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Purification and Properties of Bilirubin Oxidase from *Myrothecium verrucaria*

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ABSTRACT

Bilirubin oxidase was purified from a culture filtrate of *Myrothecium verrucaria* Mv 2,1089 by DEAE-cellulose and Sephadex G-100 column chromatographies. The purified enzyme had a specific activity of 30 U/mg protein and showed a single band on polyacrylamide gel electrophoresis.

Some of the general properties of this bilirubin oxidase were as follows: the optimum pH for the enzyme reaction was 7.5 and the optimum temperature was 50°C. The enzyme was stable at pH ranging from 9.0 to 9.5. The mol wt was calculated to be 61,900–62,700 by SDS-PAGE and gel-filtration technique. The apparent K_m value of the bilirubin oxidase was calculated to be 9.4×10^{-5} mol/L. The enzyme activity was greatly reduced by incubation of bilirubin oxidase with Fe^{2+}, Hg^{+}, NaN_3, NH_4^+, and Zn^{2+}. The enzyme reaction was inhibited in the presence of Ca^{2+}, Hg^{+}, Zn^{2+}, Fe^{2+}, and BSA.

Index Entries: Bilirubin oxidase, *Myrothecium verrucaria*.

INTRODUCTION

Bilirubin oxidase (BOX, EC 1.3.3.5) from *Myrothecium verrucaria*, which Murao S. and Tanaka N. reported in 1981, is a copper-containing enzyme that catalyzes the oxidation of bilirubin to biliverdin and further to a color-

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less, unknown substance (*1–3*). This enzyme has been used for measuring total and direct bilirubin in serum (*4–6*).

Recently, we have succeeded in the isolation of a rich BOX-producing strain from several strains of *Myrothecium verrucaria*. The optimum conditions of enzyme production were investigated and the results were reported in a previous paper (*7*). In this study, the purification and some of the general properties of this BOX are reported.

MATERIALS AND METHODS

Strain: Myrothecium verrucaria Mv 2,1089 was obtained from the Institute of Microbiology, Academia Sinica.

Chemicals: Bilirubin (Sigma, St. Louis, MO); mol wt markers (MW-SDS-70, Sigma; Cat. No. 104540, Boehringer, Mannheim GmbH, Germany); carrier ampholytes for isoelectric focusing-Pharmalyte™ (Pharmacia Chemicals, Uppsala, Sweden).

Culture medium: 20% potato extract containing 0.25% glucose, 0.25% peptone, and 0.05% Triton X-100 (pH 6.0).

DEAE-Cellulose column: DEAE-Cellulose was treated with 0.5 mol/L HCl and 0.5 mol/L NaOH successively for 20 min and then equilibrated with 0.05 mol/L, pH 9.2 carbonate buffer overnight. The DEAE-cellulose was packed into a column (5.0×20 cm).

Sephadex G-100 column: Sephadex G-100 was treated with 0.2 mol/L NaOH and equilibrated with 0.02 mol/L, pH 9.2 carboante buffer overnight. The gel was then packed into a column (2.6×60 cm).

Culture procedure for BOX production: The procedure for BOX production was carried out as the previous paper described (*7*). The strain was aerobically cultured at 25°C for 96 h.

Purification of BOX: After cultivation, the mycelia in the culture medium can be removed by either centrifugation (10,000 rpm for 10 min) or filtration through a nylon net. Carbonate buffer (500 mL, 0.2 mol/L, pH 9.2) was added to 1500 mL of culture filtrate and then allowed to flow through a DEAE-cellulose column at a flow rate of 40–50 mL/h. The enzyme was eluted with 0.1 mol/L and then with 0.2 mol/L carbonate buffer (pH 9.2). The active fractions were collected, of which 10–30 mL was placed onto a Sephadex G-100 column. The enzyme was eluted with 0.02 mol/L carbonate buffer (pH 9.2) at a flow rate of 15–20 mL/h and the active fractions were pooled. The purified enzyme was dialyzed against glycerol and stored at −18°C.

Determination of BOX activity: The BOX activity was determined as described previously (*7*). One unit was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of bilirubin/min at pH 8.1 at 25°C.

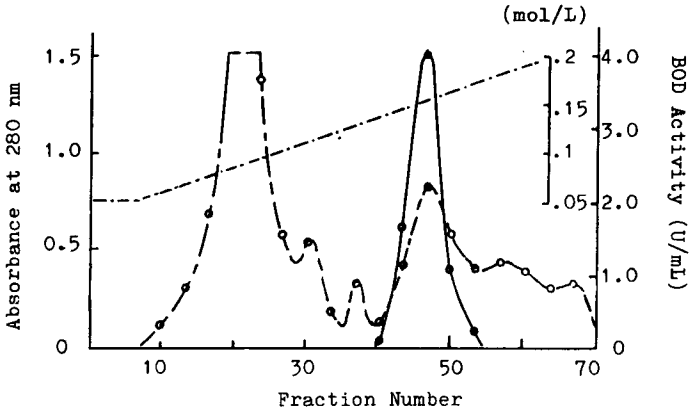

Fig. 1. Elution pattern of DEAE-cellulose column chromatography. Column size, 2.5×30 cm; flow rate, 20 mL/h; gradient elution: —·—, 0.05–0.2 mol/L, pH 9.2 carbonate buffer; ---, absorbance at 280 nm; ——, BOX activity.

