INTERACTION OF β-LACTAMASE OF *STREPTOMYCES CACAOI*

I. CLAVULANIC ACID AND PS-5

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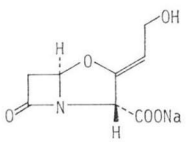
Inactivation of a β-lactamase of *Streptomyces cacaoi* by clavulanic acid and PS-5 was investigated and compared with that of a β-lactamase of *Bacillus cereus*. Inhibition of the enzymes induced by clavulanic acid and the β-lactam antibiotic PS-5 was found to be progressive with time. However, the degree of inhibition of the β-lactamase from *S. cacaoi* increased more progressively with time than that of the enzyme from *B. cereus*. Conformative response constants were determined. As compared with clavulanic acid, over ten times higher concentrations of PS-5 were necessary to give a similar degree of inhibition. At lower concentrations, both clavulanic acid and PS-5 behaved as competitive inhibitors. *Ki* values calculated from the integrated form of the LINEWEAVER-BURK type were 1.1×10^{-7} M and 7.6×10^{-6} M for clavulanic acid and PS-5, respectively.

Streptomyces produce β-lactamases with a great variety of properties constitutively with no apparent relation with resistance to β-lactam compounds[1,2]. The roles of these enzymes are not known at the present time. In order to clarify their roles in *Streptomyces* and the relationships between β-lactamases in *Streptomyces* and those in pathogenic bacteria, we have studied the effects of some β-lactamase inhibitors on the enzymatic activity of the β-lactamase from *Streptomyces cacaoi*. This paper describes the results obtained with clavulanic acid and PS-5 and the accompanying paper reports those with CP-45,899, oganomycins (cephamycins) and izumenolide. This enzyme catalyzes the hydrolysis of methicillin and cloxacillin at two-thirds to one-third the rate of benzylpenicillin[3]. In this respect, it relates to some β-lactamases from Gram-negative bacteria.[4]



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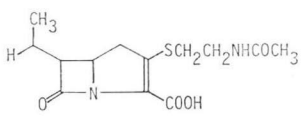


(II)

Materials and Methods

β-Lactamases

The β-lactamase from *Streptomyces cacaoi* was prepared as described in a previous paper[3]. A β-lactamase from *Bacillus cereus* was obtained from Calbiochem[5] and was used after exhaustive dialysis against 0.1 M sodium phosphate buffer, pH 7.0.

β-Lactams

Sodium clavulanate (**I**) and PS-5 (**II**) were the generous gifts from Beecham Pharmaceuticals and Sanraku-Ocean Co., respectively.

