INACTIVATION OF STAPHYLOCOCCAL PENICILLINASE BY DICLOXACILLIN

HITOSHI SAGAI and TETSU SAITO

Research Laboratories, Toyo Jozo Co., Ltd.
Ohito-cho, Shizuoka-ken, 410-23, Japan

(Received for publication February 12, 1973)

Staphylococcal penicillinase was inactivated by treatment with a relatively low concentration of methyldichlorophenyl-isoxazolyl penicillin (dicloxacillin). Inactivated enzyme was isolated by gel-filtration and reactivated by incubation at 37°C. It is suggested that the inactivated enzyme is penicilloyl enzyme which is readily hydrolyzed to active enzyme.

The rates of hydrolysis of β-lactamase-sensitive penicillins (such as benzylpenicillin and aminobenzylpenicillin) by penicillinase were greatly decreased by the addition of β-lactamase resistant penicillins[1,2]. GOUREVITCH and his coworkers reported that the inactivation of cell-bound staphylococcal penicillinase occurred when the enzyme was preincubated with dimethoxyphenyl penicillin (methicillin), and the amount of inactivated enzyme corresponded to the amount of hydrolyzed methicillin[3]. On the other hand, RICHMOND demonstrated that the purified exo-enzyme of staphylococci degraded more than 85% of added methicillin without inactivation of the enzyme[4].

In this paper, we deal with the inactivation of the extracellular staphylococcal enzyme by dicloxacillin and propose a tentative mechanism to account for the inactivation.

Materials and Methods

Drugs: Benzylpenicillin potassium salt, aminobenzylpenicillin (ampicillin) sodium salt, methyl-chlorophenylisoxazolyl penicillin (cloxacillin) sodium salt and dicloxacillin sodium salt (Toyo Jozo Co., Ltd.) were used.

Organism: *Staphylococcus aureus* 0003 was used as the penicillinase source. The strain was of clincal origin and its penicillinase was inducible.

Preparation of penicillinase: Partially purified enzyme was prepared according to the method reported by RICHMOND[4]. The supernatant fluid of methicillin-induced *S. aureus* 0003 culture was treated with phosphocellulose, and enzyme was eluted by 2.0 M tris-HCl buffer (pH 7.5). This enzyme preparation was used throughout this study. Its specific activity was approximately 0.45 units/mcg of protein when ampicillin was used as substrate.

Assay of enzyme activity: Penicillinase activity was measured in units, as defined by POLLOCK and TORRIANT[5]: 1 unit=1 μmole of penicillin hydrolyzed/hour at 30°C and pH 5.8. Enzyme activity was assayed iodometrically by the method of PERRET[6], except that 0.1 M phosphate buffer pH 5.8 was used instead of pH 6.5 buffer. MICHAELIS constants and degradation of dicloxacillin were measured by the microiodometric method[7].

Preincubation of penicillinase with a high concentration of phenylisoxazolyl penicillins: Approximately 15 units of penicillinase were preincubated with 4 ml of phenylisoxazolyl penicillins (5 mg/ml) at 30°C. After preincubation, ampicillin or benzylpenicillin was added as substrate to the mixture at the final concentration of 1 mg/ml, and penicillinase activity was measured.

Preincubation of penicillinase with various concentrations of dicloxacillin: Two hundred units of penicillinase/ml were incubated with various concentrations of dicloxacillin for 30 minutes at 30°C. An aliquot (0.1 ml) of the mixture was added to a solution of 3.4 mg ampicillin/ml to measure residual activity. This concentration of ampicillin as substrate was used for measurement of enzyme activity unless otherwise stated.

Isolation of the inactivated enzyme and reactivation of enzyme: Enzyme (approximately 600 units/ml) was incubated with 100 mcg of dicloxacillin/ml for 30 minutes at 30°C. Five milliliters of the mixture were gel-filtered through a Sephadex G 25 column (2.0×45 cm) with 0.5 M phosphate buffer pH 5.8 at 0~4°C. The collected fraction was incubated at various temperatures and reactivated activity was measured after additional incubation with substrate (ampicillin) for 10 minutes.

Results

General Properties of the Enzyme

Enzyme eluted from a phosphocellulose column was sensitive to low ionic strength as described by RICHMOND[4]. MICHAELIS constants and relative V_{max} values for some penicillins are shown in Table 1. Ampicillin was hydrolyzed by the enzyme as well as benzylpenicillin. The optimal pH for ampicillin hydrolysis was found to be 5.8~6.7.

