Bacterial Monoamine Oxidases

Part I. Purification and Crystallization of Tyramine Oxidase of *Sarcina lutea*

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A procedure for obtaining crystalline preparations of tyramine oxidase of *Sarcina lutea* has been developed. The procedure included fractionation with ammonium sulfate, treatment with protamine sulfate and separation by column chromatographies on DEAE-cellulose, hydroxylapatite and sephadex G-150. The specific activity of enzyme was increased 5,700~6,000-fold through the procedure, over the crude cell extract. Crystals were prepared from solutions of the purified enzyme by adding solid ammonium sulfate. The crystals appeared as minute, highly refractive needles, with a bright yellow color.

INTRODUCTION

Monoamine oxidase is one of the enzymes which catalyze the oxidation of tyramine, tryptamine, norepinephrine and other monoamines of natural origin, according to the following equation:

The monoamine oxidase is characterized by its location in mitochondria of animal organs, its susceptibility to certain heavy metals and its resistance to carbonyl reagents such as hydrazine, hydroxylamine and semicarbazide, as well as carbon monoxide. These characters enable one to distinguish monoamine oxidase from the other amine oxidases such as those of kidney, pea seedlings, plasma¹⁾ and *Aspergillus niger*.^{2~5)}

Due to difficulties of solubilizing the monoamine oxidase and retaining its soluble character during further treatments, the details of mechanism of the enzymatic oxidations of monoamines have remained to be elucidated, and the studies with pure enzyme will be necessary in this effort.

In the course of investigations on the nature of amine oxidases of microorganisms, we have obtained, from cells of *Sarcina lutea* grown on nutrient broth, a crystalline protein fraction which catalyzes the oxidation of tyramine in a manner reminiscent of the monoamine oxidase of animal organs. The enzyme has been observed to be inhibited by iproniazid, but not by isoniazid, hydrazine, hydroxylamine and semicarbazide. The present paper describes the purification and the crystallization of the tyramine oxidase of *Sarcina lutea*.

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EXPERIMENTAL

Organism and Medium. *Sarcina lutea* (IAM 1099) was used throughout this work. The basal medium for cultures consisted of 1.0% of peptone, 1.0% of meat extract, 0.5% of NaCl and 0.05% of yeast extract in tap water. The pH of the medium was adjusted to 7.0.

Chemicals. Hydroxylapatite was prepared according to the method of Tiselius, Hjerten and Levin.⁶⁾ Purified beef liver catalase was obtained from the Nutritional Biochemicals Corporation, U.S.A. All of the other chemicals used were commercial products.

Cultivation. *Sarcina lutea* was picked up from the basal medium agar slant and inoculated in the subculture (500 ml of the basal medium in 2-liter flask). The incubation was carried out at 30°C for 24 hours with reciprocal shaking. The subcultures were in turn inoculated into the jar fermenter containing 25 liters of the basal medium. The incubation was carried out at 30°C for 24 hours with aeration (25 liters per minute). The cells grown were harvested by the continuous-flow centrifuge and washed with distilled water.

Analytical Procedures

1) **Enzyme Assay and Protein Determination.** The enzyme assays were based on the initial rate of oxygen uptake by reaction mixture containing enzyme, 50 μ g of catalase, 5 μ moles of tyramine and 30 μ moles of phosphate buffer, pH 7.0, in a total volume of 3 ml. A unit of enzyme activity was defined as the amount of enzyme which gave an oxygen uptake of 1 μ l per 30 minutes (calculated from the initial rate). Specific activity was expressed as units per mg of protein. The protein was determined by measuring the absorbancy at 280 m μ . A $E_{cm}^{1\%}$ value of 15.0 was used throughout.

2) **Spectrophotometric Determination.** The spectrophotometric determinations were carried out using the Shimadzu model QR-50 spectrophotometer.

3) **Manometric Measurement.** The oxygen uptake was measured in air, by the conventional Warburg apparatus at 30°C. The volume of the reaction mixture was 3.0 ml and KOH was present in the center cup.

RESULTS

Purification of Enzyme

All operations were carried out around 5°C throughout the purification procedures.

