Serratia marcescens-Lytic Enzyme Produced by *Micromonospora* sp. Strain No. 152

Keitarou SUZUKI, Masaru UYEDA and Motoo SHIBATA

Laboratory of Medicinal Microbiology, Faculty of Pharmaceutical Sciences,
Kumamoto University, Kumamoto 862, Japan

Received November 5, 1984

A strain of *Micromonospora* sp. producing a lytic enzyme toward *Serratia marcescens* was isolated from soil. The lytic enzyme, called 152-enzyme, was purified from the culture filtrate by salting-out with ammonium sulfate, DEAE-cellulose column chromatography, and gel filtration on Sephadex G-75. The molecular weight of 152-enzyme was 17,000 and the isoelectric point was pH 7.3. The 152-enzyme showed lytic activity toward *S. marcescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Escherichia coli*, and *Bacillus subtilis*, but was completely inert toward *Staphylococcus aureus*. The enzyme also showed caseinolytic activity. The lytic and caseinolytic activities of 152-enzyme were maximum around pH 11.0 and at 60°C. Both activities were inhibited by DFP and API-2c. Liberation of amino groups from cell walls of *P. aeruginosa* by incubation with 152-enzyme suggested that the enzyme was a kind of cell wall-lytic peptidase.

Numerous bacteriolytic enzyme have been reported,^{1~9)} but most of them lyse Gram-positive bacteria but not Gram-negative bacteria. The surface structures of Gram-negative bacteria are exceedingly complex. The inner layer is composed of peptidoglycan inclosed by the outer layer of mostly lipopolysaccharide, lipoprotein, and lipid.^{10~12)} Ghuysen wrote that the outer multiplet-track layers of the envelope function as a barrier and prevent most lytic enzymes from reaching the underlying peptidoglycan.¹³⁾ Only a few lytic enzymes^{14,15)} were reported to lyse Gram-negative bacteria. Such enzymes may be convenient for the elucidation of the cell wall structure, and may also have some clinical significance in infectious diseases caused by low-toxic Gram-negative bacteria.¹⁶⁾

To obtain a new lytic enzyme active toward *S. marcescens*, we screened various microorganisms, and a lytic enzyme was found in the culture filtrate of *Micromonospora* sp. strain No. 152, which was isolated from a soil sample. The purified enzyme showed lytic activity toward various Gram-negative bacteria. By comparing biochemical and physicochemi-

cal characteristics of the enzyme with those of other known enzymes, we found that this is a new lytic enzyme. Hereafter, this enzyme will be referred to as 152-enzyme. In this paper, the purification procedure and some properties of 152-enzyme are described.

MATERIALS AND METHODS

Microorganisms and materials. *Bacillus subtilis* IFO 3007, *Escherichia coli* IFO 3301, *Proteus vulgaris* IFO 3167, *Pseudomonas aeruginosa* IFO 3448, *Serratia marcescens* IFO 3046, and *Staphylococcus aureus* IFO 3060 were cultures kept in our laboratory. API-2c was prepared from the culture filtrate of *Streptomyces griseoincarnatus* strain No. KTo-250 by the procedure described in our previous paper.¹⁷⁾ Benzalkonium chloride (Takeda Chemical Ind., Ltd.), brij-35 (Wako Pure Chemical Ind., Ltd.), chlorhexidine (Sumitomo Chemical Ind.), diisopropylfluorophosphate and ethylenediaminetetraacetic acid (Katayama Chemicals, Ltd.) and Pharmalyte (Pharmacia Fine Chemicals) were purchased. Other reagents were guaranteed grade.

Cultural conditions and preparation of the cells. To prepare the substrate for the lytic enzyme, *S. marcescens* was aerobically grown in a medium containing 1% peptone, 0.5% Ehrlich meat extract, and 0.3% NaCl (pH 7.0) at 37°C. After 24 hr of cultivation, the cells were harvested

by centrifugation under cooling, washed twice with chilled deionized water, and lyophilized. The cells were employed as the substrate of a lytic reaction system.

