PS-5 INHIBITION OF A β-LACTAMASE FROM *PROTEUS VULGARIS**

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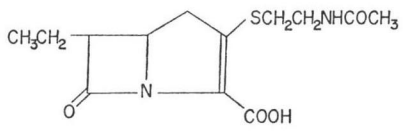
Inhibition of *Proteus vulgaris* β-lactamase by a new β-lactam antibiotic, PS-5 was studied kinetically. There were two stages of inhibition. In the early stage, PS-5 inhibited the β-lactamase by formation of a MICHAELIS-complex, and showed a competitive inhibition pattern with K_i-value of 0.22 μM (substrate, cephaloridine). After the formation of a MICHAELIS-complex between PS-5 and the enzyme, PS-5 showed a characteristic progressive inhibition pattern with time. Maximum inactivation was obtained after several minutes of preincubation of the enzyme with PS-5; as hydrolysis of PS-5 progressed, the enzyme activity was gradually recovered. Reactivation by an excess of substrate (cephaloridine) was not substantially realized in the presence of PS-5. PS-5 was very slowly hydrolyzed by the enzyme, showing a triphasic pattern in its reaction curve.

Recently new classes of β-lactam antibiotics, nocardicins[1], clavulanic acid[2], olivanic acids[3], epithienamycins[4] and thienamycin[5], have been found. The nocardicins have mono-β-lactam rings. Clavulanic acid has an oxa-penam structure and the others are a series of compounds with related structures possessing a carbapenem-nucleus in their molecules. Olivanic acids and clavulanic acid were demonstrated to be potent β-lactamase inhibitors and detailed kinetic features with clavulanic acid were given by CHARNAS *et al.*[6], FISHER *et al.*[7], DURKIN *et al.*[8] and LABIA *et al.*[9] On the other hand, only brief kinetic studies were disclosed with olivanic acids in the patent specification[10].

PS-5[11] is a new β-lactam antibiotic possessing the carbapenem-nucleus (Fig. 1). PS-5 has been shown to be produced by *Streptomyces cremeus* subsp. *auratilis*[12] or *Streptomyces fulvoviridis*[13]. The antibiotic has been shown to have potent antimicrobial activity against Gram-positive and negative bacteria[14]. The antibiotic also showed a synergistic antimicrobial activity in combination with other β-lactam antibiotics against *Enterobacter, Serratia* and *Proteus* species[15]. The minimum inhibitory concentration (MIC) of cephaloridine against *Proteus vulgaris* is 2,500 μg/ml, however, this value decreases to 7.8 μg/ml in combination with 2 μg/ml of PS-5 sodium (MIC-value of PS-5 sodium alone is 6.25 μg/ml). *Proteus vulgaris* P-5[16] produces a β-lactamase which belongs to Class Ic according to the classification of RICHMOND and SYKES[17]. The synergistic effect of PS-5 is considered to be caused by the inhibition of the β-lactamase. This paper deals with the kinetic studies of the *P. vulgaris* β-lactamase inhibition by PS-5.

Fig. 1. Structure of PS-5.
3-(2-Acetamidoethyl)thio-6-ethyl-7-oxo-1-azabicyclo [3,2,0] hept-2-ene-2-carboxylic acid.



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Materials and Methods

β-Lactamase

Purified β-lactamase of *Proteus vulgaris* P-5 (PV-enzyme) was prepared as previously described[16]. The enzyme used was homogeneous by the criterion of electrophoresis and gel filtration and had a specific activity of 495 units/mg protein, where 1 unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 μmole of cephaloridine per minute at 26°C and pH 7.0.

Determination of enzyme activity

β-Lactamase activity was determined by measuring the decrease of absorbance of cephaloridine at 255 or 290 nm with a Hitachi 200-10 spectrophotometer equipped with a thermostatted cell holder, which maintained a temperature of 26°C. The molecular absorbances before (ϵ_0) and after reaction (ϵ_∞) (complete destruction of cephaloridine) have been compared and the values of $\Delta\epsilon(=\epsilon_0-\epsilon_\infty$, M^{-1} cm^{-1}) are 9,050 at 255 nm and 2,056 at 290 nm.

