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Antibacterial Activities of Methylelaiophylin

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Abstract

Methylelaiophylin generated superoxide radicals in *Bacillus subtilis* and showed antibacterial activity against a broad range of gram positive bacteria. The inhibition of DNA synthesis was more sensitive than one of RNA synthesis. A recombination-deficient mutant strain of *B. subtilis* was 2-fold more sensitive than a wild strain, and this sensitivity was reduced in the presence of an antioxidant, dithiothreitol. Methylelaiophylin generated superoxide radicals in *B. subtilis* lysates, and this suggests that the antibacterial activity of methylelaiophylin is related to the generation of active oxygen species in the cells.

Key words : *methylelaiophylin, superoxide radicals, antibacterial activity*

Introduction

Several antitumor agents such as anthracyclines[1,2], streptonigrin[3], etc. produce oxygen radicals, for instances, superoxide anion (O_2^-), singlet oxygen, peroxide and hydroxyl radical. These free radicals are capable of causing strand scission of DNA, resulting antitumor action. This suggests that the screening for antibiotics producing such radicals will lead to the finding of new antitumor antibiotics.

In the course of screening for superoxide radical generating substances[4], methylelaiophylin, a derivative of elaiophylin, was isolated as active principles from *Streptomyces melanosporofacience*. Elaiophylin is a 16-membered macrolide antibiotic, isolated from cultures of *Streptomyces melanosporus*, which was first reported by Arcamone *et al.*[5] and exhibit activity against gram-positive bacteria and fungi. Closely related to elaiophylin, are the azalomycins F3, F4 and F5[6,7], copiamycin, neocopiamycin[8,9], and guanidylfungins A and B[10,11].

In this publication, we report the antibacterial activity, the generation of superoxide radical, and the mode of action of methylelaiophylin.

Materials and Methods

Antimicrobial agent

Methylelaiophylin was isolated from the powered rhizomes of *Streptomyces melanosporofacience*.

Test organisms

All strains used in this study were laboratory standard strains obtained from American Type Culture Collection (ATCC) and Korean Collection for Type Cultures (KCTC). All isolates were stored frozen at −70℃.

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Susceptibility tests

Antimicrobial susceptibility of the test organisms was determined by the paper disc agar diffusion method. Briefly, test strains of 10^7 CFU/mℓ were inoculated on Mueller Hinton medium (Difco Laboratories, Detroit, MI) containing serially diluted antibiotics. Test strains were grown at 37℃ for 18 h in Mueller Hinton medium.

Assay of antibacterial activity against rec⁻ strain

Antibacterial activity was measured by the paper disc method on nutrient agar using *Bacillus subtilis* as the test organism[12]. Melted agar medium prewarmed at 60 ℃ with spore suspensions of the wild type strain (rec⁺) and the supersensitive mutant strain (rec⁻) at 10^4 spores/mℓ was poured into a plastic plates and solidified at room temperature. After paper discs (8 ㎜ ϕ) containing samples were placed on agar plates, the plates were incubated overnight at 37℃, and inhibition diameters of the plates were measured.

Assay of superoxide radicals

The generation of superoxide radicals was determined by the nitro blue tetrazolium (NBT) assay[13]. *B. subtilis* KCTC 3069 (rec⁻) was homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.15 M potassium chloride and 1 mM EDTA, and centrifuged at 5,000 rpm for 20 min. The supernatant was used as cell lysate. The reaction mixture (1 mℓ) containing the cell lysate (0.8 ㎎ protein/mℓ), 0.05% NBT (Sigma) and various concentrations of drug was incubated in the presence or absence of superoxide dismutase (SOD, Sigma, from bovine erythrocytes ; 130 μm/mℓ) at 37℃ for 30 min, followed by addition of 2 mℓ of 1 N HCl to the mixture, and was centrifuged at 5,000 rpm for 20 min. The residue was washed with 1 N HCl and dissolved in 1 mℓ of hot pyridine (Sigma). The optical density of supernatant was measured by spectrophotometer (Shimadzu UV-265FW) at 515 nm.

