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PROPERTIES OF CRYSTALLINE TYRAMINE OXIDASE FROM *SARCINA LUTEA*

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SUMMARY

A procedure is described for the preparation of crystalline tyramine oxidase from cells of *Sarcina lutea* grown in a synthetic medium supplemented with tyramine. The crystalline enzyme preparation appears homogeneous in ultracentrifuge and in acrylamide gel electrophoresis. The molecular weight is determined as 129 000. The enzyme exhibits a typical flavoprotein spectrum and FAD content of the enzyme is calculated to be two moles per mole of enzyme. The enzyme does not contain any significant quantity of firmly bound heavy metal ions.

INTRODUCTION

Monoamine oxidase (monoamine:oxygen oxidoreductase, EC 1.4.3.4) has been found in the mitochondria of liver, kidney and other organs[1]. A soluble monoamine oxidase with different substrate specificity has been found in various bacteria[2-4]. These monoamine oxidases are distinguished from other diamine oxidases by their substrate and inhibitor specificities[1,5]. Monoamine oxidases have been reported to contain FAD as the prosthetic group[6,7], whereas diamine oxidases are a copper and pyridoxal phosphate dependent enzyme[8-13].

In the previous paper, the authors reported that tyramine oxidase occurs in cells of *Sarcina lutea* grown in a bouillon–peptone medium[14]. The enzyme was crystallized and its specificities for substrate and inhibitor were investigated[14-17]. In the present paper, we describe an improved method for the preparation of crystalline tyramine oxidase from cells of *S. lutea* grown in a synthetic medium supplemented with tyramine. Yields of the crystalline enzyme by this improved method are 20 to 30-fold greater than those obtained by the older method[14]. Investigations on the physico-chemical properties and the prosthetic group of this enzyme are also presented.

MATERIALS AND METHODS

Chemicals

Hydroxylapatite was prepared according to the method of TISELIUS, HJERTEN AND LEVIN[18]. Purified beef liver catalase was obtained from the Nutritional Biochemicals Corporation, U.S.A. All other chemicals used were commercial products.

Enzyme assay

The enzyme was assayed manometrically at 30° in a reaction mixture containing enzyme, 50 μg of catalase, 5 μmoles of tyramine and 30 μmoles of phosphate buffer (pH 7.0), in a total vol. of 3.0 ml. A unit of enzyme activity was defined as the amount of enzyme which gave an oxygen uptake of 1 μl/30 min (calculated from the initial rate). Specific activity was expressed as units/mg protein.

Protein determination

The protein was determined spectrophotometrically by measuring its absorbance at 280 mμ. An E value of 27.0 for 1 mg/ml and for 1-cm light path which was obtained by absorbance and dry weight determinations was used throughout.

Metal determination

The metal content of the enzyme was determined spectrophotometrically. Recrystallized enzyme prepared with deionized-distilled water and metal-free reagents was used. The enzyme preparation was dialyzed for 24 h against 0.01 M phosphate buffer (pH 7.0), containing $1 \cdot 10^{-4}$ M dithiothreitol and $1 \cdot 10^{-3}$ M EDTA; then dialyzed for 12 h against the buffer, containing $1 \cdot 10^{-4}$ M dithiothreitol alone. The dialyzed enzyme preparation, at a concentration of 1.0–1.5 mg of protein per ml, was analyzed with a Nippon Jarrell-Ash Model AA-1 atomic absorption spectrophotometer.

RESULTS

Purification of tyramine oxidase

S. lutea (Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan, 1099) was inoculated into a 3-l flask containing 700 ml bouillon–peptone medium. The subculture was carried out at 30° for 24 h under reciprocal shaking. This subculture was, in turn, inoculated into a 30-l jar fermentor containing 20 l bouillon–peptone medium and culturing was carried out for 24 h under aeration (20 l/min). The cells were harvested by a continuous-flow centrifuge, washed with distilled water and transferred into the jar fermentor containing 20 l of a synthetic medium. The synthetic medium consisted of 3.0% glucose, 0.15% tyramine, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl and 0.02% yeast extract in tap water (pH 7.0). Culturing in the synthetic medium was carried out at 30° for 12 h under aeration. The cells were again harvested and washed with distilled water.

All subsequent procedures were performed at 0–5°.

Step 1. Enzyme extraction. The cell paste (1500 g) obtained from 100 l of the synthetic medium was suspended in 0.01 M phosphate buffer (pH 7.0), to give a suspension of about 1 g/5 ml. This suspension was divided into 500-ml portions and

each portion was subjected to ultrasonic oscillation (20 kcycles) for 1 h. The cells and debris were removed by centrifugation at 10 000 × *g* for 20 min.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to 5 l of the crude extract to 0.30 satn. After standing overnight, the precipitate was removed by centrifugation at 20 000 × *g* for 20 min. The ammonium sulfate concentration of the supernatant was increased to 0.60 satn. by the addition of solid ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation at 20 000 × *g* for 20 min and dissolved in a small amount of 0.01 M phosphate buffer (pH 7.0). The enzyme solution was dialyzed for 48 h against four changes of 10 l of 0.01 M phosphate buffer (pH 7.0).