Table 1
Purification of Bilirubin Oxidase

	Total volume, mL	Total activity, U	Total protein, mg	Specific activity, U/mg	Recovery, %
Culture broth	2100	2575	1136.1	2.3	100
DEAE-cellulose I	950	1900	137.8	13.8	73.8
DEAE-cellulose II	190	1832	66.9	27.4	71.1
Sephadex G-100	510	1421	42.8	33.2	55.2

RESULTS AND DISCUSSION

DEAE-Cellulose column chromatography: Placed on a column, the enzyme was eluted with 0.05 mol/L of carbonate buffer and then with a concentration gradient of 0.05–0.2 mol/L. The elution pattern of the enzyme activity and proteins is shown in Fig. 1. Based on the result, we used stage elution, i.e., 0.1 mol/L buffer for removing part of proteins and then 0.2 mol/L buffer for eluting BOX. The original specific activity of BOX can be increased more than 10 times (Table 1). This step can be repeated by using a smaller column (e.g., 1.5×10 cm) in order to make the enzyme solution concentrated.

Gel filtration with Sephadex G-100: The elution curve of BOX on Sephadex G-100 column is shown in Fig. 2. The specific activity of BOX was further increased and 30 U/mg protein or more can be obtained. The enzyme solution was concentrated by dialysis against glycerol, which is of benefit to enzyme storage at low temperature.

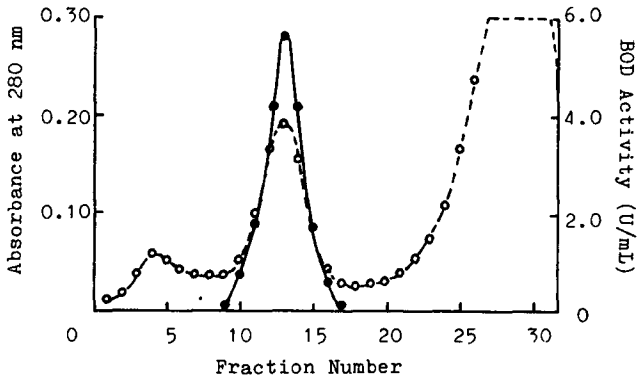

Fig. 2. Elution curve of Sephadex G-100 column chromatography. Column size, 2.5×60 cm; flow rate, 15 mL/h; ---, absorbance at 280 nm; ——, BOX activity.

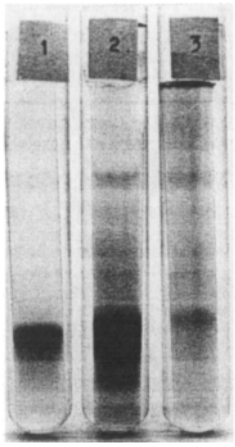

Fig. 3. Polyacrylamide gel electrophoresis of BOX. 1: Purified enzyme; 2: crude enzyme; 3: crude enzyme (Sigma).

Polyacrylamide gel electrophoresis: The purified enzyme was analyzed by PAGE. Coomassie brilliant blue was used for protein staining and a single band was obtained (Fig. 3).

Effect of pH on BOX activity: As shown in Fig. 4, the optimum pH for the enzyme reaction was 7.5 in either 0.1 mol/L Tris-HCl or phosphate buffer. Incubated at 50°C for 30 min, BOX is stable in pH between 9.0 and 9.5 in 0.1 mol/L carbonate buffer (Fig. 5).

Effect of temperature on BOX activity: As shown in Fig. 6, the optimum temperature for the enzyme reaction (within a minute) was 50°C. After incubation at 60°C for 10 min, the BOX remains at 35% of its initial activity (Fig. 7).

Molecular weight of BOX: The mol wt of BOX was determined to be 61,900–62,700 by the use of SDS-PAGE and gel-filtration methods (Figures 8, 9).

