ANTIMICROBIAL ACTION OF ε-POLY-L-LYSINE

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The antimicrobial spectrum of ε-poly-L-lysine (n=25~30, ε-PL) was investigated by comparison with α-poly-L-lysine (n=50, α-PL). ε-PL showed antimicrobial activity against Gram-positive and -negative bacteria at concentrations of 1~8 μg/ml. α-PL was less active than ε-PL. A chain length of at least 10 L-lysine monomers was found to be optimum for antimicrobial activity. Chemical modification of the amino groups of ε-PL lowered its antibacterial activity. Studies on the mode of action of ε-PL suggest that adsorption of ε-PL to the bacterial cell surface plays an important role in its antibacterial activity.

Many peptide antibiotics have been isolated from microorganisms. These antibiotics generally consist of more than two different amino acids and sometimes contain unusual amino acids. We have isolated a novel homopolymer of L-lysine, ε-poly-L-lysine (n=25~30, ε-PL) from culture filtrates of *Streptomyces albulus*[1,2,3]. We have reported that ε-PL showed antiphage action, depending more on the phage morphology than the phage nucleic acid. Furthermore, the antiphage action was significantly enhanced by the presence of ferrous ions[4]. KATCHALSKI *et al.* reported that homopolymers of basic amino acids exhibited wide biological effects[5], but there are limited studies of the antibiotic activity of such homopolymers. In this paper, a detailed analysis of the antimicrobial effects of ε-PL, α-poly-L-lysine (n=50, α-PL) and of related compounds will be described.

Materials and Methods

ε-PL (n=25~30) used in this study was obtained from *S. albulus*[1]. α-PL (n=50) was purchased from Sigma Co. Ltd. [^{14}C]-ε-PL was prepared from [^{14}C]-L-lysine as previously described[6] using washed mycelium of *S. albulus*.

Antimicrobial Activity Measurement

Microorganisms tested in this study were listed in Table 1. Minimum inhibitory concentrations (MIC) were determined by the broth dilution method as follows; bacteria were inoculated into nutrient broth (Difco) containing 1×10^6 cells/ml and incubated aerobically at 30°C for 18 hours. Fungi were tested in medium consisting of 1.0% yeast extract, 0.2% glucose and 0.5% NaCl (pH 5.5), with a 2.0% inoculum and incubated at 30°C for 24 hours.

Chemical Modification of ε-PL

Free amino groups of ε-PL and various carboxyl groups of other compounds were conjugated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the elimination reagent. A typical experiment was carried out as follows; the reaction mixture (5 ml) containing 100 mg of ε-PL, 50 mg of EDC and a carboxylic acid (molar ratio to lysine residues of ε-PL was in the range of 1:1~1:5) was incubated for 3 hours at 37°C, desalted by gel chromatography over Sephadex G-25. The fractions containing modified ε-PL were collected and evaporated to small volume. Addition of organic solvent

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Table 1. Antimicrobial spectrum of ε-PL and α-PL.

Test organisms	MIC (μg/ml)		Test organisms	MIC (μg/ml)	
	ε-PL	α-PL		ε-PL	α-PL
Penicillium urticae IFO 7011	>256		*Proteus vulgaris* IFO 3851	2	32
P. chrysogenum IFO 4897	256		*Serratia marcescens* IFO 12648	8	>64
Aspergillus niger van. Tieghem IFO 4416	>256		*Aerobacter aerogenes* IFO 3317	8	32
Fusarium oxysporum IFO 5880	>256		*Alcaligenes faecalis* IFO 12669	8	>64
Saccharomycopsis lipolytica IFO 0746	256		*Bacillus brevis* IFO 3331	3	8
Candida tropicalis IFO 0589	128		*B. subtilis* IFO 12210	1	4
C. albicans	128		*B. cereus* IFO 3514	16	32
C. utilis	128		*Arthrobacter simplex* IFO 12609	8	
Saccharomyces cerevisiae	128		*A. globiformis* IFO 12140	8	16
Escherichia coli K-12 IFO 3301	1	8	*Corynebacterium xerosis* IFO 12684	2	4
E. coli F-2	2	8	*Micrococcus aurantiacus* IFO 12422	8	8
E. coli B IFO 13168	1	8	*M. roseus* IFO 3768	3	
E. coli C IFO 13892	1	4	*M. lysodeikticus* IFO 3333	2	
Pseudomonas putida IFO 3738	2	8	*M. luteus* IFO 3232	4	4
P. aeruginosa IFO 3923	3	16	*Staphylococcus aureus* IFO 3060	4	16
			Mycobacterium tuberculosis 607	32*	

* Determined at 24 hours after incubation.