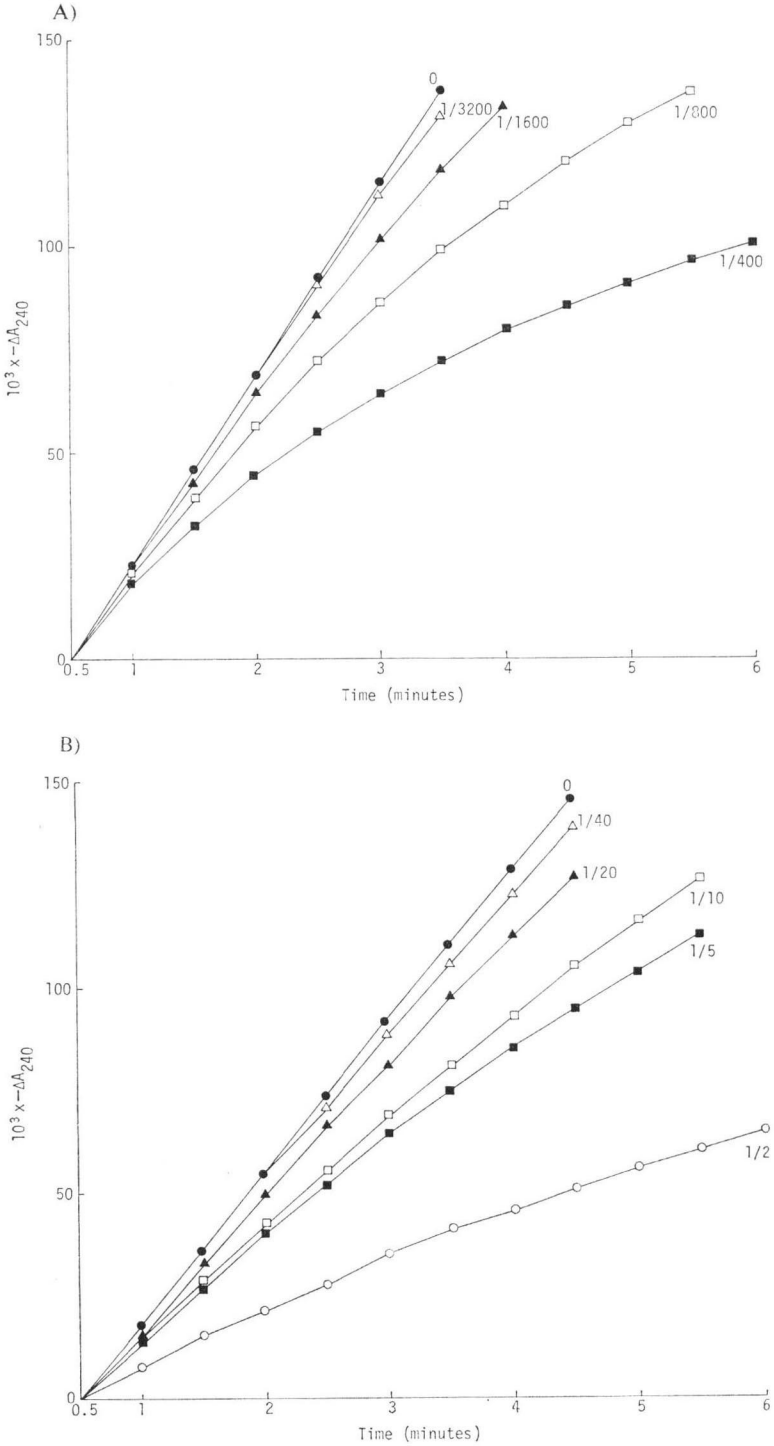
Measurement of Enzymatic Activity

Benzylpenicillin solution (1 mM, 1.49 ml) in 0.1 M sodium phosphate buffer, pH 7.0 and varied concentrations of inhibitors in the same buffer (1.49 ml) were poured into a 1.0 cm cuvette and maintained at 30°C in a Hitachi type 200-20 spectrophotometer. The reaction was started by the addition

Fig. 1. Time course of hydrolysis of benzylpenicillin in the presence of various concentrations of clavulanic acid.

(A): *S. cacaoi* β-lactamase. (B): *B. cereus* β-lactamase.

The numbers in the figure are the molar ratios of the inhibitor to benzylpenicillin. The concentration of benzylpenicillin was 0.497 mM.



of 20 μl of *B. cereus* β-lactamase or *S. cacaoi* β-lactamase in the same buffer to make the final volume of 3.0 ml, and then the change in absorbance at 240 nm was recorded at 10 second intervals against a reference cuvette containing no enzyme solution.

Results and Discussion

Hydrolysis of clavulanic acid was followed by change in the absorption spectrum of clavulanic acid in the range of 230 nm to 360 nm. No apparent change was observed in the absorption spectrum in 0.1 M sodium phosphate buffer of pH 7.0 following 1 hour incubation with the β-lactamases of *B. cereus* and *S. cacaoi*. Thus, clavulanic acid seems to be resistant to hydrolysis catalyzed by these enzymes. This is also the case with other β-lactamases[6~8].