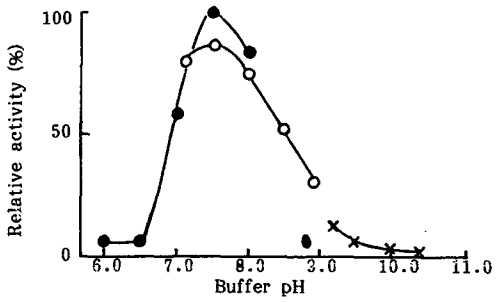

Fig. 4. Effect of pH on BOX activity: ●——●, phosphate buffer; ○——○, Tris-HCl; ×——×, carbonate buffer.

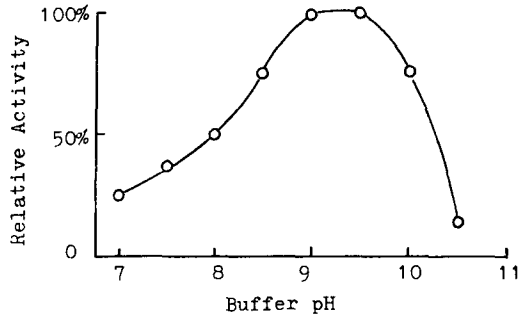

Fig. 5. Effect of pH on stability of BOX: pH 7.0–9.0, 0.1 mol/L Tris-HCl; pH 9.0–10.5, 0.1 mol/L carbonate buffer. After incubation under different pH at 50°C for 30 min, the activity of BOX was determined.

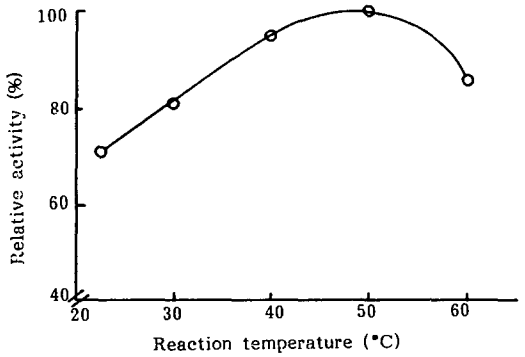

Fig. 6. Effect of Temperature on the enzyme reaction.

Isoelectric point of BOX: Using a glycerol gradient electrofocusing method (*8*), with carrier ampholyte of pH 2.5–5.0, it was shown that the isoelectric point of BOX is about pH 4.2 (Fig. 10).

K_m *Value of BOX:* The apparent K_m value of BOX was determined in 0.1 mol/L, pH 8.1 Tris-HCl buffer (bilirubin: 20–100 μmol/L) and calculated to be approx 9.4×10^{-5} mol/L from the Lineweaver-Burk plot (Fig. 11).

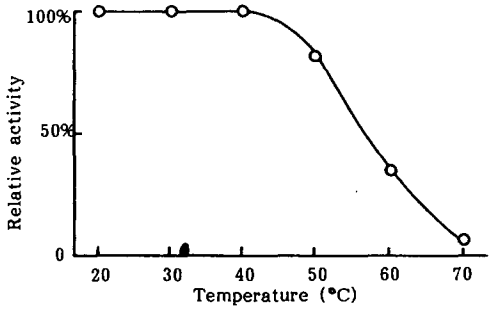

Fig. 7. Thermal stability of BOX. After incubating for 10 min at different temperatures, the BOX activity was determined.

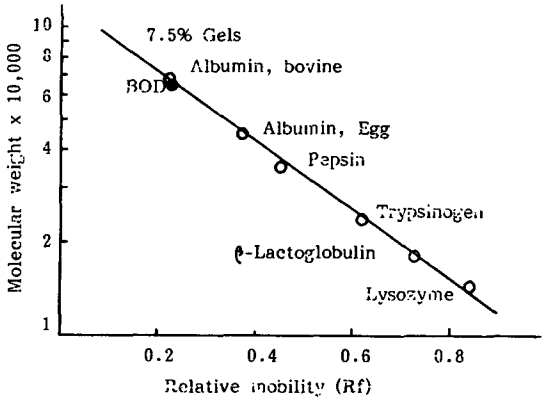

Fig. 8. Estimation of mol wt of BOX by SDS-PAGE: albumin, bovine (66,000); albumin, egg (45,000); pepsin (34,700); trypsinogen (24,000); β-lactoglobulin (18,400); lysozyme (14,300); BOX (62,700).

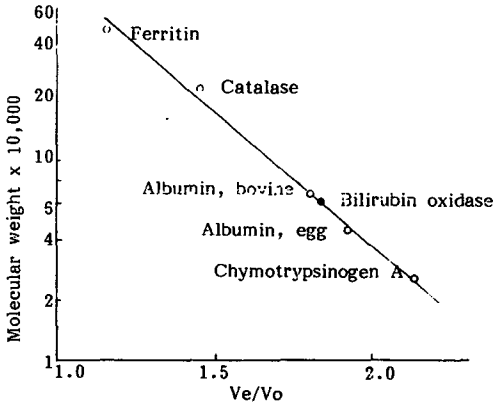

Fig. 9. Estimation of mol wt of BOX by gel filtration on Sephadex G-200: Column size, 1.0×40 cm; flow rate, 3 mL/h; ferritin (450,000); catalase (240,000); albumin, bovine (68,000); albumin, egg (45,000); chymotrypsinogen A (25,000); BOX (61,900).