Reaction of β-lactamase

Reactions were carried out in a total volume of 3.0 ml consisting of 20 μl of enzyme solution, various concentrations of PS-5 sodium, 0.5 mM cephaloridine and 0.1 M sodium phosphate buffer (pH 7.0) in a cuvette with a light path of 1.0 cm at 26°C. For a measurement of competitive inhibition of β-lactamase by PS-5, the reaction was started by the addition of the enzyme and the initial hydrolysis rate of cephaloridine was measured spectrophotometrically over the first 0.5 minute.

Inactivation of β-lactamase by PS-5

The enzyme (0.27 unit) was preincubated with 0.31×10^{-2} mM to 6.24×10^{-2} mM PS-5 sodium in 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 26°C and the remaining enzyme activity was determined from the initial hydrolysis rate of cephaloridine after adding 2.9 ml of cephaloridine (final concentration, 0.5 mM) in 0.1 M phosphate buffer (pH 7.0) at various time intervals.

Stopped-flow experiments

Stopped-flow measurements were carried out using a stopped-flow spectrophotometer, Union Giken RA 1100. The drive syringes and the mixing chamber were maintained at 26°C. Kinetic data were recorded on a storage oscilloscope and later, the stored traces were recorded on a pen recorder. One sample syringe contained the substrate solution and varying concentration of PS-5 and another syringe contained the enzyme solution. Equal volumes of the two solutions were mixed in a chamber with a dead time of 10 milliseconds.

Estimation of kinetic constants

Computerized calculations of MICHAELIS-MENTEN constants of the enzyme were performed using the integrated HENRI-MICHAELIS-MENTEN equation (1)[18]

$$(t_j-t_i)/(S_i-S_j)=K_m\cdot\ln(S_i/S_j)/V_{max}/(S_i-S_j)+1/V_{max} \quad (1)$$

where V_{max} is the maximum velocity, and S_i and S_j are the substrate concentrations at reaction time t_i and t_j, respectively. $Y=(t_j-t_i)/(S_i-S_j)$ (ordinate) and $X=\ln(S_i/S_j)/(S_i-S_j)$ (abscissa) were plotted. The slope A and the ordinate-intercept B were calculated by a least-square method with a computer (IBM, CALL, TSS). K_m-value and V_{max}-value are given by A/B and 1/B, respectively. This method was first applied to a β-lactamase reaction by FUKAGAWA and ISHIKURA[19] and the Program TI6BLASE is available from ISHIKURA on request. The data are: number of data, $\Delta\epsilon$ (difference of extinction before and after complete hydrolysis of a substrate (β-lactam antibiotic), A_∞ (absorbance at complete hydrolysis) and pairs of data (t_n, S_n).

Chemicals

Cephaloridine (CER) was obtained from Shionogi & Co., Ltd. PS-5 sodium was prepared in our laboratories as described in the previous paper[12].

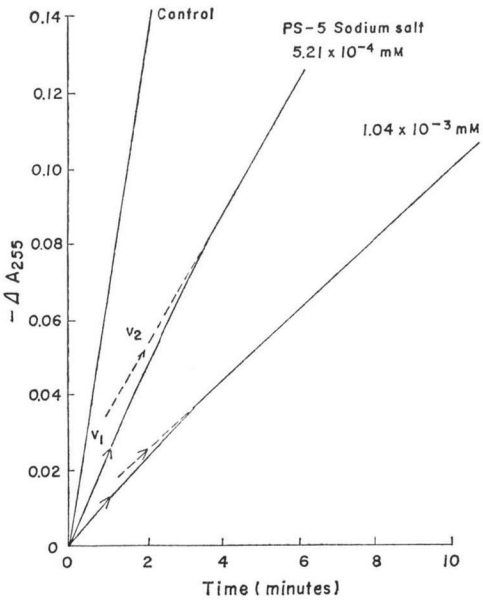
Results

Tracing of β-Lactamase Reaction with or without PS-5

Progressive curves of the β-lactamase reaction were taken in the absence or presence of various concentrations of PS-5 sodium with cephaloridine as the substrate. Marked inhibition of the β-lactamase was observed at PS-5 concentrations over 5.21×10^{-4} mM. Close observations of inhibition reactions were made, and it was revealed that the inhibition reaction had a biphasic pattern, that is, in the first phase, the inhibition appeared immediately after the addition of the enzyme and in the second phase the inhibition progressed with time. After approximately 3 minutes, it reached an apparent steady state. Extrapolating the progressive curve between 3 and 4 minutes, an apparent steady state rate, v_2 could be calculated (Fig. 2, dotted line). This overall steady state appeared before significant depletion of the substrate took place.