Step 3. Protamine treatment. 28 ml of a solution containing 2800 mg of protamine sulfate was added to 400 ml of the dialyzate with stirring. After 30 min, the precipitate was removed by centrifugation at 20 000 × *g* for 30 min.

Step 4. DEAE-cellulose column chromatography. The supernatant (412 ml) obtained in the preceding step was applied to a DEAE-cellulose column (6 cm × 60 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.0). The enzyme solution was placed on the column and the column was washed with 0.1 M phosphate buffer (pH 7.0), which removed much of the inactive protein. The enzyme was then eluted with 0.1 M phosphate buffer (pH 7.0), containing 0.15 M NaCl, at a flow rate of 1 ml/min, in 10-ml fractions. The elution of protein was followed by measurement of its absorbance at 280 mμ as well as by the determination of enzyme activity. The fractions containing enzyme activity were combined to give 500 ml which was concentrated by the addition of solid ammonium sulfate to 0.70 satn. The precipitate was collected by centrifugation at 20 000 × *g* for 20 min, dissolved in 0.01 M phosphate buffer (pH 7.0), and dialyzed for 48 h against four changes of 3 l of 0.03 M phosphate buffer (pH 7.0).

Step 5. Hydroxylapatite column chromatography. The dialyzed enzyme solution (30 ml) was applied to a hydroxylapatite column (5 cm × 8 cm) which was equilibrated with 0.03 M phosphate buffer (pH 7.0). The enzyme solution was placed on the column and the column was developed with 0.03 M phosphate buffer (pH 7.0). The buffer was allowed to flow at a rate of 10 ml/h and 5-ml fractions were collected. The enzyme was not adsorbed under these conditions, but much inert protein including most of the green protein, was adsorbed and separated from the enzyme. The fractions containing enzyme activity were combined to give 280 ml and concentrated by the addition of solid ammonium sulfate to 0.70 satn. The precipitate was collected by centrifugation at 20 000 × *g* for 20 min and dissolved in 0.01 M phosphate buffer (pH 7.0).

Step 6. Sephadex G-150 filtration. The enzyme solution was subjected to Sephadex G-150 filtration. The Sephadex was packed into a column (2 cm × 100 cm) and equilibrated with 0.01 M phosphate buffer (pH 7.0). The enzyme solution was then placed on the column and the buffer was allowed to flow at a rate of 10 ml per h. 5-ml fractions were collected. This filtration yielded a single, symmetric protein peak and the enzyme activity was entirely associated with this protein peak. Fractions containing this enzyme activity were pooled to give 100 ml, which was then concentrated by the addition of ammonium sulfate to 0.70 satn. The precipitate was collected by centrifugation at 20 000 × *g* for 20 min and dissolved in a minimum amount of 0.01 M phosphate buffer (pH 7.0), containing $1 \cdot 10^{-4}$ M dithiothreitol.

