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Affinity Chromatography of Neutral Metalloendopeptidase Produced by *Streptomyces griseoruber* on *N*-Benzyloxycarbonylglycylleucyl-aminohexylamino-Sepharose

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A neutral metalloendopeptidase recovered from culture broth of *Streptomyces griseoruber* was purified by affinity chromatography on *N*-benzyloxycarbonylglycylleucylaminohexylamino-Sepharose (Z–Gly–Leu–AH-Sepharose) to electrophoretic homogeneity.

The enzyme was adsorbed on this adsorbent from phosphate buffer (pH 5.6) and eluted with acetate buffer (pH 4.1) containing 2 M urea. The enzyme was inactivated by ethylenediaminetetraacetate but not by sulfhydryl reagents or phenylmethanesulfonyl fluoride. The enzyme showed the maximum caseinolytic activity in the region of pH 6.0—7.0 and was stable within the pH range of 5.0—7.0. The molecular weight was estimated to be 52000. The enzyme preferentially hydrolyzed Z–Gly–Leu–NH_2 and Z–Gly–Phe–NH_2 among the synthetic substrates tested in this work.

Based on taxonomic studies, the producing organism was identified as *Streptomyces griseoruber*.

Keywords——*N*-benzyloxycarbonylglycylleucylaminohexylamino-Sepharose; *Streptomyces griseoruber*; affinity chromatography; metalloendopeptidase

N-Benzyloxycarbonylglycylleucylaminohexylamino-Sepharose (Z–Gly–Leu–AH-Sepharose) has been used as an affinity adsorbent for an alkaline metalloendopeptidase named alkinonase A[1] and a serine proteinase named alkaline proteinase S[2] produced by *Streptomyces violaceorectus*. The affinity of both enzymes for the adsorbent was inferred from the hydrolysis of the synthetic substrate Z–Gly–Leu–NH_2 by these enzymes. The former hydrolyzed Z–Gly–Leu–NH_2 to Z–Gly and Leu–NH_2 and the latter gave Z–Gly–Leu and ammonia. Since neutral metalloendopeptidases of microbial origin[3] such as thermolysin[4] and neutral metalloendopeptidase from *Bacillus subtilis*[4b,5,6] hydrolyzed Z–Gly–Leu–NH_2 to give Z–Gly and Leu–NH_2, Z–Gly–Leu–AH-Sepharose was applied to the purification of neutral metalloendopeptidase of *Streptomyces* origin.

The details of the purification and characterization of the enzyme and taxonomy of the producing organism, tentatively designated as strain H 1229 MY 3, are presented in this paper.

Materials and Methods

Materials——*N*-Benzyloxycarbonyl amino acids and peptides such as Z–Gly, Z–Gly–Leu, Z–Gly–Leu–NH_2 and Z–Gly–Phe–NH_2 were products of the Peptide Institute, Protein Research Foundation, and AH-Sepharose was obtained from Pharmacia Fine Chemicals Co. All other materials were commercial products of analytical grade and amino acids were all of the L-form, unless otherwise specified.

Taxonomic Studies——The organism was isolated from a soil sample collected in Miyazaki Prefecture. The methods and media recommended by the International Streptomyces Project (ISP)[7] were primarily used along with several supplemental tests. Color names were determined according to "Guide to Color Standard"[8] and the procedures of Becker *et al.*[9] were used for the preparation of whole cell hydrolysate and for chromatographic detection of the isomers of diaminopimelic acid.

Cultivation of Strain H 1229 MY 3——The inoculum seed was developed by transferring spores of strain H 1229 MY 3 to a 500 ml Erlenmeyer flask containing 100 ml of a medium consisting of 1.0% soluble starch and 0.2% yeast extract (pH 7.0—7.2). The flask was incubated at 27 °C for 24 h on a rotary shaker at 170 rpm. Two ml of the seed culture was used to inoculate a 500 ml Erlenmeyer flask containing 100 ml of a producing medium with the following composition: 1.5% soluble starch, 1.0% glucose, 2.0% soybean meal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl, 0.00012% $CaCl_2 \cdot 2H_2O$ and 0.00008% $ZnSO_4 \cdot 7H_2O$ (pH 7.6 before sterilization). The culture was conducted at 27 °C for 4 d on the same shaker.

