Purification and Some Properties of Bilirubin Oxidase of *Myrothecium verrucaria* MT-1

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Bilirubin oxidase was purified from the culture filtrate of *Myrothecium verrucaria* MT-1 by a procedure involving ammonium sulfate precipitation, charcoal treatment, and QAE-Sephadex A-50 and Sephadex G-100 column chromatographies. The purified enzyme was homogeneous on disc gel electrophoresis.

Copper and carbohydrate were contained in the enzyme. The enzyme was inhibited by Fe^{2+} and compounds that complex with copper. Bilirubin, biliverdin, hemin and chlorophyllin which consist of tetrapyrrole, and substrates of laccase were oxidized by the enzyme. Bilirubin was oxidized more rapidly than other substances. Bilirubin oxidase differed from laccase in reactivity with substances consisting of tetrapyrrole. Substances consisting of tetrapyrrole were oxidized only a little by laccase but rapidly oxidized by bilirubin oxidase. The apparent K_m value for bilirubin was calculated to be $190\text{ }\mu\text{M}$.

In the previous communications,^{1,2)} we reported the isolation and classification of a bilirubin oxidase producing microorganism and some properties of the enzyme were also reported. From taxonomic studies, the strain was identified as *Myrothecium verrucaria*. Bilirubin oxidase was produced in a high yield in potato-glucose medium at 25°C for 62 hr in a jar fermentor. It was found that strains belonging to the genus *Myrothecium* also produced bilirubin oxidase. In the present paper, the purification and some properties of bilirubin oxidase from *Myrothecium verrucaria* MT-1 are reported.

MATERIALS AND METHODS

Chemicals. All chemicals used in this work were commercial products.

Microorganism and cultural conditions. The microorganism used was *Myrothecium verrucaria* MT-1 which had been isolated from soil.^{1,2)} The medium and cultural conditions used for the production were described in the previous paper.²⁾

Enzyme assay. Bilirubin oxidase activity was assayed by

the method described in the previous paper.¹⁾ The specific activity was expressed as the enzyme activity per mg protein.

Assay of protein. Protein concentration was determined spectrophotometrically assuming that $E_{1\text{cm}}^{1\%}$ at 280 nm was 10.0.

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was carried out at pH 9.5.³⁾ A sample was applied on the top of the stacking gel and run at a constant current of 3 mA per tube for 1 hr. Staining of the gel was carried out using Coomassie Brilliant Blue for detecting protein bands and periodic acid-Schiff (PAS) for glycoprotein bands.⁴⁾

Isoelectric focusing. Isoelectric focusing analyses were performed according to the method of Vesterberg and Svensson.⁵⁾ A sample of the enzyme protein was applied to a column of carrier ampholytes with a pH range of 3.5~5.0. Constant potentials, 300 V for the first 3 hr and 700 V for the following 40 hr, were applied to the column at 0°C and the column was drained and fractionated into 2 ml fractions.

Determination of molecular weight by gel filtration. Gel filtration was performed using Sephadex G-100 ($2.5 \times 92\text{ cm}$) following the method of Andrews.⁶⁾ Sephadex G-100 (Pharmacia) was equilibrated with $0.03\text{ M Na}_2\text{CO}_3\text{--NaHCO}_3$ buffer (pH 9.3) containing 0.1 M NaCl . Cytochrome *c*, ovalbumin and bovine serum albumin were

FIG. 1. Polyacrylamide Gel Disc Electrophoresis of Laccase.

Electrophoresis was carried out using 7% gel at pH 8.0. The protein (about 20 μ g) was stained with Coomassie Brilliant Blue.

used as the reference proteins.

Amino acid composition. Purified enzyme protein was hydrolyzed with 6.0N HCl at 110°C in evacuated sealed tubes for 24, 48 and 72 hr, respectively. The hydrolyzate was rapidly dried *in vacuo* and then dissolved in 90 mM citrate buffer (pH 2.2). Amino acids in this solution were analyzed with a Hitachi KLA-5 amino acid analyser. The amounts of cysteine and tryptophan were determined according to the methods of Moore⁷⁾ and Goodwin,⁸⁾ respectively.

Laccase of *Polyporus versicolor* IFO 9791. Laccase of *Polyporus versicolor* was obtained by the procedure of Fähræus and Reinhammar.⁹⁾ Homogeneity of the purified enzyme was examined by means of polyacrylamide gel disc electrophoresis.³⁾ As shown in Fig. 1, a single protein band was obtained, so it was regarded as being homogeneous.

RESULTS

Purification of the enzyme

After the cultivation, the culture broth was centrifuged and the clear supernatant was used as the starting material for the purification.

Step 1. Ammonium sulfate precipitation. To 3,000 ml of the culture supernatant was added 1,820 g of finely powdered ammonium

FIG. 2. Elution Pattern of QAE-Sephadex A-50 Column Chromatography.