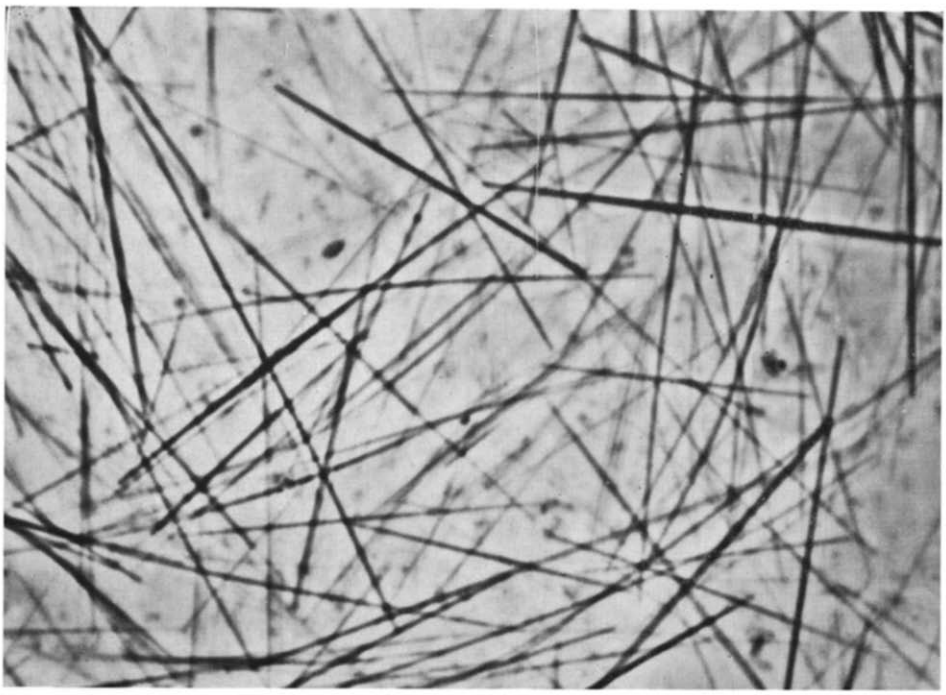

Fig. 1. Photomicrograph of crystalline tyramine oxidase from *S. lutea*. Magnified 600-fold.

Step 7. Crystallization. Solid fine-powdered ammonium sulfate was cautiously added to the purified enzyme solution (4.0 ml) until a faint turbidity appeared. Crystallization of the enzyme began about 6 h afterwards and was virtually complete within a week. For recrystallization, the crystals were dissolved in a minimum amount of 0.01 M phosphate buffer (pH 7.0), containing $1 \cdot 10^{-4}$ M dithiothreitol. Solid ammonium sulfate was added in the manner described above.

Fig. 1 is a photomicrograph of crystalline tyramine oxidase of *Sarcina lutea* which appears as fine needles with a bright-yellow color. A summary of the purification procedures of the enzyme is shown in Table I. Yields of the crystalline enzyme

TABLE I

PURIFICATION OF TYRAMINE OXIDASE FROM *S. lutea*

Step	*Fraction*	*Total protein (mg)*	*Total units*	*Specific activity*
1	Crude extract	126 600	1338 000	10.6
2	Ammonium sulfate	55 600	1158 000	20.8
3	Protamine sulfate	4 060	1098 000	270
4	DEAE-Sephadex	464	780 000	1 680
5	Hydroxylapatite	158.4	756 000	4 780
6	Sephadex G-150	63.2	720 000	11 400
7	Crystals (I)	38.8	588 000	15 100
	Crystals (II)	22.4	340 000	15 200

by this improved procedure from cells grown in the synthetic medium supplemented with tyramine, were about 20–30 times higher than those obtained by the older procedure from cells grown in the bouillon–peptone medium[14].

Properties

Homogeneity

The specific activity of enzyme achieved after the second crystallization was not altered upon further recrystallization. The recrystallized enzyme preparation gave a single band on acrylamide gel electrophoresis carried out at pH 8.3 (Fig. 2). The enzyme preparation sedimented as a single symmetric peak in the ultracentrifuge in 0.01 M phosphate buffer (pH 7.0), containing $1 \cdot 10^{-4}$ M dithiothreitol, at 20° (Fig. 3).

Fig. 2. Acrylamide gel electrophoresis of tyramine oxidase. Electrophoresis was carried out according to DAVIS[19]. The stocking and running gels were polymerized in a Pyrex tube (5 mm × 65 mm). Recrystallized enzyme (83.6 μg) was applied to the stocking gel and subjected to the electrophoresis at 4.0 mA for 60 min. After the electrophoresis, the gel was stained with 1% naphthol blue-black, destained electrophoretically, and stored in 7% acetic acid.

Fig. 3. Sedimentation patterns of tyramine oxidase. Recrystallized enzyme was used at a concentration of 0.42% in 0.01 M phosphate buffer (pH 7.0), containing $1 \cdot 10^{-4}$ M dithiothreitol. Photographs were taken 16 (A), 32 (B), 48 (C) and 64 (D) min after reaching 59 780 rev./min. The sedimentation is left to right.

Molecular weight

Extrapolation of the data obtained from four ultracentrifuge runs to zero protein concentration gave an $s^0_{20,w}$ of $5.60 \cdot 10^{-13}$ (cm/sec). A diffusion constant, $D_{20,w}$, of $4.20 \cdot 10^{-7}$ (cm^2/sec) was determined for a 4.30 mg/ml solution of protein. A value of 129 000 was calculated for the molecular weights of the enzyme, assuming a partial specific volume of 0.75.

Absorption spectra

Fig. 4 shows the absorption spectra of tyramine oxidase from *Sarcina lutea*. The visible spectrum of the enzyme was very similar to that of free FAD except that the absorption peaks showed a slight shift to 368 and 466 mμ (Curve I in Fig. 4).