Fig. 2. Time courses of inhibited reactions of PV-enzyme by PS-5.

Reaction mixtures contained 0.02 unit of PV-enzyme, 0.1 mM cephaloridine and PS-5 (not added in a control) in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction was started by the addition of the enzyme. Amounts of hydrolyzed cephaloridine were expressed by the difference of absorbance at 255 nm ($-\Delta A_{255}$).



The apparent steady state velocity (v_2) had been first considered to be a true steady state rate, because such a phenomenon is observed in reactions with inhibitors of very low K_i-value ($< 10^{-9}$ M), and double reciprocal plots (1/S *vs.* 1/v) using the velocities determined in such manner that v_2 was determined, had been expected to give a linearity. However, repeated experiments showed that the double reciprocal plots never give a linearity. On the other hand, double reciprocal plots (1/S *vs.* 1/v), using the initial velocities that occurred during the first 0.5 minute (v_1, Fig. 2) were linear and gave a competitive inhibition pattern. From this plotting, a K_m-value of 0.11 mM with cephaloridine and a K_i-value of $2.0 \sim 2.1 \times 10^{-4}$ mM for PS-5 were obtained. To determine the K_i-value more accurately, a graphical method given by DIXON[20] was adopted with a series of PS-5 concentrations. The regression line of the plot (1/v *vs.* PS-5) was obtained by a least-square method with a computer calculation. The K_i-value of 2.276×10^{-4} mM was directly obtained from the point of intersection. The competitive inhibition pattern was also demonstrated from the DIXON plot.

Kinetics of β-Lactamase Reaction by Integrated HENRI-MICHAELIS-MENTEN Equation

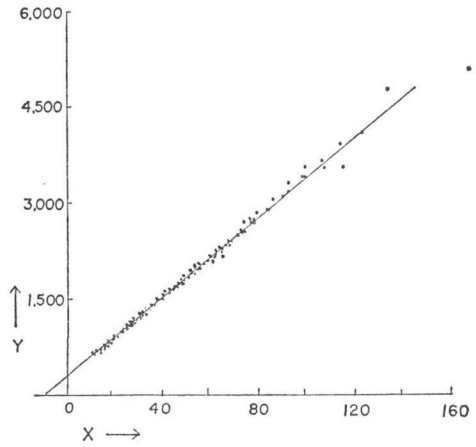
Pairs of data, (t_n, S_n) (S_n is the substrate concentration at time t_n), which were obtained from a single progressive curve of β-lactamase reaction were input in a computer and X *vs.* Y plot (see Materials and Methods) were made by the computer. In the reaction without PS-5, the plotting showed good linearity with a greater than 99.8% correlation coefficient (Fig. 3a). A K_m-value of 0.1129 mM was obtained. On the other hand, in the reaction inhibited by PS-5, no straight line was obtained and the curve was somewhat concave upward (Fig. 3b).

Fig. 3. Kinetics of cephaloridine hydrolysis by PV-enzyme.

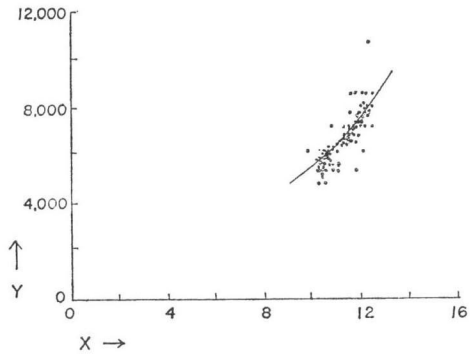
(a) Without PS-5 (Control): Twenty two pairs of data (t_n, S_n) ($n=0\sim21$) from the reaction time 0 ($S_0=0.0923$ mM) to 105 seconds ($S_{21}=0.0069$) were taken at 5-second intervals.

The plot was made by a computer on the basis of integrated HENRI-MICHAELIS-MENTEN equation.