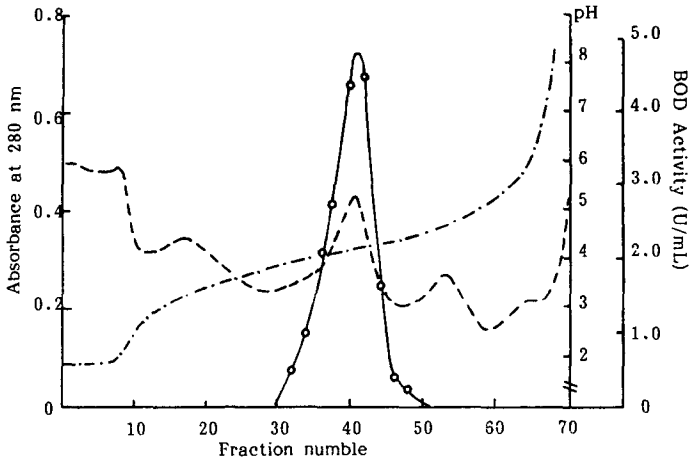

Fig. 10. Elution curve of isoelectric focusing: Carrier ampholyte, pH 2.5–5.0; power load, 4 W (700 V, 5–6 mA). Focusing took 48 h at 4°C. —·—, pH; ---, absorbance at 280 nm; ——, BOX activity.

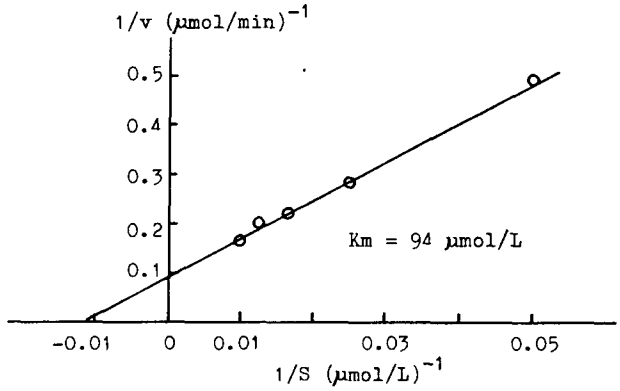

Fig. 11. Lineweaver-Burk plot for BOX.

Effect of metallic ions and compounds on BOX activity: The compounds shown in Table 2 were added to substrate separately and their effect on the enzyme reaction was assayed. The results showed that the enzyme reaction was inhibited by Ca^{2+}, Hg^{+}, Zn^{2+}, Fe^{2+}, and BSA. After incubation with each compound shown in Table 3 at 30°C for 60 min, the BOX activity was then determined. As shown in Table 3, the enzyme activity was greatly reduced by incubation of BOX with Fe^{2+}, Hg^{+}, NaN_3, NH_4^{+} and Zn^{2+}.

Stability: BOX in 50% glycerol-carbonate buffer (0.02 mol/L, pH 9.2), kept at −18°C or below, was stable for at least 6 mo.

Table 2
Effect of Metallic Ions and Compounds on the Enzyme Reaction

Compound, 1 mmol/L	Relative activity, %
–	100
$ZnSO_4$	6.3
NaN_3	60.3
$CaCl_2$	4.8
$FeCl_3$	33.3
$BaCl_2$	88.9
EDTA	100
$MgSO_4$	100
NaCl[a]	92.1
NH_4Cl[a]	84.1
Hemoglobin[a]	100
Heparin[a]	100
BSA[a]	7.7

[a]NaCl, 10 mmol/L; NH_4Cl, 10 mmol/L; Hemoglobin, 14.7 mg/L; Heparin, 0.1 g/L; BSA, 2.0 g/L.

Table 3
Effect of Metallic Ions and Compounds on the BOX Activity

Compound, 10 mmol/L	Relative activity, %
–	100
$ZnSO_4$	35.1
NaN_3	29.7
$CaCl_2$	100
$FeCl_2$	0
$BaCl_2$	100
EDTA	100
$MgSO_4$	100
NaCl[a]	51.3
NH_4CL[a]	35.1
$CuSO_4$	64.9
HgCl	8.1
Hemoglobin[a]	100
Heparin[a]	100
BSA[a]	100

[a]NaCl, 0.1 mol/L; NH_4Cl, 0.1 mol/L; Hemoglobin, 14.7 mg/L; Heparin, 0.1 g/L; BSA, 2.0 g/L.

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