Isolation of Crude Enzyme——The cultured broth filtrate (2200 ml) was brought to 70% saturation with ammonium sulfate and the pH was adjusted to 7.0 by the addition of 1 N NH_4OH. The precipitate was collected by centrifugation, redissolved in distilled H_2O (30 ml) and dialyzed against distilled H_2O containing $CaCl_2 \cdot 2H_2O$ (5 mg/l) with 94% recovery of the caseinolytic activity. The crude enzyme thus obtained was subjected to affinity chromatography.

Preparation of Z–Gly–Leu–AH-Sepharose——The details were given in the previous paper.[1] Briefly, a mixture of AH-Sepharose (20 ml as H_2O-swelled gel, equivalent to 5 mg as dry weight), Z–Gly–Leu (150 mg in 20 ml of 40% aqueous *N*, *N'*-dimethylformamide, pH 5.0) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (456 mg) was left at room temperature overnight with gentle shaking.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[10] using bovine serum albumin as a standard.

Assay of Caseinolytic Activity——Proteolytic activity against casein was measured by the casein-275 nm method of Hagihara *et al.*[11] with some modification as described in the previous paper.[3a] One unit of caseinolytic activity was defined as the amount of enzyme giving an absorbance equivalent to 1 μg tyrosine per min at 37 °C.

Hydrolysis of Synthetic Substrates——The hydrolysis rates of various synthetic substrates by the enzyme were determined according to the previous method[1] except that 50 mM Tris-HCl (pH 7.0) was used instead of 50 mM Tris-HCl (pH 9.0).

Electrophoresis——Electrophoresis was performed in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn.[12] Bovine serum albumin (MW 68000), α-chymotrypsinogen (MW 25700) and lysozyme (MW 14300) were used as reference proteins to determine the molecular weight of the enzyme.

Results

Taxonomic Studies of Strain H 1229 MY 3

Morphological characteristics were observed with light and scanning electron microscopes. The organism produced an abundant substrate mycelium which did not fragment into spores on any of the media tested. Aerial mycelium was monopodially branched and bore moderately long spore chains with 10—50 spores arranged in open or tight spirals on oatmeal agar, inorganic salts–starch agar and glycerol–asparagine agar. The spore surface was smooth and the oval spores measured 0.56—0.66 μm × 0.78—0.92 μm. Physiological characteristics are listed in Table I. LL-Diaminopimelic acid was detected in the whole cell hydrolysate.

By consulting "Bergey's Manual of Determinative Bacteriology"[13] and "ISP reports" by Shirling and Gottlieb,[14] *Streptomyces griseoruber* was selected as the most similar species to strain H 1229 MY 3, even though the production of melanoid pigments by this species on tyrosine agar and tryptone–yeast extract broth had been reported.

Purification of Neutral Metalloendopeptidase

The crude enzyme prepared by ammonium sulfate precipitation was dialyzed against 1/15 M phosphate buffer (pH 5.6) and adsorbed on Z–Gly–Leu–AH-Sepharose (5.0 cm × 0.6 cm, diam.) from the same buffer. The column was washed with a sufficient amount of the starting buffer, and the enzyme was eluted from the column with 1/10 M acetate buffer (pH 4.1) containing 2 M urea with 48% recovery of the caseinolytic activity (Fig. 1). The active fractions were pooled and dialyzed against 1/15 M phosphate buffer (pH 6.0). The purification of the enzyme is summarized in Table II.

Homogeneity and Molecular Weight Estimation

The purified enzyme after affinity chromatography showed a single band on SDS–polyacrylamide gel electrophoresis (Fig. 2). The molecular weight was estimated to be 52000

Table I. Physiological Properties of Strain H 1229 MY 3

	Characteristics
Spore wall ornamentation	Smooth
Spore chain morphology	Spiral
No. of spores per chain	10—50
Color of substrate mycelium	Yellowish white to pale yellowish brown
Color of mature sporulated aerial mycelium	Grayish brown to brownish gray
Soluble pigments	Negative
Melanoid pigments	Positive on ISP-6, negative on ISP-1 and 7
Starch hydrolysis	Positive
Gelatin liquifaction	Doubtful
Action on skimmed milk	
Coagulation	Negative
Peptonization	Positive
Utilization of sugars	
L-Arabinose	Positive
D-Xylose	Positive
D-Glucose	Positive
D-Fructose	Positive
Sucrose	Negative
Isoinositol	Positive
L-Rhamnose	Positive
Raffinose	Positive
D-Mannitol	Negative
Cellulose	Negative
Cell wall type	I (LL-Diaminopimelic acid)

Table II. Purification of Neutral Metalloendopeptidase from *Streptomyces griseoruber* H 1229 MY 3

Enzyme fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Broth filtrate (2200 ml)	4570	668000	146	100
$(NH_4)_2SO_4$ ppt.	1000	628000	608	94
Z–Gly–Leu–AH–Sepharose affinity chromatography	170	303000	1790	45

by gel electrophoresis and Sephadex G-75 gel filtration.