$X=\ln(S_i/S_j)/(S_i-S_j)$, $Y=(t_j-t_i)/(S_i-S_j)$



(b) With PS-5, 5.2×10^{-3} mM: Twenty seven pairs of data (t_n, S_n) ($n=0\sim26$) from the reaction time 0 ($S_0=0.0993$ mM) to 130 seconds ($S_{26}=0.0791$ mM) were applied in the same manner as (a).



These results indicated that the whole progressive curve of the inhibited reaction obeyed the MICHAELIS-MENTEN equation throughout the entire reaction, but that of the reaction inhibited by PS-5 did not obey the MICHAELIS-MENTEN equation, because a plot of a reaction obeying MICHAELIS-MENTEN equation should give a linearity regardless of the mechanism of inhibition defined by DIXON (competitive, noncompetitive, uncompetitive or mixed type). The concave upward pattern of the inhibited reaction suggested that PS-5 inhibition included a progressive process with time which was caused by a slow reaction of PS-5 with the enzyme.

Competitive Inhibition at the Early Stage of Inhibition

In a rapid equilibrium system, the velocity of substrate decomposition (v) in the presence of competitive inhibitor (I) is given by

$$v=V_m\cdot S/K_m/(1+S/K_m+I/K_i) \qquad (2)$$

where S is the substrate concentration, K_m is the MICHAELIS-MENTEN constant and K_i is the inhibition constant. In a series of concentrations of PS-5 (as the inhibitor), the initial velocities of decomposition of cephaloridine (as the substrate) were spectrophotometrically determined and v_0/v_i was plotted against PS-5 concentration, where v_0 was the velocity without PS-5 (control) and v_i was the velocity of the inhibited reaction with PS-5. In these experiments, the reactions were started by the addition of the enzyme. The obtained data were compared with calculated v_0/v_i from equation (2), where K_m-value of 0.11 mM and K_i-value of 0.22×10^{-3} mM were adopted (Fig. 4). The experimentally obtained data were well fitted to the theoretical line. These results indicate that equilibrium between the enzyme and cephaloridine or PS-5 is rapidly attained and MICHAELIS-MENTEN equation based on a rapid equilibrium system is applicable in the initial stage of the reactions inhibited by PS-5.

Stopped-flow Experiments

For confirming the rapid equilibrium between *P. vulgaris* β-lactamase and PS-5, inhibited reactions were analyzed using a stopped-flow spectrophotometer with 10-millisecond dead time. Progres-

Fig. 4. Inhibited reactions were compared to the theoretical line calculated from the equation (2) (see text).

The reaction mixture contained 0.04 unit of PV-enzyme, cephaloridine (0.1 or 0.5 mM) and varying amounts of PS-5 sodium in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction was started by the addition of the enzyme. Initial velocities (0~0.5 minute) were spectrophotometrically determined and compared with the calculated velocities on the basis of the following equations:

$$v = V \cdot S/K_m/(1+S/K_m+I/K_i)$$

v_0 is the velocity without PS-5 and v_i is the inhibited velocity by PS-5.

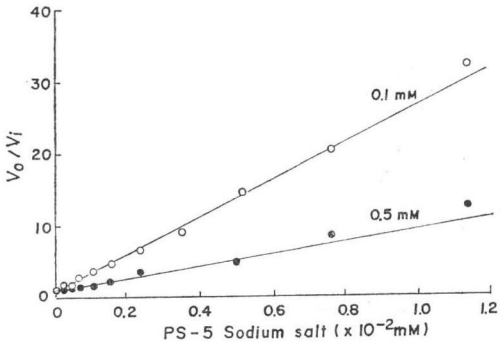
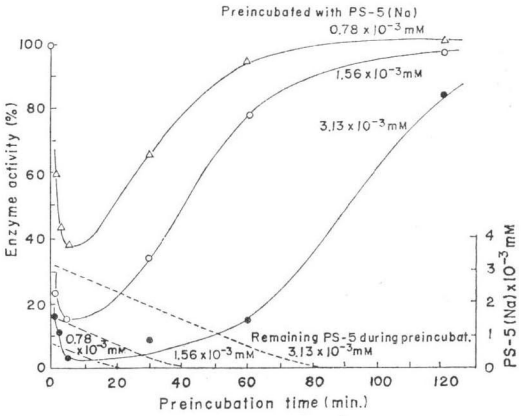


Fig. 5. Inactivation of PV-enzyme by PS-5.

