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Screening of Compactin-Resistant Microorganisms Capable of Converting Compactin to Pravastatin

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Abstract. A simple method of using compactin for effective screening of microbial strains with high hydroxylation activity at the 6β position of compactin was developed. Agar plates containing different carbon sources and 500 μg compactin mL^{-1} were used to screen the microorganisms that can convert compactin to pravastatin. About 100 compactin-resistant strains were isolated from the Basal agar containing 7% (w/v) mannitol as a carbon source, in which two bacteria, *Pseudomocardia autotrophica* BCRC 12444 and *Streptomyces griseolus* BCRC 13677, capable of converting compactin to pravastatin with the yield of 20 and 32% (w/w), respectively, were found. High-performance liquid chromatography using C-18 column and two sequential mobile phases, 30% and 50% (v/v) acetonitrile, was also established to simultaneously determine the concentration of compactin and pravastatin in the culture broth. As such, about 2% of target microorganisms could be obtained from the screening program.

Statin drugs are currently the most therapeutically effective drugs to reduce the level of low-density lipoprotein in the bloodstream of a patient at risk for cardiovascular disease. The statin drugs disrupt the synthesis of cholesterol by competitively inhibiting the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase [2]. Among the statin drugs, pravastatin exhibits an important therapeutic advantage, i.e., it selectively inhibits cholesterol synthesis in the liver and small intestine but leaves cholesterol synthesis in the peripheral cells substantially unaffected [12, 14].

In the early days of its development as a statin drug, pravastatin was obtained from compactin by means of chemical synthesis [7], but more recently, due to problems of high cost and the occurrence of stereoisomers, the most economically feasible method of making pravastatin is by enzymatic hydroxylation of compactin at the C-6 position. Compactin is produced by the fermentation of *Penicillium citrinum* [4, 5]. However, microorganisms seldom are found to have a potent hydroxylating ability on compactin at the 6β-position. *Streptomyces carbophilus* is one of the most effective microorganisms, which has shown the hydroxylation activity performed by a cytochrome P450 system [6]. *Actinomadura sp.* strain 2966 appears to have another cytochrome P450 system in the conversion of compactin to pravastatin [8]. Although some microorganisms have the ability to convert compactin, the resulting products other than pravastatin, such as 3α- and 3β-hydroxycompactin, are found in the hydroxylation of compactin by *Syncephalastrum nigricans* and *Mucor hiemalis* [9].

Microorganisms sensitive to compactin are one of the problems in the production of pravastatin. A high concentration of compactin in the culture broth not only inhibits the cell growth but also causes cell lysis during the conversion of compactin to pravastatin [5, 7]. Therefore, establishment of a simple and rapid method for the screening of compactin-resistant strains with the capability of converting compactin to pravastatin is performed in this study.

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Materials and Methods

Microorganisms, chemicals, and culture media. Microorganisms were isolated from soils in the National Chung Hsing University (Taichung, Taiwan) and from wastewater in the petroleum plant of Chinese Petroleum Co. (Taipei, Taiwan), and obtained from the Bioresources Collection and Research Center (BCRC, Hsinchu, Taiwan). Soil was put through a 5-mm sieve to remove roots and large particulates and stored at 4°C until use. Bacteria were identified by 16S rRNA gene sequence alignment and biological characterization. For 16S rRNA gene sequencing, the genomic DNA was extracted from the cells and 16S rRNA gene was amplified by PCR using primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) [15]. PCR amplification was performed using a Thermocycler model 9600 (Perkin-Elmer, Norwalk, CT, USA). DNA was amplified for 25 cycles of denaturation at 94°C for 1.5 min, annealing at 65°C for 2 min, and elongation at 72°C for 2 min. The final extension step at 72°C for 5 min was included. The PCR product was purified and then ligated into pGEM-T Easy vector (Promega Co., Madison, WI, USA). The nucleotide sequences were determined by Protech (Taipei, Taiwan). The obtained sequences were compared to those in the National Center for Biotechnology Information nucleotide sequence database by using the BLAST algorithm [1]. The biological characteristics and cellular fatty acids composition of the strain were performed by BCRC (Taiwan). Compactin and pravastatin were purchased from Standard Chem. & Pharm. Co. (Tainan, Taiwan). Basal medium [1% (w/v) yeast extract, 0.15% (w/v) soy meal, 0.05% (w/v) polyethyleneglycol, 0.1% (w/v) KH_2PO_4, and 0.1% (w/v) KCl; pH 7.0] containing 7% (w/v) of mannitol, glycerol, glucose, or lactose was used for the isolation and cultivation of compactin-resistant strains.