Extraction. *Sarcina lutea* was grown on 200 liters of the basal medium at 30°C in the jar fermenter. The cells were harvested by the continuous-flow centrifuge and washed with distilled water. The cells were suspended in 0.01 M phosphate buffer, pH 7.0, to give a suspension of 1 g of wet cells per 5 ml of the buffer. The suspension was subjected to the action of the Kaijo-denki ultrasonic oscillator (20 kc) for 1 hour. The cells and debris were removed by centrifugation at 10,000 $\times$ g for 20 minutes.

First Ammonium Sulfate Fractionation. To 27 liters of the cell extract was added solid ammonium sulfate to 0.80 saturation. After standing overnight, the precipitate was collected by centrifugation at 20,000 $\times$ g for 20 minutes and dissolved in 0.01 M phosphate buffer, pH 7.0, and the solution was dialyzed for 48 hours against four changes of 15 liters of 0.01 M phosphate buffer, pH 7.0.

Second Ammonium Sulfate Fractionation. Solid ammonium sulfate to 0.40 saturation was added to the dialyzate (3,030 ml) from the preceding step and the pH was adjusted to 7.0 by the addition of ammonia. After standing overnight, the precipitate was removed by centrifugation at 20,000 $\times$ g for 20 minutes. The ammonium sulfate concentration was then increased to 0.60 saturation by the addition of solid ammonium sulfate and the pH was again adjusted to 7.0.

After standing overnight, the precipitate was collected by centrifugation at 20,000 $\times$ g for 20 minutes and dissolved in a small amount of 0.01 M phosphate buffer, pH 7.0. The enzyme solution was dialyzed for 48 hours against four changes of 10 liters of 0.01 M phosphate buffer, pH 7.0.

Protamine Treatment. To 800 ml of the dialyzate was added 80 ml of a solution containing 4,800 mg of protamine sulfate, with

6) A. Tiselius, S. Hjerten and O. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

stirring. After 30 minutes, the precipitate was removed by centrifugation at $20,000\times g$ for 30 minutes.

DEAE-Cellulose Column Chromatography. The supernatant (835 ml) obtained from the preceding step was subjected to DEAE-cellulose column chromatography. The adsorbent was packed into a column (6.0×53 cm) and equilibrated with 0.01 M phosphate buffer, pH 7.0. The supernatant was passed through the column and the column was treated with 0.01 M phosphate buffer, pH 7.0, followed with 0.1 M phosphate buffer, pH 7.0, which removed much of the inactive protein. The enzyme was then eluted stepwise with 0.05 M and 0.15 M NaCl in 0.1 M phosphate buffer, pH 7.0, at a flow rate of 1 ml per minute in fractions consisting of 15 ml. The elution of protein was followed by the measurement of absorbancy at $280\text{ m}\mu$ as well as by the determination of enzyme activity. The fractions containing enzyme activity were combined to give 1,040 ml which was concentrated by the addition

of solid ammonium sulfate to 0.70 saturation. The precipitate obtained by centrifugation ($20,000\times g$, for 20 minutes) was dissolved in 0.01 M phosphate buffer, pH 7.0, and dialyzed for 48 hours against four changes of 3 liters of 0.01 M phosphate buffer, pH 7.0. The elution pattern of the chromatographed enzyme is shown in Fig. 1.

Third Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the dialyze (82 ml) to give 0.40 saturation and the pH was adjusted to 7.0. After standing overnight, the precipitate was removed by centrifugation at $20,000\times g$ for 20 minutes. The ammonium sulfate concentration was then increased to 0.55 saturation by the addition of solid ammonium sulfate and the pH was again adjusted to 7.0. After standing overnight, the precipitate was collected by centrifugation at $20,000\times g$ for 20 minutes and dissolved in a small amount of 0.01 M phosphate buffer, pH 7.0. The solution was dialyzed for 48 hours against four changes of 3 liters of 0.01 M phosphate buffer,

FIG. 1. Chromatography of the Crude Tyramine Oxidase of *Sarcina lutea* on DEAE-Cellulose.

Protein, 16.7 g, containing 1.14×10^5 units of activity, was applied to a column (6.0×53 cm), equilibrated with 0.01 M phosphate buffer, pH 7.0. Fractions of 15 ml were collected at a flow rate of 1 ml per minute.

FIG. 2. Chromatography of the Partially Purified Tyramine Oxidase on Hydroxylapatite.

Protein, 830 mg, containing 7.11×10^4 units of activity, was applied to a column (5×15 cm), equilibrated with 0.003 M phosphate buffer, pH 7.0. Fractions of 10 ml were collected at a flow rate of 10 ml per hour.

pH 7.0.