The enzyme (0.13 unit) was preincubated with PS-5 sodium; 3.13×10^{-3} mM, 1.56×10^{-3} mM or 0.78×10^{-3} mM in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 26°C. The remaining enzyme activity was determined at each preincubation time by the addition of 1 ml of cephaloridine solution at the final concentration of 0.1 mM.

Remaining PS-5 during the preincubation was spectrophotometrically determined; 3.13×10^{-3} mM, 1.56×10^{-3} mM, 0.78×10^{-3} mM (Initial concentration of PS-5).



sive curves of the initial reaction that occurred during the first 15 seconds were analyzed on the basis of equation (1) with or without PS-5, and good linearity of plotting X *versus* Y was obtained. Initial velocities that occurred during the first 2 seconds were determined with a series of concentrations of PS-5, and DIXON plotting was made. From the plotting, a K_i-value of 2.17×10^{-4} mM was obtained, which showed a good coincidence with that obtained from the experiment with the usual spectrophotometer. A competitive inhibition pattern was confirmed from the plotting.

Inactivation of *P. vulgaris* β-Lactamase by PS-5

P. vulgaris β-lactamase was preincubated with PS-5 for various time intervals, and the remaining activities were determined spectrophotometrically by measuring the initial velocities after adding cephaloridine (final concentration, 0.5 mM) to the reaction mixture (Fig. 5). The inhibition observed without preincubation is due to competitive inhibition, thereafter, PS-5 clearly inactivated the β-lactamase. The maximum inactivation was attained after approximately 5-minute preincubation, thereafter, the activity began to be recovered. During the preincubation of the enzyme with PS-5, PS-5 was very slowly hydrolyzed. (The time course of remaining PS-5 is roughly drawn linear in Fig. 5, although, actually linearity does not exist in the first 5 minutes and in the last stage of the reaction as seen in Fig. 8.) Complete recovery of the enzyme activity was observed only more than 60 minutes after the complete hydrolysis of PS-5.

The enzyme was preincubated with PS-5 in 7.5 to 150-fold molar excess. The rate of loss of the enzyme activity was dependent on the concentration of PS-5. Inactivation during 5-minute preincubation in varying concentration of PS-5 was shown in Fig. 6. Ninety five per cent of inhibition was

Fig. 6. Effect of PS-5 concentration on the inactivation of PV-enzyme by PS-5.

The enzyme (0.27 unit) was preincubated with varying concentrations of PS-5 in 0.1 ml of sodium phosphate buffer (pH 7.0) for 5 minutes at 26°C, then 2.9 ml of cephaloridine solution were added to the reaction mixture at the final concentration of 0.5 mM, and the remaining enzyme activity was spectrophotometrically determined. The molar ratio (PS-5/Enzyme) was 150 at 6.4×10^{-2} mM of PS-5 sodium.

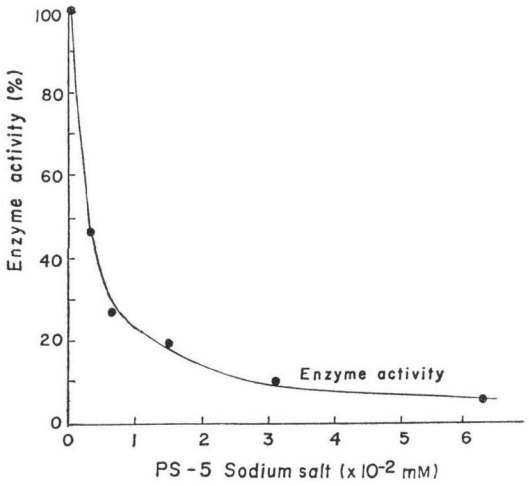
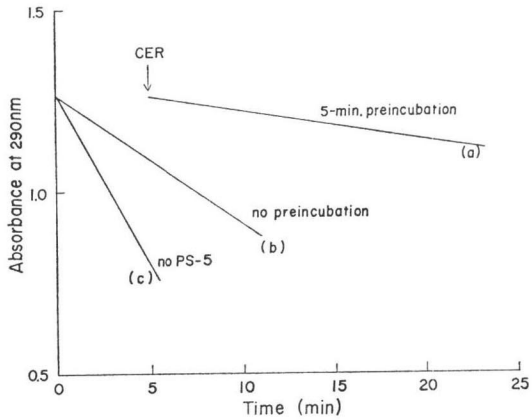


Fig. 7. Absence of a reactivation phenomenon by competing substrate (cephaloridine) after 5-minute preincubation of *P. vulgaris* β-lactamase with PS-5.