Determination of antibacterial action

Bacilus subtilis KCTC 3069 (rec⁻) was grown at 37℃ in a test tube containing Davis-Mingioli minimal medium with glucose (20 g/ℓ), asparagine (0.1 g/ℓ), Difco casamino acids (2 g/ℓ), and thymidine (2 ㎎/ℓ). When the culture reached on optical density (590 nm) of 0.2, it was divided in four parts. To two of these, precursors of DNA (2 mCi/ℓ of [^{3}H]dThd, Amersham, +2 ㎎/ℓ of unlabeled thymidine, Sigma) and RNA (1 mCi/ℓ of [^{3}H]uridine, Amersham, +10 ㎎/ℓ of unlabeled uridine, Sigma). The third cultures was used to monotor cell density. After 10 min of incorporation, each culture was further divided into three parts : untreated control, 1 ㎎/ℓ of methylelaiophylin and positive control. The positive controls were 25 ㎎/ℓ of nalidixic acid (as an inhibitor of DNA synthesis) and 2.5 ㎎/ℓ of rifampicin (as an inhibitor of RNA synthesis). At various times, up to 60 min, 0.1 mℓ samples were added to 2 mℓ of ice-cold 5% TCA and filtered on glass fibers (GF/C, Whatman). Samples containing tritiated leucine were heated for 15 min at 75℃ before filtering. The filters were placed in 5 mℓ of Insta-Fluor (Packard, IL) and counted in a liquid scintillation spectrometer (Packard)[14].

Results and Discussion

Activity of methylelaiophylin against a mutant and a wild type strain of *Bacillus subtilis*

Methylelaiophylin showed an antibacterial activity against gram(+) bacteria as determined by the agar dilution method (Table 1). Many kinds of gram positive bacteria were sensitive, but all gram negative bacteria tested were insensitive to this compound at 100 μg/mℓ. The following yeasts and fungi were insensitive to the methylelaiophylin at the highest concentration tested, 100 μg/mℓ : *Candida albicans* KCTC 1940, *Saccharomyces cerevisiae* KCTC 1552, *Aspergillus niger* KCTC 2119, *Penicillium citrinum* KCTC 1255. MIC values determi-

ned by methylelaiophylin are showed 1.56 μg/mℓ against *B. subtilis* ATCC 6633 (rec$^+$ strain) and 0.78 μg/mℓ against *B. subtilis* KCTC 3069 (*rec*$^-$ strain), which is deficient in enzyme for DNA recombination.

Table 1. Antibacterial activities of methylelaiophylin.

	Test organisms	MIC (μg/mℓ)
Gram(+)	*Bacillus cereus* KCCM 11774	1.56
	Bacillus circulans IAM 1140	3.13
	Bacillus subtilis KCTC 3069	0.78
	Bacillus subtilis ATCC 6633	1.56
	Bacillus thuringiensis KCTC 1033	1.56
	Entrococcus lactis KCTC 1913	1.56
	Lactobacillus brevis KCTC 3102	0.39
	Pediococcus acidilactici KCTC 1626	0.78
	Staphyloccus aureus KCTC 1621	6.25
Gram(−)	*Enterobacter cloacae* KCTC 2361	−
	Escherichia coli KCTC 1682	−
	Klebisiella pneumoniae KCTC 2208	−
	Pseudomonas aeruginosa KCTC 2004	−

The values of minimum inhibitory concentration (MIC) were determined by serial agar dilution method.

A significant difference was observed in the sensitivity to methylelaiophylin between wild *rec*$^+$ and mutant *rec*$^-$ strains of *Bacillus subtilis*. *B. subtilis* KCTC 3069, a *rec*$^-$ strain, was about 2-fold more sensitive than *B. subtilis* ATCC 6633, a *rec*$^+$ strain, to this compound (Table 1). Melted agar medium prewarmed at 60℃ with spore suspensions of the wild type strain (*rec*$^+$) and the supersensitive mutant strain (*rec*$^-$) at 10^4 spores/mℓ was poured into a plastic plates and solidified at room temperature. After paper discs (8 ㎜, ϕ) containing samples were placed on agar plates, the plates were incubated overnight at 37℃, and inhibition diameters of the plates were measured. As shown in Fig. 2, methylelaiophylin inhibited the growth of *rec*$^-$ strain more potently than that of *rec*$^+$ strain. Since recombination deficient mutants have been known to show higher sensitivity against antibiotics which can cause DNA damage, this suggests that antibiotics induce the DNA damage, through cleavable complex formation with the DNA gyrase[15,16,17,18]. However, DNA strand scission by methylelaiophylin was not observed practically when analyzed by agarose gel electrophoresis (data not shown). Those results are corresponded with observation that antibacterial activity (MIC) against the *rec*$^-$ mutant was more effective than that of *rec*$^+$ strain.

