

PROPERTY OF THE β -LACTAMASE
FROM *STREPTOMYCES* E750-3

HIROSHI OGAWARA, TAKEHITO MINAGAWA
and HIROKO NISHIZAKI

Second Department of Biochemistry,
Meiji College of Pharmacy
Nozawa-1, Setagaya-ku, Tokyo 154, Japan

(Received for publication June 26, 1978)

β -Lactamase activity has been found in a wide variety of microorganisms including *Streptomyces*, which are among the non-pathogenic microorganisms. In *Streptomyces*, at least three quarters of strains recently isolated from soil¹⁾ as well as strains isolated some 30 years ago²⁾ produced β -lactamase extracellularly and constitutively. Moreover, the strains isolated recently and those isolated 30 years ago did not differ significantly in the proportion that produced β -lactamase and in their enzyme activities.

Recently many β -lactam antibiotics³⁻⁵⁾ and β -lactamase inhibitors⁶⁻¹⁰⁾ containing a β -lactam moiety in their structures have been isolated from culture filtrates of many *Streptomyces* strains and related organisms, and some *Streptomyces* strains produce β -lactamase and an inhibitor simultaneously (OGAWARA, unpublished result). The definite elucidation of the relationships between these compounds in *Streptomyces* may throw some light on the understanding of the supposed roles of β -lactamases in the distant past and of the origin of the roles of these enzymes in bacterial resistance.¹¹⁾ In this report, we describe the isolation and the properties of β -lactamase from *Streptomyces* strain E750-3, which is a recently isolated strain.¹⁾

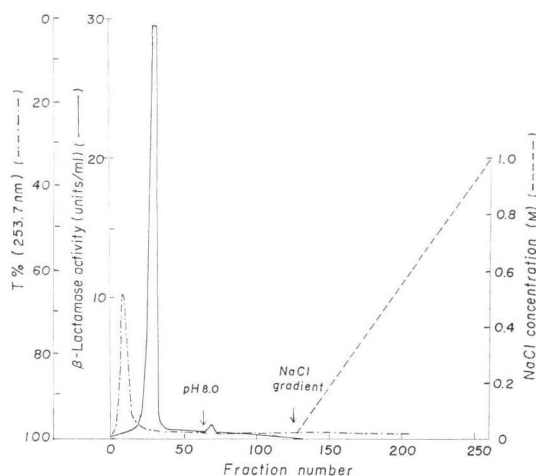
The organism was grown in shaken culture in a medium consisting of peptone (Polypeptone, Daigo Eiyu), 3%; yeast extract (Oriental), 0.2%; glycerol, 2.5%; CaCO₃, 0.6%; and silicone (KM-70, Shin Etsu Kagaku), 0.02%. The pH was adjusted to 6.8 before autoclaving. The medium for the seed culture consisted of starch, 3%; glucose, 0.5%; meat extract (Wako Pure Chemicals), 1%; peptone, 1%; NaCl, 0.3%; and silicone, 0.02%. The pH was 6.8.

A 2-day culture grown in the seed medium on a rotary shaking machine at 220 rpm was inoculated into 100 ml of culture medium as described above, in 500 ml Erlenmeyer flasks, incubated on a rotary shaker for 5 days at 27°C,

and the mycelium removed by centrifugation. To the supernatant (19.8 liters) solid ammonium sulfate was added to make 50% saturated solution at 0°C. After standing overnight at 4°C, the precipitate was collected by centrifugation at 12,000×g for 10 minutes and dissolved in 0.1 M sodium phosphate buffer, pH 7.0. The solution was then divided into eight equal fractions and each was passed through a column of Sephadex G-75 (86.5×5 cm) followed by a column of Sephadex G-50 (87×5 cm). Fractions containing β -lactamase activity, which was determined by using a modified method^{12,13)} of PERRET,¹⁴⁾ were pooled and concentrated to about 30 ml by ultrafiltration through UM-10 membrane on an Amicon-apparatus. The concentrated solution was then applied to a column of CM-cellulose (CM23, Whatman, 30×1.6 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 5.0, and the β -lactamase activity eluted in a linear gradient manner in a total volume of 1 liter from 0 to 0.3 M NaCl in the same buffer. This procedure was repeated once more. The enzymatically active fractions were pooled, concentrated as above and dialyzed against deionized water and the pH was adjusted to 7.0. The enzyme was finally purified by passing through a column of cephalixin-CH-Sepharose 4B¹⁵⁾ (15.5×1.6 cm) equilibrated with 0.05 M sodium

Fig. 1. Chromatography of β -lactamase from *Streptomyces* E750-3 by cephalixin-CH-Sepharose 4B column.

The eluate was collected in 6.7 ml fractions. The purest enzyme was obtained from the fractions 22~34.



phosphate, pH 7.0. The enzyme was retarded but not adsorbed and was separated from the bulk of the contaminants as shown in Fig. 1. The final yield was 2 mg protein. The protein was determined by the method of LOWRY *et al.*¹⁵⁾ using serum albumin as a standard.

The purified enzyme had a specific activity of 980 units/mg protein. Here, 1 unit was defined as the amount of the enzyme which catalyzed the hydrolysis of 1 μ mole of benzylpenicillin per minute at pH 7.0 and 30°C. This was about 40 times higher than that of the enzyme isolated by JOHNSON *et al.*¹⁶⁾ The molecular weight determined by gel filtration through Sephadex G-75 was about 20,000. The pH optimum was around 7.0 and the optimum temperature was between 30° and 35°C. Treatment of the enzyme for 2 minutes at 60°C at pH 7.0 reduced the activity to almost zero. Metallic ions such as CuSO₄, ZnSO₄, CoSO₄ and EDTA at 1 mM and NaCl at 100 mM had no effect on the enzymatic activity. On the other hand, *p*-chloromercuribenzoate at 1 mM reduced the activity to 60% by 5-minute treatment.

