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Characterization and Culture Optimization of Regiospecific Cyclosporin Hydroxylation in Rare Actinomycetes Species

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Abstract Cyclosporins are a family of clinically-important immunosuppressive cyclic peptides produced by *Tolypocladium inflatum*. The structural modification of cyclosporins *via* hydroxylation at various positions of N-methyl leucines in cyclosporin A leads to a dramatic change of their bioactive spectra. Among over 100 soil actinomycetes screened, two actinomycetes species, *Sebekia benihana* and *Pseudonocardia autotrophica*, were identified to contain superior cyclosporin A hydroxylation activities. A HPLC-based cyclosporin A hydroxylation assay revealed that each strain possesses distinctive hydroxylation specificity and regiospecificity; mono-hydroxylation at the 4th N-methyl leucine of cyclosporin A by *S. benihana*, and di-hydroxylations at both 4th and 9th N-methyl leucines of cyclosporin A by *P. autotrophica*. The conversion yields for cyclosporin A hydroxylation by both *S. benihana* and *P. autotrophica* were significantly improved from less than 10% and 18% up to 58% and 45%, respectively, in the optimized culture containing molybdenum with 0.05 g/l of cyclosporin A concentration. An ancymidol-specific inhibition of cyclosporin hydroxylation also suggested that the regiospecific cyclosporin hydroxylation might be catalyzed by a putative cytochrome P450 mono-oxygenase enzyme.

Key words: Cyclosporin, hydroxylation, actinomycetes, bioconversion

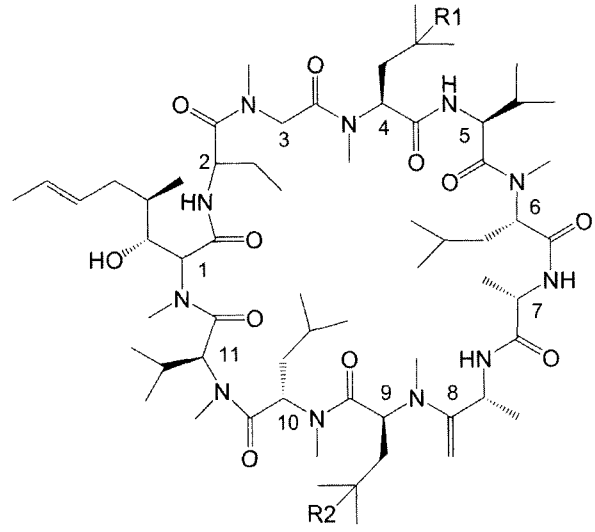

Fig. 1. Chemical structures of cyclosprin A and its hydroxylated forms; cyclosporine (R1-H, R2-H), mono-hydroxylated cyclosporin A at 4th N-methyl leucine (R1-OH, R2-H), di-hydroxylated cyclosporin A at both 4th and 9th N-methyl leucines (R1-OH, R2-OH).

Cyclosporins, typically produced by *Tolypocladium inflatum*, are a family of cyclic peptides consisting of 11 amino acids; four N-methyl leucines, two alanines, N-methyl-4-(2-butenyl)-4-methyl threonine, sarcosine, valine, N-methyl-valine, and one of various amino acids (aminobutyric acid in cyclosporine A) (Fig. 1) [12, 19, 22]. Cyclosporins have been known to possess various biological activities including antiviral, antifungal, and antiprotozoan activities, and are currently used as a very potent immunosuppressor drug [13, 14, 17, 20], even though patients administrated with cyclosporins show the symptoms of various side effects including kidney and liver toxicity, hypertension, and hirsutism [1, 2, 3, 13, 21]. Recently, it has been suggested that the structural modification of cyclosporins *via* hydroxylation at various positions of N-methyl leucines in cyclosporin A leads to a dramatic change of their bioactive spectra [16]. Specifically, the regiospecific hydroxylation at the gamma

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position of 4th and/or 9th N-methyl leucine leads to significant loss of the immunosuppressor activity, while maintaining the hair-growth stimulating effect [8, 9], implying the possibility of developing a novel hair-growth stimulating substance using hydroxylated cyclosporins [8, 9]. Traditional chemical modification of structurally complex compounds like cyclosporins has been previously reported to result in a non-regiospecific modification as well as inefficient conversion yield [11]. Unlike chemical modification, a bioconversion approach using a specific enzyme or microorganism should generate a highly regioselective product in a more environment-friendly process. One of the most challenging tasks in a bioconversion system including cyclosporin hydroxylation using a biological system is, however, to isolate and optimize a specific enzyme or microorganism suitable for a high efficiency bioconversion process [10]. In this short communication, we report the isolation, characterization, and culture optimization of potentially important rare actinomycetes strains that possess the cyclosporin hydroxylation activity, which might be catalyzed by a putative cytochrome P450 mono-oxygenase enzyme.