Screening of pravastatin-producing strains. Enrichment of microorganisms transforming compactin was performed with 1% inocula from both the soils and wastewater using Basal medium containing different carbon sources and 100 μg compactin mL^{-1}. The cultures were grown at 28°C with rotary shaking (220 rpm). After three successive transfers (with a 1:10 dilution in every transfer), the enrichment cultures obtained from the soils and wastewater were used for the isolation of pure cultures. Pure culture with a similar morphology was obtained in Basal agar incubated at 28°C. A single colony from enrichment cultures and BCRC strains was picked and inoculated to different Basal agar plates containing 7% (w/v) of various carbon sources and different concentrations of compactin in the range of 0.1 to 1 mg mL^{-1} and incubated at 28°C for 3 to 10 days. To determine the hydroxylation activity of isolated microorganisms toward compactin, colonies of compactin-resistant strains were transferred from agar plates into a 100-mL Erlenmeyer flask containing 20 mL of the Basal medium with 7% (w/v) of different carbon sources and incubated at 28°C for 2 days on a rotary shaker (220 rpm). Five milliliters of the seed culture were inoculated into 100 mL of the Basal media containing 7% (w/v) of different carbon sources. After incubating at 28°C for 2 days, 500 μg compactin mL^{-1} were added into the culture broth to observe the conversion of compactin to pravastatin.

HPLC analysis. Concentrations of compactin and pravastatin in culture broth were analyzed by the HPLC method. The HPLC system was a 625 LC System equipped with 717 plus Autosampler and 996 Photodiode Array Detector (Waters, Milford, MA). A VERCOPAK-C18 column (3.2 × 250 mm, Vercotech, Taipei, Taiwan) was used and UV detection was set at 235.9 nm for compactin and pravastatin. The column was eluted with 30% (v/v) acetonitrile and 10% (v/v) methanol for 20 min; or 30% (v/v) acetonitrile for 15 min, followed by 50% (v/v) acetonitrile for 25 min. Elution was performed at room temperature and a velocity of 1 mL min^{-1}. Pravastatin from Standard Chem. & Pharm. Co. was used as an internal standard.

Table 1. Bioconversion of compactin by compactin-resistant microorganisms

Microorganism	Residual compaction in culture broth (%)
Streptomyces regensis BCRC 11890	88
Pseudonocardia autotrophica BCRC 12444	80
Streptomyces griseolus BCRC 13677	68
Mucor hiemalis BCRC 32824	85
Rhodosporidium fluviale BCRC 22986	29
Xanthomonas compestries NCHU 1126	90
Amycalatopsis sulphurea NCHU 1345	90
Bacillus megaterium NCHU 1546	25
Salmonella enterica NCHU 1586	45

Cells were cultivated in Basal medium containing 500 μg compactin mL^{-1} at 28°C for 10 days.

Results and Discussion

Only a few microorganisms have been found to have the capability to hydroxylate compactin at the 6β position [13]. Moreover, the most effective microorganisms for this type of hydroxylation suffer from a low tolerance to a high concentration of compactin and produce a large amount of byproducts [13]. It is, therefore, necessary to establish an effective method to maximize the rate of isolating microorganisms with the ability to convert compactin to pravastatin. To enhance the rate of isolating pravastatin producers, compactin was used as a selective pressure to minimize the growth of undesired candidates. In the preliminary screening of microorganisms capable of converting compactin to pravastatin, microorganisms from enrichment cultures and BCRC were plated on the agar plate containing 7% (w/v) of different carbon sources and 0.1 to 1 mg compactin mL^{-1}. Numerous colonies were found from the plates supplemented with compactin less than 250 μg mL^{-1}, whereas no colony was found in the agar plate containing 1 mg compactin mL^{-1}. Therefore, Basal agar plates containing 500 μg compactin mL^{-1} were used for the screening of compactin-resistant strains. About 1000 compactin-resistant strains were obtained from various sources, in which about 100 strains were isolated from the Basal agar plate containing 7% (w/v) mannitol as a carbon source.