Table 1. Kinetic study of penicillinase from *Staphylococcus aureus* 0003

	Substrate		
	Benzyl-penicillin	Ampicillin	Dicloxa-cillin
Km (μM)	12	45	172
V_{max}	31.8	(100)	0.38

MICHAELIS constants (Km) and relative V_{max} values were determined by the method of LINEWEAVER and BURK[10]

Effect of Preincubation of the Enzyme in a High Concentration of Dicloxacillin or Cloxacillin

A high concentration (5 mg/ml) of phenylisoxazolyl penicillins was used to examine inhibition of hydrolysis of benzylpenicillin or ampicillin under conditions similar to those of GOUREVITCH [8]. When dicloxacillin was added simultaneously with the substrate, inhibition of hydrolysis was observed as shown in Table 2. The inhibition was enhanced by preincubation with phenylisoxazolyl penicillins. Inhibition of hydrolysis of both substrates by dicloxacillin was greater than that by cloxacillin.

Table 2. Inhibition of penicillinase by phenylisoxazolyl penicillin.

Substrate		Benzylpenicillin		Ampicillin	
Inhibitor		Dicloxa-cillin	Cloxa-cillin	Dicloxa-cillin	Cloxa-cillin
Preincubation time (min.)	0	17.0	3.4	78.9	60.3
	15	98.0	55.8	98.6	78.9
	30	99.7	62.6	99.3	86.7
	60	99.7	68.2	99.1	91.6

Penicillinase was preincubated with dicloxacillin or cloxacillin (5 mg/ml) at 30°C for the indicated times, and penicillinase activity was assayed by the iodometric method. Ampicillin or benzylpenicillin (final concentration 1 mg/ml) was used as subrtrate. Numbers indicate percent inhibition.

Effect of the Concentration of Dicloxacillin on Inhibition of Penicillinase Activity

Enzyme was preincubated with various concentrations of dicloxacillin for 30 minutes at 30°C. As shown in Fig. 1, inhibition of enzymatic activity on ampicillin reached a plateau at a concentration of 100 mcg dicloxacillin/ml. The degree af inhibition was 80 % at this point. On the other hand, the inhibition was less than 10 % without preincubation. The time course of the reaction is shown in Fig. 2.

These data strongly suggested that dicloxacillin inactivated the enzyme rather than competiti-

vely inhibited it.

Isolation of the Inactivated Enzyme and its Reactivation

Preincubated mixture was filtered through a Sephadex G 25 column to eliminate the possibility of competitive inhibition by free dicloxacillin. The concentration of dicloxacillin in the filtered enzyme fraction was determined to be less than 0.4 mcg/ml (8.1×10^{-7} M) by the cylinder cup assay method with *Sarcina lutea* ATCC 9341 as test organism. Although the enzyme was separated from free dicloxacillin, it possessed low penicillinase activity when compared with a fraction not treated with dicloxacillin (Table 3).

Fig. 1. Effect of dicloxacillin concentration on inhibition of penicillinase activity

(a) Penicillinase was preincubated with the indicated concentration of dicloxacillin for 30 minutes at 30°C and activity was assayed

(b) Enzyme activity was measured in the mixture of ampicillin and dicloxacillin without preincubation

Ampicillin was used as substrate.

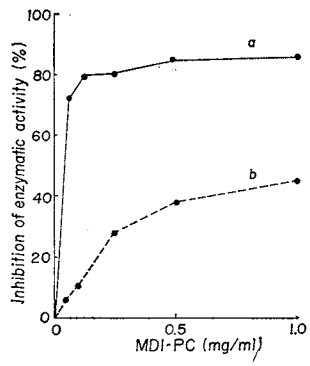


Fig. 2. Timda course of ampicillin degradation in the presence of dicloxacillin

(a) Enzyme activity was assayed after the preincubation with dicloxacillin (100 mcg/ml).

(b) Enzyme activity was assayed in the presence of dicloxacillin (100 mcg/ml) without preincubation.

(c) Control. Activity was assayed in the absence of dicloxacillin.