Screening test for microorganisms which produced *S. marcescens*-lytic enzyme. To obtain microorganisms producing lytic enzymes toward *S. marcescens*, soil samples were screened on *S. marcescens* cells-agar plates. To sterilized medium consisting of 0.8% agar and 0.1% K_2HPO_4 was added about 0.5% intact cells of *S. marcescens*, and the mixture was poured into a Petri dish as the bottom layer. After the layer solidified, 5 ml of 1.5% agar medium was poured over the bottom layer and a dilute suspension of soil was spread on the agar plate. The uniformly opaque agar plate was incubated at 28°C for 3 to 6 days and the colonies which were surrounded by zones of lysis were picked up on the agar slants of 0.1% dextrin, 0.1% peptone, 0.1% K_2HPO_4 , 0.5% *S. marcescens* cells, and 1.5% agar (pH 7.0). The ability of selected microorganisms to produce lytic enzymes was compared by cultivating them aerobically in a medium of 2% glucose, 3% starch, 1% corn steep liquor, 1% soybean flour, 0.5% peptone, 0.3% NaCl, and 0.5% $CaCO_3$ (pH 7.0) at 28°C for 1 to 5 days on a rotary shaker (180 rpm, 5 cm radius).

Assay of enzyme activity. Bacteriolytic activity was determined by measuring the degree of lysis of lyophilized cells suspended in 0.1 M Tris-HCl buffer (pH 8.0), to give an optical density of approximately 0.6 at 660 nm using a photometer ANA-7A, Tokyo Photoelectric Co., Ltd. To 2 ml of this suspension was added 1 ml of enzyme solution and the mixture was incubated at 37°C for 60 min. The optical density at 660 nm was measured before and after incubation. One unit of lytic activity was defined as the amount of enzyme causing a decrease in optical density of 0.001 per min.

Caseinolytic activity was measured in a mixture of 2 ml of enzyme solution and 1 ml of 0.2 M Tris-HCl buffer (pH 8.0) that was incubated at 37°C for 3 min, then 1 ml of 2% casein solution was added. After 15 min of incubation at 37°C, 2 ml of 12% perchloric acid was added, and the mixture was filtered after standing at room temperature for 30 min. The filtrate was assayed for enzyme activity by measuring the optical density at 660 nm according to Lowry's colorimetry.¹⁸⁾

Disc electrophoresis. Polyacrylamide gel disc electrophoresis was done at pH 9.4 (Tris-glycine buffer),¹⁹⁾ and pH 4.0 (β -alanine-acetate buffer).²⁰⁾ A sample (15 μ g protein) was put on the top of the stacking gel (7.5% acrylamide) and run at the constant current of 3 mA per gel column for 2 hr. After electrophoresis, the gel was stained with amino black 10B and destained with 7% acetic acid solution.

Isoelectric focusing on polyacrylamide gel was done using carrier ampholite (pH 3~10, Pharmalyte) by the method of Wrigley.²¹⁾ A sample solution was layered on

top of the gel (5% acrylamide, 2% ampholite) under a protecting layer of 2% carrier ampholite solution. Isoelectric focusing was done at 200 V for 3 hr using 0.02 M phosphoric acid as the anode solution and 1 M sodium hydroxide as the cathode solution. The gel was stained with 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid. The pH range in the gel was determined by migration distances of marker proteins (Oriental Yeast Co., Ltd.).

Other methods. Cell walls of *Pseudomonas aeruginosa* IFO 3448 were prepared by the method of Yoshimoto *et al.*¹⁵⁾ Detection of reducing power and amino groups was done by the methods of Somogyi and Nelson²²⁾ and Yemm and Cocking,²³⁾ respectively. The cell walls were hydrolyzed with 3 N HCl at 100°C for 2 hr for determination of total reducing power and with 6 N HCl at 115°C for 12 hr for total amino groups.

RESULTS AND DISCUSSION

Results of screening test

Four strains of actinomycetes producing lytic enzymes were obtained from about 500 test samples of soil. Strain No. 152 was finally chosen as the best producer of the lytic enzyme, therefore, further studies were made using this strain. Strain No. 152 forms well-developed, branched mycelium without aerial mycelium. A single sessile spore is produced on substrate mycelium. On most media, raised, folded, orange-colored growth developed well or moderately. In some cases, the orange color of the growth later turned to black; but no soluble pigment was produced. From these properties, the strain was concluded to be a *Micromonospora* species.

