Journal of Microbiology and Biotechnology
Vol. 1, No. 2, 85-89, 1991

Purification and Characterization of Proteinaceous β-Lactamase Inhibitor from the Culture Broth of *Streptomyces* sp. SMF-19

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Received 22 February 1991 / Accepted 27 May 1991

The aim of this study is to elucidate characteristics of β-lactamase inhibitor produced by *Streptomyces* sp. SMF-19 isolated from soil was found to produce proteinaceous extracellular β-lactamase inhibitor. The β-lactamase inhibitor was purified through ammonium sulfate fractionation, gel filtration, anion exchange chromatography and fast performace liquid chromatography. The molecular weight of the β-lactamase inhibitor was estimated to be about 48,000 by SDS-PAGE. The mode of inhibition against penicillin G as a substrate was uncompetitive. The β-lactamase inhibitor was stable in wide pH range but unstable at high temperature above 50°C.

β-Lactam antibiotics have been used widely in clinics due to their low toxicity and their selective effectiveness on Gram positive bacteria. However, pathogenic bacteria resistant to the β-lactam antibiotics have occurred coincidently with the wide use of the antibiotics. It is well known that the resistants produce various types of β-lactamase which hydrolyze the β-lactam ring of β-lactam antibiotics (1). These facts give rise to increase interests in the development of β-lactamase inhibitors as antibiotic-protecting agents. As a result, natural products, such as clavulanic acid (10), olivanic acid (3), thienamycin (6), ps-5(9), and izumenolide (8) were isolated to have either inhibitory activities to β-lactamase or both of the inhibitory and antimicrobial activities. Recently Doran *et al.* (4) isolated proteinaceous β-lactamase inhibitor from the culture broth of *Streptomyces clavuligerus* and determined the amino acid sequence of the protein.

In this study the proteinaceous β-lactamase inhibitor, which was produced by *Streptomyces* SMF-19, was purified and its characteristics were investigated.

MATERIALS AND METHODS

Solid Samples and Media Used

Soil samples used for the isolation of Actinomycetes were collected from different sites in Korea. Medium used for the isolation of Actinomycetes from the soil was formulated as described in elsewhere (5). The rich medium for stock culture was formulated as follows: 0.1 % beef extract, 0.1% yeast extract, 0.2% peptone, 1% dextrose and 1.5% agar (pH 7.2). The β-Lactamase inhibitor production medium was formulated as followed: 2% glycerol, 1.5% soytone, 0.2% yeast extract, 0.12% K_2HPO_4, 0.06% $CaCO_3$, 0.001% $CoCl_2$, 0.0025% $FeSO_4$.

Selection of Microorganism Producing β-Lactamase Inhibitor

Soil samples were dried at 80℃ for 20 min and suspended in a sterile distilled water and then spreaded on the Actinomycetes isolating media containing cyclohexamide (50 μg/ml). Colonies developed spores after 4-5 days incubation were picked and transferred to the agar plates of rich medium. The selected colonies were inoculated on the agar discs (5 mm in radius and 5 mm in thickness) of β-lactamase inhibitor producing medium. The inoculated agar discs were incubated for 3 days at 30℃ in a humid chamber and then placed on the agar plates of rich medium where penicillin G (50 mg/l), β-lactamase (0.24 unit/ml of Bactopenase derived from *Bacillus cereus*), and penicillin G resistant (ATCC 6538P) of *Stapylcoccus aureus* were pregnated. After incubation at 37℃ for 1 day, colonies which showed growth inhibitory clear zone around the agar pieces were

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Key words: *Streptomyces* sp. SMF-19, β-lactamase inhibitor

selected as candidate microorganisms to produce β-lactamase inhibitor.

Production and Purification of β-Lactamase Inhibitor

The stock culture was transferred into a rich liquid medium and cultured in a shaking incubator at 30℃ for 48 h and then inoculated into the β-lactamase inhibitor production medium with 5% inoculum size. Fermentation was carried out with a stirred jar fermentor (Korea Fermentor Co., 5 *l*) where temperature was maintained at 30℃. Aeration and agitation was controlled to give 0.5 vvm, 300 rpm, respectively. The culture broth was harvested and the cell free culture broth was collected by centrifugation at 6000 g for 10 min. The β-lactamase inhibitor was purified from the cell free culture broth through ammonium sulfate fractionation, gel filtration, anion exchange chromatography and fast performance liquid chromatography (Pharmacia Co.).