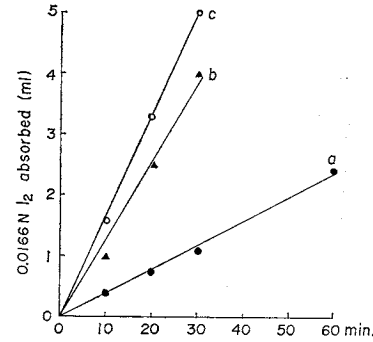


Table 3. Recovery of inactivated- and reactivated-penicillinase

Fraction	Untreated				Dicloxacillin treated			
	Volume (ml)	Total protein (mg)	Total activity (unit)	Recovery (%)	Volume (ml)	Total protein (mg)	Total activity (unit)	Recovery (%)
Partially purified enzyme	5	7.62	3240	(100)	5	7.62	3140	(100)
Dicloxacillin treated*	—	—	—	—	5	—	432	13.8
Sephadex eluate**	20	7.28	3220	99.4	20	7.48	840	26.8
37°C treated***	20	—	2980	91.9	20	—	2800	89.2

* Penicillinase was incubated with 100 mcg of dicloxacillin/ml for 30 minutes at 30°C.
** Enzyme was gel-filtered through a Sephadex G 25 column at 0~4°C.
*** Sephadex eluate fraction was incubated for 40 minutes at 37°C.

Incubation of the filtered enzyme fraction at 37°C resulted in rapid recovery of penicillinase activity (Fig. 3). An accurate kinetic study on reactivation of the enzyme with respect to temperature could not be made in our system since reactivated activity was assayed by a further incubation with ampicillin for 10 minutes at 30°C. However it is clear that reactivation of the enzyme depends on temperature as shown in Fig. 3.

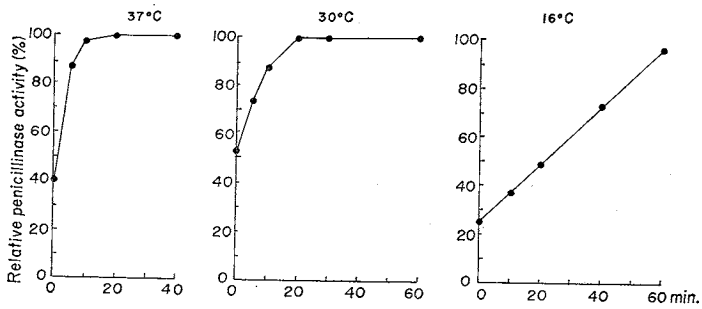
Hydrolysis of Dicloxacillin by the Enzyme

The rate of hydrolysis of dicloxacillin was measured by the microiodometric assay method at a

concentration of 100 mcg of dicloxacillin/ml. This concentration is the same as that used for the inactivation of the enzyme. Dicloxacillin was hydrolyzed at a constant rate up to 120 minutes as shown in Fig. 4. The rate of hydrolysis of dicloxacillin was about 0.0005 of that for ampicillin (Table 4).

Fig. 3. Time course of reactivation of inactivated enzyme at various temperatures

Penicillinase was incubated with dicloxacillin (100 mcg/ml) and inactivated enzyme was isolated by gel-filtration on Sephadex G25. Inactivated enzyme was incubated at 37°C, 30°C and 16°C.



Discussion

Inactivation of penicillinase was observed by incubation with a low concentration of dicloxacillin, and the inactivated enzyme was readily reactivated by incubation in the absence of free dicloxacillin. As shown in Fig. 4, the rate of hydrolysis for dicloxacillin was constant using the low concentration of substrate. This agrees with the findings of RICHMOND, although in his experiment, he used methicillin instead of dicloxacillin.

From these results, we propose a tentative model for the inactivation of the enzyme by dicloxacillin. The inactivated enzyme is considered to be penicilloyl enzyme. On the basis of the assumptions illustrated below (where K_1, K_2, K_3 and K_4 are the rate constants of the reactions indicated by arrows), the reaction may progress from state 1 to state 2 during the period of preincubation with dicloxacillin. Thus penicilloyl enzyme would be obtained if the rate of hydrolysis of penicilloyl enzyme would be very slow at the low temperature employed.

$$\text{Dicloxacillin} + \text{Enzyme} \underset{K_2(\text{large})}{\overset{K_1(\text{large})}{\rightleftarrows}} \text{dicloxacillin : Enzyme} \xrightarrow{K_3(\text{small})}$$

(State 1)

$$\text{Dicloxacillin} = \text{Enzyme (penicilloyl enzyme)} \xrightarrow{K_4(\text{very small})}$$

(State 2)

Penicilloic acid + Enzyme

State 1: No covalent bond is formed between dicloxacillin and enzyme.

State 2: Covalent bond is formed between dicloxacillin and enzyme.