Course of lytic enzyme production by strain No. 152 culture

The cultural conditions, including the composition of the medium, were examined to obtain the highest production of lytic enzyme, and the optimal conditions are summarized in Table I. Changes in lytic activity and pH in the culture filtrate, and growth of mycelium are shown in Fig. 1. Almost linear growth was observed until 4 days after inoculation, and the growth reached a maximum after 5 days of cultivation. The accumulation of lytic activity began on the third day of cultivation, the lytic activity reached a maximum after 4 days and

thereafter its activity gradually decreased.

Production and purification of 152-enzyme

Strain No. 152 was cultivated for 4 days, and the mycelia and other residues were removed by filtration. To the clear filtrate was added solid ammonium sulfate to give 80% saturation. After standing overnight at 5°C, the precipitate was collected by centrifugation, dissolved in a minimal volume of 0.02 M Tris-HCl buffer (pH 8.0) and then dialysed against the same buffer for 2 days to remove ammonium sulfate. This dialysate solution was put on a DEAE-cellulose column which had been equilibrated with the above dialysing buffer. A lytic enzyme was eluted with the

same buffer containing linear concentrations of NaCl from 0 to 1 M. The elution pattern is shown in Fig. 2. The active fractions (tubes 41 to 47) were combined and solid ammonium sulfate was added to 80% saturation. The resulting precipitate was dissolved in a minimal volume of 0.02 M Tris-HCl buffer (pH 8.0) containing 0.01 M KCl, and then dialysed against the same buffer for 2 days at 5°C. Then the crude solution thus obtained was put on a Sephadex G-75 column previously equilibrated with the above dialysing buffer and eluted with the same buffer. With the active fractions, gel filtration was repeated under the

TABLE I. CULTURAL CONDITIONS FOR THE PRODUCTION OF 152-ENZYME

Strain used:	<i>Micromonospora</i> sp. strain No. 152
Seed culture medium:	glucose 2.0, starch 3.0, corn steep liquor 1.0, soybean flour 1.0, peptone 0.5, NaCl 0.3, CaCO ₃ 0.5 (%)
Main culture medium:	glucose 1.0, peptone 1.0, yeast extract 0.1, NaCl 0.1, K ₂ HPO ₄ 0.1, MgSO ₄ ·7H ₂ O 0.05 (%)
Initial pH:	7.0
Culture volume:	40 ml medium/200 ml flask
Inoculum size:	2 ml/40 ml medium (5% seed)
Culture temperature:	28°C
Agitation:	180 rpm

FIG. 1. Production by *Micromonospora* sp. Strain No. 152 in Culture.

●, lytic activity; ○, growth; □, pH.

FIG. 2. Column Chromatography of 152-Enzyme on DEAE-cellulose.

Column size, 2.5 × 20 cm; flow rate, 15 ml/hr; fraction size, 10 ml; buffer system, Tris-HCl buffer (0.02 M, pH 8.0) and the same buffer containing linear concentrations of NaCl from 0 to 1.0 M; ●, lytic activity; ○, absorbance at 280 nm; ---, NaCl concentration.

TABLE II. SUMMARY OF PURIFICATION STEPS OF 152-ENZYME FROM CULTURE FILTRATE

Fraction	Volume (ml)	Total lytic activity (unit)	Specific activity (unit/OD at 280 nm)	Recovery (%)	Purification rate
Culture filtrate	900	32,130	2.5	100.0	1.0
Ammonium sulfate ppt.	56	13,334	8.5	46.7	3.4
DEAE-cellulose eluate	78	7,426	31.7	23.1	12.7
1st Sephadex G-75 eluate	34	2,268	169.3	7.1	68.0
2nd Sephadex G-75 eluate	24	2,177	226.4	6.8	107.0

FIG. 3. Refiltration of 152-Enzyme on Sephadex G-75. Column size, 1.9×90 cm; flow rate, 10 ml/hr; fraction size, 5 ml; buffer, Tris-HCl (0.02 M, pH 8.0) containing 0.1 M KCl; ●, lytic activity; ○, absorbance at 280 nm.

same conditions. A typical elution pattern is shown in Fig. 3. The active fractions (tubes 23 to 26) were combined and dialysed against water to remove any salt, and then lyophilized.

Homogeneity of the purified 152-enzyme was examined by polyacrylamide gel disc electrophoresis at pH 9.4 and pH 4.0. As shown in Fig. 4, the enzyme migrated as a single, clear band, so it was regarded as homogeneous.