(ethanol - ether mixture) yielded the modified ε-PL. Phenylazobenzoyl-ε-PL was prepared according to the method of IKENAKA[7]. Dinitrophenylation of ε-PL was performed as previously reported[3].

Partial Hydrolysis of ε-PL

Oligomers of L-lysine were prepared as previously described[3]. The composition of the oligomers was determined by the ninhydrin method.

Uptake of ε-PL by *Escherichia coli* K-12

[^{14}C]-ε-PL was added to mid to late logarithmic cultures of *E. coli* K-12 in Davis medium; 0.2% glucose, 0.1% ammonium sulfate, 0.05% sodium citrate, 0.7% K_2HPO_4, 0.2% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$, supplemented with 0.5% Casamino Acids, and further incubated at 30°C. At times indicated an aliquot was transferred into TB buffer (10 mM Tris-HCl, pH 7.3, 0.5 mM $MgCl_2$, 0.15 M NaCl) containing unlabeled ε-PL (1 mg/ml). A few minutes later, the cells were filtered on membranes (Toyo, 0.45 μm) and washed with TB (5~10 ml). It was demonstrated that this treatment removed only radiolabeled ε-PL attached to the cell surface. Radioactivity was measured by liquid scintillation using ACS-II (Amersham) as the scintillation fluid.

Experiment on Bacterial Respiration

Oxygen uptake by the test organism was measured using a Warburg manometer. The incubation mixture (final volume 3.0 ml) consisted of 1/30 M phosphate buffer (pH 7.1), 0.04 M glucose, ε-PL and bacterial cells; a filter strip moistured with KOH solution was placed in the center well. Incubation was at 37°C and measurements were at intervals of 10 minutes.

Incorporation of Labeled Precursors into Macromolecules

[*U*-^{14}C]Phenylalanine (513 Ci/mol), [2-^{14}C]thymine (49.6 Ci/mol) and [*U*-^{14}C]uridine (551 Ci/mol) were purchased from Amersham International Ltd., and [1-^{14}C]glucosamine (7 Ci/mol) was purchased from New England Nuclear. The test organism *E. coli* K-12 was cultivated in Davis medium supplemented with 0.5% Casamino Acids, 2 μg/ml thymine and 10 μg/ml uridine. To 100 ml of fresh medium 1.0 ml of an 18-hour culture of the organism was inoculated and incubated with shaking at 37°C. 0.1 μCi/ml of radiolabeled substrate was added at the mid-logarithmic phase (0.3 OD at 660 nm). 0.5 ml samples were placed in cold 10% trichloroacetic acid (TCA), the precipitates were harvested on glass microfiber filters (Whatman GF/C) and washed successively with chilled 5% TCA, ethanol, and ether and dried. Radioactivity was determined as described above.

Transmission Electron Microscopy

An overnight culture of *E. coli* K-12 was harvested, suspended in phosphate buffered (1/30 M, pH 7.1, PB) - saline (PBS) and treated with ε-PL (50 μg/ml) at 30°C for 2 hours. The cells were washed with PBS by centrifugation. The cell paste was fixed in soft agar (1% agar) and the agar block treated with 5% glutaraldehyde in water for 3 hours and washed with PB. Post fixation was done in 2% osmium tetroxide in PB for 2 hours. Thin sections were obtained from SPURR resin embedded material and stained with uranyl acetate and lead citrate. The samples were examined with a Hitachi H-300 transmission electron microscope operating at 70 kV.

Results and Discussions

Antimicrobial Activity of ε-PL

The growth inhibitory potencies of ε-PL against various microorganisms are shown in Table 1. ε-PL showed a wide antibacterial spectrum, inhibiting both Gram-positive and -negative bacterial growth at concentrations of 1~8 μg/ml except for *Mycobacterium tuberculosis*. Although some yeasts were inhibited at 128 μg/ml of ε-PL, most fungi and yeasts were not affected at 256 μg/ml. Compared with the α-PL (n=50), ε-PL (n=25~30) was more effective against bacteria.