Effects of pH on Caseinolytic Activity and Enzyme Stability

The optimum hydrolysis of casein was observed in the region of pH 6.0—7.0 (Fig. 3). The enzyme was stable within the pH range of 5.0—7.0 as shown in Fig. 4.

Effect of Various Chemicals

The enzyme was inactivated completely by ethylenediaminetetraacetate, iodine and *N*-bromosuccinimide. No inhibition by phenylmethanesulfonyl fluoride or *p*-chloromercuribenzoate was observed (Table III). The caseinolytic activity of the enzyme lost by the treatment with 1 mM ethylenediamine tetraacetic acid (EDTA) was completely recovered by the addition of 1 mM $ZnCl_2$ to the substrate solution. $CoCl_2$ was also effective at the same concentration, to a lesser extent (Fig. 5).

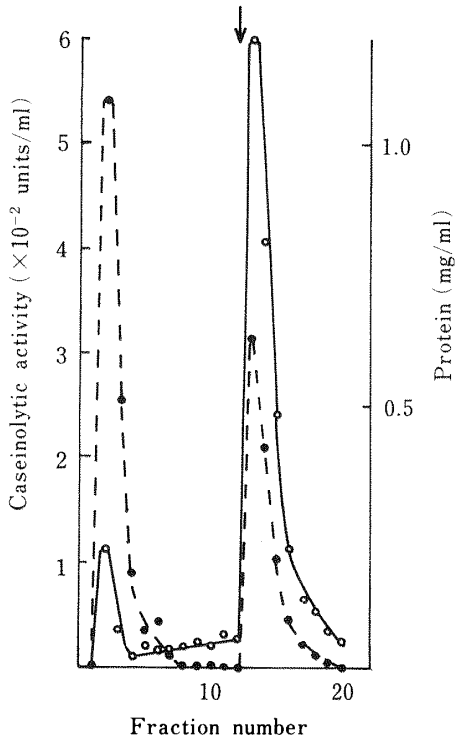

Fig. 1. Affinity Chromatography of Neutral Metalloendopeptidase from *Streptomyces griseoruber* H1229MY3

The crude enzyme obtained by ammonium sulfate precipitation (20 mg as protein) was dialyzed against 1/15 M phosphate buffer (pH 5.6) and charged on a Z-Gly-Leu-AH-Sepharose column (5.0 cm × 0.6 cm, diam.). The arrow indicates change of the buffer from 1/15 M phosphate buffer (pH 5.6) to 1/10 M acetate buffer (pH 4.1) supplemented with 2 M urea. The eluate was collected in 4.0 ml fractions.

○, caseinolytic activity; ●, protein concentration.

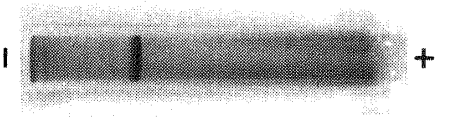

Fig. 2. SDS-Polyacrylamide Gel Electrophotogram of the Purified Enzyme

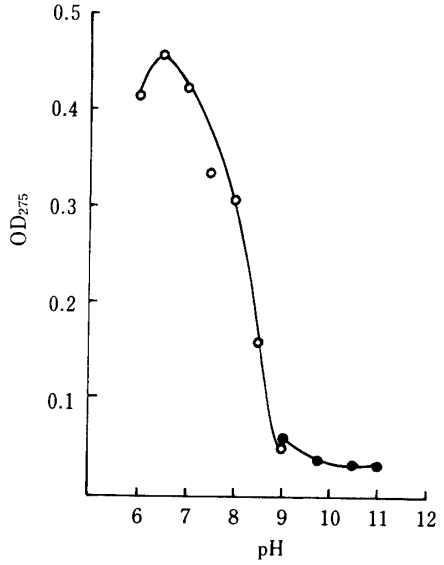

Fig. 3. Effect of pH on Caseinolytic Activity

A mixture of the aqueous enzyme solution (5.0 μg/ml) and 1% casein solution (1 ml) in 0.1 M Tris-HCl buffer (pH 6.0—9.0) or 0.1 M carbonate buffer (pH 9.0—11.0) was incubated at 37 °C for 20 min.