The enzyme (0.13 unit) and PS-5 (9.36×10^{-3} μmoles) were preincubated in 0.6 ml of 0.1 M phosphate buffer (pH 7.0) for 5 minutes at 26°C. After adding 2.4 ml of CER (final concentration 0.5 mM) to the mixture, the enzyme reaction was traced spectrophotometrically (Curve a). Reactions of curve b and c were started by the addition of the enzyme. The final concentration of PS-5 was 3.13×10^{-3} mM.



obtained at the concentration of 6.24×10^{-2} mM PS-5 (PS-5/Enzyme = 150).

Reactivation of Inactivated Enzyme

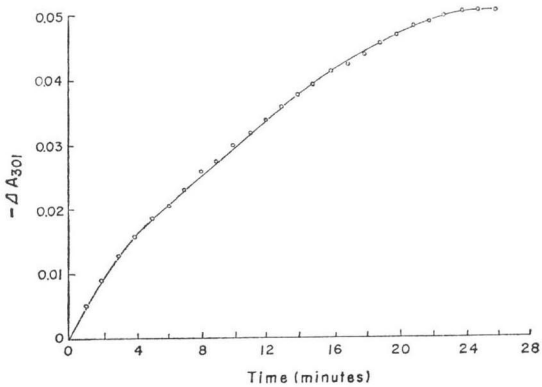
The enzyme was preincubated with a 75-fold molar excess of PS-5 for 5 minutes and the enzyme reaction was spectrophotometrically traced after the addition of cephaloridine (0.5 mM). Approximately 9% of the enzyme activity remained after the preincubation and the enzyme reactivation phenomenon by an excess of the substrate was not substantially observed over 15 minutes (Fig. 7).

Hydrolysis of PS-5 by *P. vulgaris* β-Lactamase

As the hydrolysis rate of PS-5 was very slow, a large amount of the enzyme was needed for hydrolysis of PS-5. A characteristic triphasic pattern of the progressive curve was observed, *i.e.*, initially fast, then slow, followed by a substrate-limiting phase (Fig. 8). The rate in the middle phase gave the maximum velocity (V_{max}). From

Fig. 8. Time course of decomposition of PS-5 by PV-enzyme.

The reaction mixture contained 1.8 units of PV-enzyme and 6.25×10^{-3} mM PS-5 sodium in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0). PS-5 was determined by measuring the decrease of absorbance at 301 nm. The reaction was carried out at 26°C.



the substrate-limiting phase in which the MICHAELIS-MENTEN equation seemed to be applicable, a K_m-value of 2.2×10^{-4} mM was obtained by computer-calculations based on the integrated HENRI-MICHAELIS-MENTEN equation (1). The V_{max} of PS-5 was 1/1,700 of that of cephaloridine.

Discussion

PS-5 inhibition of *P. vulgaris* β-lactamase included complicated processes. Two stages of the inhibition were observed. In the early stage, the inhibition was elucidated as a competitive inhibition based on a rapid equilibrium theory, which was confirmed by the experiments using a stopped-flow spectrophotometer. Kinetic analysis using the initial velocities that occurred during the first 0.5 minute obtained with a conventional spectrophotometer showed good coincidence with that of the stopped-flow experiments, that is, the K_i-value determined with the conventional spectrophotometer coincided with that obtained with the stopped-flow experiments. However, in the second stage, the progressive reaction curve after approximately $3 \sim 5$ minutes showed a different phenomenon. The integrated HENRI-MICHAELIS-MENTEN equation was useful to examine whether the enzyme reaction obeyed the MICHAELIS-MENTEN equation throughout the entire progressive reaction curve (time *versus* substrate concentration) or not. Inhibited reactions of PV-enzyme (*P. vulgaris* β-lactamase) by PS-5 were analyzed using the integrated equation. Although the entire time-substrate curve without PS-5 obeyed the MICHAELIS-MENTEN equation, which was demonstrated by a good linearity in the computation plotting based on the integrated HENRI-MICHAELIS-MENTEN equation, the progressive curve with PS-5 did not obey the MICHAELIS-MENTEN equation, and a concave upward curve was obtained. This indicated that V_{max} (proportional to the total enzyme) was decreasing with time, or K_m-value was changed with time, that is, the enzyme was being inactivated slowly or conformationally changed with time as the reaction was progressing.