The relationship between the molecular size of ε-PL and antibacterial activity was investigated. The antibacterial effect of various L-lysine oligomers is shown in Table 2. ε-PLs with more than 10 L-lysine residues clearly had antibacterial activity and activity was proportional to the number of residues; at least 10 L-lysine residues appear to be necessary.

Table 2. Antibacterial activity of ε-PL on *E. coli*, effect of number of lysine residue.

No. of L-lysine residue of ε-PL	MIC (μg/ml)
1	>200
2	>200
3	>200
4	>200
5	>200
6	>200
7	>125
8	>100
9	12
10	6.1
11	3.8
12	2.6
13	1.8
14	2.1
ε-PL (25~30)	1.0

Table 3. Comparison of antibacterial activities of ε-decalysine and native ε-PL.

Test organisms	MIC (μg/ml)	
	Decalysine	ε-PL
E. coli K-12	5	1
E. coli F-2	5	2
P. aeruginosa	7.5	3
P. vulgaris	5	2
A. aerogenes	7.5	8
A. faecalis	50	8
B. cereus	50	16
B. subtilis	5	1
A. simplex	1.5	8
C. xerosis	7.5	2
M. lysodeikticus	2.5	2
S. aureus	15	4

Table 4. Antibacterial activities of various derivatives of ε-PL.

Derivative	MIC (μg/ml)	
	E. coli K-12	*S. aureus*
Native ε-PL	1	4
p-Chlorobenzoyl	12	24
2,3-Dichlorobenzoyl	5.5	20
2,4-Dichlorobenzoyl	5.5	10
2,5-Dichlorobenzoyl	5.5	20
2,6-Dichlorobenzoyl	5.5	20
3,4-Dichlorobenzoyl	7.3	20
3,5-Dichlorobenzoyl	7.3	10
α-Lysyl	2.4	20
Guanidyl	2.4	16
Phenylazobenzoyl	5.5	20
Diaminobenzoyl	7.3	20
Thiophencarboxyl	3.8	20
Dinitrophenyl	10	20

Fig. 1. Uptake of ε-PL in *E. coli* K-12.

[^{14}C]-ε-PL was added to *E. coli* K-12 broth. Radioactivity incorporated into cells was measured at indicated times.

○ [^{14}C]-ε-PL, ● [^{14}C]-ε-PL+NaN_3.

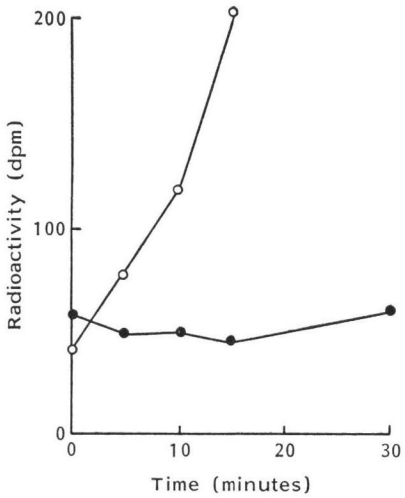
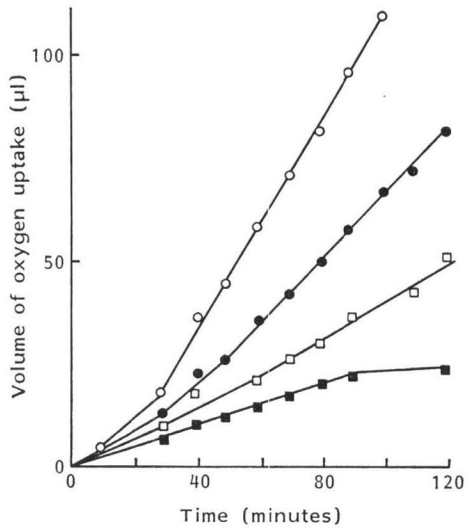


Fig. 2. Effect of ε-PL on the respiration of *E. coli* K-12 in the presence of glucose.

Population of cells in the reaction mixture was 6×10^8 cells/ml. Concentration of ε-PL; ○ 0 μg/ml, ● 8 μg/ml, □ 16 μg/ml, ■ 64 μg/ml.



The antibacterial spectrum of ε-decalysine, the shortest oligomer with activity was compared with that of ε-PL (n=25~30). The results are shown in Table 3.

Attempts to alter the antibacterial activity of ε-PL by chemical modification were carried out. Activities of the derivatives are shown in Table 4. Antibacterial activity was diminished by chemical modification of the amino groups. Conjugation of lysine to the α-amino group or addition of a guanidyl radical to the ε-amino group of ε-PL resulted in some reduction of antibacterial activity against *E. coli*. These results suggested that basic groups in the ε-PL molecule play a role in determining antibiotic activity.