○, Tris-HCl buffer; ●, carbonate buffer.

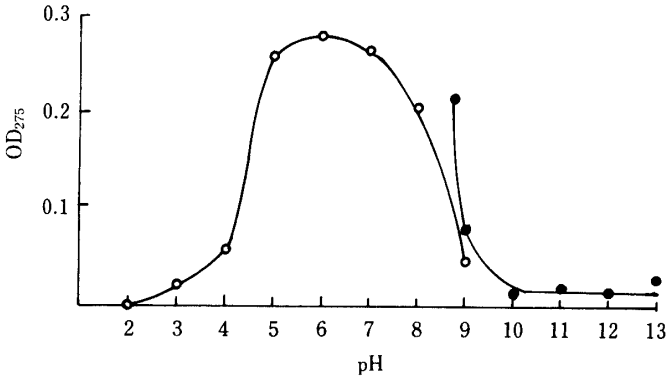

Fig. 4. Effect of pH on the Stability of the Purified Enzyme

A mixture of the aqueous enzyme solution (5.0 μg/0.1 ml) and 0.1 M buffer solution (0.1 ml) was left at room temperature for 1 h. After adjustment of the pH to 7.0 and the total volume to 1.0 ml, the enzyme solution was incubated with 1% casein solution (1 ml) in 0.1 M Tris-HCl buffer (pH 7.0) at 37 °C for 20 min.

○, phosphate-citrate buffer (pH 2.0—9.0); ●, glycine-NaOH buffer (pH 8.75—13.0).

Hydrolysis of Synthetic Substrates

Both Z-Gly-Leu-NH_2 and Z-Gly-Phe-NH_2 were hydrolyzed well by the enzyme among the synthetic substrates tested. The K_m value of the enzyme-catalyzed hydrolysis of Z-Gly-Leu-NH_2 was 3.4 mM as determined from Lineweaver-Burk plots. The results are listed in Table IV.

Discussion

Various enzyme inhibitors have been employed as specific ligands for affinity chromatog-

Table III. Effect of Various Chemicals on Casein Hydrolysis

Chemicals	Residual activity (%)
Ethylenediaminetetraacetate	7
N-Bromosuccinimide	3
Iodine	0
8-Hydroxyquinoline	90
L-Cysteine	97
Glutathione (reduced)	108
α, α′-Dipyridyl	108
Monoiodoacetate	93
p-Chloromercuribenzoate	125
Hydroxylamine hydrochloride	114
Phenylmethanesulfonyl fluoride	107

A mixture of the enzyme solution (10.0 μg/0.9 ml) in 0.1 M Tris-HCl (pH 7.0) and 10 mM inhibitor solution (0.1 ml) in the same buffer was kept at room temperature for 20 min. The mixture was incubated with 1% casein solution (1 ml) in 0.1 M Tris-HCl (pH 7.0) at 37 °C for 20 min. The absorbance at 275 nm was measured against the blank after addition of 10% TCA (3 ml) followed by centrifugation.

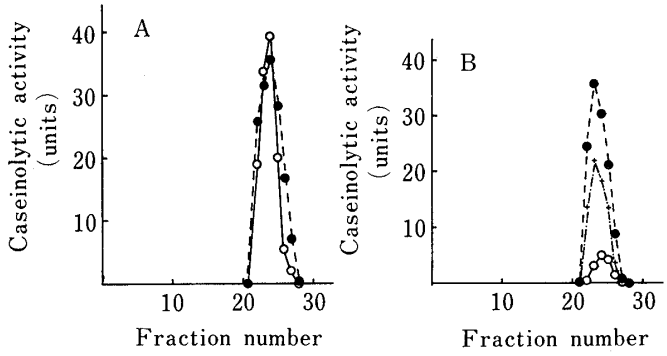

Fig. 5. Chromatography of Untreated and EDTA-Treated Enzymes on a Column of Sephadex G-10

(A) The purified enzyme (400 μg as protein) was charged on a Sephadex G-10 column (85.0 cm × 1.6 cm, diam.) which had been equilibrated with 50 mM phosphate buffer (pH 6.0). The enzyme was eluted with the same buffer and the eluate was collected in 4.0 ml fractions. The caseinolytic activity was measured by using 1% casein solution in 0.1 M Tris-HCl (pH 7.0) in the absence (○) and presence (●) of 1 mM $ZnCl_2$.