Several approaches have been reported for the detection of compactin and pravastatin, including mass spectrometry, nuclear magnetic resonance, and infrared spectroscopy [10]. However, the tedious assay proce-

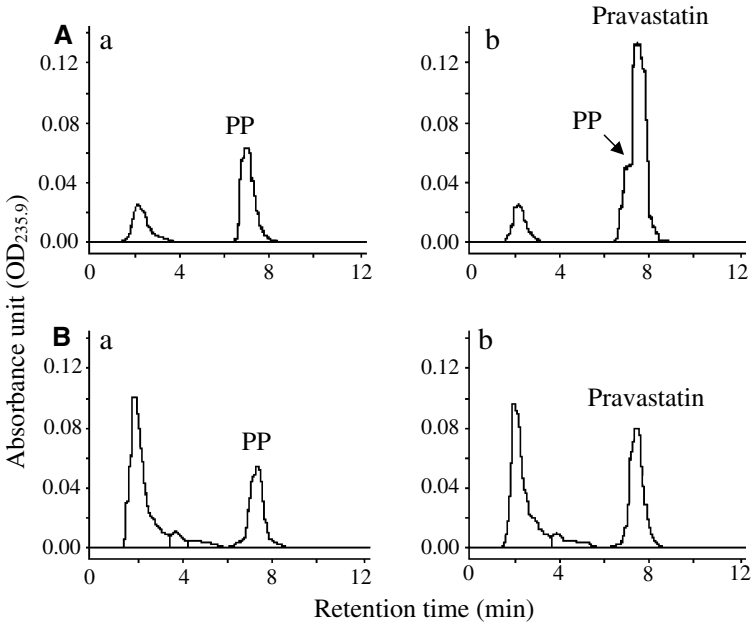

Fig. 1. HPLC analysis of the products converted from compactin by *R. fluviale* BCRC 22986 (A) and *P. autotrophica* BCRC 12444 (B) using the mobile phase of 30% acetonitrile and 10% methanol. a, without internal standard (pravastatin); b, with internal standard; PP, putative pravastatin.

dures limit their practical application. The HPLC method is frequently used to determine the concentration of pravastatin and its derivatives in human plasma and urine [3] and in culture broth [11]. To observe the transformation of compactin, compactin-resistant strains were cultivated in the Basal media containing different carbon sources at 28°C for 2 days and then allowed to grow for 10 days in the same medium containing compactin. It was observed that cell lysis of isolated microorganisms occurs in the culture broth containing compactin at a concentration higher than that for our screening program. Therefore, the concentration of 500 μg compactin mL^{-1} in Basal medium was chosen for microbial hydroxylation. Nine strains with the potential to convert compactin were identified by the HPLC method using a mobile phase of 30% (v/v) acetonitrile and 10% (v/v) methanol (Table 1). Among them, *Salmonella enterica* NCHU 1586, *Streptomyces regensis* BCRC 11890, *M. hiemalis* BCRC 32824, *Xanthomonas compestries* NCHU 1126, *Amycalatopsis sulphurea* NCHU1345, *Bacillus megaterium* NCHU 1546, and *Rhodosporidium fluviale* BCRC 22986 (Fig. 1A) showed peaks with retention time around 7.4 min. The other two strains, *Pseudonocardia autotrophica* BCRC 12444 (Fig. 1B) and *Streptomyces griseolus* BCRC 13677 isolated from the Basal agar plate containing 7% (w/v) mannitol, had peaks with a retention time that coincided with that of the internal standard, pravastatin (7.5 min), on the chromatograms. The data revealed that the transformed products from *P. autotrophica* BCRC 12444 and *S. griseolus* BCRC 13677 might be pravastatin, and the compounds involved in this catalysis could not be resolved clearly by the above HPLC method.

Although these nine organisms can convert the compactin to the products with a retention time close to that of pravastatin, it is necessary to verify if the products are pravastatin. In order to achieve observable chromatographic separation, two separation elutions with different concentrations of acetonitrile were investigated. We found the HPLC method using 30% (v/v) acetonitrile for 15 min followed by 50% (v/v) acetonitrile for 25 min could clearly separate pravastatin and compactin (substrate) with retention times of 11.5 and 32 min, respectively (Fig. 2). The results suggested that sequential use of two different mobile phases resulted in a satisfactory resolution of compactin and pravastatin in the culture broth.