Uptake of ε-PL by *E. coli* K-12

We noticed that various bacteria flocculated in the presence of ε-PL, and that large amounts of ε-PL were adsorbed. This is true also in the case of α-PL. These polymers were almost completely removed by washing with buffer (data not shown). Amounts of radioactivity not exchangeable by short term exposure (see Materials and Methods) to unlabeled ε-PL increased with time and this incorporation was completely inhibited by addition of sodium azide (Fig. 1). These results imply that ε-PL is incorporated into *E. coli* cells.

The Effect of ε-PL on Respiration of Test Organisms

The results shown in Fig. 2, indicate the relationship between oxygen uptake of *E. coli* and the concentration of ε-PL added in the medium. The presence of ε-PL decreased oxygen uptake by 10% at a concentration of 4 μg/ml and by 60% at 16 μg/ml. Fig. 3 shows the effect of preincubation of cells in the presence of ε-PL (8 μg/ml) on oxygen uptake by *E. coli*. These results indicate that the inhibitory effect of ε-PL increased with pre-incubation. One-hour pre-incubation in ε-PL reduced the oxygen uptake more than 50%, but was not completely repressed.

Fig. 3. Effect of pre-incubation with ε-PL on *E. coli* K-12 respiration.

Concentration of ε-PL in the reaction mixture was 8 μg/ml and population of cells was 6×10^8 cells/ml. ○ Control (no ε-PL), ● 0-hour pre-incubation, □ 1-hour pre-incubation, ■ 2-hour pre-incubation.

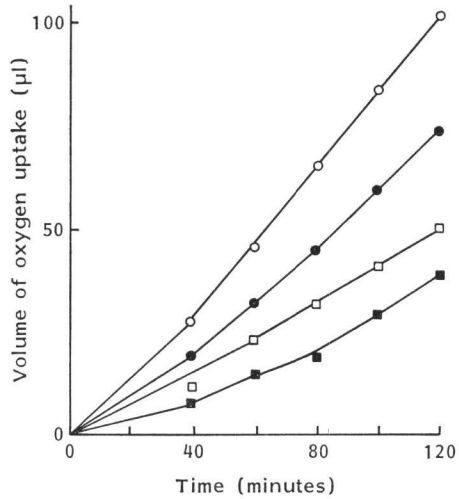


Fig. 4. Effect of ε-PL on DNA and RNA biosyntheses in *E. coli* K-12.

E. coli was cultured to mid-logarithmic phase, and ε-PL, [^{14}C]thymine and [^{14}C]uridine were added to the growing cultures. After 15 minutes, incorporation was stopped by TCA and the radioactivity of TCA-insoluble precipitates was determined. ○ [^{14}C]Thymine, □ [^{14}C]uridine.

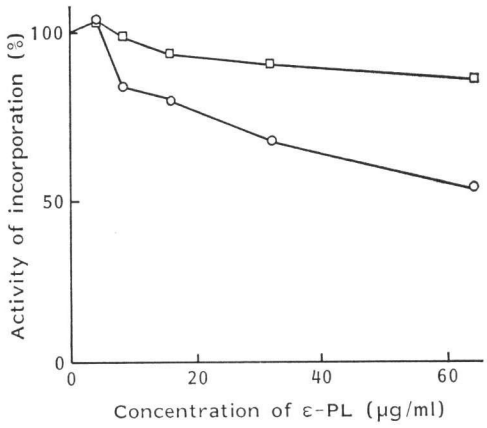


Fig. 5. Effect of ε-PL, α-PL and chloramphenicol (CM) on protein biosynthesis in *E. coli* K-12.

E. coli was cultured to mid-logarithmic phase and to the growing culture, drugs and [^{14}C]phenylalanine were added. After 15 minutes, the incorporation of [^{14}C]phenylalanine was stopped by TCA and the radioactivity of TCA-insoluble precipitates was determined. ○ ε-PL, □ α-PL, ● chloramphenicol.