(B) The enzyme (360 μg as protein) was treated with 1 mM EDTA at room temperature for 30 min prior to gel filtration. The caseinolytic activity was measured by using 1% casein solution in 0.1 M Tris-HCl (pH 7.0) in the absence of any metal ion (○) and in the presence of 1 mM $ZnCl_2$ (●) or 1 mM $CoCl_2$ (+).

raphy of microbial metalloendopeptidases. Morihara and Tsuzuki[3e] found that Z–Phe completely inhibited the hydrolysis of Z–Gly–Leu–Ala by neutral metalloendopeptidase from *Bacillus subtilis* and Fujiwara *et al.*[16] used *N*-benzyloxycarbonylphenylalanyltriethyltetraminyl-Sepharose to purify thermolysin and neutral metalloendopeptidase produced by *B. subtilis*. Recently Hiramatsu[17] reported the purification of neutral metalloendopeptidase from *Str. naraensis* using Z–Phe–AH-Sepharose as an affinity adsorbent. Phosphoramidon, *N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)leucyltryptophan, was isolated from culture broth of *Str. tanashiensis* as a powerful inhibitor of thermolysin by Suda *et al.*,[18] and Komiyama *et al.*[19] found that phosphoramidon was a suitable ligand for affinity chromatography of thermolysin and neutral metalloendopeptidase from *B. subtilis*. On the other hand, the authors[1] employed Z–Gly–Leu–AH-Sepharose in the purification of an alkaline metalloendopeptidase named alkinonase A from *Str. violaceorectus*, based on the structural resemblance of the adsorbent to Z–Gly–Leu–NH_2 which is one of the best substrates of this enzyme. Alkinonase A was adsorbed on Z–Gly–Leu–AH-Sepharose from Tris-HCl buffer (pH 9.0) and recovered by elution with phosphate buffer (pH 7.0). Although the K_m value for *Str. griseoruber* enzyme-catalyzed hydrolysis of Z–Gly–Leu–NH_2 was almost equivalent to that for the alkinonase A-catalyzed hydrolysis, the enzyme from *Str. griseo-*

TABLE IV. Hydrolysis of Synthetic Substrates

Amino acid or peptide	Hydrolysis rate (relative value)
Z–Gly–Pro–Leu–Gly	1
Z–Gly–Pro–Leu	0
Z–Gly–Leu–Tyr	1
Z–Gly↓Leu–NH_2	100
Z–Gly↓Phe–NH_2	155
Z–Pro–Leu–NH_2	1
Z–Gly–D-Leu–NH_2	3
Z–Gly–Leu	0
Z–Gly–Phe	0
Z–Glu–Tyr	0
Z–Phe–Tyr	4
Z–Gly	2
Z–Phe	0
Ac–Tyr–OEt	0
ClAc–Tyr	0

A mixture of 5 mM substrate solution (250 μl) in 50 mM Tris-HCl (pH 7.0) and the aqueous enzyme solution (25.0 μg/250 μl) was incubated at 37 °C for 10 min. After addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution[15] (500 μl), the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against the blank and the absorbance with Z–Gly–Leu–NH_2 was defined as 100. Ac–, Acetyl; ClAc–, chloroacetyl.

ruber seemed to have much higher affinity for Z–Gly–Leu–AH-Sepharose at the temperature used for affinity chromatography (4 °C) as compared with alkinonase A. Neither a lower pH value nor addition of 2 M urea was sufficient for elution of the enzyme from this adsorbent. Furthermore, urea could not be replaced by NaCl. As regards the pH of the eluting buffer, the enzyme was unstable at pH values lower than 5.0, as shown in Fig. 4, and the eluate should be immediately neutralized with 1 N NaOH to minimize inactivation of the enzyme.