Analytical Methods

Cell mass was determined as dried cell weight (D.C.W) after drying at 80℃ for 24 h. Protein concentration was analyzed by the Bradford method (2) and β-lactamase activity was estimated by the modified iodometric assay method (11). The β-lactamase inhibitory activity was calculated as follows: Inhibitory activity (%)=[(A−B)/A]×100, where A is the β-lactamase activity without the inhibitor and B is the β-lactamase activity with the inhibitor. One unit of activity was defind as the amount of inhibitor needed for the 50% inhibition of 0.96 units of β-lactamase (Bactopenase).

RESULTS AND DISCUSSION

Selection of Strains Producing β-Lactamase Inhibitor

Over 200 strains of *Streptomyces* spp. were tested to select strains producing β-lactamase inhibitors. Clear zone around the agar discs (A in Fig. 1) indicated that the growth of *St. aureus* was inhibited by penicillin G which was not inactivated by the β-lactamase, since the β-lactamase was inhibited simultaneously by β-lactamase inhibitor which was produced from the isolant. As a result, isolant SMF-19 identified tentatively as *Streptomyces* sp. was selected as a candidate to produce extracellular β-lactamase inhibitor.

Production and Purification of β-Lactamase Inhibitor

Batch culture data for the cell growth, β-lactamase inhibitor production, and pH changes are shown in Fig. 2. The activity of β-lactamase inhibitor in the culture broth increase rapidly during the exponential growth phase and gave maximal values at stationary phase. It indicated that the production of β-lactamase inhibitor

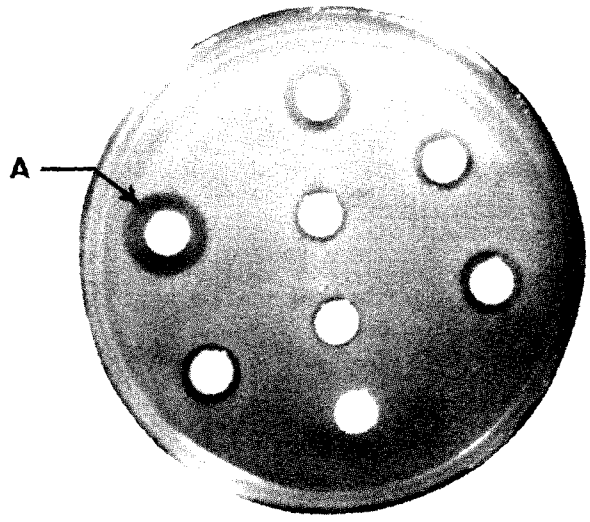

Fig. 1. Screening of *Streptomyces* sp. producing β-lactamase inhibitor. β-Lactamase inhibitoty activity revealed as clear zone around the agar disc (A).

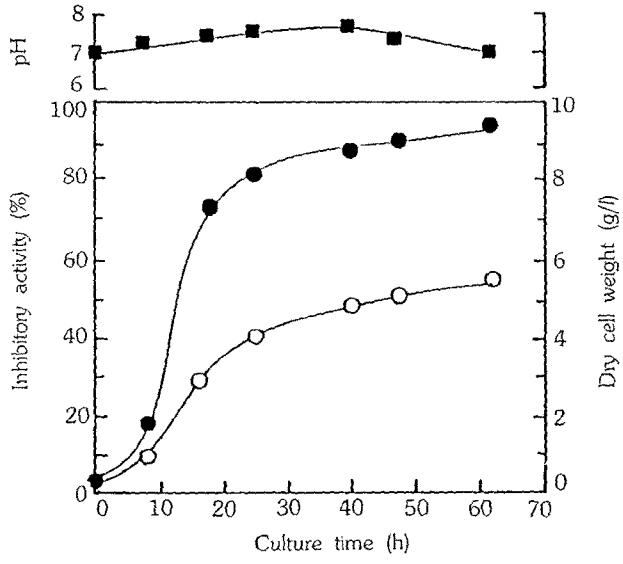

Fig. 2. The changes of biomass formation, β-lactamase inhibitor production, and pH in the batch culture of *Streptomyces* sp. SMF-19.
(●): β-lactamase inhibitor, (○); biomass (■); pH

was closely related to the cell growth.