Table II gives a summary of the purification of 152-enzyme from culture filtrate. The enzyme was purified about 107-fold on the basis of protein (unit/OD at 280 nm) with a yield of 6.8% from culture filtrate. Finally, about 4.3 mg of the enzyme from 1 liter of culture filtrate was obtained as a white powder and a 1% solution of 152-enzyme had an absorbance of 20.9 at 280 nm. The lytic activities of the

FIG. 4. Polyacrylamide Gel Disc Electrophoresis of 152-Enzyme.

Polyacrylamide gel disc electrophoresis was done at pH 9.4 and 4.0, at 3 mA per gel column. Arrow indicates the position of sample applied.

enzyme toward lyophilized cells and intact cells of *S. marcescens* were 560 unit/mg and 107 unit/mg, respectively. The caseinolytic activity of the enzyme was 29 unit/mg.

Molecular weight and isoelectric point

The molecular weight of 152-enzyme was estimated by the method of Andrews²⁴⁾ with a Sephadex G-75 column at 5°C in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.1 M KCl. The ratio of elution volume to void volume was plotted against the logarithm of molecular weight for each reference protein (cytochrome *c*, AP-I,²⁵⁾ and ovalbumin) and 152-enzyme giving an approximate molecular weight of 17,000 for 152-enzyme.

The isoelectric point of 152-enzyme was measured by electrofocusing on a polyacrylamide gel containing carrier ampholite with a pH range of 3 to 10. The pH gradient generated was determined by the migration distance of marker proteins (acetylated cyto-

chrome c). The migration distance was plotted against the isoelectric point of each marker protein and 152-enzyme, giving an approximate isoelectric point of pH 7.3 for 152-enzyme.

Effects of pH on activity

The enzyme activities were measured while changing the pH from 5.0 to 12.0. As shown in Fig. 5, the optimal pH of 152-enzyme for the lysis of *S. marcescens* cells was 11.0. The optimal pH for caseinolytic activity was 11.0, the same as that of the lytic activity.

Effects of temperature on activity

The enzyme activities determined at various temperatures from 5°C to 80°C at pH 8.0 are shown in Fig. 6. The maximal activities were

FIG. 5. Effects of pH on Activity of 152-Enzyme.

The buffers used were acetate (pH 5.0), phosphate (pH 6.0~8.0) and borate (pH 9.0~12.0) in a concentration of 0.05 M. ●, lytic activity; ○, caseinolytic activity.

FIG. 6. Effects of Temperature on Activity of 152-Enzyme.

●, lytic activity; ○, caseinolytic activity.

observed at 60°C for both lytic and caseinolytic activities of 152-enzyme.

Effect of pH and temperature on stability

Stability of 152-enzyme was tested in buffer solutions of various pHs at 37°C for 20 hr. Residual activity was measured at pH 8.0. As shown in Fig. 7, 152-enzyme was most stable at pH 7.0, keeping 73% of the initial lytic activity.

After treatment at various temperatures for 60 min at pH 8.0, each remaining activity was assayed at 37°C. As shown in Fig. 8, 152-enzyme was stable up to 50°C, but gradual inactivation occurred above 50°C, and the enzyme was completely inactivated at 70°C.

FIG. 7. Effects of pH on Stability of 152-Enzyme.

After incubation at 37°C for 20 hr, residual activity was measured at pH 8.0. ●, lytic activity; ○, caseinolytic activity.

FIG. 8. Effects of Temperature on Stability of 152-Enzyme.

After incubation at various temperatures for 60 min at pH 8.0, residual activity was measured at 37°C. ●, lytic activity; ○, caseinolytic activity.

Effects of metal ions on activity

The lytic and caseinolytic activities of 152-enzyme were measured with 1 mM metal ion in the standard assay solution. The reaction was initiated by addition of substrate after the enzyme was preincubated with metal ion at 37°C for 10 min. As shown in Table III, the lytic activity of 152-enzyme was inhibited completely by Zn^{2+} , Cu^{2+} , and Pb^{2+} ions. On the other hand, the caseinolytic activity of the enzyme was not affected by the metal ions tested.