To isolate the microorganisms possessing cyclosporin hydroxylation activity, over 100 actinomycetes species were screened, some of which were purchased from the Korean Cell Type Collection (KCTC) and others isolated from various soil samples. Each actinomycetes strain was inoculated into 25 ml of GSMY (glucose 0.7%, yeast extract 0.45%, malt extract 0.5%, soluble starch 1.0%, and calcium carbonate 0.005%) medium and cultured for 24 h at 26°C, 200 rpm [4, 6, 7]. The cyclosporin A was then added into each culture at the final concentration of 0.1 g/l. Each culture was further incubated at the same condition as described above for 5 more days, followed by equal volume extraction with ethyl acetate. A HPLC assay for cyclosporin A hydroxylation was performed using a reverse-phase C_{18} column (4.6×250 mm, 5 μm, Symmetry, Milford, U.S.A.) with a two-buffer gradient system; 25% methanol (buffer A) and 100% acetonitrile (buffer B). One cycle of buffer B gradient was programmed to start 40% for 4 min, 61% for 20 min, 100% buffer B for 40 min, and 40% for 45 min. The column temperature was maintained at 70°C, and the cyclosporin and its derivatives were monitored at 210 nm. The flow rate was kept constant at 1 ml/min during the entire HPLC assay. The standards of cyclosporin A and its hydroxylated form (hydroxylation at the gamma position of 4th N-methyl leucine of cyclosporin A which was kindly provided by LG Household & HealthCare) were confirmed to be eluted at 30 min and 25 min, respectively (Fig. 2A).

Among over 100 actinomycetes samples tested, two rare actinomycetes species, *Sebekia benihana* (KCTC 9610) and *Pseudonocardia autotrophica* (KCTC 9441), were identified to possess cyclosporin A hydroxylation activities. As shown in Fig. 2, *S. benihana* was able to convert about 10% of cyclosporin A to the hydroxylated form in a 5-day culture. The hydroxylated form of cyclosporin A in *S. benihana* culture, which was eluted as a single peak at approximately 25 min, was determined to be exactly the same as the standard form of the 4-gamma-hydroxylated cyclosporin A (Fig. 2B). In the case of the *P. autotrophica* culture, unlike *S. benihana*, about 18% of cyclosporin A was converted to the two hydroxylated forms, eluted at approximately 25 min and 23 min, respectively. They were determined to be mono- and di-hydroxylated forms of cyclosporin A; 4-methyl leucine-gamma-hydroxylated cyclosporin A and 4, 9-methyl leucine-gamma-di-hydroxylated cyclosporin A, respectively (Fig. 1). Although both rare actinomycetes strains were proved to possess cyclosporin hydroxylation activities, the *in vivo* cyclosporin A conversion yield was less than 18% (Fig. 2C). To improve the conversion yield for cyclosporin A hydroxylation, both the culture condition and the cyclosporin

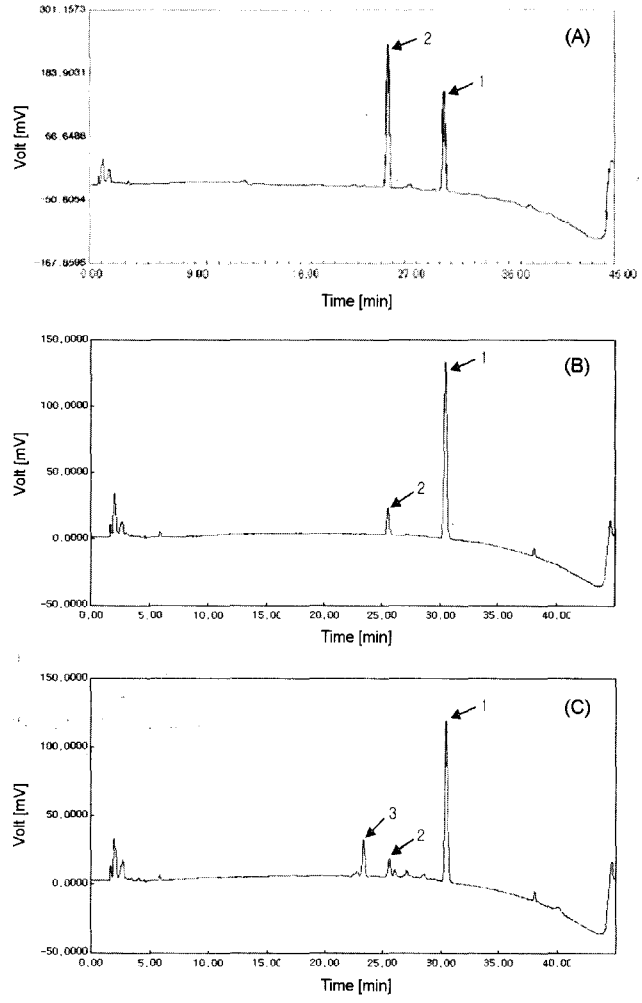

Fig. 2. HPLC chromatograms of cyclosporin A conversions by standard (A), *S. benihana* (B), and *P. autotrophica* (C): Peak #1, Cyclosporin A (CyA); peak #2, 4-mLeu-γ-Hydroxy CyA; peak #3, 4, 9-γ-Dihydroxy CyA.