When the β-lactamase inhibitory activity reached maximum, the culture broth was harvested by centrifugation and protein was fractionated by the addition of ammonium sulfate at 4℃. Precipitate obtained between 60 and 80% ammonium sulfate saturation revealed the activity of β-lactamase inhibitor. The active fraction was desalted through dialysis and then applied to the column of Sephadex G-100 equilibrated with 0.02 M Tris-HCl

buffer (pH 7.8). The column was eluted with the same buffer where the flow rate was maintained at 12.0 ml/h and fractions of 6.0 ml were collected (Fig. 3). The active fraction was concentrated through ultrafiltration and applied to the anion exchange chromatography of DEAE-Sephadex A-50 equilibrated with the same buffer system used for gel filtration. After washing the column with the same buffer, a linear gradient of 0 to 1.0 M NaCl in the buffer was applied. The flow rate was 30.0 ml/h and fractions of 10 ml were collected. Active β-lactamase inhibitor was eluted from 0.4 M NaCl (Fig. 4). The active fraction was concentrated and injected into a mono Q-type anion exchanger column for fast performance liquid chromatography. Elution was performed with the same NaCl gradient used for anion exchange chromatography in 0.02 M Tris-HCl buffer (pH 7.5). The purified proteinaceous β-lactamase inhibitor was obtained from fast performance liquid chromatography. The purity was checked with SDS-PAGE (7) and the results are shown in Fig. 5. The results indicated that β-lactamase inhibitor obtained through the purification steps was pure and that the molecular weight was estimated to be about 48,000 by SDS-PAGE. The purification fold and final yield were 15.9 and 1.1% respectively (Table 1).

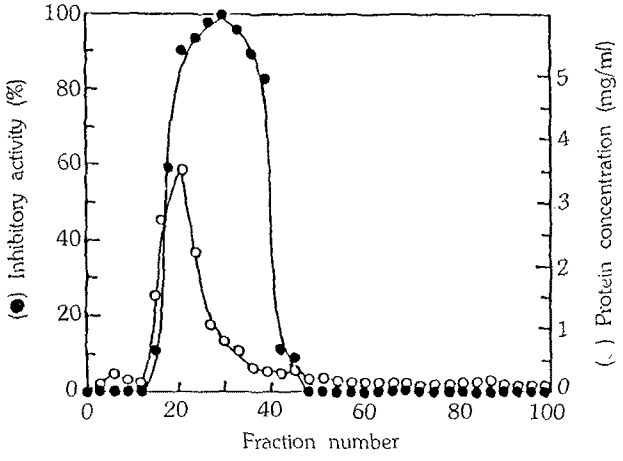

Fig. 3. Gel filtration chromatography of β-lactamase inhibitor on Sephadex G-100.
The sample solution was applied to the column (φ2.2×60 cm) equillibrated with 0.02 M Tris·HCl buffer (pH 7.8) and then eluted with the same buffer. The flow rate was 12.0 ml/h and fractions of 6.0 ml were collected.

