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Screening and Isolation of Penicillinase Inhibitor, KA-107

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It is known that penicillin resistance of bacteria is mainly caused by the inactivation of penicillin by penicillinase derived from such strains. We have developed a screening procedure for penicillinase inhibitors. Several microorganisms were found to produce such inhibitors, and from the culture filtrate of *Streptomyces gedanensis* ATCC 4880 a penicillinase inhibitor, named KA-107, was isolated. The characteristics of this inhibitor were revealed by an in vitro test by using penicillinase derived from penicillin resistant *Staphylococcus aureus*, FS-1277. When KA-107 was used in combination with penicillin-G, ampicillin, D- or L-phenethicillin, the growth inhibitory activity of these penicillins was maintained.

The progressive increase in the incidence of antibiotic-resistant microorganisms is one of the most important problems in antibiotic chemotherapy today. In recent years, inactivating enzymes for various antibiotics have been discovered in resistant strains of microorganisms, and these enzymes are considered to be one of the major factors in the emergence of this resistance. Development of a substance that exerts an effect on an enzyme which inactivates antibiotics is considered to be a useful approach for control of antibiotic-resistant strains. In the case of penicillin (PC), it has been shown that the antibiotic is inactivated by penicillinase (PCase) derived from PC-resistant strains. Thus, if a substance which inhibits the action of PCase could be developed, the lack of effectiveness of PC against PC-resistant strains could conceivably be reversed when used with such a PCase inhibitor. Consequently, we have established a screening method to detect PCase inhibitory substances among the metabolites of various microorganisms (4).

We will discuss mainly a PCase inhibitory substance, named KA-107, that is produced by a strain of *Streptomyces gedanensis*.

MATERIALS AND METHODS

Strains. We utilized *Staphylococcus aureus* FS-1277, a PC-resistant strain, kindly supplied by S. Mitsuhashi of the Department of Bacteriology, School of Medicine, Gunma University as the PCase-producing strain.

Production and supply of PCase. The optimum conditions for PCase production were studied as follows: *S. aureus* FS-1277 was inoculated into 100 ml of medium containing 1.0% polypeptone and 0.5% NaCl in a 500-ml flask and preincubated on a reciprocal shaking machine at 27 C. A 48-h culture was then transferred into each flask of production medium at a 2% inoculum rate and cultivated at 27 C for 72 h. Penicillin G (PC-G) was used as a PCase inducer. A 48-h culture of *S. aureus* FS-1277 was transferred into a 30-liter jar fermentor containing 20 liters of medium made up of 0.2% glucose, 0.2% KH_2PO_4, 0.01% $MgSO_4$, 1.0% yeast extract, and 200 U of PC-G per ml, and the fermentation was carried out at 27 C for 48 h. The exo-PCase (FS-1277 PCase) from the culture filtrate was obtained as a crude powder by salting out with ammonium sulfate, dialyzing and lyophilizing.

Estimation of PCase activity. PCase activity was estimated by a modified Pollock's bio-assay method (8) by using *S. aureus* FDA 209-P as the test organism. PCase activity was calculated by Pollock units (9) with NBCo penicillinase (*Bacillus cereus* PCase) derived from *B. cereus* 569/H used as the reference standard.

Screening procedure of PCase inhibitors (INH). The screening procedure consisted of a 10-min preincubation at 37 C of FS-1277 PCase with test culture filtrates, followed by addition of PC-G, and incubation for another 10 min at 37 C. The reaction was terminated by heating at 80 C for 1 min. PCase inhibitory activity was estimated by measuring residual PC content in the reaction mixtures by a paper disc method using *S. aureus* FDA 209-P as the test organism. The test system consisted of 100 U of PC-G per ml and 5 U of PCase per ml. One unit of PCase inhibitory activity was defined as the amount of PCase inhibitor necessary to cause a 50% inhibition of PCase activity.

Growth inhibition experiment. *S. aureus* FS-1277 culture (0.1 ml) in the stationary growth phase was inoculated into 5 ml of a medium containing 0.1%

glucose, 0.2% yeast extract, 0.3% NaCl, 11 mg of $CaCl_2$ per liter, 44 mg of KH_2PO_4 per liter, 95 mg of $MgCl_2$ per ml, 2.5 ml of 1 N NaOH per liter, and various concentrations of PC-G or semi-synthetic PC's with or without KA-107 in a Monod tube and all were incubated with shaking at 30 C. Bacterial growth was checked at intervals by determining optical density at 660 nm in a Coleman spectrophotometer.

RESULTS

Studies on the conditions of FS-1277 PCase production. FS-1277 PCase is an inducible enzyme. Therefore, the effect of concentration and addition time of the inducer for PCase production was studied. The highest production of FS-1277 PCase was obtained when 200 U of PC-G per ml was added to the culture media at 0 h and the mixture was incubated for 72 h (Table 1). The effect of various medium constituents on PCase production was investigated and yeast extract in the media was found to be excellent for the production of FS-1277 PCase.

