Rifamycin B oxidase from *Monocillium* spp., a new type of diphenol oxidase

Moon H. Han, Baik-Lin Seong, Hyeung-Jin Son and Tae-Ick Mheen

Biotechnology Research Department, The Korea Advanced Institute of Science and Technology, PO Box 131, Dong Dae Mun, Seoul, Korea

Received 9 November 1982

It was found that enzyme from a microbial strain, *Monocillium* spp. ATCC 20621, catalyzed the oxidative reaction of rifamycin B to form rifamycin O. The identification of the reaction products suggested that the reaction proceeded by the oxidative cyclization of rifamycin B to give rifamycin O, which spontaneously hydrolyzed to rifamycin S in neutral aqueous milieu. The characteristic of the enzyme was different as compared with that of other polyphenol oxidases such as laccase. It is proposed that this new type of enzyme be classified into a subgroup EC 1.10.3.6 with a trivial name rifamycin B oxidase.

Polyphenol oxidase *Rifamycin B oxidase* *Biotransformation* *Antibiotic* *Rifamycin* *Enzyme*

1. INTRODUCTION

Studies on the biosynthesis of rifamycin B indicated that rifamycin SV is the precursor of rifamycin B in *Nocardia mediterranei* culture. [^{14}C]Rifamycin SV was converted in high yield into labelled rifamycin B by either growing culture or by washed mycelia of this microorganism [1,2]. It was supported by the isolation of a mutant of *N. mediterranei* producing rifamycin SV [3]. In the final step of rifamycin B biosynthesis, rifamycin SV or its oxidized form, rifamycin S, was converted into rifamycin B, but it is not known whether the intermediate rifamycin O was involved or not.

We attempted to determine other microbial species which could catalyze the conversion of rifamycin B to rifamycin O or rifamycin S, which is the reverse reaction of rifamycin B biosynthesis, and were able to isolate two kinds of Fungi Imperfecti, *Humicola* spp. and *Monocillium* spp., which are now assigned the no. ATCC 20620 and 20621, respectively.

Here, this new type of enzyme of *Monocillium* spp. ATCC 20621 was purified and enzymic characteristics, including substrate specificity, were compared with those of other similar enzymes known.

2. MATERIALS AND METHODS

2.1. *Materials*

All the chemicals including NAD^+, $NADP^+$, FAD, pyrogallol and resorcinol were purchased from Sigma, Bacto-beef and yeast extract were obtained from Difco. Thin-layer chromotography sheet (no. 13181) was purchased from Eastman. Rifamycin B was prepared from culture broth of *N. mediterranei* as in [4]. Rifamycin O, refamycin S and rifamycin SV were prepared by the improved synthetic method [5]. Diethylaminomethyl rifamycin SV was synthesized from rifamycin S by Mannich reaction [6] and 3-formylrifamycin SV was prepared by the oxidative cleavage of DEAM-rifamycin SV [7].

2.2. *Enzyme preparation*

The microbial cells of *Monocillium* spp. ATCC 20621 were grown in a 28-l jar fermentor (NBS model CMF-128, USA) with a working volume of 15 l with an agitation speed of 300 rev./min and an aeration rate of 1.5 vvm at 30°C for 4 days. The culture medium was composed of Bacto-beef (1.0%), yeast extract (1.0%) and dextrose (1.0%).

