PURIFICATION OF β-LACTAMASE FROM *STREPTOMYCES CELLULOSAE* BY AFFINITY CHROMATOGRAPHY ON BLUE SEPHAROSE

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A β-lactamase from culture supernatant of *Streptomyces cellulosae* was purified about 1,450-fold to apparent homogeneity in polyacrylamide gel electrophoresis and isoelectric focusing on polyacrylamide gel sheet. The methods used were ammonium sulfate precipitation, CM-52 cellulose ion-exchange chromatography and affinity chromatography on Blue Sepharose CL-6B. The molecular weight was determined to be approximately 27,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This value was in good agreement with the previous value determined by gel filtration on Sephadex G-75. The isoelectric point was pH 9.5. The enzyme behaved primarily as penicillinase and apparent K_m value for benzylpenicillin was 500 μM. The β-lactamase of *S. cellulosae* interacted strongly with blue dextran and $NADP^+$-agarose but not with Sepharose. In addition, the presence of $NADP^+$ but not NAD^+ and ATP diminished sharply the intrinsic fluorescence intensity of the enzyme and the apparent association constant was calculated to be 1.4×10^3 M^{-1}. The β-lactamase decreases its enzymatic activity against benzylpenicillin in the presence of $NADP^+$. From these results, it is suggested that this β-lactamase has a dinucleotide binding fold.

β-Lactamases are produced by a wide range of different prokaryotic cells with great variety in chemical, physical and enzymatic properties[1]. β-Lactamases are called as such on the basis of only one common property: they catalyze the hydrolysis of β-lactam ring of penicillins and cephalosporins to produce antibacterially inactive products, penicilloic acids and cephalosporoic acids. This provides the pathogenic bacteria with resistant ability against β-lactam compounds. At the same time, however, these enzymes are produced by non-pathogenic micro-organisms as well. We have previously shown that at least three-quarters of *Streptomyces* strains isolated recently[2] and also those isolated thirty years ago[3] produced β-lactamase constitutively irrespective of their resistance to benzylpenicillin. Another non-pathogenic prokaryote, the cyanobacteria, is also reported to produce β-lactamase[4]. The roles of these enzymes are not known completely in non-pathogenic micro-organisms.

β-Lactamases in *Streptomyces* have many different properties and can be divided into five groups on the basis of their substrate specificity and physico-chemical properties*. Some enzymes have a high affinity to blue dextran and are sometimes eluted along with blue dextran in the void volume position from a Sephadex column[3]. Considering that *Streptomyces* produce many β-lactam compounds[5~9] and some of them show antibacterial activity and some act as β-lactamase inhibitors, such diverse properties of β-lactamases suggests that *Streptomyces* protect themselves from β-lactam compounds, their own metabolites, by degrading with enzymes having a β-lactamase activity from many different origins. This paper describes the purification of a β-lactamase from *Streptomyces* by affinity chromatography on Blue Sepharose CL-6B and some properties. As far as we know, this is

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the first time that it has been shown that β-lactamase interacts strongly with blue dextran and a dinucleotide such as $NADP^+$.

Experimental Procedures

Materials

Blue Sepharose CL-6B and Sepharose 4B were purchased from Pharmacia, CM-52 cellulose from Whatman, $NADP^+$-agarose, NAD^+ (yeast, grade III), $NADP^+$ (sodium salt), ATP (equine muscle), bovine serum albumin and Tris from Sigma, pI marker from Oriental Yeast Co., sodium dodecyl sulfate (SDS)* and acrylamide from Nakarai Chemicals Ltd., and N,N′-methylene bis(acrylamide) and N,N,N′,N′-tetramethylethylenediamide from Wako Pure Chemicals. Myoglobin (Mw=17,800), chymotrypsinogen A (25,000), and ovalbumin (45,000) were obtained from Schwarz/Mann and DNase I (31,000) from Worthington. These proteins were used for the molecular weight standards. Chromogenic cephalosporin 87/312 was a kind gift from Dr. M. MATTHEW of Glaxo Research Laboratory. *Streptomyces cellulosae* KCC S-0127 was a courteous gift from Dr. A. SEINO of Kaken Chemical Co. and was maintained on slants of agar-GAA. Composition of agar-GAA and medium E used for shaken culture was described previously[3].

Methods

Growth of Organism

Streptomyces cellulosae KCC-0127 (drived from ATCC 3313) was grown on a rotary shaking machine (200 rpm) in 500-ml Erlenmeyer flasks containing 100 ml of medium E at 27°C. Two ml of a 2-day culture was inoculated into 100 ml of the same medium, and grown for 5 days. The mycelia were removed by centrifugation and the supernatant was used as an enzyme source.