From the observation of the time course of the initial part ($0 \sim 1$ minute) of the inactivation experiments in Fig. 6, pseudo-first-order rate constants of 0.012 second^{-1} (0.72 minute^{-1}) (PS-5 1.56×10^{-2} mM) and 0.0243 second^{-1} (1.46 minute^{-1}) (PS-5 3.12×10^{-2} mM) were obtained. From the pseudo-first-order rate constant, the second-order rate constant could be calculated to be $7.7 \sim 7.8 \times 10^2$ M^{-1} second^{-1}, which was considered to be a slow reaction because general enzyme reactions had the value ranging $1.2 \times 10^4 \sim 10^9$ M^{-1} second^{-1} [21]. This fact suggested a slow complex-formation. An exponential inactivation rate constant (k′) of 0.24 minute^{-1} (at PS-5 6.25×10^{-3} mM) was obtained using the same data as in Fig. 8 at the first 5 minutes according to the hysteretic enzyme model, or Mechanism II defined by FRIEDEN[22] (Fig. 9). This value was comparable with that obtained in Fig. 6 (0.72 minute^{-1} at PS-5 1.56×10^{-2} mM corresponded to 0.29 minute^{-1} at PS-5 6.25×10^{-3} mM).

Assuming, for simplicity, that complications of PS-5 degradation and enzyme reactivation were not present during the first 5-minute preincubation, the data of Fig. 6 were analyzed in terms of irreversible inactivation, applying equation (1) of KITZ and WILSON[23] to the following scheme:

$$E + I \overset{K_I}{\rightleftharpoons} EI \overset{k_3}{\rightarrow} E' + I_p \qquad E' \rightarrow E$$

$$\epsilon = (E) + (EI) = E_0 - (E')$$

$$-\ln(\epsilon/E_0) = k_3 \cdot t/(1 + K_I/I) \qquad (3)$$

Fig. 9. Estimation of an exponential inactivation rate constant (k′) defined by FRIEDEN.

v_t is the velocity at time t, v_f is the velocity at the steady state ($5 \sim 10$ minutes), v_0 is the velocity at $t = 0$ and k′ is a complex rate constant of FRIEDEN.

The velocities were measured from the slope during every 0.5 minute using the same data as in Fig. 8.

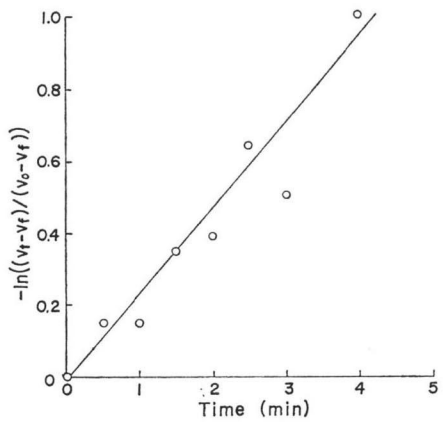


Table 1. Estimation of kinetic parameters in irreversible inactivation according to the equation of KITZ and WILSON.

I[a] ($\times 10^{-2}$ mM)	1/I (mM^{-1})	$(\epsilon/E_0)^b_{apparent}$	$(V/V_{max})^c$	$(V_{exp}/V_0)^d$	$(\epsilon/E_0)^e_{corrected}$	$(-5)/\ln(\epsilon/E_0)_{corrected}$
0		1.0	0.819	1		
0.312	320.5	0.47	0.755	0.921	0.510	7.425
0.624	160.3	0.27	0.700	0.855	0.316	4.340
1.56	64.1	0.17	0.574	0.700	0.243	3.534
3.12	32.1	0.10	0.442	0.540	0.185	2.963
6.24	16.0	0.05	0.303	0.370	0.135	2.497

a Initial PS-5 concentration
b From Fig. 6 (ϵ=active enzyme, E_0=total enzyme)
c Calculated from equation 2 using PS-5 concentration after dilution.
d Fraction of initial activity expected if no inactivation has occurred=$(V/V_{max})/0.819$.
e True fraction of active enzyme=$(\epsilon/E_0)_{apparent}/(V_{exp}/V_0)$.