13 ml fractions were collected. ○—○, absorbance at 280 nm; ●—●, bilirubin oxidase activity.

FIG. 3. Elution Pattern of Sephadex G-100 Column Chromatography.

The flow rate was 20 ml per hr and 6.8 ml fractions were collected. ○—○, absorbance at 280 nm; ●—●, bilirubin oxidase activity.

sulfate to 80% saturation under stirring and the mixture allowed to stand overnight. The precipitates were collected by filtration with Hyflosupercel, dissolved in 400 ml of water and dialyzed against three changes of 10 liters of the 10 mM Na_2CO_3 – NaHCO_3 buffer (pH 9.2) for 24 hr and then clarified by centrifugation.

Step 2. Charcoal treatment. The dialyzed solution was decolorized by treatment with charcoal powder twice [0.7% (w/v) pH 8.8 and 0.2% (w/v) pH 7.4].

Step 3. QAE-Sephadex A-50 column chromatography. The enzyme solution was placed on a QAE-Sephadex A-50 column (4 × 15 cm) equilibrated with 15 mM Na_2CO_3 – NaHCO_3 buffer (pH 9.3). Then the enzyme was eluted with a linear gradient of Na_2CO_3 – NaHCO_3 buffer of concentrations from 0.015 M to 0.8 M.

The elution pattern of the enzyme is shown in Fig. 2. Active fractions were pooled and concentrated on a Diaflo PM-10 membrane filter.

Srep 4. Gel filtration by Sephadex G-100. The enzyme solution was placed on a Sephadex G-100 column (2.5 × 98 cm) equilibrated with 30 mM Na₂CO₃-NaHCO₃ buffer (pH 9.4). The elution pattern of the enzyme is shown in Fig. 3. The purified enzyme was submitted to polyacrylamide gel electrophoresis. Staining of the gels were carried out with Coomassie Brilliant Blue for detecting protein and periodic acid-Schiff (PAS) for detecting glycoprotein. A single band was obtained¹⁾ respectively and the protein band agreed with the glycoprotein band. Bilirubin oxidase was analyzed with an atomic absorption spectroscopy and it was found that it possesses about 1 atom of copper per molecule.

Estimation of molecular weight of the enzyme

From the results shown in Fig. 4, the molecular weight of the enzyme was estimated to be approximately 52,000.

Isoelectric point

Electrofocusing with a carrier ampholyte (pH 3.5~5.0) showed that the enzyme has an isoelectric point around pH 4.1.

Amino acid composition

As shown in Table I, half cystine was not present.

FIG. 4. Estimation of Molecular Weight of the Enzyme by Gel Filtration on Sephadex G-100.

A, bovine serum albumin; B, bilirubin oxidase; C, ovalbumin; D, cytochrome *c*.

Stability

After the purified enzyme was incubated in 0.1 M phosphate buffer, pH 8.0, at various

TABLE I. AMINO ACID COMPOSITION OF BILIRUBIN OXIDASE

Amino acid	Number of residues/molecule
Lysine	7
Histidine	35
Arginine	21
Aspartic acid	56
Threonine	35
Serine	21
Glutamic acid	42
Proline	35
Glycine	35
Alanine	42
1/2 Cystine	0
Valine	35
Methionine	7
Isoleucine	14
Leucine	28
Tyrosine	21
Phenylalanine	28
Tryptophan	14
Total	476

FIG. 5. Effect of Temperature on Stability of the Enzyme.

After treatment at various temperatures for 15 min at pH 8.0, the remaining activity was measured.

FIG. 6. Effect of pH on Stability of the Enzyme.

The enzyme, in 0.1 M McIlvaine (●), sodium phosphate (○), Na₂CO₃-NaHCO₃ (◐), and Na₂B₄O₇-Na₂CO₃ (◑) buffer, was preincubated at 37°C for 1 hr. After adjustment of the pH to 8.4, the residual activity was measured.

temperatures for 15 min, the remaining enzyme activity was assayed. As shown in Fig. 5, bilirubin oxidase was stable up to 37°C, but above 37°C gradual inactivation occurred. As shown in Fig. 6, bilirubin oxidase was stable in the pH range of 5.0 to 9.7 for 60 min at 37°C. When the enzyme solution was preserved at 5°C for 5 days, the enzyme was unstable below pH 9.0 but stable in the range of pH 9.2 to 9.7.

Effect of temperature and pH on the enzyme activity

As shown in Fig. 7, the optimum temperature for the enzyme reaction was about 40°C. Bilirubin is not dissolved in acid solution, so the effect of pH on the enzyme activity was investigated on the alkaline side. As shown in Fig. 8, the better pH for the enzyme reaction was about pH 8.0.