Assay Methods for β-Lactamase

β-Lactamase activity was determined iodometrically at pH 7.0 and 30°C using a slight modification[10,11] of the method of PERRET[12], and the micro-iodometric assay of NOVICK[13] was used for the kinetic measurement of the enzyme reaction. One unit of β-lactamase was defined as the amount of the enzyme that catalyzed the hydrolysis of 1 μmol of benzylpenicillin per minute at 30°C and pH 7.0.

Isoelectric Focusing

Isoelectric focusing was carried out by an LKB 2117 Multiphor apparatus on a sheet of polyacrylamide gel. The detailed procedure was described in a previous paper[3]. The isoelectric point was determined by comparison with the standard pI markers. The pIs of the proteins used are: cytochrome c (horse), 10.6; myoglobin (sperm whale), 8.7; myoglobin (horse), 7.6; cytochrome c_2 (*R. rubrum*), 6.2; and cytochrome c′ (*R. rubrum*), 5.6.

Protein Determination

Protein was determined using the method of LOWRY *et al.*[14] with crystalline bovine serum albumin as a standard.

SDS Polyacrylamide Gel Electrophoresis

This was performed by the modified method[15] of LAEMMLI and FAVRE[16].

Amino Acid Analysis

After exhaustive dialysis against deionized water, the sample was hydrolyzed in a constant boiling HCl for 20 hours at 110°C. Amino acid analysis was performed by the Hitachi 835 Amino Acid Analyser.

Fluorescence Spectrophotometry

Hitachi 204-S spectrophotofluorimeter was used. In all experiments, both excitation and emission slits were 10 nm. Emission spectra were obtained at 28°C by an excitation at 290 nm, and monitoring was done at the peak near 334 nm. Data were obtained at an enzyme concentration of about 50 nM

* The abbreviation used is: SDS, sodium dodecyl sulfate.

in 0.01 M Tris-HCl buffer of pH 7.5 and treated as in previous papers[17,18].

Results and discussion

Purification of the Enzyme

All the procedures were carried out at 4°C. To the culture supernatant (4,600 ml) solid ammonium sulfate (650 g) was added. After standing for 3 hours, the precipitate was removed by centrifugation, and to the supernatant further ammonium sulfate (2,900 g) was added and the solution was kept standing overnight. The supernatant was removed (1,000 units) by centrifugation and the precipitate was dissolved in water and dialyzed against three changes of 5 liters of water and one change of 5 liters of 50 mM sodium phosphate, pH 6.0. The precipitate was removed by centrifugation. From 400 ml of the supernatant (18.5 units/ml), 380 ml (7,022 units) was passed through a column of CM-52 cellulose equilibrated with 50 mM sodium phosphate, pH 6.0, and the column was washed with the same buffer. In a pass-through fraction and a washing, 1.28 g of protein was recovered. Then, a linear gradient elution consisting of 500 ml each of the same buffer and 0.5 M NaCl in the same buffer was applied, and fractions were collected as shown in Fig. 1 (a). Fractions 22~27 contained 980 units of β-lactamase and fractions 35~38 contained 910 units of β-lactamase. Main fractions (28~31) were combined and concentrated to 4 ml (2,940 units) and equilibrated with the buffer described below by ultrafiltration through UM-10 membrane on an Amicon-apparatus. The concentrate (3.6 ml) was passed through a column of Blue Sepharose CL-6B. After washing, a linear gradient (100 ml each) of 0~1.0 M NaCl in the same buffer was applied. The elution pattern was shown in Fig. 1 (b). The main fractions 96~109 were combined and the properties were determined. The results of the purification was summarized in Table 1.

Fig. 1(a). Chromatography on CM-52 cellulose of ammonium sulfate precipitate fraction.

The sample containing 1.75 g protein and 7,022 units β-lactamase was applied to a column (1.6×40 cm) equilibrated with 50 mM sodium phosphate, pH 6.0. After washing with 200 ml of the same buffer, a linear gradient was applied as described in the text. Fractions of 250 drops were collected.