K_I is the dissociation constant for the initial reversible complex, and k_3 is the first-order rate constant for the conversion of the reversible complex to the irreversibly inhibited enzyme. E_0 is the total enzyme concentration, E is the active enzyme and E′ is the inhibited enzyme. I_p is the degradation product of I (PS-5). The equation (3) can be inverted, as in the LINEWEAVER-BURK plotting procedure to give:

$$(-5)/\ln(\epsilon/E_0)=(K_m/V_{max})(1/I)+1/V_{max} \quad (4)$$

where K_m and V_{max} replace K_I and k_3 of KITZ and WILSON, respectively. $(-5)/\ln(\epsilon/E_0)$ replaces $1/k_{app}$ of KITZ and WILSON.

The apparent remaining enzyme activities of Fig. 6 must first be corrected to account for reversible inhibition by residual PS-5 before employing the LINEWEAVER-BURKE plot. The required data were calculated using the same data as in Fig. 6 and were shown in Table 1. The K_m and V_{max} for the inactivation were estimated from the intercepts of abscissa and the ordinate of a plot of $(-5)/\ln(\epsilon/E_0)$ *versus* 1/I by a computer-assisted least square method. The K_m and V_{max} values obtained were 6.3×10^{-3} mM and 0.415 $minute^{-1}$ (at 2.7 enzyme units/ml), respectively. This K_m is approximately 30 times the value for the K_i of reversible inhibition or the K_m for PS-5 degradation (0.22×10^{-3} mM). Thus the data of Fig. 6 indicate that the reversible inhibition complex (MICHAELIS complex) cannot lead directly to the inactivated enzyme. Some complex other than that observed for reversible inhibition must precede the inactivation, and it is conceivable that enzyme with less activity, undergoing probably conformational changes, is preceding the inactivation.

The recovery of enzyme activity during the preincubation as shown in Fig. 5 seemed not to have a parallel relation with the decomposition of PS-5. When preincubation was started in the concentration of 1.56×10^{-3} mM, the concentration of PS-5 became 0.78×10^{-3} mM (half the initial concentration) after 15 minutes, and at this time, CER hydrolysis velocity (v_i) gives 0.167 of V_{max} by adding 0.1 mM CER based on equation (2). The V_{max} of CER hydrolysis without PS-5 (V_0) is calculated to be 0.467 of V_{max} at 0.1 mM CER from the equation (2). Consequently the inhibition per cent $(100(v_0-v_i)/v_0)$ at 0.78×10^{-3} mM PS-5 was expected to be 65%. On the other hand, the observed inhibition per cent was 83%. Similarly, when the preincubation was started at the concentration of 3.13×10^{-3} mM PS-5, the expected inhibition and the observed inhibition per cent were 79% and 93% at the time of 40-minute preincubation, respectively. Furthermore, at the time when PS-5 was substantially depleted (specific UV-absorption at 301 nm was less than the detection limit), considerable inhibition still remained, and over 60 minutes were required for the 100% recovery of the enzyme activity. This reactivation process had approximately 0.03~0.05 $minute^{-1}$ of a first-order rate constant (Fig. 5). This reactivation rate is substantially slower than the turnover rate for PS-5 degradation calculated from Fig. 8 (approximately 2~3 $minute^{-1}$). This also shows that inactivated enzyme is not an intermediate in the main degradation reaction.

The delay of recovery of enzyme activity after the substantial depletion of PS-5 might be due to a conformational change of the enzyme, but a possibility that some part of the delay is caused by reaction products of the β-lactamase may not be denied. Unfortunately products from both a normal MICHAELIS-complex and a transiently bound enzyme-PS-5 complex are not yet confirmed. The fact that substantial reactivation of the enzyme activity by the addition of excess substrate was not observed provides an additional evidence that some inactivated form other than a MICHAELIS-complex exists (Fig. 7).

A possible speculation made from the above considerations is that the formation of inactivated enzyme, which may be a conformational change, slowly occurred after the rapid formation of a MICHAELIS-complex, and some modified complex may be formed preceding the inactivated enzyme.

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