To determine the ability of *P. autotrophica* BCRC 12444 and *S. griseolus* BCRC 13677 in the conversion of compactin to pravastatin, cells were cultivated in the different Basal media containing 500 μg compactin mL^{-1} at 28°C. A low yield (<5%, w/w) was found for the cells cultivated in the Basal media containing glucose, glycerol, or lactose as a carbon source, whereas about 20% and 32% (w/w) of compactin were stoichiometrically converted to pravastatin without a detectable byproduct by *P. autotrophica* BCRC 12444 and *S. griseolus* BCRC 13677, respectively, in a 9-day incubation in the Basal medium containing mannitol as a carbon source (Fig. 3). The data showed that mannitol may serve as a good carbon source for the cells involved in the conversion of compactin to pravastatin. Although *S. griseolus* BCRC 13677 had a higher conversion ability, pravastatin rapidly de-

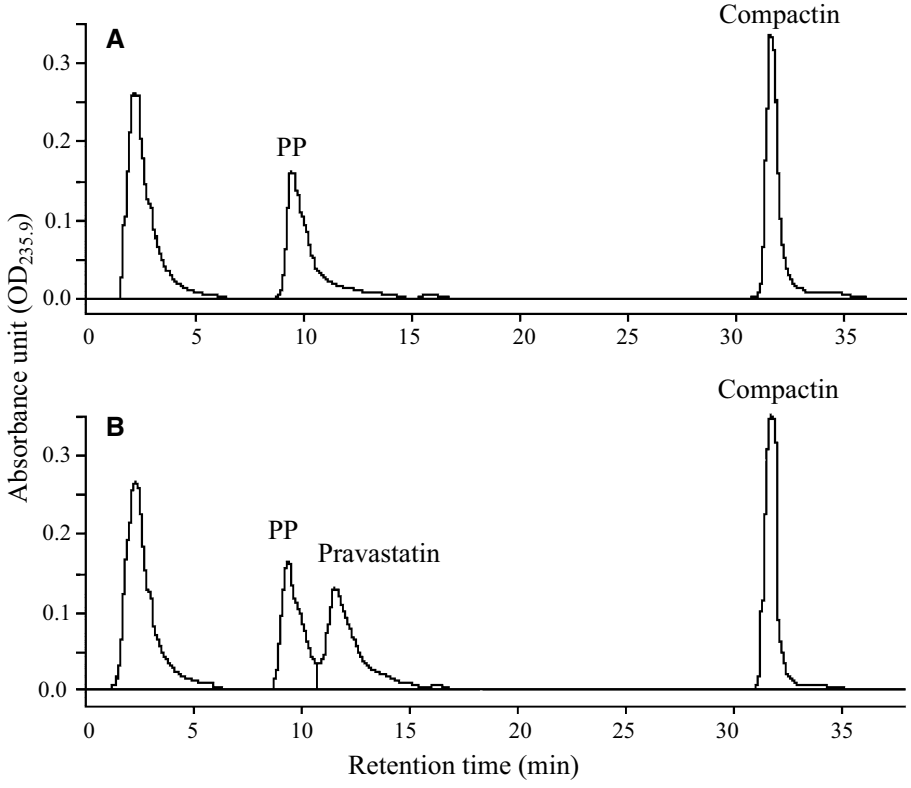

Fig. 2. HPLC analysis of the products converted from compactin by *R. fluviale* BCRC 22986 using two sequential mobile phases, 30% acetonitrile for 15 min followed by 50% acetonitrile for 25 min. (A) Without the addition of internal standard (pravastatin). (B) With the addition of pravastatin. PP, putative pravastatin.

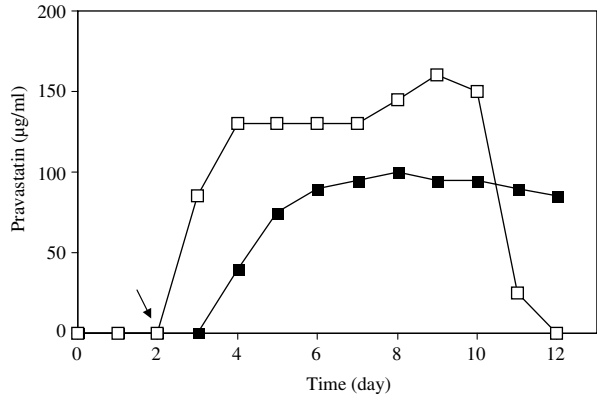

Fig. 3. Conversion of the compactin to pravastatin by *S. griseolus* BCRC 13677 (open squares) and *P. autotrophica* BCRC 12444 (closed squares). Arrow indicates the time for the addition of compactin. Data presented were the means of three independent tests.

creased after a 10-day incubation, demonstrating that pravastatin could be further converted to other products by the cells.