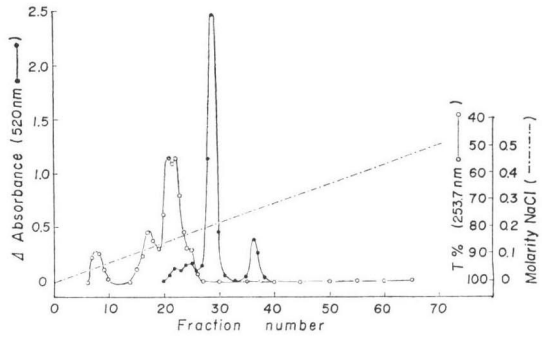
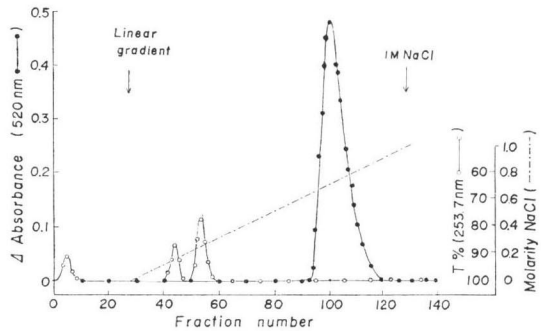


Fig. 1(b). Affinity chromatography on Blue Sepharose CL-6B of fraction 28~31 of Fig. 1 (a).

The sample containing 17.4 mg protein and 2,650 units β-lactamase was applied to a column (0.9× 13 cm) equilibrated with 10 mM Tris-HCl, pH 7.5. After washing with 50 ml of the same buffer, a linear gradient was applied as described in the text, and then 1 M NaCl in the same buffer. Fractions of 30 drops were collected. The enzymatic activity in the ordinate was expressed as the consumed amounts of iodine determined by the optical absorbance at 520 nm.



Physico-chemical Properties

The purity of the enzyme was checked by gel electrophoresis. In analytical polyacrylamide gel electrophoresis the enzyme migrated as a

Table 1. Purification of β-lactamase from culture supernatant of *Streptomyces cellulosae*

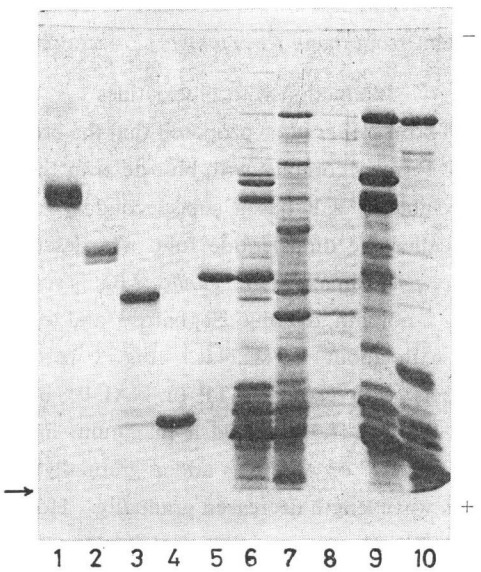
Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification	Recovery* (%)
Culture supernatant	4,600	12,400	9,660	0.78	1.0	100
Ammonium sulfate	400	1,840	7,400	4.02	5.2	76.6
CM-52 cellulose						
Fr. 22~27	88	85.4	980	11.4	14.6	10.7
Fr. 28~31**	67	19.4	4,000	205	263	43.6
Fr. 35~38	65	21.4	910	42	53.8	9.9
UM-10	4	—	2,940	—	—	32.0
Blue Sepharose	28	1.8	2,050	1,130	1,450	24.8

* This value is not actual recovery but is calculated considering the amount used in the experiment.
** This fraction was used for further purification.

single band in a β-alanine-acetate buffer (pH 4.5) system, which was coincident with an enzymatically active band detected with chromogenic cephalosporin 87/312. Gel electrophoresis in Tris-glycine buffer (pH 8.3) containing sodium dodecyl sulfate system resulted in the appearance of a single band with very minor bands (Fig. 2). The molecular weight calculated from the mobilities in gel electrophoresis was 27,000. This value was in good agreement with the value determined by gel filtration on Sephadex G-75[3]. In isoelectric focusing on a sheet of polyacrylamide gel, the enzyme migrated as a single band with isoelectric point of about 9.5, which was also coincident with an enzymatically active band detected with chromogenic cephalosporin 87/312. The pH-activity curve showed the optimal pH of near 7 and a relatively sharp decrease on either side of this region. Because of the shortage of the sample, only a preliminary amino acid composition could be obtained.

Fig. 2. SDS-polyacrylamide gel electrophoresis of β-lactamase fractions.