K_m value

From the Lineweaver-Burk plot inserted in Fig. 9, the apparent K_m value for bilirubin

was calculated to be 190 μM .

Substrate specificity

Substances consisting of tetrapyrrole and substrates of laccase were tested for their reactivity against bilirubin oxidase. Laccase of *Polyporus versicolor* was tested in the same way. As shown in Table II, bilirubin was

FIG. 9. Lineweaver-Burk Plots of Bilirubin Oxidase.

The enzyme activity was determined under the standard assay conditions.

TABLE II. SUBSTRATE SPECIFICITY OF BILIRUBIN OXIDASE AND LACCASE

Manometric experiments were performed to determine the amount of molecular oxygen. The rate of oxidation of each substrate by bilirubin oxidase is expressed relative to the rate of bilirubin oxidation (100%) and the rate of oxidation by laccase is expressed relative to the rate of *p*-phenylenediamine oxidation (100%). Bilirubin oxidase was assayed in 0.2M sodium phosphate buffer, pH 8.0, and laccase in 0.2M triethylamine-acetic acid buffer, pH 5.6. Bilirubin, biliverdin and hemin are dissolved only on the alkaline side, so these substances were assayed in 0.2M sodium phosphate buffer, pH 8.0.

FIG. 7. Effect of Temperature on Activity.

The enzyme activity was measured at pH 8.4 at various temperatures.

FIG. 8. Effect of pH on Activity.

The enzyme activity was measured with 0.1M buffer as indicated. KH_2PO_4 - $\text{Na}_2\text{B}_4\text{O}_7$ buffer was used from pH 8.0 to 9.2, Na_2CO_3 - NaHCO_3 buffer from pH 9.2 to 10 and $\text{Na}_2\text{B}_4\text{O}_7$ - Na_2CO_3 buffer from pH 10 to 10.5.

Substrate	Relative activity (%)	
	Bilirubin oxidase	Laccase
Bilirubin	100	$\pm^a$
Biliverdin	50	$\pm^a$
Chlorophyllin	50	$\pm^a$
Hemin	10	$\pm^a$
Vitamin B ₁₂	0	0
Hemoglobin	0	0
Hydroquinone	20	100
Catechol	67	83
Pyrogallol	10	58
<i>N,N</i> -Dimethyl- <i>p</i> -phenylenediamine	23	54
<i>p</i> -Phenylenediamine	15	100

^a Oxidized only a little.

TABLE III. EFFECT OF METAL IONS
AND COMPOUNDS THAT COMPLEX
WITH COPPER ON THE ENZYME
ACTIVITY

The enzyme was preincubated for 10 min with the metal ion or compounds that complex with copper before the addition of substrate.

Compound (1 mM)	Relative activity (%)
None	100
Mn ²⁺	100
Cd ²⁺	100
Sr ²⁺	100
Cu ²⁺ (0.1 mM)	100
Fe ²⁺	0
Ni ²⁺	100
Mg ²⁺	100
Ca ²⁺	100
Li ²⁺	100
Potassium cyanide (0.1 mM)	0
Sodium azide (0.1 mM)	58
Thiourea (0.1 mM)	85

oxidized more rapidly than other substances by bilirubin oxidase. Bilirubin oxidase differed from laccase in reactivity with substances consisting of tetrapyrrole. That is to say, substances which consisted of tetrapyrrole were oxidized only a little by laccase but rapidly oxidized by bilirubin oxidase.

Effect of metal ions and compounds that complex with copper on the enzyme activity

The effect of metal ions and compounds that complex with copper on the enzyme activity were examined. After preincubation of the enzyme with each reagent for 10 min at 30°C, the remaining activity was determined (Table III). The enzyme activity was inhibited by Fe²⁺, potassium cyanide, sodium azide and thiourea.

DISCUSSION

From 3 liters of culture filtrate of

Myrothecium verrucaria MT-1, bilirubin oxidase was purified. The purified enzyme was homogeneous on disc gel electrophoresis and contained copper and carbohydrate. This enzyme was inhibited by Fe²⁺ and compounds that complex with copper. Biliverdin, hemin and chlorophyllin which consist of tetrapyrrole, and substrates of laccase were oxidized. Bilirubin was oxidized more rapidly than other substances. Bilirubin oxidase differed from laccase in reactivity with substances consisting tetrapyrrole. Substances consisting of tetrapyrrole were oxidized only a little by laccase but rapidly oxidized by bilirubin oxidase.

The presence of bilirubin oxidase activity was expected in the brain of guinea pig, but the pure enzyme was not obtained.¹⁰⁾ Therefore, the properties of bilirubin oxidase were not clear. But we purified bilirubin oxidase produced by *Myrothecium verrucaria* MT-1 and described the properties of the bilirubin oxidase.

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