When the hydrolysis of benzylpenicillin by the *S. cacaoi* enzyme was followed in the presence of various concentrations of clavulanic acid, inhibition increased gradually with time (Fig. 1A). Curvature was more significant with higher concentrations of the inhibitor. The inhibition of the β-lactamase activity was also observed with the enzyme from *B. cereus*. However, about 100 times higher concentrations were needed to cause a similar degree of inhibition. In addition, the curvature was not so significant as in the case of *S. cacaoi* enzyme (Fig. 1B).

By using the method of ZYK and CITRI[9], the enzyme was preincubated with clavulanic acid at 30°C for various length of time, and then the remaining enzyme activity was measured by the addition of benzylpenicillin as a substrate. As illustrated in Figs. 2A and 2B, the enzyme activity was inactivated to an extent depending on both the length of time of preincubation and the concentration of the inhibitor. From the rate constants of the inactivation (*Ka*), the apparent conformative response constant (*Kcr*), a value numerically equal to the concentration of the inhibitor which caused half-maximal change of the conformation induced through specific interaction at the active site[9], was calculated to be 5.7×10^{-5} M

Fig. 2. Effect of preincubation with clavulanic acid on the enzyme activity.
(A): *S. cacaoi* β-lactamase. (B): *B. cereus* β-lactamase.
The enzyme was incubated with clavulanic acid for indicated period and then the enzyme activity was measured by adding benzylpenicillin solution to the above mixture. The numbers in the figure are the molar ratios of the inhibitor to benzylpenicillin. The concentration of benzylpenicillin was 0.333 mM.

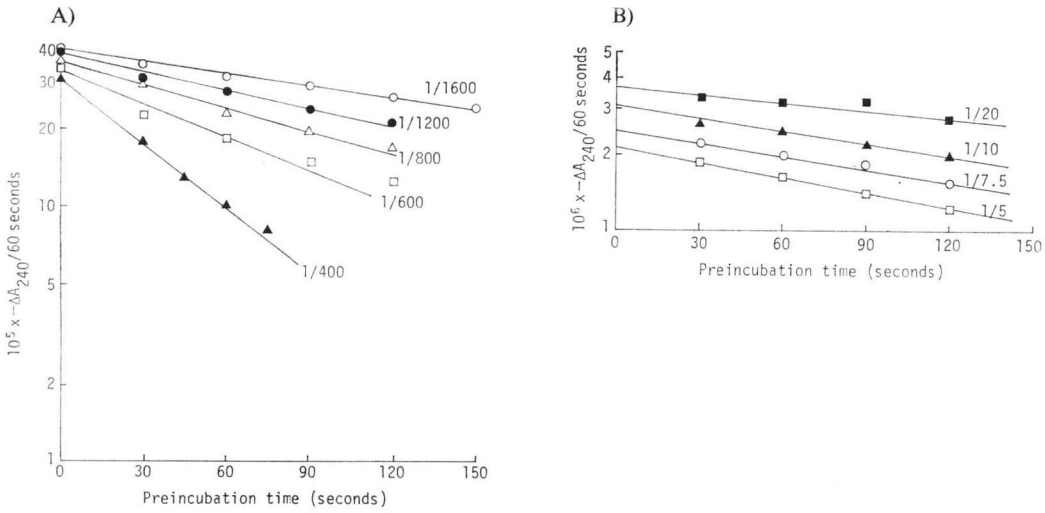
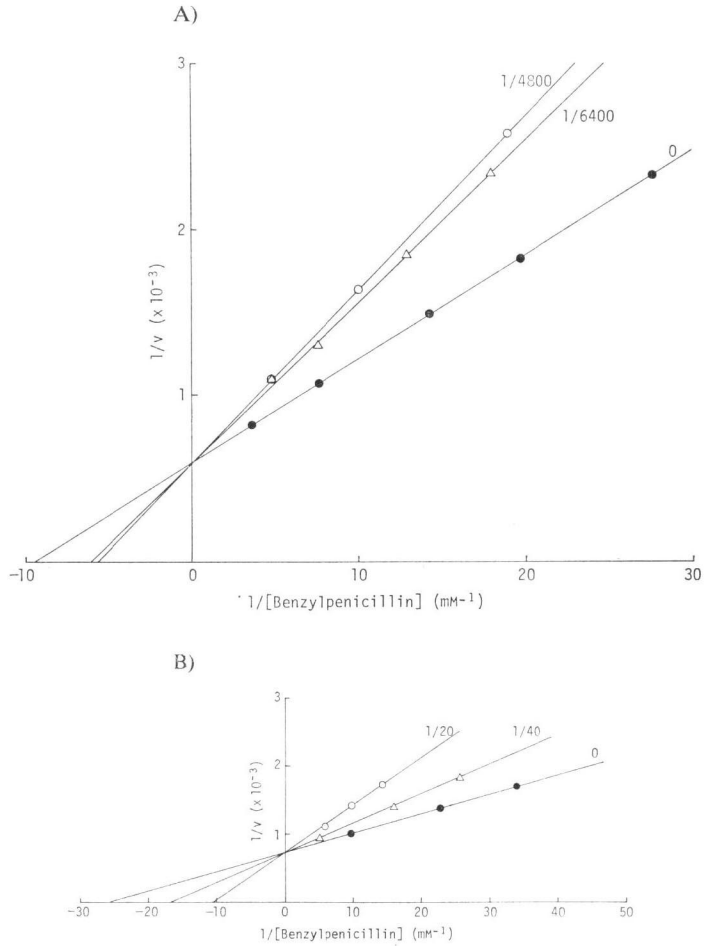