According to this model, it could also be propossed that the penicillinase-resistance of dicloxacillin is not due to the resistance of the β-lactam ring but to the slow rate of hydrolysis of the penicilloyl enzyme.

Another proposed mechanism of inactivation of the enzyme is a conformational change of the enzyme brought about by dicloxacillin. CITRI and GARBER[8,9] reported on the interaction

Fig. 4. Degradation of dicloxacillin (MDI-PC) by staphylococcal penicillinase

Degradation of dicloxacillin was measured by the microiodometric assay method, 100 mcg of dicloxacillin/ml was used as substrate.

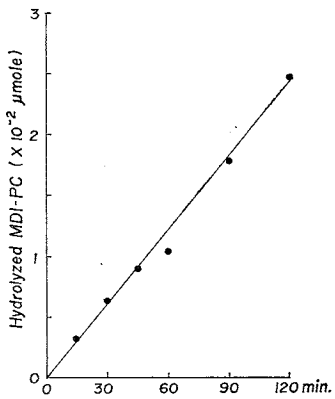


Table 4. Rates of hydrolysis of ampicillin and dicloxacillin

Substrate	Hydrolysis rate
Dicloxacillin	0.207 μmole/mcg protein·hr
Ampicillin	0.417 μmole/mcg protein·hr

The rate of hydrolysis of dicloxacillin was determined by the microiodometric method, using 100 mcg of dicloxacillin/ml as substrate.

The rate of hydrolysis of ampicillin was detemined by the iodometric method, using 3.4 mg of ampicillin/ml as substrate.

of penicillinase from *Bacillus cereus* with methicillin or oxacillin. They concluded that the interaction of the enzyme with the antibiotics caused conformational changes in enzyme as evidenced by increased susceptibility of the enzyme to iodine, urea and heat. However, inhibition of enzyme activity by methicillin or oxacillin was competitive and the inactivation of the enzyme was not observed. Our data shows that staphylococcal penicillinase is inactivated by dicloxacillin. Thus interaction of dicloxacillin with staphylococcal penicillinase may be different from that of oxacillin with *B. cereus* penicillinase.

Precipitation of protein was observed during the incubation of high concentrations of the enzyme with high concentrations of dicloxacillin. The supernatant fluid was gel-filtered and then incubated at 37°C, but no reactivation of enzymatic activity was observed. Precipitated protein was dialyzed and incubated at 37°C, but it also was not reactivated. Only 20 % reactivation was obtained when dialyzed insoluble protein was treated with 0.5 M hydroxylamine and the reagent was removed by dialysis. These facts indicate that further inactivation of the enzyme occurs in the presence of high concentrations of dicloxacillin. GOUREVITCH also demonstrated an inactivation of penicillinase[3]. His experimental condition may correspond to our experiment in which we used high concentrations of dicloxacillin.

References

1) HAMILTON-MILLER, J. M. T. & J. T. SMITH: Inhibition of penicillinase from gram-positive and gram-negative bacteria by substrate analogues. Nature 201 : 999~1001, 1964
2) SMITH, J. T.; J. M. T. HAMILTON-MILLER & R. KNOX: Isoxazolyl penicillin and penicillinase. Nature 195 : 1300~1301, 1962
3) GOUREVITCH, A.; T. A. PURSIANO & J. LEIN: Inactivation of staphylococcal penicillinase by reaction with resistant penicillins. Nature 195 : 496~497, 1962
4) RICHMOND, M. H.: Purification and properties of the exopenicillinase from *Staphylococcus aureus*. Biochem. J. 88 : 452~459, 1963
5) POLLOCK, M. R. & A. M. TORRIANI: Purification et caractéristiques physiochimiques de la pénicillinase de *Bacillus cereus*. C. R. Acad. Sci., Paris 237 : 276, 1953
6) PERRET, C. J.: Iodometric assay of penicillinase. Nature 174 : 1012~1013, 1954
7) NOVICK, R. P.: Micro-iodometric assay for penicillinase. Biochem. J. 83 : 236~240, 1962
8) GARBER, N. & N, CITRI: The interaction of penicillinase with penicillins. I. Effect of substrates and of a competitive inhibitor on native and urea-treated enzyme. Biochim. Biophys. Acta 62 : 385~396, 1962
9) CITRI, N. & N. GARBER: The nature of resistance of a penicillin to hydrolysis by penicillinase. J. Pharm. Pharmacol. 14 : 784~793, 1962
10) LINEWEAVER, H. & D. BURK: The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56 : 658~666, 1934