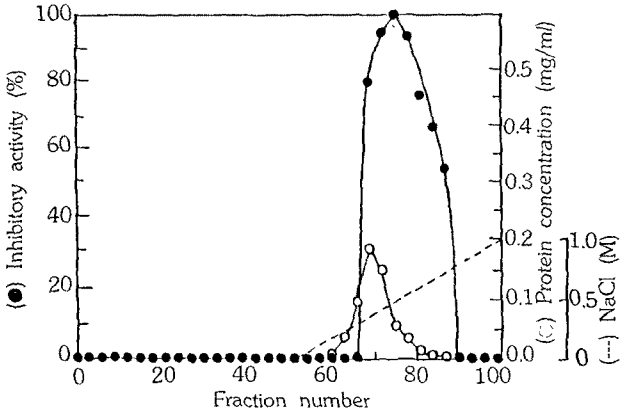

Fig. 4. Anion exchange chromatography of β-lactamase inhibitor on DEAE Sephadex A-50.
The sample solution was applied to the column (φ3.0×20 cm) equillibrated with 0.02 M Tris·HCl buffer (pH 7.8) and then eluted with the same buffer. The flow rate was 30.0 ml/h and fractions of 10.0 ml were collected.

Characteristics of β-Lactamase Inhibitor

In order to know the inhibition mode of β-lactamse inhibitor, the activity of bactopenase against penicillin G was tested with different concentrations of β-lactamase inhibitor. The V_{max} and K_m value of the bactopenase against penicillin G was estimated to be 25 μmol/min and 12.5 mM, respectively. As shown in Fig. 6, it was clear that the inhibition mode of the β-lactamase inhibitor was uncompetitive. The inhibition constant (K_i) was

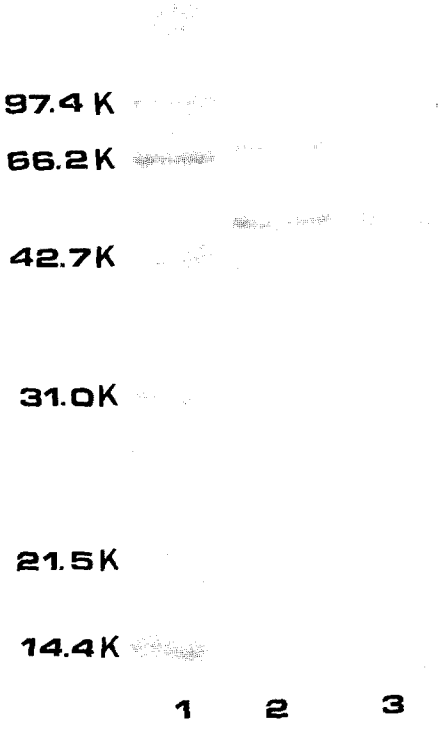

Fig. 5. SDS-polyacrylamide gel electrophoresis of β-lactamase inhibitor.
Lain 1; molecular weight standard
Lain 2; after gel filtration and ion exchange chromatography
Lain 3; purified β-lactamase inhibitor (obtained from FPLC)

Table 1. Purification of β-lactamase inhibitor

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification yield (%)	Fold
Culture broth	129,410	3,234.6	40.0	100.0	1.0
Ammonium sulfate fractionation	105,340	1,980.1	53.2	81.4	1.3
Gel filtration	26,335	70.6	373.0	20.4	9.3
Anion exchange chromatography	7,098	7.0	1,014.0	5.5	25.3
FPLC	1,422	2.2	637.7	1.1	15.9

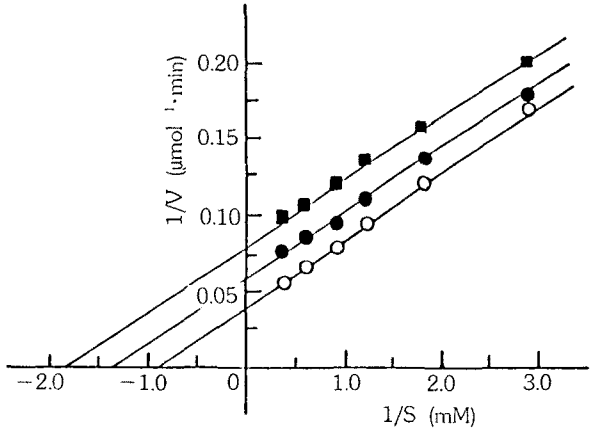

Fig. 6. Inhibition mode of β-lactamase inhibitor against bactopenase.
Lineweaver-Burk plots of penicillin G concentration against rate of hydrolysis by bactopenase in the absence and in the presence of β-lactamase inhibitor
(○); in the absence of β-lactamase inhibitor
(●); in the presence of β-lactamase inhibitor (1.49 μg)
(■); in the presence of β-lactamase inhibitor (2.98 μg)