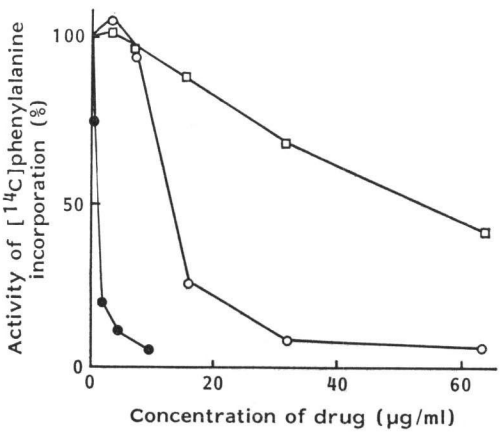
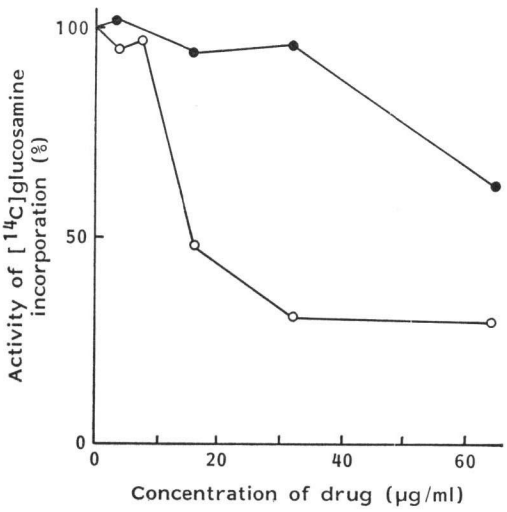


Fig. 6. Effect of ε-PL and α-PL on cell wall biosynthesis in *E. coli* K-12.

E. coli was cultured to mid-logarithmic phase, and to the growing culture, drugs and [^{14}C]glucosamine were added. After 1 hour, the radioactivity of TCA-insoluble precipitates was determined. ○ ε-PL, ● α-PL.



The Effect of ε-PL on the Biosyntheses of Macromolecules in *E. coli* K-12

The incorporations of [^{14}C]thymine and -uridine into TCA insoluble fractions of *E. coli* were not significantly inhibited by ε-PL (Fig. 4).

As shown in Fig. 5, the incorporation of [^{14}C]phenylalanine was inhibited 75% at 16 μg/ml of ε-PL and about 90% inhibition was observed about 32 μg/ml. Similar results were found using [^{14}C]-L-lysine as the precursor. Inhibitory effect of ε-PL was stronger than that of α-PL. These results agreed with the data of Table 1. Although inhibition by ε-PL was not as complete as with chloramphenicol (CM), the dose response curves were similar. Such inhibition could result from the interaction of ribosome with ε-PL.

Table 5. Effect of ε-PL on the short term uptake of phenylalanine and lysine and protein synthesis in *E. coli* K-12.

Substrate	Relative activity (%)	
	Uptake	Synthesis
Phenylalanine	127	8
Lysine	89	29

Concentration of ε-PL was 32 μg/ml. Uptake was measured for 20~30 seconds in buffer.

The incorporation of [^{14}C]glucosamine was also tested. As shown in Fig. 6, glucosamine incorporation was inhibited 70% at 32 μg/ml of ε-PL. In this case, ε-PL was more effective on inhibition than α-PL.

Protein synthesis was affected most strongly by ε-PL, whereas effects on uptake of precursor into cells were minimal in short term incorporation studies (20~30 seconds) (Table 5).

Electron Microscopic Studies

Recently, VAARA *et al.*[5,9] reported that various polycations including α-PL, sensitized enteric bacteria to several antibiotics. They suggested that this was due to an effect on the outer membrane by such polycations.

The morphological appearance of ε-PL-treated *E. coli* K-12 cells was studied by thin-section electron microscopy. Plate 1 shows untreated cells with typical undulating outer membrane and defined cytoplasmic structure. Plate 2 shows cells treated with ε-PL (50 μg/ml, 2 hours).

Thus, we would like to conclude that the electrostatic adsorption of ε-PL to the cell surface followed by stripping of the outer membrane, followed by abnormal distribution of the cytoplasm would produce the physiological damage to the ε-PL treated cell.

The mechanisms of antimicrobial action of α- and ε-PL were considered to be similar based on

Plate 1. Thin-section electron micrographs of normal *E. coli* K-12 cells.

