Enzymatic Degradation of Colistin

Isolation and Identification of α -N-Acyl α,γ -Diaminobutyric Acid and Colistin Nonapeptide

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Received November 22, 1972

Colistin, a fatty acyl peptide antibiotic, was attacked by proteolytic enzymes such as papain, ficin and bromelain, and as degradation product, a peptide portion retaining the ring structure of colistin was liberated. In contrast, an analogous antibiotic polymyxin B showed a characteristic resistance to the catalytic activity of papain.

Colistin nonapeptide and α -N-fatty acyl α,γ -diaminobutyric acid were obtained as products from the above enzymatic hydrolyzates of colistin and their chemical and physicochemical properties were investigated.

Contrary to colistin, this colistin nonapeptide was inactive to *Escherichia coli*. NIHJ and to many other strains even at a concentration of 800 mcg/ml by the agar dilution method. As α -N-fatty acyl α,γ -diaminobutyric acid which is rest part of colistin was added to colistin nonapeptide, antimicrobial activity of colistin nonapeptide did not increase.

Colistin, a fatty acyl oligopeptide antibiotic, is composed of 10 moles of amino acids such as L- α,γ diaminobutyric acid (Dab), L-threonine (L-Thr), L-leucine (L-Leu) and D-leucine (D-Leu) as well as one mole of 6-methyl octanoic acid (MOA) or isooctanoic acid (IOA). Suzuki *et al.*^{1~4)} clarified the primary structure of both homologs, recognizing the difference only in fatty acid component attached to the colistin decapeptide; MOA in colistin A while IOA in colistin B. Each fatty acid is linked to α -amino group of N-terminous Dab of colistin decapeptide, which is constituted of a cyclic heptapeptide moiety and straight tripeptide moiety attached to it. The above two colistin homologs, though different in fatty acid component, have practically the same antimicrobial activity with each other.⁵⁾

Despite its basic nature, colistin is not attacked by trypsin or chymotrypsin, while rapidly degraded by bacterial subtilopeptidase "nagarse." Ito *et al.*⁶⁾ have revealed that, during the course of colistin production, two kinds of colistin inactivating enzymes were liberated from cells of the strain producing

this antibiotic, *Bacillus polymyxa* var. *colistinus* KOYAMA and that the main portion of these enzymes having a strong caseinolytic activity was very similar to "nagarse" regarding the catalytic and immunological characteristics.

Paulus and Gray,⁷⁾ on the other hand, indicated that polymyxin B, a colistin analog differing from colistin only in D-amino acid component,⁵⁾ *i.e.* D-phenylalanine instead of D-Leu in the cyclic heptapeptide moiety of colistin, was cleaved into two fragments by catalytic action of papain or ficin, where α -N-fatty acyl Dab and branched cyclic nonapeptide, the so-called polymyxin nonapeptide, were regarded to be produced from polymyxin B. The fact that the cyclic heptapeptide moiety of colistin is hardly degraded by the action of proteolytic enzymes such as pronase, nagarse and papain was further pointed out by Suzuki *et al.*³⁾ in the course of their studies on the structure of colistin. Based upon the results obtained by these authors, colistin was then subjected to the enzymatic hydrolysis, using plant proteinases, such as papain, ficin

and bromelain and, same as in the case of polymyxin B mentioned above, α -N-fatty acyl Dab and colistin nonapeptide were isolated as degradative products by ficin or papain (Fig. 1), whereas a peptide different from colistin nonapeptide was obtained by bromelain hydrolysis. The results obtained are dealt with in this paper.

FIG. 1. Enzymatic Degradation of Colistin by Ficin.

F.A.: 6-methyl octanoic or isooctanoic acid.

MATERIALS AND METHODS

Colistin and polymyxin B. Colistin sulfate (19600 u/mg, Kayaku Antibiotics Research Laboratory Co., Ltd.) was used throughout this study, and polymyxin B hydrochloride (10000 u/mg, Taito Pfizer Co., Ltd.) was obtained from commercial source.

Enzymes. Nagarse (Nagase Sangyo Co., Ltd.), pronase (Kaken Chemical Co., Ltd.), papain (Kata-yama Kagaku Co., Ltd.) and bromelain (Nutritional Biochemicals Corporation) were purchased from market.

Other reagents. 2,4-Dinitrofluorobenzene was obtained from Tokyo Kasei Kogyo Co., Ltd. and silica gel H for thin-layer chromatography from E. Merk Co., Ltd.