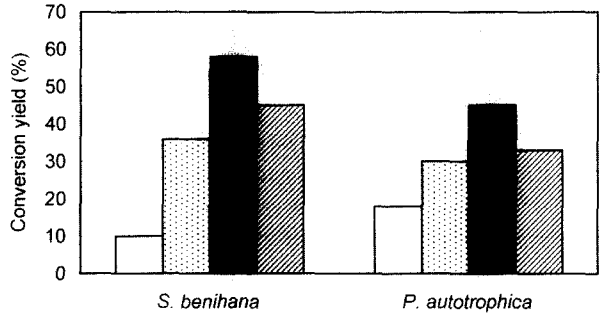

Fig. 3. Optimization of medium composition with molybdenum and cyclosporin A concentration.
Symbols: □, culture without molybdenum and with cyclosporin A concentration of 0.1 g/l; ▒, seed culture with molybdenum of 0.2 mg/l and cyclosporin A concentration of 0.1 g/l; ■, seed culture with molybdenum of 0.2 mg/l and cyclosporin A concentration of 0.05 g/l; ▨, seed culture with molybdenum of 0.2 mg/l and cyclosporin A concentration of 0.01 g/l.

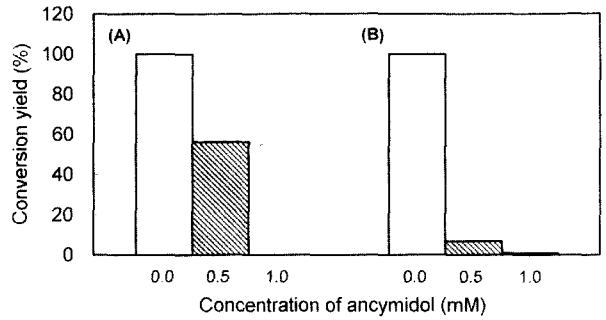

Fig. 4. Inhibition of cyclosporin A conversion by cytochrome P450 inhibitor, ancymidol, in *S. benihana* (A) and *P. autotrophica* (B).

A concentration were further optimized. Each of *S. benihana* or *P. autotrophica* was cultured first in GSMY medium without cyclosporin A for 48 h as a seed culture to maximize the cell growth. The seed culture (10%) was then transferred to the modified GSMY medium containing 0.2 mg/l of $(NH_4)_6Mo_7O_{24}$ as a conversion culture. After 48 h of incubation, the cyclosporin A was then added as a substrate into a conversion medium at the cyclosporin A concentration of 0.1, 0.05, or 0.01 g/l. After 5 more days of incubation, each culture was analyzed for cyclosporin A hydroxylation using HPLC. In both *S. benihana* and *P. autotrophica* cultures, the highest cyclosporin A conversion yields were achieved with the 0.05 g/l of cyclosporin A concentration, generating more than 45% of hydroxylated forms of cyclosporin A (Fig. 3). These results suggest that some key factors such as well-grown actinomycetes seed culture, appropriate medium composition, and optimum concentration of cyclosporin A are very critical to optimize the *in vivo* cyclosporin hydroxylation process.

Based on the recent genome sequencing analysis of several actinomycetes species, there are multiple cytochrome P450 mono-oxygenase genes present in the chromosome, some of which are involved in the hydroxylation of various secondary metabolites with unique substrate specificities [4]. Considering the complex chemical structure of cyclosporin and regiospecific hydroxylation pattern, it is very likely that cyclosporin A hydroxylation may be catalyzed by a novel type of cytochrome P450 mono-oxygenase enzyme only present in *S. benihana* or *P. autotrophica*. To test this hypothesis, a well-known cytochrome P450 mono-oxygenase inhibitor, ancymidol [18], was added into the cyclosporin conversion culture of *S. benihana* or *P. autotrophica*. As shown in the Fig. 4, the cyclosporin A conversion was significantly inhibited by ancymidol in each culture, implying that the cyclosporin A hydroxylation might be carried out by a novel type of cytochrome P450 mono-oxygenase enzyme only present in either *S. benihana* or *P. autotropica*.

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