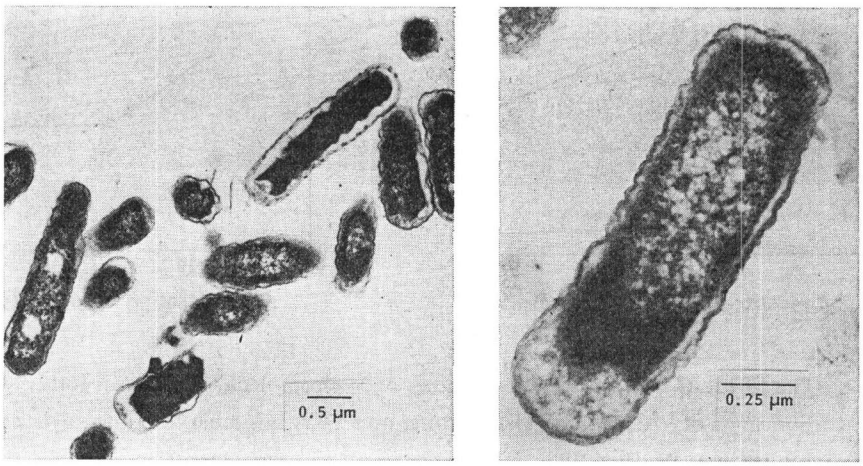
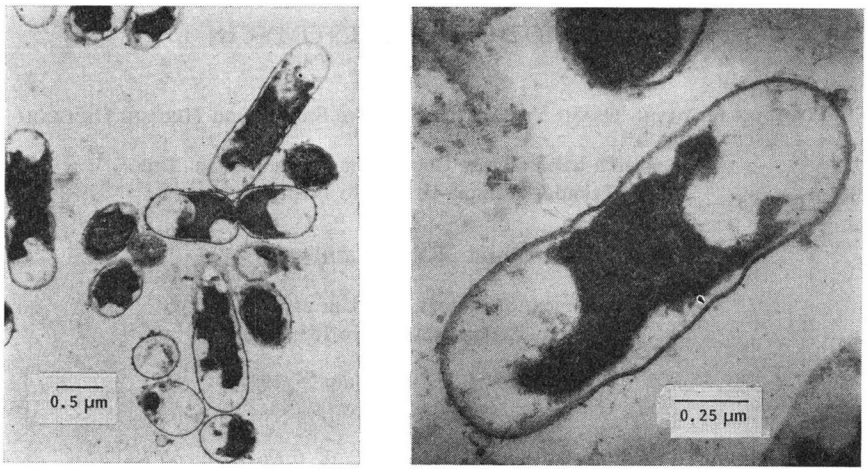


Plate 2. Thin-section electron micrographs of *E. coli* K-12 cells treated with ε-PL (50 μg/ml, 2 hours).



their known chemical properties, although some qualitative differences were noticed in their biological activities on *E. coli* and bacteriophages[4].

Acknowledgments

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References

1) SHIMA, S. & H. SAKAI: Polylysine produced by *Streptomyces*. Agric. Biol. Chem. 41: 1807~1809, 1977
2) SHIMA, S. & H. SAKAI: Poly-L-lysine produced by *Streptomyces*. II. Taxonomy and fermentation studies. Agric. Biol. Chem. 45: 2497~2502, 1981
3) SHIMA, S. & H. SAKAI: Poly-L-lysine produced by *Streptomyces*. III. Chemical studies. Agric. Biol. Chem. 45: 2503~2508, 1981
4) SHIMA, S.; Y. FUKUHARA & H. SAKAI: Inactivation of bacteriophages by ε-poly-L-lysine produced by *Streptomyces*. Agric. Biol. Chem. 46: 1917~1919, 1982
5) KATCHALSKI, E.; L. BICHOWSKI-SLOMNITZKI & B. E. VOLCANI: The action of some water-soluble poly-α-amino acids on bacteria. Biochem. J. 55: 671~680, 1953
6) SHIMA, S.; S. OSHIMA & H. SAKAI: Biosynthesis of ε-poly-L-lysine by washed mycelium. Nippon Nogeikagaku Kaishi 57: 221~226, 1983
7) IKENAKA, T.: Chemical modification of Taka-amylase A. II. Phenylazobenzoylation of Taka-amylase A. J. Biochem. 46: 297~304, 1959
8) VAARA, M. & T. VAARA: Polycation sensitize enteric bacteria to antibiotics. Antimicrob. Agents Chemother. 24: 107~113, 1983
9) VAARA, M. & T. VAARA: Polycation as outer membrane-disorganizing agents. Antimicrob. Agents Chemother. 24: 114~122, 1983