D-Trehalose medium has been used in an extensive screening program to discover microorganisms having strong hydroxylation activity at the 6β position of compactin. A potent converter, *Streptomyces carbophilus*, which produces only limited byproducts, has been found [13]. In this approach, target microorganisms were obtained at the rate of about 2% from the enrichment cultures, indicating that Basal medium containing 7% (w/v) mannitol and 500 μg compactin mL^{-1} in combination with our HPLC method could be used for the effective screening of the microorganisms with the ability to convert compactin to pravastatin. Although the maximal yield of pravastatin from compactin by isolated microorganisms reached only 32%, no detectable byproduct was found in the 9-day incubation. Molecular cloning of genes involved in the cytochrome P450 monooxygenase system from *S. griseolus* BCRC 13677 and *P. autotrophica* BCRC 12444 into a bacteria strain with a high tolerance to compactin is currently in progress to develop a high-efficiency biotransformation system for use in the drug industry.

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Literature Cited

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

2. Arai M, Serizawa N, Terahara A, Tsujita Y, Tanaka M, Masuda H, Ishikawa S (1988) Pravastatin sodium (CS-514), a novel cholesterol-lowering agent which inhibits HMG-CoA reductase. Sankyo Kenkyusyo Nenpo 40:1–38
3. Bauer S, Mwinyi J, Stoeckle A, Gerloff T, Roots I (2005) Quantification of pravastatin in human plasma and urine after solid phase extraction using high performance liquid chromatography with ultraviolet detection. J Chromatogr B Anal Technol Biomed Life Sci 818:257–262
4. Endo A, Kuroda M, Tsujita Y (1976) ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinium*. J Antibiot (Tokyo) 29:1346–1348
5. Hosobuchi M, Kurosawa K, Yoshikawa H (1993) Application of computer to monitoring and control of fermentation process: Microbial conversion of ML-236B Na to pravastatin. Biotechnol Bioeng 42:815–820
6. Matsuoka T, Miyakoshi S, Tanzawa K, Nakahara K, Hosobuchi M, Serizawa N (1989) Purification and characterization of cytochrome P-450sca from *Streptomyces carbophilus*. ML-236B (compactin) induces a cytochrome P-450sca in *Streptomyces carbophilus* that hydroxylates ML-236B to pravastatin sodium (CS-514), a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase. Eur J Biochem 184:707–713
7. Park JW, Lee JK, Kwon TJ, Yi DH, Kim YJ, Moon SH, Suh HH, Kang SM, Park YI (2003) Bioconversion of compactin into pravastatin by *Streptomyces* sp. Biotechnol Lett 25:1827–1831
8. Peng Y, Yashphe J, Demain AL (1997) Biotransformation of compactin to pravastatin by *Actinomadura* sp. 2966. J Antibiot (Tokyo) 50:1032–1035
9. Serizawa N, Nakagawa K, Tsujita Y, Terahara A, Kuwano H (1983) 3 alpha-Hydroxy-ML-236B (3 alpha-hydroxycompactin), microbial transformation product of ML-236B (compactin). J Antibiot (Tokyo) 36:608–610
10. Serizawa N, Nakagawa K, Tsujita Y, Terahara A, Kuwano H, Tanaka M (1983) 6 alpha-Hydroxy-iso-ML-236B (6 alpha-hydroxy-iso-compactin) and ML-236A, microbial transformation products of ML-236B. J Antibiot (Tokyo) 36:918–920
11. Serizawa N, Serizawa S, Nakagawa K, Furuya K, Okazaki T, Terahara A (1983) Microbial hydroxylation of ML-236B (compactin), studies on microorganisms capable of 3,â-hydroxylation of ML-236B. J Antibiot (Tokyo) 36:887–891
12. Serizawa N, Matsuoka T (1991) A two component-type cytochrome P-450 monooxygenase system in a prokaryote that catalyzes hydroxylation of ML-236B to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Biochim Biophys Acta 1084:35–40
13. Serizawa N (2000) Biocatalytic production of pravastatin, an anticholesterol drug. In: Patel RN (ed) Stereoselective biocatalysis. New York: Marcel Dekker, pp 703–711
14. Tsujita Y, Kuroda M, Shimada Y, Tanzawa K, Arai M, Kaneko I, Tanaka M, Masuda H, Tarumi C, Watanabe Y (1986) CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. Biochim Biophys Acta 877:50–60
15. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703