Effects of various reagents on activity

The effects of various reagents on 152-enzyme was investigated by determining the remaining lytic and caseinolytic activities after incubation of the enzyme with reagents at pH 8.0 and 37°C for 10 min. The results are summarized in Table IV. Both activities of 152-enzyme were inhibited by DFP and API-2c¹⁷⁾ which is a specific inhibitor for microbial serine proteases, and only the lytic activity was also affected by the other reagents tested. It was thought that these reagents and metal ions such as Zn^{2+} , Cu^{2+} , and Pb^{2+} did not produce direct effects on the enzyme itself, but altered the surface structure of *S. marcescens* cells, because the caseinolytic activity of the enzyme was not affected by them.

Effects of the lytic enzyme on various bacteria

Susceptibility of lyophilized cells of various bacteria to 152-enzyme was tested. The results in Table V show the relative values for lysis, when the lytic activity of the enzyme toward *S. marcescens* was regarded as 100. Among Gram-negative bacteria, *P. aeruginosa* and *E. coli* as well as *S. marcescens* were lysed by 152-enzyme, and *P. vulgaris* was weakly lysed by the enzyme. *B. subtilis* was rather more susceptible to the enzyme than *S. marcescens*, but *S. aureus* was quite resistant.

Digestion of cell walls of *P. aeruginosa* by 152-enzyme

About 100 mg of *P. aeruginosa* cell walls was suspended in 10 ml of 0.1 M phosphate

TABLE III. EFFECTS OF METAL IONS ON ACTIVITY OF 152-ENZYME

Metal ion (1 mM)	Relative lytic activity (%)	Relative caseinolytic activity (%)
None	100	100
KCl	113	101
NaCl	108	102
CaCl ₂	142	115
MnCl ₂	73	98
MgSO ₄	74	108
FeSO ₄	49	85
ZnSO ₄	8	79
CuSO ₄	0	95
Pb(CH ₃ COO) ₂	0	95

TABLE IV. EFFECTS OF VARIOUS REAGENTS ON ACTIVITY OF 152-ENZYME

Reagent	Concentration (%)	Relative lytic activity (%)	Relative caseinolytic activity (%)
None		100	100
EDTA	1 mM	201	99
DFP	1 mM	0	9
PCMB	1 mM	144	95
SDS	1 mM	202	83
Brij-35	1 mM	160	93
API-2c	100 µg/inc. mix.*	25	30
Benzalkonium chloride	100 µg/inc. mix.*	42	98
Chlorhexidine	100 µg/inc. mix.*	40	91

EDTA, ethylenediaminetetraacetic acid; DFP, diisopropylfluorophosphate; PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate; API-2c, alkaline protease inhibitor.

* Incubation mixture (3 ml).

TABLE V. EFFECTS OF 152-ENZYME ON VARIOUS BACTERIA

Bacteria	Relative lytic activity (%)
<i>Serratia marcescens</i>	100
<i>Pseudomonas aeruginosa</i>	96
<i>Escherichia coli</i>	91
<i>Proteus vulgaris</i>	39
<i>Staphylococcus aureus</i>	0
<i>Bacillus subtilis</i>	187

FIG. 9. Digestion of Cell Walls of *Pseudomonas aeruginosa* by 152-Enzyme.

●, amino groups; ○, reducing power; □, turbidity.

buffer (pH 8.0), and incubated at 40°C with 350 μ g (196 unit) of 152-enzyme. Aliquots of the reaction mixture were periodically withdrawn, and the amino groups and reducing power liberated were measured. Figure 9, shows the course of changes in turbidity, and the increase in amino groups and reducing power during incubation of cell walls with 152-enzyme. 152-Enzyme liberated amino groups with a decrease in optical density, but no significant release of reducing power was observed. The enzyme was inhibited by DFP and it liberated amino groups from cell walls. These results suggest that 152-enzyme is a kind of endopeptidase in the serine protease group.

152-Enzyme showed two activities, lytic and caseinolytic, as in cases of *Sorangium* α -, β -enzyme,^{26,27)} *Myxobacter* AL-1 enzyme,²⁸⁾ MR-enzymes from *Streptomyces albus* G,^{29,30)} and Fl-enzyme from *Streptomyces griseus*.³¹⁾ However, 152-enzyme was distinguished from these lytic enzymes because these lytic enzymes are inactive toward Gram-negative bacteria such as *S. marcescens*, *P. aeruginosa*, *E. coli*, and *P. vulgaris*, which 152-enzyme lyses. A lytic enzyme from *Clostridium perfringens*,³²⁾ B-enzyme from *Bacillus subtilis* YT-25,¹⁴⁾ and *Pseudomonas aeruginosa*-lytic enzyme from *Streptomyces griseus* P-51¹⁵⁾ act on Gram-negative bacteria. 152-Enzyme was also distinguished from these enzymes, since they are glucosaminidases or muramidases.