Gel electrophoresis and sample preparation were performed by the modified method[15] of LAMMLI and FAVRE[16]. Gel 1, ovalbumin; gel 2, DNase I; gel 3, chymotrypsinogen A; gel 4, myoglobin; gel 5, Blue Sepharose Fr. 96~109; gel 6, CM-52 Fr. 28~31; gel 7, ammonium sulfate precipitate; gel 8, culture supernatant; gel 9, CM-52 Fr. 35~38; and gel 10, CM-52 Fr. 22~27. The arrow indicates the position of marker dye.



The results were expressed as the relative molar ratio to leucine (=100): Asp, 120; Thr, 100; Ser, 320; Glu, 180; Gly, 300; Ala, 150; Cys, 80; Val, 210; Ile, 60; Leu, 100; Tyr, 70; Phe, 160; Lys, 110; His, 130; Arg, 40.

Substrate Specificity

The substrate specificity was determined using benzylpenicillin, ampicillin, carbenicillin, cloxa-

cillin, cephaloridine and cephazoline as substrates. When the hydrolysis rate of benzylpenicillin was expressed as 100%, that of ampicillin, carbenicillin, cloxacillin, cephaloridine and cephazoline was 36.9, 3.7, 7.3, 1.0, 3.9%, respectively. K_m value for benzylpenicillin was found to be 500 μM. It is known that penicillinases of Gram-negative bacteria are strongly inhibited competitively by the presence of cloxacillin or methicillin[10]. However, rate of hydrolysis of benzylpenicillin by this enzyme was not much retarded by the presence of cloxacillin or methicillin. The relative rate of hydrolysis of benzylpenicillin (0.2 mM) in 0.1 M sodium phosphate, pH 7.0, was found to be 100 (benzylpenicillin only), 52 (plus cloxacillin, 0.1 mM) and 85 (plus methicillin, 0.1 mM). These results, coupled with the fact that certain penicillinases of Gram-negative bacteria can degrade cephaloridine at a rate comparable with benzylpenicillin whereas the enzyme of *S. cellulosae* can not degrade cephaloridine so rapidly, indicate that the construction mode of the active site is markedly different in these two kinds of penicillinases, although the amino acid residues involved directly in the catalysis and/or binding of substrate are not necessarily different[19].

Effects of Inhibitors

The effects of various organic and inorganic compounds on the enzymatic activity were summarized in Table 2. Iodine, *p*-chloromercuribenzoate and N-bromosuccinimide inhibited strongly the enzymatic activity and Cu^{2+} inhibited slightly. As stated in a previous paper[2], most β-lactamases in *Streptomyces* are strongly inactivated by *p*-chloromercuribenzoate. In this respect, the enzyme of *S. cellulosae* is not an exception. On the other hand, sensitivity to iodine and N-bromosuccinimide of the *S. cellulosae* enzyme is different from many *Streptomyces* β-lactamases.

Table 2. Effect of various compounds on the enzymatic activity of β-lactamase from *S. cellulosae**

Reagents	Concentration (mM)	Activity remaining (%)
I_2	0.5	0
NaCl	100	104
$CuSO_4$	1	57
$CoSO_4$	1	96
$ZnSO_4$	1	72
p-Chloromercuribenzoate	1	0
EDTA·2Na	1	94
N-Bromosuccinimide	1	0

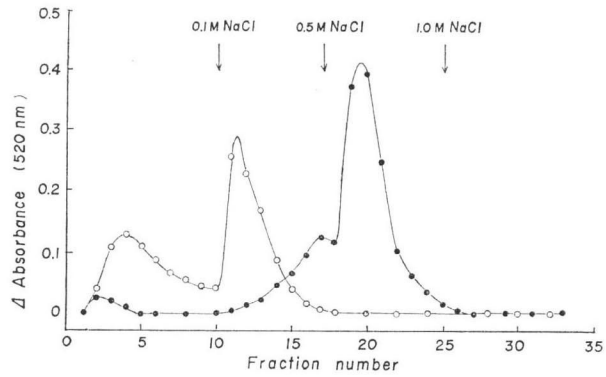
* The enzyme (about 210 nM) was incubated in 0.1 M sodium phosphate, pH 7.0, with various compounds for 10 minutes. The β-lactamase activity remaining was determined using an aliquot.