Substrate specificity of FS-1277 PCase. PC-G and various kinds of semi-synthetic PC's were used as substrates, and the substrate specificity of both FS-1277 PCase and *B. cereus* PCase was studied. PC-G, ampicillin, D- and L-phenethicillin were completely inactivated by both enzymes, but there was little, if any, inactivation of methicillin, oxacillin, cloxacillin, and dicloxacillin (Fig. 1).

Screening for INH. PCase inhibitory activity of culture filtrates of various microorganisms such as *Streptomyces*, fungi, and bacteria were examined. Four strains of *Streptomyces*, *S. gedanensis* ATCC 4880, *S. olivaceus* NRRL B-1125, OS-3301, and OS-3391, were found to be producers of PCase inhibitor (Table 2). Among these strains, the culture filtrate of *S. gedanensis* showed the highest activity. This PCase inhibitory substance, which was designated as KA-107, has been studied in some detail.

TABLE 1. *Effect of concentration and addition time of inducer (PC-G) on FS-1277 PCase production*[a]

Time of PC-G added (h)	Amt. of PC-G added (U/ml)	FS-1277 PCase produced (U/ml)
0	1,000	1.0
	500	1.2
	200	17.2
	120	16.0
	60	1.1
	10	1.1
	0	0.9
24	200	2.8
48	200	0.0

[a] Medium: 1.0% polypeptone, 0.5% NaCl.

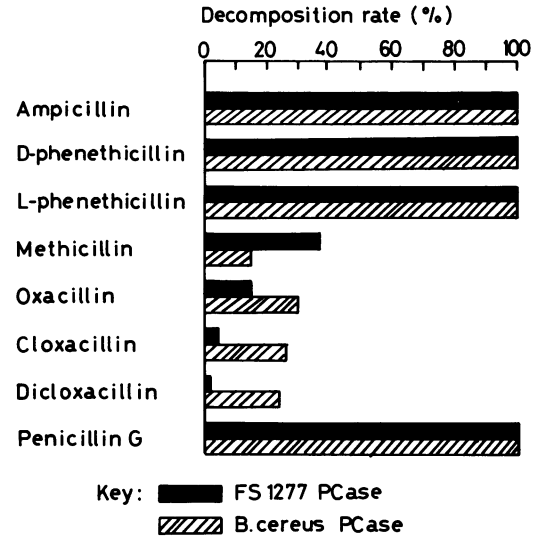

FIG. 1. *Comparison of substrate specificity between FS-1277 PCase and B. cereus PCase.*

TABLE 2. *PCase inhibitory activity of culture broth from positive Streptomyces strains*

Strains	PCase inhibitory activity (U/ml)
Streptomyces olivaceus NRRL B-1125.	0.5
Streptomyces gedanensis ATCC 4880 ..	1.7
Streptomyces sp. no. OS-3301	1.2
Streptomyces sp. no. OS-3391	0.9

Preparation of KA-107. *S. gedanensis* was grown at 27 C in a 30-liter jar fermentor in 20 liters of medium containing 2% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dry yeast, and 0.3% $CaCO_3$. Production of KA-107 started 20 h after cultivation and reached its peak in 45 to 55 h (Fig. 2). After filtration through a bed of Celite 545, KA-107 was obtained as a brownish crude powder by salting out with 80% saturated ammonium sulfate, dialyzing, and lyophilizing. This powder was dissolved in water, and chromatographed on diethylaminomethyl-cellulose. The column was washed with 0.1 M of NaCl in 0.01 M of phosphate buffer (pH 7.0) and developed with 0.3 M of NaCl in 0.01 M of phosphate buffer. The active fraction was precipitated with 35 to 55% saturated ammonium sulfate, dialyzed, purified by Sephadex G-75 column chromatography, and lyophilized as a white amorphous powder.

Action of KA-107, PCase inhibitor. Hamilton-Miller (2) demonstrated the synergism be-

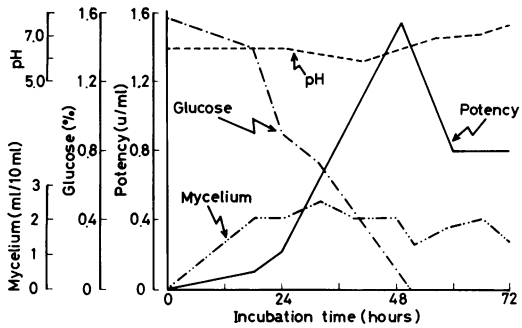

FIG. 2. *A typical time course of KA-107 fermentation. A 48-h culture was transferred into 20 liters of medium in a 30-liter jar fermentor and the fermentation carried out under the following conditions: temperature, 27 C; aeration, 10 liters per min; agitation, 240 rpm; pressure, 0.5 kg/cm².*