Fig. 3. The double reciprocal plots of the inhibition by clavulanic acid.
(A): *S. cacaoi* β-lactamase. (B): *B. cereus* β-lactamase.
The numbers in the figure are the molar ratios of the inhibitor to benzylpenicillin (0.333 mM).



and 9.6×10^{-6} M for the enzyme of *B. cereus* and *S. cacaoi*, respectively. This indicates that the *S. cacaoi* enzyme can be changed in its conformation by lower concentrations; in other words, *S. cacaoi* enzyme appears to behave more flexibly towards clavulanic acid.

In contrast to the above finding, that is, the progressive inactivation of the enzyme, when lower concentrations of clavulanic acid was used, it behaved as a competitive inhibitor of the two enzymes. The double reciprocal plot is characteristic of the enzyme being inhibited competitively (Figs. 3A and 3B). However, the concentration of the inhibitor causing a similar pattern differed greatly between the two enzymes. *Ki* values calculated from the integrated form of the LINEWEAVER-BURK type were 1.3×10^{-5} M and 1.1×10^{-7} M for the enzymes from *B. cereus* and *S. cacaoi*, respectively. These values together with *Kcr* values indicate that clavulanic acid binds to *S. cacaoi* enzyme more strongly than to *B. cereus* enzyme.

The β-lactam compound PS-5 (reference 10) also could not be hydrolyzed significantly by the *S. cacaoi* enzyme, since there was no apparent change in absorption at 301 nm in 0.1 M sodium phosphate buffer of pH 7.0 in the presence of the enzyme. In contrast, the *B. cereus* enzyme could hydrolyze PS-5

at about one 2,400th the rate of benzylpenicillin. In addition, the enzyme activity of *B. cereus* was affected significantly only at very high concentrations of PS-5. However, hydrolysis of benzylpenicillin by the *S. cacaoi* enzyme was inhibited strongly by the presence of PS-5 (Fig. 4). As in the case with clavulanic acid, the inhibition increased with time and the curvature was more significant at higher con-

Fig. 4. Time course of hydrolysis of benzylpenicillin by *S. cacaoi* β-lactamase in the presence of various concentrations of PS-5.

The numbers of the figure are molar ratios of the inhibitor to benzylpenicillin (0.497 mM).