Interaction with Nucleotides

THOMPSON *et al.*[20] proposed that the protein which forms a complex with blue dextran that is dissociable by salt has a supersecondary structure called the dinucleotide fold. As described above, β-lactamase of *S. cellulosae* binds strongly to a column of Blue Sepharose and eluted only with about 0.7 M NaCl (Fig. 1 (a)). Accordingly, we examined whether the β-lactamase interacts with NAD^+, ATP or $NADP^+$ by fluorescence titration. The enzyme solution in 10 mM Tris-HCl, pH 7.5, showed a maximum fluorescence spectrum at 334 nm when excited at 290 nm. When NAD^+ or ATP was added gradually to the enzyme solution, the fluorescence intensity at maximum wave length decreased gradually. However, analysis of these data by the method of CHEN and COHEN[21] indicates no interaction between the enzyme and NAD^+ or ATP. On the other hand, the enzyme solution was passed through a column of $NADP^+$-agarose, the enzyme was eluted only just with 0.5 M NaCl, while the enzyme was applied to a column of Sepharose 4B, the enzyme was passed through or eluted completely with less than 0.1 M NaCl (Fig. 3). Furthermore, when $NADP^+$ was added gradually to the enzyme solution, the fluorescence intensity at maximum wave length decreased sharply (Fig. 4). Treatment of the data as in previous papers[17,18] gave an association constant of

Fig. 3. Chromatography of β-lactamase from *S. cellulosae* on Sepharose 4B (0.3 ml, ○——○) and $NADP^+$-agarose (0.3 ml, ●——●) equilibrated with 10 mM Tris-HCl, pH 7.5.

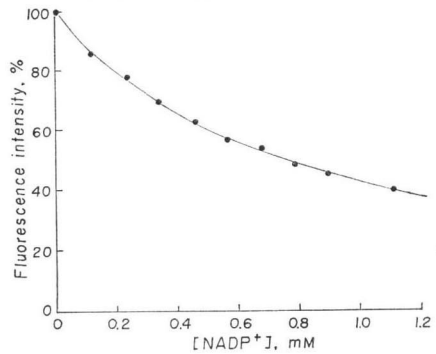
After washing with 7 ml of the same buffer, the column was eluted with 5 ml each of 0.1 M NaCl, 0.5 M NaCl and 1.0 M NaCl in the same buffer. Fractions of 10 drops were collected and the β-lactamase activity in an aliquot was determined and expressed in the ordinate as the consumed amounts of iodine measured by the optical absorbance at 520 nm.



about 1.4×10^3 M^{-1}. These results indicate that the β-lactamase of *S. cellulosae* interacts strongly with $NADP^+$ and has a dinucleotide fold, even though its physiological role is unknown yet. However, it is very interesting in this connection that the β-lactamase shows a small but definitive decrease of its enzymatic activity in the presence of $NADP^+$. Because some but not all the β-lactamases from *Streptomyces* interact strongly with blue dextran[3,22], these enzymes should also interact with some dinucleotides, even if their properties such as molecular weights and isoelectric points are quite different from each other.

Fig. 4. The fluorescence titration with $NADP^+$.

$NADP^+$ solution was added gradually to an enzyme solution of about 50 nM in 0.01 M Tris-HCl buffer of pH 7.5. Fluorescence emission spectra were monitored at the peak near 334 nm at 28°C by an excitation at 290 nm.



The finding that the β-lactamase has a dinucleotide fold is quite interesting also from the point of the view of evolution or source of β-lactamases in pathogenic bacteria. As stated above, the properties of β-lactamases are quite diverse. Consequently, it is possible that β-lactamases now have been evolved convergently from many different proteins into one common property: hydrolysis of β-lactam ring. If one accepts the idea that the enzymes inactivating antibiotics in pathogenic bacteria may be derived from antibiotic-producing *Streptomyces*[23], these divergent properties are easily understood. The reason is that the *Streptomyces* must protect themselves at any cost against β-lactam compounds which are their own metabolites. If they did not do this they would kill themselves by the binding of these β-lactam compounds to their own targets, penicillin-binding proteins (manuscript submitted). The synthesis of some β-lactamases is controlled also in *Streptomyces* by a plasmid[24] and/or a transposon[25]. Thus, such β-lactamases may be found in a few years in pathogenic as well as non-pathogenic micro-organisms.