All the purification steps were conducted in a

Published by Elsevier Biomedical Press

cold room (5°C) unless otherwise stated. Cultured fungal mycelium pellets were separated by filtration and ground with Jank-Kunkel Ultra-Turrax. Wet cell cake (45 g) containing 1800 units of enzyme activity was recovered from 1 l culture broth with 99% recovery of enzyme activity. The cell cake was homogenized with acid-washed glass beads (75–150 μM) in 500 ml of phosphate buffer (50 mM, pH 7.0). Protein precipitates with active enzyme fraction, appearing in 30–70% ammonium sulfate, were collected from the supernatant. The precipitates were redissolved in 10 mM phosphate buffer (pH 7.0) followed by ultra-filtration with Amicon millipore filter. The crude enzyme solution (2.0 g) was applied to a DEAE-cellulose column (4 × 40 cm) and equilibrated with 20 mM phosphate buffer (pH 7.2). The column was then washed with the equilibration buffer at a flow rate 40 ml/h until A_{280} was <0.05, and followed by linear NaCl gradient elution (0–0.5 M). The active fractions were pooled and ultrafiltered. The sample was applied on the Sephadex G-100 column (2 × 50 cm) and equilibrated with 10 mM phosphate buffer (pH 7.0). Active fractions were collected and used for other experiments.

2.3. *Assay methods*

Two different methods were used for the determination of rifamycin B oxidase activity: Firstly, the enzyme activity was measured by the spectrophotometric method. The enzyme solution was added to 2 ml rifamycin B (2 mM phosphate buffer, pH 7.8). After incubation for 15 min at 40°C with vigorous stirring, usine a star-shaped magnetic bar (Nalge), the reaction mixture was boiled for 5 min in a water bath to stop the enzyme reaction with simultaneous hydrolysis of rifamycin O to rifamycin S. The hydrolysis of rifamycin O was completed in a given assay condition as visualized in thin-layer chromatography (TLC). The reaction mixture was cooled with running water and diluted 2-fold with 0.1 M phosphate buffer (pH 7.8). The absorbance of rifamycin S in the reaction mixture was measured at 525 nm ($E^{1\%}_{1cm} = 68$) in phosphate buffer (pH 7.8). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 μmol of rifamycin B/h. The enzyme activity was also measured by the consumption of dissolved oxygen with Gilson Model 5/6H oxygraph at 37°C with various substrates.

2.4. *Electrophoretic analysis*

Electrophoretic analysis on 7.5% acrylamide gel (0.75 × 8.5 cm) was performed in Tris–glycine buffer (pH 8.3) as in [8]. For the activity-staining of rifamycin B oxidase activity, the gel was immersed in 2 mM rifamycin B solution for 20 min and incubated in 0.1 M phosphate buffer (pH 7.8) containing 50% glycerol for 1 h at 30°C.

2.5. *TLC method*

Eastman Chromagram sheet no. 13181 was used with a developing solvent system of chloroform–acetone (1 : 1). During the enzyme reaction, in 2 mM rifamycin B (0.1 M phosphate buffer, pH 7.5) with 25 units of the enzyme, 5 ml aliquots were pooled, acidified with d-HCl, and extracted with 1 ml ethyl acetate. The organic layer was pooled and developed twice on TLC.

3. RESULTS

The result of a typical purification procedure for the enzyme is shown in table 1. About 95% of the

Table 1

Purification steps of rifamycin B oxidase from *Monocillium* spp ATCC 20621

Purification step	Total protein (mg)	Total act. (units)	Spec. act. (units/mg protein)	Yield (%)	Purification (-fold)
Homogenization	2638	5540	2.1	100	1.0
Fractionation by ammonium sulfate	620	4432	7.14	80	3.4
DEAE-cellulose	45.4	3601	79.3	65	37.8
Sephadex G-100	8.11	1551	191.2	28	91.0