Thus, all data reported above suggest that 152-enzyme from *Micromonospora* sp. strain No. 152 is a unique lytic enzyme which lyses Gram-negative bacteria such as *S. marcescens* and *P. aeruginosa*. The mode of action and substrate specificity of 152-enzyme are now under investigation, and the result will be described in our next paper.

REFERENCES

- 1) M. McCarty, *J. Exp. Med.*, **96**, 555 (1952).
- 2) Y. Satomura, S. Okada and J. Fukumoto, *Nippon Nôgeikagaku Kaishi*, **31**, 281, 801 (1957).
- 3) K. Kato, S. Kotani, T. Matsubara, J. Kotani, S. Hamamoto, H. Chimori and I. Kazekawa, *Biken's J.*, **5**, 117 (1962).
- 4) J. M. Ghuysen, D. J. Tipper, C. H. Birge and J. L. Strominger, *Biochemistry*, **4**, 2245 (1965).
- 5) S. Kotani, *Protein, Nucleic Acid and Enzyme*, **13**, 1136 (1968).
- 6) T. Yoshimoto, T. Nakanishi, J. Fukumoto and D. Tsuru, *Agric. Biol. Chem.*, **35**, 1775 (1971).
- 7) K. Yokogawa, S. Kawata and Y. Yoshimura, *Agric. Biol. Chem.*, **36**, 2055 (1972).
- 8) K. Hayashi, T. Kasumi, N. Kubo and N. Tsumura, *Agric. Biol. Chem.*, **45**, 2289 (1981).
- 9) S. Ogata, Y. Tahara, S. Noji, S. Hayashida and M. Hongo, *Nippon Nôgeikagaku Kaishi*, **57**, 17 (1983).
- 10) J. P. H. Pelzer, D. Maass and W. Weidel, *Biochem. Biophys. Acta*, **46**, 68 (1961).
- 11) C. A. Schnaitman, *J. Bacteriol.*, **108**, 553 (1971).
- 12) H. D. Heilman, *Eur. J. Biochem.*, **31**, 456 (1972).
- 13) J. M. Ghuysen, *Bacteriol. Rev.*, **32**, 425 (1968).
- 14) S. Murao and Y. Takahara, *Agric. Biol. Chem.*, **38**, 2305 (1974).
- 15) T. Yoshimoto, S. Hayashida, H. Yamasaki and T. Tsuru, *J. Ferment. Technol.*, **53**, 703 (1975).
- 16) J. Honma, *Jap. J. Bacteriol.*, **27**, 629 (1972).
- 17) K. Suzuki, M. Uyeda and M. Shibata, *Agric. Biol. Chem.*, **42**, 1539 (1978).
- 18) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 19) B. J. Davis, *Ann. New York Acad. Sci.*, **121**, 404 (1964).
- 20) R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).
- 21) C. W. Wrigley, "Methods in Enzymology," ed. by W. B. Jakoby, Vol. 22, Academic Press, New York, 1971, p. 559.
- 22) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
- 23) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).
- 24) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
- 25) M. Uyeda, K. Suzuki and M. Shibata, *Agric. Biol.*

- Chem.*, **40**, 1479 (1976).
- 26) D. R. Whitaker, L. Jurašek and C. Roy, *Biochem. Biophys. Res. Commun.*, **24**, 173 (1966).
- 27) D. R. Whitaker, "Methods in Enzymology," Vol. 19, Academic Press, 1970, p. 599.
- 28) J. C. Ensign and R. S. Wolfe, *J. Bacteriol.*, **91**, 524 (1968).
- 29) J. F. Petit, E. Munoz and M. Ghuysen, *Biochemistry*, **5**, 2764 (1966).
- 30) J. M. Ghuysen, L. Dierickx, J. Coyette, M. Leyh-Bouille, M. Guinand and J. N. Campbell, *Biochemistry*, **8**, 213 (1969).
- 31) T. Yoshimoto and D. Tsuru, *J. Biochem.*, **72**, 379 (1972).
- 32) H. H. Martin and S. Kemper, *J. Bacteriol.*, **102**, 347 (1970).
-