Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 5, 398-401 (1988)

Characters of β-Lactamase Inhibitor Produced by *Streptomyces* sp.

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放線菌의 一株가 生成하는 β-Lactamase Inhibitor의 特性

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***Streptomyces* sp. producing β-lactamase inhibitor were isolated from soil. The culture conditions for the production of the β-lactamase inhibitor were evaluated and isolation produre of the β-lactamase inhibitor from the culture broth was also established. Some characters of the partially purified β-lactamase were determined.**

β-Lactam antibiotics have been used widely in clinics due to their low toxicity and their selective effectiveness on Gram positive bacteria. However resistance strains to the β-lactam antibiotics have been thought to occur coincidently with the wide uses of the antibiotics. It is well known that the resistant strains produce β-lactamase which hydrolyze the β-lactam ring resulted in the lose of the antimicrobial activity. A great efforts have being done to develop new β-lactam antibiotics not to be inhibited by the β-lactamase. A compound, which shows on inhibitory activity against to the β-lactamase, is very useful for the treatment of infections of the resistant bacteria. The research for naturally β-lactamase inhibitors has being intensively carried out, compounds, such as, clavulanic (1), olivanic acids(2-4), epithienamycins(5), ps-5(6), izumenolide (7) were reported and it has been well realized that the β-lactamase inhibitors having antimicrobial activity will be the most important antibiotics in future.

Materials and Methods

Isolation of aerobic actinomycetes

Soil samples collected from different sites in Korea were used as major sources of aerobic actinomycetes. The soil samples were dried at 80°C for 20 min and suspended in a sterile saline solution and then plated on a rich agar medium which was formulated as followed: 1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% K_2HPO_4, 0.05% $MgSO_47H_2O$, 0.02% $CaCO_3$, 0.001% $FeSO_4$ $7H_2O$, and agar 2% (pH 7.4 before sterilization).

After 7-10 days incubation at 25°C, bacterial colonies having spores were picked and transferred to agar slants containing 0.1% beef extract, 0.1% yeast extract, 0.2% peptone, 0.5% NaCl, and 1.5% agar (pH 7.4 before sterilization). The isolates of actinomycetes were submergically cultured for 5 days at 28°C in 250ml flasks containing 50 ml of liquid fermentation media having the following compositions: 3.5% maltose, 1.2% Na-casinate, 1.2% peptone, 0.05% Na_2SO_4, 0.001% $CoCl_2$ $6H_2O$, and 0.05% $CaCO_3$. (pH 7.2 before autoclaved).

Screening for actinomycetes producing β-lactamase inhibitors

β-Lactamase producing actinomycetes were screened with the methods described by Butterworth *et al.* (2). Fermentation samples were immersed to the 5 mm filter discs (Whatman) and then placed on the plates of rich media where Penicillin

Key words: β-Lactamase inhibitor, β-lactam antibiotics, *Streptomgces* sp.
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G (100 units/m*l*) and Penicillin G. resist strain of *Escherichia coli* (about 10^8 cells/m*l*) were pregnated. After incubation at 37 °C for 1 day, samples showed growth inhibitory clear zone around the disc were selected as a culture to produce β-lactamase inhibitors.

Determination of antimicrobial activities

Antimicrobial activities of the selected cultures were determined by the paper disc bioassay method using *Bacillus subtilis* and *Escherichia coli* as test organisms.

Mass culture conditions

Cells grown in fermentation media were stocked in a deep freezer (-60 °C). The stocked culture was transferred to 200 m*l* of fermentation medium contained in 1 liter baffled flasks and cultured at 30 °C for 24 hours using a gyrorotory shaking incubator. The seed culture was inoculated into a 4 liter jar fermentor (B. Braun, E model). Temperature was maintained to 30 °C and pH was controlled to 7.0 by automatic addition of 1 N NaOH or 1N HCl. The agitation speed was 150 rpm and aeration was changed to give 70% saturation of dissolved oxygen tension (DOT).