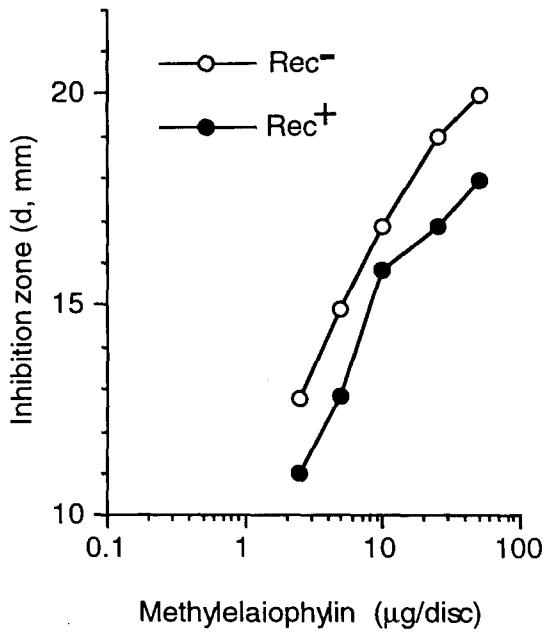

Fig. 1. Antibacterial activities of methylelaiophylin against *Bacillus subtilis* wild type strain (rec$^+$; ●) and mutant strain (rec$^-$; ○).
Activity is expressed as diameter of growth inhibition zone surrounding paper discs on the assay plate.

Generation of superoxide radicals in *Bacillus subtilis* cell lysate by methylelaiophylin

To obtain some insights into the correlation between

antibacterial activity and active oxygen radical formation, the sensitivity of *B. subtilis* KCTC 3069 to methylelaiophylin was compared in the presence and absence of dithiothreitol (DTT). Since the antibacterial activities caused by active oxygen species can be prevented by an antioxidative agent, we examined the effect of DTT on the antibacterial activity of methylelaiophylin against *B. subtilis* KCTC 3069, which is known to be vulnerable to oxygen stress. *B. subtilis* KCTC 3069 (1×10^3 cells /mℓ) was incubated for 1 day with designated concentrations of methylelaiophylin in the presence or absence of 250 μM DTT, and then viable cells were counted to determine IC_{50} values. The IC_{50} values in the absence of DTT were 0.15 μg/mℓ and those in the presence of DTT were 1.18 μm/mℓ for methylelaiophylin, indicating a 8-fold more resistance to the methylelaiophylin in the presence of DTT. This result suggests that the action of methylelaiophylin, at the least, may be correlated with

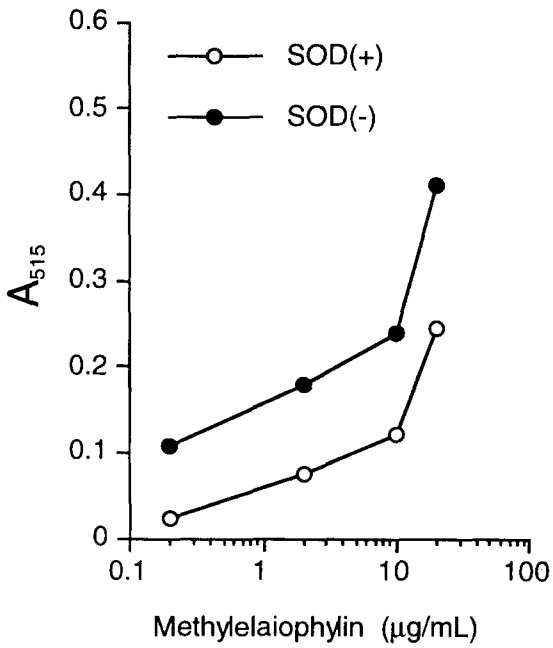

Fig. 2. Superoxide radical generation in *B. subtilis* KCTC 3069 cell lysate by methylelaiophylin.

Table 2. Effect of dithiothreitol (DTT) on the antibacterial activity of methylelaiophylin against *B. subtilis* KCTC 3069.

	methylelaiophylin	
	−DTT	+DTT
IC_{50} (μg/mℓ)	0.15	1.18

B. subtilis KCTC 3069 ($1\times a1/10^3$ cells/mℓ) was incubated for 1 day with various concentrations of methylelaiophylin in the presence or absence of 250 μM DTT, and then viable cells were counted to determine IC_{50}.

their action as active oxygen radical generators.

