MICROBIAL TRANSFORMATION OF RIFAMYCIN B: A NEW SYNTHETIC APPROACH TO RIFAMYCIN DERIVATIVES

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Rifamycin B is a metabolic product of *Nocardia mediterranei*, while rifampicin is a semisynthetic antibiotic derived from rifamycin B. Rifampicin is presently in therapeutic use as an antituberculosis drug. Ever since the discovery of the rifamycins was announced by SENSI *et al.*[1], synthetic processes for rifamycin derivatives has depended on only chemical means.[2,3,4] Rifamycin S prepared from rifamycin B by a chemical process, is known to be the key intermediate in the synthesis of rifampicin[5,6] and other derivatives of therapeutic value.[7]

During our studies on rifamycins, we have endeavored to develop a biological process for preparation of rifamycin derivatives. We thus have examined various soil samples to obtain microorganisms active on rifamycin B.

The isolation procedure was as follows. The agar medium contained (g/liter); glucose 10, peptone 10, yeast extract 10, rifamycin B 0.5 and agar 20. The pH was adjusted to 7.0 before sterilization. Various dilutions of soil were streaked, after incubation at 30°C for 3~4 days, colonies with a red halo was isolated, the red color showing deep contrast with the ambient yellow color of rifamycin B. The red colored substance was tentatively identified as rifamycin S due to its high absorption at 525 nm in neutral aqueous solution. A color change was further used as a method of identification *i.e.*, the change in the absorption peak of rifamycin S to 405 nm (pale yellow) at acidic condition. The dissociation-association equilibrium of the phenolic OH group (*pKa* 7.2)[7] in the chromogenic naphthalenic moiety is thought to be responsible for the color change. After isolating the candidate colonies several drops of acetate buffer (0.1 M, pH 4.6) were layered in the red halo region. The colonies without color change were discarded. Since this isolation procedure could have failed since some other microbial pigments could show a pH dependence of color, a more reliable method was introduced. Mild reducing agents such as ascorbic acid are known to reduce the quinone form of rifamycin S to the hydroquinone form of rifamycin SV. This reaction accompanies the color change to deep yellow of rifamycin SV (445 nm) from the red color of rifamycin S (525 nm) in neutral aqueous solution.[7] Thus, the red halo region in agar plate was treated with several drops of 0.5% ascorbic acid (0.1 M phosphate buffer, pH 7.3) and a colony was isolated which showed the predicted color changes described above.

The isolated microorganism was identified as *Monocillium* sp. based on the physiological and morphological characteristics described in BARNETT and HUNTER's reference book[8] and SAKSENS's paper.[9] It showed following the morphological and cultural characteristics. After 5~7 days of growth on YM agar media at 30°C, conidiophores were septate consisting of a pedicel, and a swollen vesicle which bears a long chain of conidia formed basipetally. Conidia (phialospores) were single-celled, hyaline, ovoid to ellipsoid and smooth. The cultural and morphological characteristics in various media after incubation for 5~7 days at 30°C are summarized in Table 1. The optimum growth temperature and pH were 28~30°C and 7.5~8.5, respectively. The carbohydrate utilization pattern of this organism are summarized in Table 2.

Cells of *Monocillium* sp. were produced by growth in a medium containing (g/liter); glucose 10, yeast extract 10, and peptone 10. After adjusting the pH to 7.0, the medium was autoclaved at 121°C for 15 minutes. Each flask was inoculated with 5% (v/v) inoculum which had been grown for 2 days at 30°C in the same medium. The flasks were incubated at 30°C in the same medium. The flasks were incubated at 30°C on a reciprocal shaker at 300 strokes per minute. After 4 days of culture, the wet cells were obtained by centrifugation. The enzyme responsible for the biotransformation of rifamycin B was an intracellular one since no enzyme activity was detected in the supernatant fluid. The wet cell paste was disrupted by grinding with sea sand and centrifuged at 5,000 rpm for 30

Table 1. Morphological and cultural characteristics of *Monocillium* sp. ATCC 20621 on various media.

Mycological agar plate	The colony is light pink, flat, velvety and scallop-edged. As conidia develop, a depressed dark green central zone is formed. The reverse color is orange. A red-brown pigment is produced.
Wort agar plate	A moderately fast growing colony is flat, scallop-edged, pink and has a depressed center. The texture is velvety. The reverse color is orange. No pigment is produced.
Corn meal agar plate	The organism has a moderately fast growth rate with a colony that is flat and entire-edged. Dark green conidia are produced abundantly.
Yeast extract - malt extract (YM) agar plate	The organism has an intermediate growth rate with a colony that is flat, light brown, and scallop-edged. As conidia develop, the central zone becomes dark green. The reverse color is brown.
Malt extract agar plate	The rapidly growing colony is white, flat and entire-edged. The texture is velvety. A dark green central zone and orange reverse color are developed after 5~7 days.
Czapek-Dox agar plate	The organism has a moderate growth rate with a colony that is white, flat and entire-edged. The texture is compact wooly. A greenish black color appears as conidia develop.
Gelatin agar plate	The colony is flat and white with a slightly brown central zone. As conidia develop, a dark green color appears. The texture is compact wooly.
Sabouraud - maltose agar plate	A white, flat, slightly folded colony is formed. The texture is velvety. Reverse color is not developed.