Isolation of β-lactamase inhibitors

Isolation of β-lactamase inhibitor from the culture broth was conducted as followed as; 4 liters of cell free culture broth was mixed throughly with 200 grams of activated charcoal and allow to stand for 2 hours. The mixture was filtered through filter paper (Whattman No. 4). The filter cake of charcoal was washed with 3 volume of deionized water and then eluated with 2 volume of ammonia-ethanol mixture (pH was adjusted to 12 with 28% NH_4OH). The eluent was evaporated under reduced pressure to dry and the residues were extracted with 20 m*l* deionized water. The extracts were loaded on ion-exchangers, DEAE-sepharose CL-6B (Cl form) packed in a column 2 × 40 Cm and eluated with deionized water. The active fractions were collected and concentrated under reduced pressure. The concentrated active fraction was followed to add two volumes of ethanol and allowed in a refrigerator, 10 °C, for few days. Precipitates obtained from the water ethanol precipitations were desolved in deionized water and recrystallized by the same conditions. The crystals were harvested and dried under vacuum and stored for subsequent use.

Results and Discussions

Selection of β-lactamase producing *Actinomycetes*

About 5000 cultures were picked from the soil samples and the cultures were tested in order to select cultures producing β-lactamase inhibitors. Colonies having distinct actinomycetes characters could be isolated from the isolating rich medium. The isolated cultures were incubated in the fermentation medium and analyzed the β-lactamase inhibitory activities. The total frequency to isolate cultures producing β-lactamase inhibitors was about 1%, hence 50 cultures were selected as the first step using filter disc method. The selected cultures were reexamined in order to select the best culture to produce β-lactamase. Isolated culture No. 209 was selected based upon their production of β-lactamase inhibitors and antimicrobial activities. It was interest to note that culture of No. 209 strain showed strong inhibition on the both growth of G^- (*E. coli*) and G^+ (*B. subtilis*) bacteria. The microscopic observation of the isolated culture No. 209 is shown in Figure 1. It shows gray aerial mycelia, rectus-flexible and spores. It produced purple pigment on rich solid medium. The other characters were those of *Streptomyces* sp., hence the isolate was tentativly designated as *Streptomyces* sp.

β-Lactamase inhibitor fermentation

β-Lactamase inhibitor fermentation was carried out using the fermentation medium. It was found that disaccharides viz, maltosesucrose, and lactose were good substrates for the production of

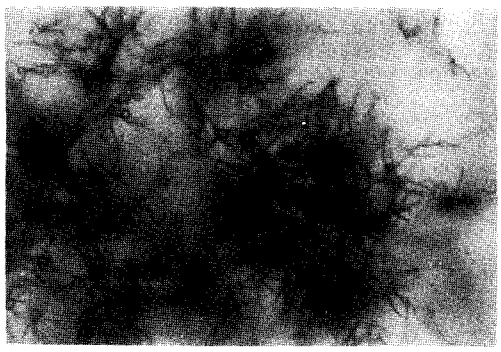

Fig. 1. Microscopic observation of β-lactamase inhibitor producing microorganism.

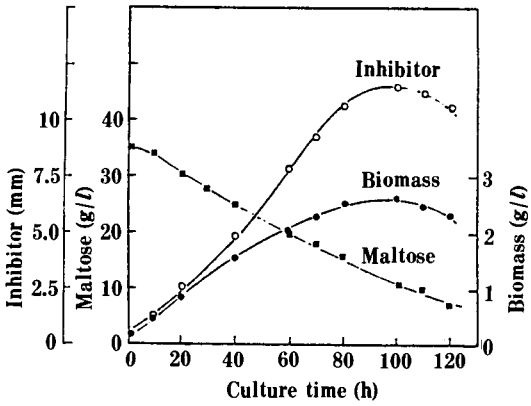

Fig. 2. Changes of maltose concentration and the formation of biomass and β-lactamase inhibitor in the culture of *Streptomyces* sp. KL 209.