Purification of ficin. As commercial preparation of ficin (Tokyo Kasei Kogyo Co., Ltd.) was very crude, its purification was required for the experimentation. The commercial ficin preparation was dissolved in 2% in 1/1000 M ethylenediaminetetraacetate (EDTA) solution with stirring, and insoluble pellets were centrifuged at 3000 rpm for 15 min and discarded, and then the supernatant was brought to 50% saturation with crystalline ammonium sulfate. The enzyme thereby precipitated was collected by centrifugation, dissolved in

a small amount of deionized water and dialyzed against running deionized water for 12 hr at 5°C. The dialyzed was clarified by centrifugation at 13000 rpm for 30 min and lyophilized. Pale straw-colored ficin was obtained in about 50% yield.

Isolation of fatty acyl Dab and colistin nonapeptide from colistin hydrolyzate by ficin. Colistin sulfate, dissolved in 1% in 0.1 M phosphate buffer, pH 7.0, was hydrolyzed by purified ficin preparation for 24 hr

at 37°C. Colistin hydrolyzate was clarified by centrifugation after the enzyme protein used was coagulated by boiling of the reaction mixture, and after acidified to pH 2.0 with 1 N HCl, the clarified solution was followed by extraction several times with a half volume of *n*-butanol to dissolve fatty acyl Dab. At the paper chromatography using solvent system of *n*-butanol, acetic acid and water (3:1:1) and detecting the spots with ninhydrin reaction, fatty acyl Dab migrated to the spot of *R_f* 0.76. The residual aqueous solution was adjusted to pH 8.0 with 1 N NaOH, and was repeatedly extracted by *n*-butanol to remove the remaining colistin. After again adjusting the aqueous layer to pH 7.0, it was passed through Amberlite IRA-410 column (OH type). The effluent was adjusted to pH 5.0, concentrated *in vacuo* and lyophilized. Pale colored amorphous powder was obtained and recognized as colistin nonapeptide from its peptide nature. Flow sheet of this isolation procedure is indicated in Fig. 2.

Purification of colistin nonapeptide by the counter current distributor. In this experiment, fully automatic counter current distributor was used. This apparatus (Mitamura Riken Kogyo Co., Ltd.) was equipped with 150 of extraction tube of 20 ml in content. For the separation of colistin nonapeptide, the following solvent system was used; *n*-butanol, *sec*-butanol and 0.1 N HCl, 6:30:40. After mixing these solvent, clear underlayer was poured into the all extraction tubes

FIG. 2. Isolation and Purification Process of α -N-Acyl-Dab (Fraction I) and Colistin Nonapeptide (Fraction II).

in 10 ml per tube, then 500 mg of crude colistin nonapeptide was dissolved in the second extraction tube. Continuous extraction with top layer of the above mixed solvent was carried out for 1100 transfers with recyclicalization of top layer.

Distribution of colistin nonapeptide in extraction tubes was quantitatively traced by colorimetric method with ninhydrin reagent applied to the aliquot of underlayer solution in each tube. The fractions containing colistin nonapeptide were collected in a separating funnel and discarded the top solvent layer after standing. The underlayer was adjusted to pH 5.5 with dil. NaOH, concentrated *in vacuo*, and desalted with Biogel P-2 column chromatography, followed by lyophilization. White fluffy pure colistin nonapeptide hydrochloride was obtained.

Preparation of various dinitrophenylated (DNP) amino acids. γ -DNP-Dab and α,γ -di-DNP-Dab were prepared by the method of Kurihara *et al.*,⁸⁾ while α -DNP-Dab by the method of Suzuki.⁹⁾ DNP-Thr and DNP-Leu were prepared by Sanger's method¹⁰⁾ from L-Thr, L-Leu.

Preparation of dinitrophenylated (DNP) colistin nonapeptide. Fifty milligrams of colistin nonapeptide was dissolved in 1 ml of 5% NaHCO₃ solution, and added to 2.1 ml of ethanol solution of 2,4-dinitrofluorobenzene (DNFB) of which concentration was 0.1 ml of DNFB in 2 ml of ethanol. Dinitrophenylation of the peptide was carried out under the dark con-

dition with shaking at 30°C for 4 hr. The DNP-peptide precipitated was filtered off through the glass filter, washed with a small amount of ether, subsequently with a trace amount of water and again with ether, then followed by drying. About 61.6 mg of DNP-colistin nonapeptide was obtained.

Identification of DNP-amino acid containing in peptides derived from colistin degradation. Dinitrophenylated peptides obtained by dinitrophenylation of peptides which were isolated from various enzyme hydrolyzates of colistin were subjected to hydrolysis with 6 N HCl for 16 hr at 110°C in a sealed tube in nitrogen atmosphere. The acid hydrolyzates thus obtained were dried up *in vacuo*, and the resultant DNP amino acids were analyzed by thin-layer chromatography on silica gel plate, using the solvent system of chloroform, methanol and acetic acid (90:5:1).