Hydroxylapatite Column Chromatography. The dialyzed enzyme solution (50 ml) was subjected to hydroxylapatite column chromatography. The hydroxylapatite was packed into a column (5×15 cm) and equilibrated with 0.003 M phosphate buffer, pH 7.0. The enzyme solution was placed on the column and developed with 0.003 M and 0.03 M phosphate buffers, pH 7.0. The buffers were allowed to flow at a rate of 10 ml per hour and 10-ml fractions were collected. The elution pattern of the enzyme is shown in Fig. 2. The fractions containing enzyme of specific activity greater than 200 were pooled to give 460 ml which was concentrated by the addition of solid ammonium sulfate to 0.70 saturation. The precipitate obtained by centrifugation ($20,000 \times g$ for 20 minutes) was dissolved in a minimum amount of 0.01 M phosphate buffer, pH 7.0.

First Sephadex Filtration. The enzyme solution (13 ml) was subjected to sephadex G-150 filtration. The sephadex G-150 was packed

FIG. 3. First Filtration of the Purified Tyramine Oxidase on Sephadex G-150.

Protein, 160 mg, containing 5.11×10^4 units of activity, was applied to a column (2×100 cm), equilibrated with 0.01 M phosphate buffer, pH 7.0. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

into a column (2×100 cm) and equilibrated with 0.01 M phosphate buffer, pH 7.0. The buffer was allowed to flow at a rate of 10 ml per hour and 5-ml fractions were collected. The elution pattern of the enzyme is shown in Fig. 3. Filtration resulted in the separation of two colored proteins: a leading green protein, followed by yellow protein. The enzyme activity was entirely associated in the yellow protein. The yellow fractions containing enzyme of specific activity greater than 300 were combined to give 90 ml which was concentrated by the addition of solid ammonium sulfate to 0.70 saturation. The precipitate was collected by centrifugation ($20,000 \times g$ for 20 minutes) and dissolved in a minimum amount of 0.01 M phosphate buffer pH 7.0.

Second Sephadex Filtration. The enzyme solution (5.5 ml) was again subjected to the sephadex G-150 filtration. Conditions of the second filtration were the same as those described in the first filtration and the elution pattern of the enzyme is shown in Fig. 4. One sym-

FIG. 4. Second Filtration of the Purified Tyramine Oxidase on Sephadex G-150.

Protein, 84 mg, containing 4.14×10^4 units of activity, was applied to a column (2×100 cm), equilibrated with 0.01 M phosphate buffer, pH 7.0. Fractions of 5 ml were collected at a flow rate of 10 ml per hour.

metric protein peak was eluted and the enzyme activity coincided almost exactly with the protein peak. The enzyme fractions were pooled to give 100 ml which was concentrated by the addition of solid ammonium sulfate to 0.70 saturation. The precipitate was collected by centrifugation ($20,000 \times g$, for 20 minutes) and dissolved in a minimum amount of 0.01 M phosphate buffer, pH 7.0. The enzyme solution was dialyzed for 24 hours against two changes of 3 liters of 0.01 M phosphate buffer, pH 7.0, containing 0.001 M dithiothreitol.

Fourth Ammonium Sulfate Fractionation. To the dialyzed enzyme solution (3.5 ml) was added solid ammonium sulfate to 0.35 saturation and the pH was adjusted to 7.0. After standing overnight, the precipitate was removed by centrifugation at $20,000 \times g$ for 20 minutes. The ammonium sulfate concentration was then increased to 0.40 saturation by the addition of solid ammonium sulfate and the pH was again adjusted to 7.0. After standing overnight, the precipitate was collected by centrifugation at $20,000 \times g$ for 20

minutes and dissolved in a minimum amount of 0.01 M phosphate buffer, pH 7.0, containing 0.001 M dithiothreitol.

Crystallization. Solid fine-powdered ammonium sulfate was cautiously added to the purified enzyme solution (1.5 ml) until a faint turbidity appeared. After 30 minutes, the precipitate was removed by centrifugation at $25,000 \times g$ for 20 minutes. Saturated ammonium sulfate solution was added dropwise to the supernatant to appearance of permanent turbidity and the mixture was kept in an ice bath. Crystallization of the enzyme began after about 6 hours and virtually completed within a week. For recrystallization, the crystals were dissolved in the minimum amount of 0.01 M phosphate buffer, pH 7.0, containing

FIG. 5. Photomicrograph of the Crystalline Tyramine Oxidase of *Sarcina lutea*. (magnified 600-fold).