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References

1) CITRI, N.: Penicillinase and other β-lactamases. *in* "The Enzymes" (BOYER, P. D. *ed.*), Vol. 4, pp. 23~46, Academic Press Inc., New York, 1971
2) OGAWARA, H.: Production and property of β-lactamases in *Streptomyces*. Antimicr. Agents & Chemoth. 8: 402~408, 1975
3) 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. Antimicr. Agents & Chemoth. 13: 865~870, 1978
4) KUSHNER, D. J. & C. BREUIL: Penicillinase (β-lactamase) formation by blue-green algae. Arch. Microbiol. 112: 219~223, 1977
5) NAGARAJAN, R. L.: β-Lactam antibiotics from *Streptomyces*. *in* "Cephalosporins and Penicillins" (FLYNN, E. H. *ed.*), pp. 636~661, Academic Press Inc., New York, 1972
6) UMEZAWA, H.; S. MITSUHASHI, M. HAMADA, S. IYOBE, S. TAKAHASHI, R. UTAHARA, T. OSATO, Y. YAMAZAKI, H. OGAWARA & K. MAEDA: Two β-lactamase inhibitors produced by a *Streptomyces*. J. Antibiotics 26: 51~54, 1973
7) 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
8) OKAMURA, K.; S. HIRATA, Y. OKAMURA, Y. FUKAGAWA, Y. SHIMAUCHI, K. KOUNO, T. ISHIKURA & J. LEIN: PS-5, a new β-lactam antibiotic from *Streptomyces*. J. Antibiotics 31: 480~482, 1978
9) KAHAN, J. S.; F. M. KAHAN, R. GOEGELMAN, S. A. CURRIE, M. JACKSON, E. O. STAPLEY, T. W. MILLER, A. K. MILLER, D. HENDLIN, S. MOCHALES, S. HERNANDEZ, H. B. WOODRUFF & J. BIRBAUM: Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J. Antibiotics 32: 1~12, 1979
10) OGAWARA, H.; K. MAEDA & H. UMEZAWA: A β-lactamase of *Escherichia coli*. Biochim. Biophys. Acta 289: 203~211, 1972
11) OGAWARA, H. & H. UMEZAWA: Affinity labeling of an *Escherichia coli* β-lactamase. Biochim. Biophys. Acta 327: 481~489, 1973
12) PERRET, C. J.: Iodometric assay of penicillinase. Nature 174: 1012~1013, 1954
13) NOVICK, R. P.: Micro-iodometric assay for penicillinase. Biochem. J. 83: 236~240, 1962
14) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the FOLIN phenol reagent. J. Biol. Chem. 192: 265~275, 1951
15) HORIKAWA, S. & H. OGAWARA: Membrane-bound penicillin-binding proteins of *Escherichia coli*. Comparison of a strain carrying an R-factor and the parent strain. J. Antibiotics 31: 1283~1291, 1978
16) LAEMMLI, V. K. & M. FAVRE: Maturation of the head of bacteriophage T4. J. Mol. Biol. 80: 575~599, 1973
17) OGAWARA, H.: A specific cephalosporin-binding protein of *Citrobacter freundii*. Biochim. Biophys. Acta 420: 155~164, 1976
18) OGAWARA, H.: Penicillin-binding proteins of *Escherichia coli*. Comparison of a strain carrying an R-factor and the parent strain. Biochim. Biophys. Acta 491: 223~231, 1977
19) OGAWARA, H. & H. UMEZAWA: Active sites and conformation of β-lactamases. *in* "Microbial Drug Resistance" (MITSUHASHI, S. & H. HASHIMOTO *ed.*), Vol. 1, pp. 375~389, University of Tokyo Press, Tokyo, 1975
20) THOMPSON, S. T.; K. H. CASS & E. STELLWAGEN: Blue-dextran-Sepharose: An affinity column for the dinucleotide fold in protein. Proc. Natl. Acad. Sci. U.S.A. 72: 669~672, 1975
21) CHEN, R. F. & P. F. COHEN: Quenching of tyrosine fluorescence in proteins by phosphate. Arch. Biochem. Biophys. 114: 514~522, 1966
22) OGAWARA, H.; S. HORIKAWA, S. SHIMADA-MIYOSHI & K. YASUZAWA: β-Lactamases in *Streptomyces*. *in* "Microbial Drug Resistance" (MITSUHASHI, S. *ed.*), Vol. 2, pp. 331~333, Japan Scientific Societies Press, Tokyo, 1979

23) BENVENISTE, R. & J. DAVIES: Aminoglycoside antibiotic-inactivating enzymes in actinomyces similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70: 2276~2280, 1973

24) OGAWARA, H. & S. NOZAKI: Effect of acriflavine on the production of β-lactamase in *Streptomyces*. J. Antibiotics 30: 337~339, 1977

25) MATSUBARA-NAKANO, M.; Y. KATAOKA & H. OGAWARA: Unstable mutation of β-lactamase production in *Streptomyces lavendulae*. Antimicr. Agents & Chemoth. in press