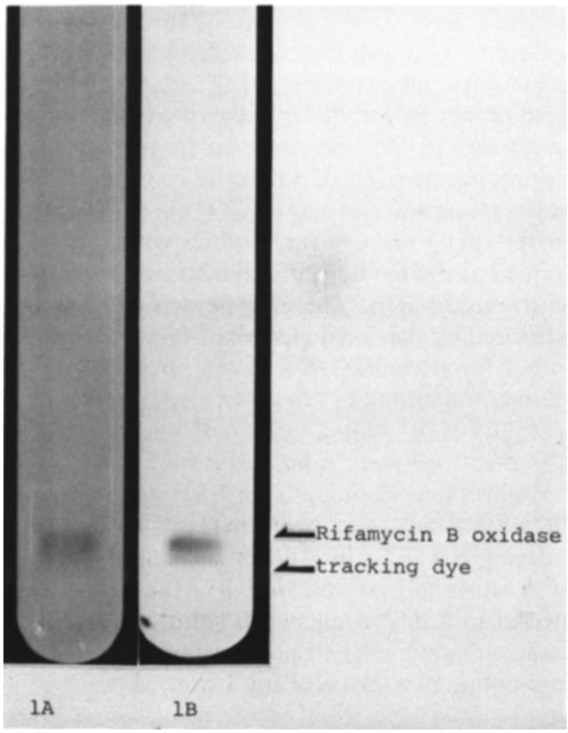

Fig. 1. Polyacrylamide gel electrophoresis of purified enzyme and activity staining. The red colour of rifamycin S, the hydrolyzed product of rifamycin O at this pH easily visualized the location of active enzyme band (1A). The protein stained with 0.5% amido block 10B appeared as a single band (1B).

initial enzyme activity was recovered by homogenization with glass beads. During DEAE-cellulose column chromatography, the active fractions were eluted with NaCl solution in a gradient between 0.15 –0.25 M. Gel filtration on Sephadex G-100 showed the coincidence of the protein and the activity band suggesting that the enzyme was nearly pure. Analysis by polyacrylamide gel electrophoresis confirmed the purity of the enzyme obtained by the present purification procedure. The location of the enzyme was indicated by its coincidence with a band stained for activity (fig. 1).

Thin-layer chromatography on a time course of enzyme reaction confirmed the identity of the reaction products (fig. 2). Rifamycin S (purple, R_f 0.7) and rifamycin O (pale yellow, R_f 0.8) were identified along with unconverted rifamycin B (yellow, R_f 0.1) at the initial stage of enzyme reaction. Rifa-

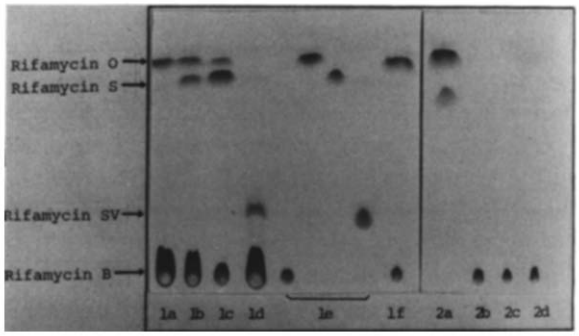

Fig. 2. Identification of reaction products by thin-layer chromatography. (1a) 5 min; (1b) 30 min; (1c) 2 h; (1d) reduced with ascorbic acid after reaction for 30 min; (1e) standards of rifamycin derivatives. $R_f = 0.1$, rifamycin B (yellow); $R_f = 0.25$, rifamycin SV (yellow); $R_f = 0.7$, rifamycin S (purple); $R_f = 0.8$, rifamycin O (pale yellow); (1f) shows the reaction products, the enzyme reaction being carried out at pH 5.0 (0.1 M acetate buffer). Apparent inhibition by NADH is tested. (2a) Standard samples rifamycin O + rifamycin S; (2b) rifamycin O + NADH; (2c) rifamycin O + mercaptoethanol; (2d) rifamycin O + cysteine.

Table 2

Effect of electron acceptors and donors on the rifamycin B oxidase activity

Electron donor or acceptor	Conc. (mM)	Enzyme activity (μmol/h)
None	0.0	6.8
NAD^+	0.5	6.5
$NADP^+$	0.5	6.8
FAD	0.5	7.1
NADH	0.5	2.3
NADPH	0.5	2.3
Cysteine	20.0	4.2
Mercaptoethanol	150.0	0.85

mycin O and rifamycin B spots were attenuated with the intensification of the rifamycin S spot as the reaction proceeded.