TABLE I. SUMMARY OF PURIFICATION AND CRYSTALLIZATION OF TYRAMINE OXIDASE OF *Sarcina lutea*.

	Total protein (mg)	Total units	Specific activity
Cell extract	330,000	160,000	0.48
Ammonium sulfate (I)	248,000	151,000	0.61
Ammonium sulfate (II)	48,000	124,000	2.60
Protamine sulfate	16,700	114,000	6.80
DEAE-cellulose	2,030	81,200	40.0
Ammonium sulfate (III)	830	71,100	84.0
Hydroxylapatite	160	51,100	320
Sephadex G-150 (I)	84	41,400	490
Sephadex G-150 (II)	67	40,300	600
Ammonium sulfate (IV)	10	27,400	2,740
Crystallization	5	19,650	3,930
Recrystallization	3	12,450	4,150

0.001 M dithiothreitol. Solid ammonium sulfate was added in the same way as described above.

Fig. 5 is a photomicrograph of crystalline tyramine oxidase of *Sarcina lutea* which appeared as separate needles with a yellow color and which was easily soluble in phosphate buffer, pH 7.0, to give a bright yellow-colored solution. Table I gives a summary of the purification and crystallization of the enzyme.

DISCUSSION

There are many similarities between monoamine oxidase and diamine oxidase. They belong to the group of enzymes of oxidative deamination, including D- and L- amino acid oxidases, whose over-all reaction can be described by the same equations. However, the two enzymes are distinguished from one another not only by their substrate specificities but also by their inhibitor specificities. Unlike monoamine oxidase, diamine oxidase is efficiently blocked by acylhydrazine and other carbonyl reagents.^{1,7)}

Diamine oxidase is relatively easily soluble and several diamine oxidases, such as those of pea seedlings,⁸⁾ plasma,^{9,10)} kidney,¹¹⁾ *Aspergillus niger*³⁾ and *Trichosporon* sp.,¹²⁾ have recently been purified extensively. Their properties as well as the reaction mechanisms have been elucidated in detail.¹³⁾ Monoamine oxidase, on the other hand, seems to be connected with the structural elements of the cell

and attempts to solubilize monoamine oxidase have been unsuccessful. Monoamine oxidase has thus been purified only partially and definitive experimental results on prosthetic groups as well as the mechanisms of oxidation of monoamines have not yet been obtained.

In the course of investigations on the amine oxidases of microorganisms, we have found that the cell extract of *Sarcina lutea* grown on the nutrient broth catalyzes the oxidation of tyramine in a manner reminiscent of the monoamine oxidase. The enzyme was not inhibited by cyanide, isoniazid and other carbonyl reagents, but potentially inhibited by iproniazid. Purpose of the present work was to develop efficient procedures for purification of the enzyme in order to facilitate the investigation on the detailed mechanisms of enzymatic oxidation of monoamines.

The test with various adsorbents was initially performed to ascertain their efficiencies in purifying the enzyme of *Sarcina lutea*. The final method described in this paper, based mainly on purification with DEAE-cellulose, hydroxylapatite and sephadex G-150, yielded preparations which were purified about 6,100-fold. Crystallization was carried out on the purified enzyme preparations, by the gradual addition of solid ammonium sulfate. The crystals appeared as minute, highly refractive needles. On the crystallization steps, the specific activity of the enzyme increased again from 2,700~2,800 to 3,900~4,200.

The spectrophotometric investigations on the crystalline preparations of the enzyme of *Sarcina lutea* revealed that the enzyme was bright yellow and that the visible spectrum of the enzyme was very similar to that of flavin nucleotides except that the absorption peaks were shifted slightly to 368 and 466 m μ . Addition of tyramine to the enzyme under anaerobic conditions produced a bleaching of the visible color. Detailed investigations on prosthetic groups of the fungal amine oxidase are currently under investigation. While this work was in progress, Nara, Igaue, Gomes

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and Yasunobu,¹⁴⁾ with the use of 58-fold purified enzyme of beef liver, presented the evidence that flavin is an essential component of the monoamine oxidase of animal organs.

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