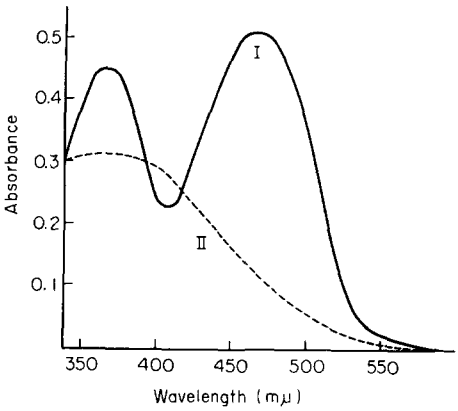

Fig. 4. Absorption spectra of tyramine oxidase. Recrystallized enzyme was used at a concentration of 0.31% in 0.01 M phosphate buffer (pH 7.0) containing $1 \cdot 10^{-4}$ M dithiothreitol. Curve I represents the native enzyme and Curve II the reduced enzyme after the addition of 1 μmole of tyramine.

Addition of tyramine to the enzyme, under anaerobic conditions, procuced a bleaching of the absorption peaks (Curve II in Fig. 4). The peaks were subsequently restored by bubbling oxygen into the solution.

The extinction coefficient of the enzyme, at 280 mμ ($E^{1\%}_{1\,cm}$), was found to be 27.0 and the ratio of $A_{280\ m\mu}/A_{466\ m\mu}$ was 16.3.

Prosthetic group

Flavin analysis. Treatment of the recrystallized enzyme with heat at 100° for 2–5 min, resulted in liberation of a yellow compound. This yellow compound was identified as FAD by paper chromatography with three different solvent systems[20]. It served as a prosthetic group of the D-amino acid oxidase[21]. Assuming the enzyme-bound FAD extinction coefficient at 466 mμ as equal to that of free FAD at 450 mμ ($\varepsilon_M = 1.13 \cdot 10^4$) (ref. 22), the FAD content was found to be 14.7 μmoles/mg of enzyme. This value corresponds to 1.9 moles of FAD per mole of enzyme.

Metal analysis. Analysis of metals known to be involved in the oxidative processes; for example, iron or copper[23], was carried out spectrophotometrically with the recrystallized enzyme. The result is shown in Table II. No metals were found to be present in concentrations exceeding 0.15 gatom/mole of enzyme.

TABLE II

METAL CONTENT OF CRYSTALLINE TYRAMINE OXIDASE

Metal	*Content (natoms/mg protein)*
Iron	0.00
Copper	0.22
Zinc	1.24
Manganese	0.24

DISCUSSION

An improved method for the preparation of crystalline tyramine oxidase from cells of *S. lutea* grown in a tyramine-supplemented medium was described. Yields of the crystalline enzyme by the present method are 20–30 times higher than those obtained by the older method with cells grown in a bouillon–peptone medium[14]. It seems that the enzyme may be adaptive in nature and that it is responsible for cell growth on a medium with tyramine as the sole nitrogen source[24].

The results described in the present paper demonstrate that tyramine oxidase is a flavoprotein containing 2 moles of FAD per mole of enzyme and that FAD is responsible for its catalytic activity. The participation of FAD in the oxidation process of amines has been demonstrated with various amine oxidases and dehydrogenases, particularly those of bacterial origin. SATAKE AND FUJITA[25] reported that the amine dehydrogenase of Achromobacter sp. required FAD for the catalytic activity. CAMPELLO, TABOR AND TABOR[26] prepared a partially purified spermidine dehydrogenase from *Serratia marcescens* and showed that the enzyme was a flavoprotein requiring FAD. We[27] previously obtained an apparently homogeneous preparation of putrescine oxidase from *Micrococcus rubens* and demonstrated that this enzyme was a flavoprotein containing 1 mole of FAD per mole of enzyme.

A preliminary experiment on metals in tyramine oxidase, described in the previous communication[15], indicated that the enzyme was a copper-flavoprotein containing 22.0 nmoles of copper per mg of protein. However, evidence for the hypothesis that metals function as electron acceptors and donors in metallo flavoproteins is, in most cases, meagre[28]. The authors carefully re-examined the presence of copper and other heavy metals in the recrystallized enzyme preparation obtained with deionized-distilled water and metal-free reagents. Analysis by atomic absorption spectrophotometry failed to detect any significant quantity of heavy metals. This result indicates that neither copper nor other heavy metals are essential for the activity of tyramine oxidase.

The effects of various chelating reagents on tyramine oxidase were described in the previous paper[16]. Of the chelating reagents investigated, only 8-hydroxyquinoline produced marked inhibition. Further investigation showed that α- and β-naphthols were also potent inhibitors and produced the same type of inhibition (competitive with the substrate) as that of 8-hydroxyquinoline (H. YAMADA AND H. KUMAGAI, unpublished observation). Inhibition of tyramine oxidase by these compounds may be related to the phenolic nature of the reagents, rather than to their capacity to chelate heavy metals. Phenols have been shown to form a charge transfer complex with FAD and inhibit other flavoproteins[29].

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