The effect of various electron acceptors such as NAD^+, $NADP^+$ and FAD on the enzymatic oxidation of rifamycin B was examined and the results are shown in table 2. Experimental results indi-

cated that the enzyme can be classified as an oxidase, not a kind of dehydrogenase since there was no requirement of electron acceptor other than oxygen.

NADH and NADPH showed an apparent inhibition on the enzyme, and it was further examined by thin-layer chromatography. The results indicated that the inhibition mode was an artifact since the reaction product rifamycin O was found to be reduced to the initial substrate rifamycin B in the presence of such electron donors. It was also confirmed by the finding that the apparent inhibition of enzyme activity in the presence of the reducing agents such as cysteine and mercaptoethanol was accompanied with the reduction of rifamycin O to rifamycin B (fig. 2).

Substrate specificity of the enzyme was compared with that of laccase by the relative oxidation rates of various rifamycin derivatives and other phenolic compounds (table 3). The enzyme rapidly oxidized hydroquinone and rifamycin B, while laccase showed relatively low activity on those compounds. This enzyme oxidized hydroquinone and rifamycin B rapidly, while laccase showed relatively low activity on those compounds. This enzyme also oxidised rifamycin SV, 3-formylrifamycin SV, pyrogallol and catechol. Resorcinol and the Mannich derivative of rifamycin SV (3-diethylaminomethyl rifamycin SV) were not attacked at all. *p*-Hydroxyphenoxyacetic acid resembling the reactive moiety of rifamycin B gave very low activity with the enzyme.

Table 3

Comparison of the substrate specificity of rifamycin B oxidase and laccase

Substrate[a]	Relative reaction rate (%)[c]	
	Rifamycin B oxidase	Laccase[b]
Rifamycin B	100.0	3.5
Rifamycin SV	54.4	29.7
DEAE-rifamycin SV	0.0	–
3-Formylrifamycin SV	2.3	–
p-Hydroxyphenoxyacetic acid	0.3	–
p-Hydroquinone	260.5	14.0
Pyrogallol	57.8	100.0
Catechol	13.6	20.5
Resorcinol	0.0	2.0

[a] The concentration of all the substrates used was 2 mM

[b] Crude preparation of laccase was obtained by ammonium sulfate precipitation of the culture filtrate of *Pleurotus ostriatus*

[c] Enzyme activity was measured with oxygraph (Gilson model 5/6H)

The UV–visible absorption spectrum of the purified enzyme exhibited a typical protein absorption spectrum with a maximum peak at 280 nm with no significant absorption in the visible region. Thus the enzyme seemed to contain no chromophogenic moiety such as flavins, heme of Cu^{3+}.

The effect of various metal ions on the enzyme activity was studied, using rifamycin B as a substrate. The enzyme activity was neither inhibited by Ag^{+} or Hg^{2+}, nor activated by Cu^{2+}. Other metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} and Mo^{6+} did not affect their enzyme activity. The addition of a metal chelating agent such as EDTA caused no marked inhibition of the enzyme activity.

4. DISCUSSION

The newly discovered enzyme catalyzed the oxidation of rifamycin B to rifamycin O which was subsequently hydrolyzed to give rifamycin S depending upon the pH value of the reaction solution. Isolation and identification of the enzyme reaction product by thin-layer chromatography and instrumental analysis, together with the balance study of the reaction, have led to the following reaction scheme (fig. 3).

The studies on the enzymatic characteristics showed that this enzyme had quite different properties as compared with other similar enzymes such as catechol oxidase (EC 1.10.3.1), laccase (EC 1.10.3.2), ascorbate oxidase (EC 1.10.3.3), *o*-aminophenol oxidase (EC 1.10.3.4) and 3-hydroxyanthranilate oxidase (EC 1.10.3.5) using diphenols and related compounds as electron donors, and oxygen as an electron acceptor [10].