β-lactamase inhibitor. Monosaccharides and polysaccharides were allowed very good cell growth but did not give to produce β-lactamase inhibitor. In selection of good nitrogen source, casinate or peptone was found to be the best organic nitrogen source. It was very interest to note that β-lactamase inhibitor and antimicrobial compound against *B. subtilis* and *E. coli* were produced simultaneously only in the medium containing maltose and peptone as carbon and nitrogen sources respectively.

Changes of the concentrations of maltose and biomass and the activities of β-lactamase inhibitor are shown in Fig. 2. It was evident that the production of β-lactamase inhibitor was closely linked to the cell growth. Rapid decreases of the activities of β-lactamase inhibitor was followed as the decreases in cell concentrations at the stationary phase.

Characters of the purified β-lactamase inhibitor

The purified β-lactamase was analyzed in order to know the chemical and biological properties. Ultra-violet absorption showed only one absorption at 267 nm and infra-red absorption spectra showed also distinct sharp peaks at 640 nm, 1075 nm, 1658 nm, 1685 nm, and 3441 nm. Those spectra were not identified with the reported β-lactamase inhibitors such as olivanic acid, thienamycin, epithienamycins, ps-5 etc.. The obtained compound did not react with hydroxylamine, which indicated that the compound did not have β-lactam ring. Color reaction with nihydrine was positive which meant that

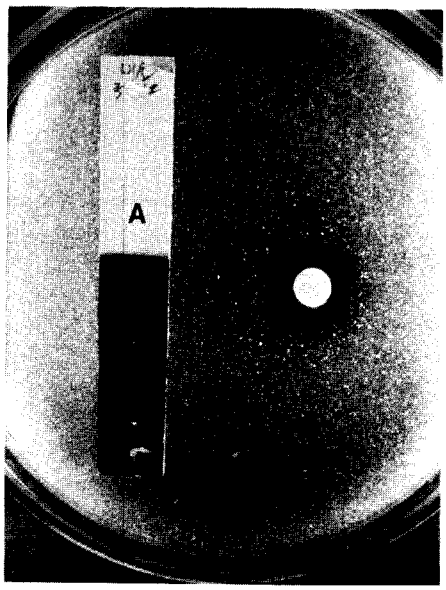

Fig. 3. [A] Silica-gel thin layer chromatogram of the isolated -lactamase inhibitor. (The spot was detected by ninhydrin). [B] Antimicrobial activity against penicillin G resistant strain of *E. coli*.

the compound has free amine radical. Molecular weight was estimated to be about 208 by field adsorption method. Although the chemical structure of the compound was not fully elucidated, it was worth to note that the compound was not β-lactam ring. Because most of the β-lactam inhibitors reported so far were β-lactam compounds, therefore, it was thought that the compound would be a new compound.

Silica gel thin layer chromatograms were made using various developing systems. As shown in Fig. 3(A), it was found that the purified sample was homogeneous. The concentrated sample separated by the TLC was tested the β-lactamase inhibitory activity using pen G resist strain (LE 392 resist to pen G 50 mg/*l*) of *E. coli*. As can be seen in Fig. 3B, it was very clear the isolated sample was pure and had a strong β-lactamase inhibitor.

요 약

β-Lactamase을 저해하는 물질을 생성하는 방선균의 일균주를 토양에서 분리하였다. 분리된 균으로부터 β-lactamase 저해물질을 생산하는 조건을 검토하였고, 아울러 배양액에서부터 동 물질을 분리정제

하여 그 특성의 일부를 조사하였다. 그 결과 이 물질은 β-lactam ring을 가지고 있지 않았으며, β-lactam 항생물질에 강한 내성을 나타내는 *E. coli* 내성균주에 강한 항생력을 나타냄을 알았다.

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(Received September 10, 1988).