Table 2. Carbohydrate utilization by *Monocillium* sp. ATCC 20621.

Utilized	Arabinose, xylose, glucose, mannose, galactose, lactose, sucrose, melibiose, salicin, dulcitol, adonitol, inositol, rhamnose, xylan, inulin, dextrin, starch
Weakly utilized	Cellulose
Not utilized	Fructose, erythritol

minutes to remove cell debris. The protein precipitate obtained by ammonium sulfate precipitate (60% saturation) was collected by centrifugation at 25,000 rpm at 4°C and redissolved in acetate buffer (0.1 M, pH 5.5). The presence of oxygen was found to be a prerequisite for the enzyme reaction since no rifamycin B was converted into rifamycin O under the oxygen free condition. The optimum pH of this enzyme was 5.0 when using 1 mM rifamycin B as substrate. A spectrophotometric assay was used for the determination of rifamycin B oxidase activity.[10]

At pH 5.0, the reaction solution became hazy due to precipitate formation. The pale yellow precipitate was recovered by filtration, washed with 0.1 M acetate buffer (pH 5.0) and a small amount of cold methanol. The identity of this material was determined by thin-layer chromatography and IR spectrum. It showed the same Rf value (0.6) and same pale yellow color as standard rifamycin O in TLC (Eastman Chromagram Sheet No. 13181) employing chloroform - acetone, 1:1 mixture as the developing solvent. Furthermore, the IR spectrum of this material was the identical to authentic rifamycin O. Precipitation of the reaction product, rifamycin O could be due to the increase of hydrophobicity produced by the disappearance of the hydrophilic carboxyl group by lactam ring formation.

The solubility of rifamycin O increases appreciably at neutral or alkaline pH due to the ioniza-

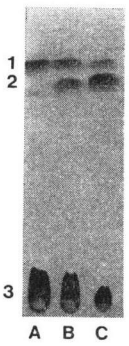
Fig. 1. Thin layer chromatogram show as the time course of the enzyme reaction.

The enzyme reaction was carried out with 2 mM rifamycin B solution in 0.1 M phosphate buffer (pH 8.0) with vigorous stirring. Aliquots of the reaction solution were pooled, acidified with dilute HCl and extracted with ethyl acetate. The organic layer was developed twice on an Eastman Chromagram Sheet No. 13181 with a solvent system of chloroform - acetone (1:1).

A) 10 minutes, B) 20 minutes, C) 50 minutes.

1) Rifamycin O (Rf 0.8, pale yellow), 2) rifamycin S (Rf 0.7, purple), 3) rifamycin B (Rf 0.1, yellow).

The color of the rifamycin derivatives turned brownish on standing.



tion of the phenolic OH group in the chromogenic moiety (*pKa* 7.9)[7]. If the enzyme reaction is carried out at pH 8.0, solubilization of rifamycin O confers an easy attack of the lactone ring by water thus giving rifamycin S with a purple color. The nonenzymatic hydrolysis reaction at pH 8.0 was readily seen by the naked eye since the initial yellow color of rifamycin B gradually changed to deep purple as the enzyme reaction proceeded. It could be also visualized by the changing behavior in the TLC pattern (Fig. 1). The reaction proceeded with the initial appearance of rifamycin O (pale yellow, Rf 0.6) followed by intensification of rifamycin S (purple, Rf 0.4) and the decline of rifamycin O. At the end of the enzyme reaction at pH 8.0, the precipitate of purple color was collected and washed with 0.1 M phosphate buffer (pH 8.0). The UV-visible and IR spectrum were identical with those of standard rifamycin S.

This result demonstrates that rifamycin O and rifamycin S can be prepared in high yield by microbial transformation of rifamycin B. This showed that the biotransformation of rifamycin B be recognized in view of the importance of rifamycin S as a key intermediate for preparation of many semisynthetic rifamycin derivatives with therapeutic use. Further improvements could be achieved by optimizing the culture conditions for enzyme production. Purification and studies on the enzymes characteristics also merit further investigation for industrial application of this enzyme.

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