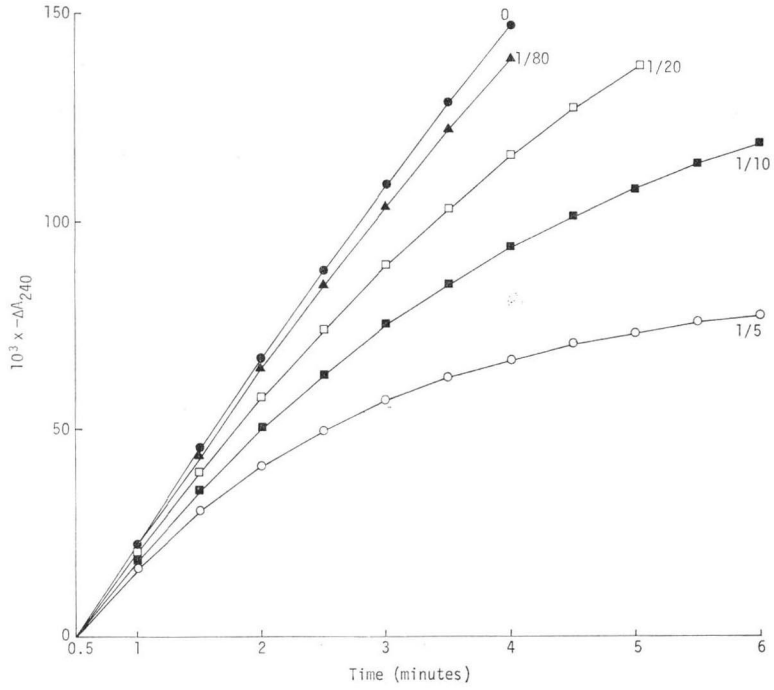


Fig. 5. Effect of preincubation with PS-5 on the enzymatic activity of *S. cacaoi* β-lactamase.

The numbers in the figure are the molar ratios of the inhibitor to benzylpenicillin (0.333 mM).

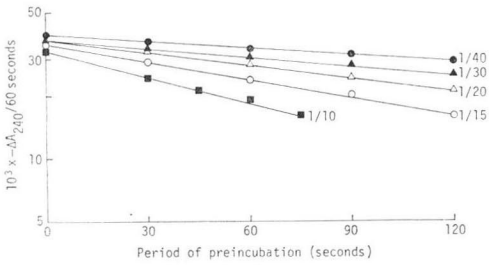
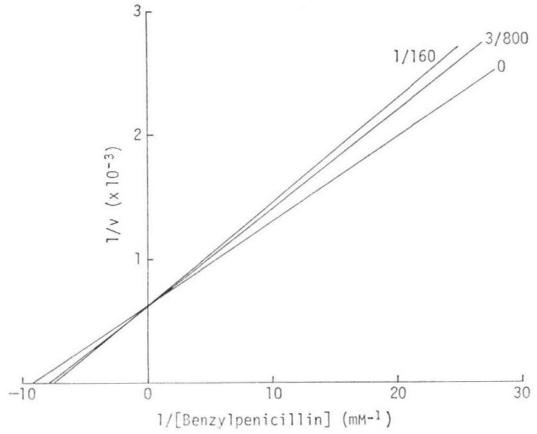


Fig. 6. Double reciprocal plots of the inhibition of *S. cacaoi* β-lactamase by PS-5.

The numbers in the figure are the molar ratios of PS-5 to benzylpenicillin (0.333 mM).



centrations of PS-5. However, as compared with clavulanic acid, over ten times higher concentrations of PS-5 were necessary to give a similar degree of inhibition. The enzyme was preincubated with PS-5 at 30°C for various length of time, and then the remaining enzyme activity was measured with benzylpenicillin as a substrate (Fig. 5). It is apparent from this that the enzyme was inactivated progressively with time. From the reaction rate of the inactivation, the conformative response constant *Kcr* was calculated to be 2.6×10^{-4} M. Similar to clavulanic acid, lower concentration of PS-5 inhibited the *S. cacaoi* enzyme competitively (Fig. 6) and the *Ki* value was calculated from the integrated form of the LINEWEAVER-BURK type to be 7.6×10^{-6} M.

Acknowledgment

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