To corfirm the generation of superoxide radicals, lysates of *B. subtilis* KCTC 3069 were treated with methylelaiophylin as described in the legend to Fig. 2, and superoxide radicals were quantified by the nitro blue tetrazolium (NBT) reduction method[13]. Superoxide radicals were generated by methylelaiophylin depending on the concentrations, and it was strongly inhibited by the addition of superoxide dismutase (Fig. 2). This result suggests that the antibacterial activity of methylelaiophylin is related to the generation of active oxygen species in the cells.

Mechanism of action of methylelaiophylin in intact bacteria

To effects of methylelaiophylin on DNA and RNA syntheses were examined by quantifying the incorporation of [^{3}H]thymidine and [^{3}H]uridine, into trichloroacetic acid (TCA)-insoluble fraction of *B. subtilis* KCTC 3069 according principally to the method reported by Selva *et al.*[14]. The inhibition of RNA synthesis was initiated at 20 min after addition, while DNA synthesis inhibited promptly (Fig. 3). This result suggests that an obstruction of RNA synthesis was less sensitive than one of DNA synthesis. However, niphithricin A, closely related to methylelaiophylin, showed the unspecific inhibition of the biosynthesis of macromolecular of *B. subtilis*[19].

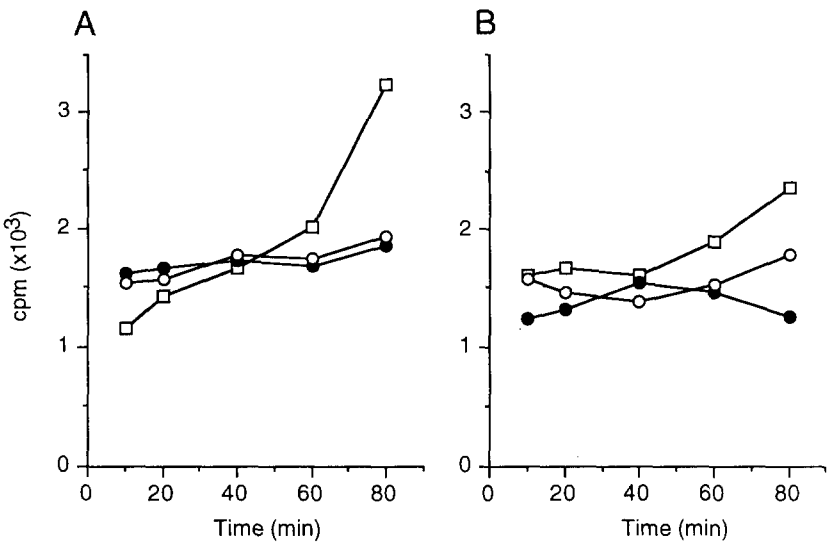

Fig. 3. Mechanism of action of methylelaiophylin in *B. subtilis* KCTC 3069.
(A) DNA synthesis, (B) RNA synthesis. □, control ; ●, positive control (A : 25 μg/mℓ nalidixic acid, B : 10 μg/mℓ rifampicin) ; ○, methylelaiophylin (20 μg/mℓ). Antibacterial agents were added at the time indicated by the arrow.

Methylelaiophylin generated superoxide radicals and their antibacterial activity in a *rec*⁻ mutant of *B. subtilis* was greatly reduced in the presence of DTT. This suggests that superoxide radical formation might be the cause for the antibacterial activity of methylelaiophylin, but the precise mechanism of action waits for further studies.

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초록 : Methylelaiophylin의 항균활성

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Methylelaiophylin은 gram 양성균에 대하여 광범위한 항균활성을 보이며, 특히 *Bacillus subtilis*에서 superoxide radicals을 발생하여 항균활성을 나타낸다. B. subtilis에대한 항균활성은 wild type strain보다 mutant strain인 rec$^-$에 대하여 더민감하며, 이러한 항균력은 항산화제인 dithiothreitol에 의하여 현저히 감소되었다. 또한 항균력은 세포내 RNA합성보다 DNA합성에 효과적으로 작용하였다. 이러한 결과들은 methylelaiophylin의 항균활성이 세포내에서 발생한 활성산소에 의하여 기인한 것임을 나타낸다.