The substrate specificity of the enzyme was also different to that of the laccase from *Pleurotus ostriatus,* and to other sources [11–13]. First of all, this enzyme is neither a cuproprotein nor a flavoprotein. From table 2, the metal ion effect, and the absorption spectrum of the protein, this enzyme appears to contain no flavins, heme, non-heme

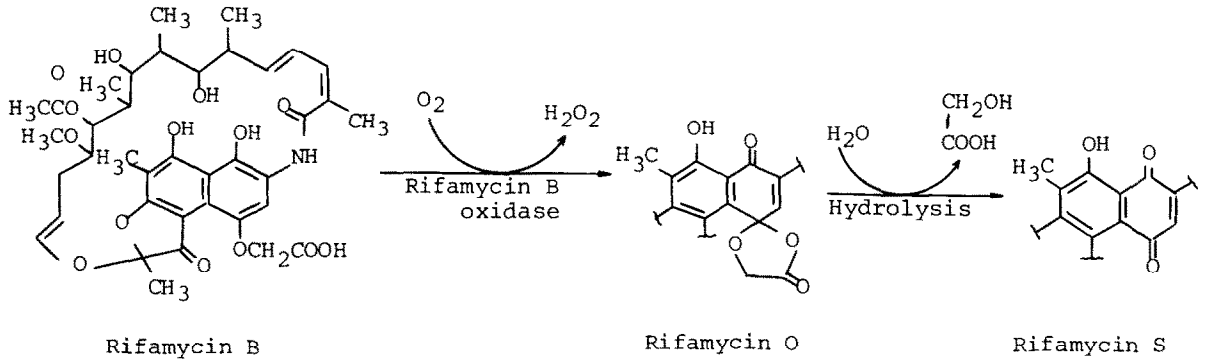

Fig. 3. Proposed mechanism of rifamycin B oxidase action. Hydrogen peroxide was detected during enzyme reaction with *p*-Hydroquinone as a substrate by peroxidase-*o*-dianisidine method [9].

iron nor other metal ions as co-factors, nor the -SH group in the active site of the enzyme.

We proposed that this new type of enzyme be assigned to subgroup EC 1.10.3.6 (acting on diphenols and related substances as electron donors, and on oxygen as an electron acceptor) with the trivial name rifamycin B oxidase.

ACKNOWLEDGEMENT

We thank Dr P. Sensi, who kindly offered us a sample of rifamycin derivatives for our work.

REFERENCES

[1] Lancini, G.C. and Sensi, P. (1967) Proc. Vth Int. Congr. Chemother. 1, 41–47.
[2] Lancini, G.C., Gallo, G.G., Sartori, G. and Sensi, P. (1969) J. Antibiot. 22, 369–377.
[3] Birner, J. Hodgson, P.R., Lane, W.R. and Baxter, E.H. (1972) J. Antibiot. 25, 356–359.
[4] Lee, J.K., Choi, C.Y., Seong, B.L. and Han, M.H. (1981) Kor. J. Appl. Microbiol. Bioeng. 9, 225–230.
[5] Seong, B.L. and Han, M.H. (1982) Chem. Lett. 5, 627–628.
[6] Maggi, N., Arioli, V. and Sensi, P. (1965) J. Med. Chem. 8, 790–793.
[7] Maggi, N., Galls, G.G. and Sensi, P. (1966) Il Farmaco Ed. Sci. 22, 316–325.
[8] Davis, B.J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[9] Hugget, A.St.G. and Nixon, D.A. (1957) Biochem. J. 66, 12–15.
[10] Bielka, H. et al. (1979) in: Enzyme Nomenclature pp. 104, IUB–Academic Press, New York.
[11] Gregory, R.P.F and Bendall, D.S. (1966) Biochem. J. 101, 569–681.
[12] Peisach, J. and Levine, W.G. (1965) J. Biol. Chem. 240, 2284–2289.
[13] Froehenr, S.C. and Eriksson, K.E. (1974) J. Bacteriol. 120, 458–465.