The interactions between the proteolytic enzymes and the two amino acid residues which form the scissile bond of the substrate are the basis of the primary specificity. The enzyme often also interact with other amino acid residues of the substrate and these interactions lead to a secondary specificity.[20] The hydrolysis of *N*-benzyloxycarbonyl peptides such as Z–Gly–Leu–Ala and Z–Gly–Leu–Phe by neutral metalloendopeptidase from *B. subtilis* was about 30-fold greater than that of Z–Gly–Leu–NH_2.[3e] The authors[1] also observed that alkinonase A hydrolyzed Z–Gly–Leu–Tyr to Z–Gly and Leu–Tyr at a rate about 35-fold greater than that for Z–Gly–Leu–NH_2. The rate of hydrolysis of Z–Gly–Leu–Tyr by neutral metalloendopeptidase from *Str. griseoruber*, however, was almost negligible compared with that of Z–Gly–Leu–NH_2 (Table IV). The factors which contribute to this effect remain to be elucidated.

References

1) Y. Inouye, Y. Kawaguchi and S. Nakamura, *Chem. Pharm. Bull.*, **32**, 2333 (1984).
2) Y. Inouye, S. Nakamura, M. Hamada, K. Aikawa, K. Kitagaki, K. Sato and E. Yasuda, *Chem. Pharm. Bull.*, **32**, 4036 (1984).
3) *a*) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka, T. Takeuchi and H. Umezawa, *Chem. Pharm. Bull.*, **17**, 714 (1969); *b*) S. Nakamura, Y. Marumoto, H. Miyata, I. Tsukada, N. Tanaka, M. Ishizuka and H. Umezawa, *ibid.*, **17**, 2044 (1969); *c*) S. Nakamura, M. Hamada, M. Ishizuka and H. Umezawa, *ibid.*, **18**, 2112 (1970); *d*) S. Nakamura, M. Hamada and H. Umezawa, *ibid.*, **18**, 2577 (1970); *e*) K.

Morihara and H. Tsuzuki, *Arch. Biochem. Biophys.*, **146**, 291 (1971); *f*) S. Nakamura, H. Fukuda, T. Yamamoto, M. Ogura, M. Hamada, M. Matsuzaki and H. Umezawa, *Chem. Pharm. Bull.*, **20**, 385 (1972).
4) K. Morihara and M. Ebata, *J. Biochem.* (Tokyo), **61**, 149 (1967); K. Morihara, H. Tsuzuki and T. Oka, *Arch. Biochem. Biophys.*, **123**, 572 (1968); K. Morihara and H. Tsuzuki, *Eur. J. Biochem.*, **15**, 374 (1970).
5) J. Feder, *Biochemistry*, **6**, 2088 (1967).
6) K. Morihara, T. Oka and H. Tsuzuki, *Arch. Biochem. Biophys.*, **132**, 486 (1969).
7) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **16**, 313 (1966).
8) S. Wada, "Guide to Color Standard," Nippon Shikisai Co., Ltd., Tokyo, 1954.
9) B. Becker, M. P. Lechevalier, R. E. Gordon and H. A. Lechevalier, *Appl. Microbiol.*, **12**, 421 (1951).
10) T. H. Lowry, N. T. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) B. Hagihara, N. Matsubara, J. Nakai and K. Okunuki, *J. Biochem.* (Tokyo), **45**, 185 (1957).
12) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
13) T. G. Pridham and H. D. Tresner, "Bergey's Manual of Determinative Bacteriology," 8th ed., The Williams and Wilkins Co., Baltimore, 1974, pp. 748—829.
14) E. B. Shirling and D. Gottlieb, *Intern. J. Syst. Bacteriol.*, **18**, 69 (1968); *idem, ibid.*, **18**, 279 (1968); *idem, ibid.*, **19**, 391 (1969); *idem, ibid.*, **22**, 265 (1972).
15) S. Moore, *J. Biol. Chem.*, **243**, 6281 (1968).
16) K. Fujiwara, K. Osue and D. Tsuru, *J. Biochem.* (Tokyo), **77**, 739 (1975).
17) A. Hiramatsu, *Agric. Biol. Chem.*, **46**, 371 (1982).
18) H. Suda, T. Aoyagi, T. Takeuchi and H. Umezawa, *J. Antibiot.*, **26**, 621 (1973).
19) T. Komiyama, T. Aoyama, T. Takeuchi and H. Umezawa, *Biochem. Biophys. Res. Commun.*, **65**, 352 (1975).
20) J. S. Fruton, "Proteases and Biological Control," ed. by E. Reich, D. B. Rifkin and E. Shaw, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1975, p. 33.