The substrate specificity of the enzyme was determined by the method of NOVICK¹⁷⁾ and the results are shown in Table 1. Benzylpenicillin was the most rapidly hydrolyzed substrate and ampicillin the next. Carbenicillin was also hydrolyzed fairly rapidly while oxacillin and cloxacillin were hydrolyzed only slowly. The enzyme did not bring about significant hydrolysis of methicillin or the cephalosporins tested. *K_m* values were in the order of 10⁻⁴ M. Cloxacillin inhibited the enzymatic activity competitively using benzyl-

penicillin as a substrate. These results indicate that the β -lactamase of *Streptomyces* E750-3 is a typical penicillinase. Here, it is interesting to note that the enzyme is inhibited strongly by clavulanic acid and other related "inhibitors", but at the same time these "inhibitors" may be hydrolyzed by the enzyme.

Acknowledgement

The authors express their great thanks to Prof. HAMAO UMEZAWA, the Director of the Institute of Microbial Chemistry, for his hearty encouragement throughout the present work, and also to the Institute of Microbial Chemistry and the Naito Foundation for their kind support of this work.

References

- OGAWARA, H.: Production and property of β -lactamases in *Streptomyces*. *Antimicrob. Agents & Chemoth.* 8: 402~408, 1975
- OGAWARA, H.; S. HORIKAWA, S. SHIMADA-MIYOSHI & K. YASUZAWA: Production and property of β -lactamases in *Streptomyces*: Comparison of the strains isolated newly and thirty years ago. *Antimicrob. Agents & Chemoth.* 13: 865~870, 1978
- UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI: New antibiotics, bleomycin A and B. *J. Antibiotics, Ser. A* 19: 200~209, 1966
- NAGARAJAN, R.; L. D. BOECK, M. GORMAN, R. L. HAMILL, C. E. HIGGINS, M. M. HOEHN, W. M. STARK & J. G. WHITNEY: β -Lactam antibiotics from *Streptomyces*. *J. Am. Chem. Soc.* 93: 2308~2310, 1971
- HASHIMOTO, M.; T. KOMORI & T. KAMIYA: Nocardicin A, a new monocyclic β -lactam antibiotic. II. Structure determination of nocardicins A and B. *J. Antibiotics* 29: 890~901, 1976
- UMEZAWA, H.; S. MITSUHASHI, M. HAMADA, S. IYOBE, S. TAKAHASHI, R. UTAHARA, Y. OSATO, Y. YAMAZAKI, H. OGAWARA & K. MAEDA: Two β -lactamase inhibitors produced by a *Streptomyces*. *J. Antibiotics* 26: 51~54, 1973
- MAEDA, K.; S. TAKAHASHI, M. SEZAKI, K. IINUMA, H. NAGANAWA, S. KONDO, M. OHNO & H. UMEZAWA: Isolation and structure of a β -lactamase inhibitor from *Streptomyces*. *J. Antibiotics* 30: 770~772, 1977
- BROWN, A. G.; D. BUTTERWORTH, M. COLE, G. HANSCOMB, J. D. HOOD, C. READING & G. N. ROLINSON: Naturally-occurring β -lactamase inhibitors with antibacterial activity. *J. Antibiotics* 29: 668~669, 1976
- JOHNSTON, D. B. R.; S. M. SCHMITT, F. A.

Table 1. Relative rate of hydrolysis of various β -lactam antibiotics by the β -lactamase of *Streptomyces* E750-3

β -Lactam antibiotics	Relative rate (Benzylpenicillin = 100)	<i>K_m</i> (M)
Benzylpenicillin	100	1.9×10^{-4}
Ampicillin	28	2.3×10^{-4}
Carbenicillin	13	
Oxacillin	6	
Cloxacillin	5	
Methicillin	0	
Cephaloridine	2	9.5×10^{-4}
Cephaloglycine	1	

- BOUFFARD & B. G. CHRISTENSEN: Total synthesis of (\pm)-thienamycin. *J. Am. Chem. Soc.* 100: 313~315, 1978
- 10) OKAMURA, K.; S. HIRATA, Y. OKUMURA, Y. FUKAGAWA, Y. SHIMAUCHI, K. KOUNO, T. ISHIKURA & J. LEIN: PS-5, a new β -lactam antibiotics from *Streptomyces*. *J. Antibiotics* 31: 480~482, 1978
- 11) OGAWARA, H.: Penicillin-inactivating enzyme, β -lactamase, with special reference to the active sites, the role of the existence and the molecular evolution. *Protein, Nucleic Acid & Enzyme (in Japanese)* 20: 1214~1227, 1975
- 12) OGAWARA, H. & H. UMEZAWA: Affinity labeling of an *Escherichia coli* β -lactamase. *Biochim. Biophys. Acta* 327: 481~489, 1973
- 13) OGAWARA, H. & H. UMEZAWA: *Bacillus cereus* β -lactamase. Reaction with N-bromosuccinimide and the properties of the product. *Biochim. Biophys. Acta* 391: 435~447, 1975
- 14) PERRET, C. J.: Iodometric assay of penicillinase. *Nature* 174: 1012~1013, 1954
- 15) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. *J. Biol. Chem.* 193: 265~275, 1951
- 16) JOHNSON, K.; J. DUSART, J. N. CAMPBELL & J. M. GHUYSEN: Exocellular β -lactamases of *Streptomyces albus* G and strain R39 and K11. *Antimicrob. Agents & Chemoth.* 3: 289~298, 1973
- 17) NOVICK, R. P.: Micro-iodometric assay for penicillinase. *Biochem. J.* 83: 236~240, 1962