. The recrystallized enzyme gave a single band on SDS-polyacrylamide gel electrophoresis carried out as described by Weber and Osborn (7), using 10% gels and an SDS-phosphate buffer system at pH 7.2.

Properties.

The sedimentation coefficient in water at 20°C, extrapolated to zero protein concentration ($S^0_{20,w}$) was found to be 6.2 S when protein concentration

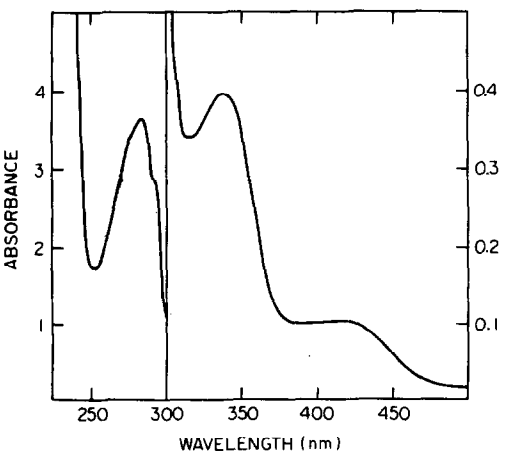

Fig. 2. Absorption spectra of aromatic L-amino acid decarboxylase from *M. percitreus*. The enzyme protein, 3.05 mg was dissolved in one milliliter of 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-4} M pyridoxal phosphate, 10^{-3} M mercaptoethanol and 10^{-4} M EDTA. The spectra were taken using the same buffer as a reference.

was varied from 2.5 mg to 5.0 mg per ml of the buffer. A diffusion coefficient $D_{20,w}$, of 6.0 x 10^{-7} cm^2 sec^{-1} was determined for a 3.31 mg/ml solution of protein. The molecular weight of enzyme was determined as 101,000 ± 5,000 from values of sedimentation and diffusion coefficients (8), by the sedimentation equilibrium analysis and by the gel filtration method with a Sephadex G-150.

Fig. 2 shows the absorption spectra of the enzyme. The visible spectrum exhibits two absorption maxima at 340 nm and 450 nm. The amount of pyridoxal phosphate bound by the enzyme was determined (9) after dialysis of the enzyme against 0.05 M potassium phosphate buffer, pH 7.0, containing 10^{-2} M mercaptoethanol, 10^{-3} M dithiothreitol, 10^{-4} M EDTA and 10^{-5} M pyridoxal phosphate. An excess concentration of pyridoxal phosphate was found inside the dialysis bag, corresponding to the binding of 1 mole of pyridoxal phosphate per mole of enzyme. Dialysis of the enzyme against the buffer without pyridoxal phosphate led to a complete loss of the activity. The dialyzed enzyme retains its original activity in the presence of excess of pyridoxal phosphate.

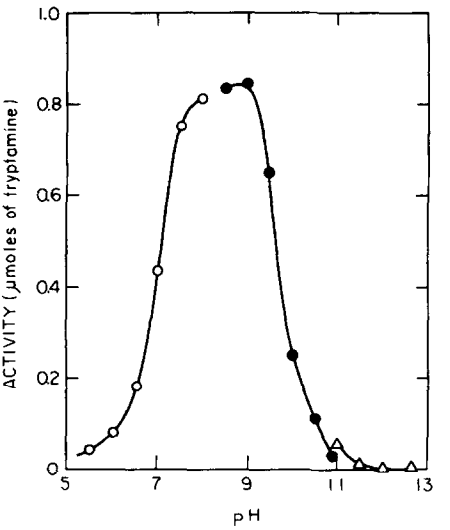

Fig. 3. Dependence upon pH of the decarboxylation of L-tryptophan by aromatic L-amino acid decarboxylase. The reaction was carried out at 30°C for 30 min in a reaction mixture containing 0.2 μmole of pyridoxal phosphate, 1.5 μg of enzyme, 4 μmoles of L-tryptophan and 50 μmoles of the following buffers in a total volume of 0.4 ml. The buffers used were KH_2PO_4-K_2HPO_4 (o---o), NH_4OH-NH_4Cl (•---•) and K_2HPO_4-KOH (Δ---Δ). The enzyme activity was determined as described in the text.

The concentration of pyridoxal phosphate to give the half maximum rate with the dialyzed enzyme was determined to be 2.0×10^{-5} M.

L-Tryptophan showed classical hyperbolic saturation kinetics with a Michaelis constant of 2.4×10^{-3} M. The maximal velocity of decarboxylation of L-tryptophan was determined to be 22.0 μmole/min/mg of protein. The optimal pH was around 9.0 (Fig. 3), in 0.125 M NH_4OH-NH_4Cl buffer. The enzyme also catalyzed decarboxylation of L-phenylalanine and L-tyrosine, at the relative reaction rates of 87 and 120, respectively, in the comparison with L-tryptophan (100).

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