tween β-lactam antibiotics by an isobologram. An isobologram for combined action of KA-107 and PC-G, ampicillin, or L-phenethicillin on *S. aureus* FS-1277, is shown in Fig. 3. Each curve indicates the minimal inhibitory concentration of PC-G, ampicillin, or L-phenethicillin combined with a different amount of KA-107 on agar media. KA-107 itself had no growth inhibitory activity against various microorganisms including *S. aureus* FDA 209-P and FS-1277, but induced a remarkable growth inhibition activity when combined in use with PC-G. Thus, the two agents appeared to act synergistically against the PC resistant strain. The action of KA-107 on bacterial growth in fluid culture media was also revealed by the following experiment. The growth of *S. aureus* FS-1277 was inhibited by 1 μg of oxacillin per ml and 5 μg of methicillin per ml, but was not inhibited by 100-μg concentrations per ml of ampicillin, D- or L-phenethicillin. The results obtained by the growth experiments indicated the substrate specificity of FS-1277 PCase for various types of semisynthetic PC's. Rapid growth of *S. aureus* was observed in 2 to 4 h in controls with no PC-G or KA-107 (Fig. 4). Similar growth was observed in the presence of 0.4 U of KA-107 per ml or 200 U of PC-G per ml. However, when 200 U of PC-G per ml of 0.4 U of KA-107 per ml were both added to the culture, growth was completely inhibited for approximately 8 to 10 h. Thereafter, propagation resumed. The resumption of bacterial growth was reproducible under the same experimental conditions. It was stilled observed even when more than 0.4 U of KA-107 per ml was added at 0 h. Similar results were obtained with ampicillin, and D- and L-phenethicillin which are also inactivated by FS-1277 PCase.

DISCUSSION

Studies on the mechanism responsible for antibiotic resistance among bacterial strains have made significant progress, and approaches from various angles have been taken to combat such strains. An outline of such research directions and approaches to new drugs against antibiotic resistant strains are: (i) discovery of new antibiotics; (ii) chemical modification of antibi-

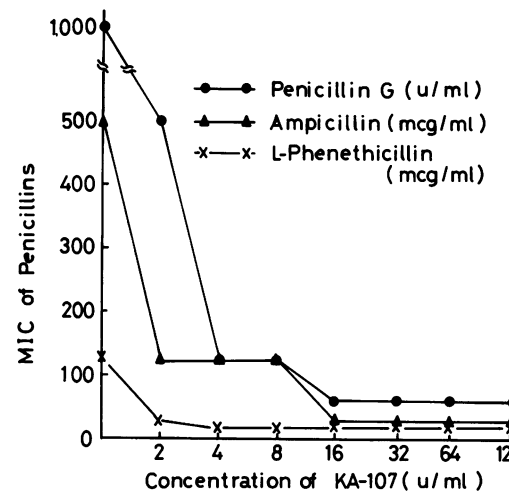

FIG. 3. *Isobologram for combined use of KA-107 and PC-G, ampicillin or L-phenethicillin on S. aureus FS-1277. Isobologram (2) was constructed from the minimum inhibitory concentration test of each penicillin in the presence of stated concentrations of KA-107. Minimal inhibitory concentration was determined by agar dilution method. S. aureus FS-1277 was incubated at 37 C for 24 h in heart infusion broth.*

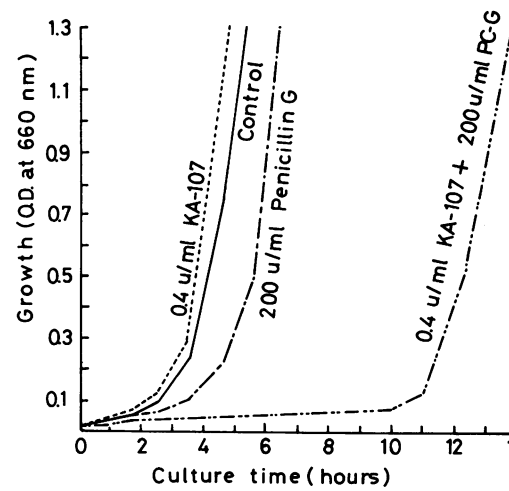

FIG. 4. *Effect of PC-G and KA-107 on the growth of S. aureus FS-1277.*

otics, semi-synthetic penicillins, 3'-deoxy kanamycin (13); (iii) drugs interfering in the resistance transfer process; drugs restoring activity at the episomal level (3, 5, 6); and drugs inhibiting some metabolic processes necessary for the transfer of resistance factors (7, 10, 11). In addition to these research programs, development of substances which act on enzymes that inactivate antibiotics must be considered to be an effective approach for control of antibiotic-resistant strains. Studies on inhibitors of enzymes that inactivate antibiotics have been reported only rarely. For example, Tanaka and co-workers (12) demonstrated the inhibition of chloramphenicol-*o*-acetyl transferase by crystal violet. The inhibition of β-lactamase activity by semi-synthetic PC's has been studied and recently screening results were obtained with such compounds as described by Cole and co-workers (1). However, they did not use metabolites of microorganisms as a source of the inhibitors. Therefore, the approach used in their studies was not the same as that used in ours.

The growth of strain FS-1277 was observed to be inhibited by the combined use of KA-107 and PC-G (Fig. 4). However, the reason that inhibition of bacterial growth occurred for only a limited period of time after addition of both PC-G and KA-107 is not apparent. The inhibitory effect of KA-107 against *S. aureus* FS-1277 PCase in vitro and in vivo will be described in detail in another paper.

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