calculated as 0.62×10^{-4} μmol. The inhibitor showed very strong inhibitory activity to β-lactamase produced from *Bacillus cereus* (bactopenase from Difco) and *Enterobacter cloace* (penicillinase type-IV from Sigma) and showed very weak inhibitory effect against *Bacillus cereus* penicillinase (type-I from Sigma). However the inhibitor showed no inhibitory effect against *Streptomyces* sp. KIS-13 β-lactamase (S.B. Moon, 1991. A thesis for the M.S. degree, Seoul National University, Seoul, Korea) which was isolated in our laboratory. The β-lactamase inhibitor was stable in a wide pH range from 4.0 to 10.0 at 37℃ and its activity was stable below 40℃ but decreased rapidly above 50℃.

Acknowledgement

The presented works were supported by a grant from the Ministry of Science and Technology, Korea. The financial supports were greatly appreciated.

REFERENCES

1. **Abraham, E.P.** 1983. History of β-lactam antibiotics, p. 1-14. *In* A. L. Demain and N.A. Solomon (ed.), *Antibiotics containing the β-lactam Structure Part 1*, Springer-Verlag, Berlin.
2. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
3. **Butterworth, D., M. Cole, G. Hanscomb, and G.N. Rolinson.** 1979. Olivanic acids, a family of β-lactam antibiotics with β-lactamase inhibitor properties produced by *Streptomyces* species I. Detection, properties and fermentation studies. *J. Antibiot.* **32**: 287-294.
4. **Doran, J.L,. B.K., Leskiw, S. Aippersbach, and E. Jensen.** 1990. Isolation, and characterization of a β-lactamase-inhibitory protein from *Sreptomyces clavuligerus* and cloning and analysis of the corresponding gene. *J. Bacreriol.* **172**(9): 4909-4918.
5. **Hunter-Cevera, J.C., M.E. Fonda, and A. Belt.** 1986. Isolation of cultures p.-3-23 *In* A. L. Demain and N.A. Solomon (ed.) *Manual of Industrial Mirobiology and Biotechnology*, American society for Microbiology, Washington, D.C.
6. **Kahan, K.S., F.M. Kahan, R. Goegelman, S.A. Currie, M. Jackson, E.O. Stapley, T.W. Miller, A.K. Miller, D. Hendlin, S. Mochals, S. Hernandez, H.B. Woodruff, and J. Birnnaum.** 1979. Thienamycin, a new β-lactam antibiotic I. Discovery, taxonomy, isolation and physical properties *J. Antibiot.* **32**: 1-12.
7. **Laemmli, U.K.** 1970. Cleavage of stuctural proteins during the assembly of the head to bacteriophage T4. *Nature* **227**: 680-685.

8. **Liu, W.C., G. Astle, J.S. Wells, W.H. Trejo, P.A. Principe, M.L. Rathnum, W.L. Parker, O.R. Kocy, and R.B. Sykes.** 1980. Izumenolide, a novel β-lactamase inhibitor produced by *Micromonospora*. 1, Detection, isolation and characterization. *J. Antibiot.* **33**: 1256-1261.
9. **Oakmura, K., S. Hirata, Y. Okamura, Y. Fukagawa, T. Ishikure, Y. Shimachi, K. Kouno, and J. Lein.** 1978. PS-5, a new β-lactam antibiotics from *Streptomyces*. *J. Antibiot.* **31**: 480-482.
10. **Reading, C. and M. Cole.** 1977. Clavulanic acid: A β-lactamase inhibiting β-lactam from *Streptomyces clavuligerus*. *Antimicrob. Agents* and *Chemother.* **11**: 852-857.
11. **Sawai, T., T. Takahashi, and S. Yamagashi.** 1978. Iodometric assay method for β-lactamase with various β-lactam antibiotics as substrates. *Antimicrob. Agents. Chemother.* **13**: 910-913.