Identification of mono-DNP-Dab by paper electrophoresis. At the silica gel thin-layer chromatography above described, it was unable to separate α -DNP-Dab from γ -DNP-Dab because of no migration. Then, the mono-DNP-Dab fraction extracted with methanol from the original point of thin-layer chromatogram of silica gel plate, was subjected to the paper electrophoresis. In this experiment, the apparatus of Toyo Roshi Co., Ltd. was employed, using Toyo Roshi No. 50 filter paper. The apparatus was operated for 6 hr under the regular electric voltage at 200 V per 15 cm on width of filter paper in a 1/30 M phosphate buffer, pH 8.0. α -DNP-Dab migrated toward the anode about 49 mm from original point, while in turn γ -DNP-Dab migrated toward the cathode about 6 mm.

Quantitative determination of DNP-amino acids. The acid hydrolyzates of DNP-peptides were developed on a thin-layer plate and the areas occupied by each DNP-amino acid were cut off, eluted by methanol and brought to a defined volume with further addition of methanol. From the optical densities at 350 nm shown by each eluate and authentic DNP-amino acid used as control, molar ratio of DNP-amino acids contained in the DNP-peptide was calculated.

Gas liquid chromatography of fatty acids obtained from acid hydrolyzate of colistin and α -N-acyl Dab. Fatty acids obtained by ether extraction from acid hydrolyzates of colistin or α -N-acyl Dab were esterified with diazomethane in the ether solution. These analytical samples were quantitatively estimated by Hitachi gas liquid chromatograph type K-53 apparatus, where carrier was celite 545, liquid phase was polydiethylene glycol succinate and analysis carried out at 95°C.

Antibacterial assay of colistin and polymyxin B.

Escherichia coli NIHJ was used as test organism for the determination of antibacterial activity of colistin, its hydrolyzates and polymyxin B. Assay was carried out by agar diffusion method using pulp disk.

Acute toxicity of colistin nonapeptide. Male D. D. strain mice, weighing from 18 to 20 g, were used in this test. Colistin nonapeptide was dissolved in the physiological saline solution and injected into the tail venous at 0.5 ml per 20 g body weight of a mouse. At each amount dosed for the test, 5 mice were employed. After 72 hr feeding, LD 50 value was calculated by the Behrens and Kärber's method.¹¹⁾

RESULTS AND DISCUSSION

1. Degradation of colistin by various proteolytic enzymes

As mentioned already, colistin receives the degradative attack by various kinds of proteases. The colistin degrading activities of these enzymes such as nagarse, pronase, papain and purified commercial ficin preparation were compared with each other, using phosphate buffer, pH 7.0, and the temperature at 37°C, where each reaction mixture was incubated for 12 hr, and then the remaining colistin activity was assayed by agar diffusion method incubating for about 20 hr at 37°C. Table I

TABLE I. ENZYMATIC INACTIVATION OF COLISTIN

Enzyme reaction was carried out in 5.0 ml of 0.05 M phosphate buffer containing each enzyme at the concentration described below and 5 mg of colistin sulfate. After incubation at 37°C for 12 hr, residual activity of colistin in each reaction mixture was assayed by microbiological method.

Enzyme	mg/ml	Residual activity of colistin (%)
Nagarse	20	9.1
Pronase	100	59.5
Papain	100	72.5
Ficin	100	45.5

shows the result, and nagarse was most active, where about 90% of total colistin activity was lost by this proteinase at a concentration of 20 mcg/ml. In contrast, pronase, papain and ficin showed a rather weak activity and

even at a concentration of 100 mcg/ml the remaining activities of colistin were 60%, 72% and 45%, respectively. Thus, the plant proteinases such as papain and ficin showed the same level in colistin degrading activity as the streptomyces enzyme, pronase. The degradation activity of plant proteases such as papain, ficin and bromelain was then examined, using as substrate colistin and polymyxin B at a concentration of 1.5 mg/ml. In this case, the incubation period for the enzyme reaction was 20 hr, during which the remaining activity of the antibiotics was determined periodically. The results obtained are indicated in Fig. 3. For both antibiotics, ficin was of the strongest degrading activity, and within 20 hr incubation, colistin and polymyxin B had lost completely their antibacterial activity, whereas papain and bromelain could attain only to an extent of 50% in colistin degradation and to about 10% or less than this level in polymyxin degradation. Despite that catalytic activity of bromelain on polymyxin B exceeded that on colistin, the reverse was the case in the degradative activity of papain, in which polymyxin B was more resistant substrate than colistin.

FIG. 3. Comparison of the Inactivation Rate of Colistin and Polymyxin B by Papain, Ficin and Bromelain.

Enzyme reaction was carried out at 37°C for 24 hr in 5.0 ml of 0.1 M phosphate buffer, pH 7.0, containing 5 mg of enzyme and 7.5 mg of antibiotic. The course of enzyme reaction was followed by the microbiological assay of remaining antibiotic activity. ---, Papain; —, Ficin; ----, Bromelain.

In order to investigate the degradative products by these enzymes, the reacted mixture

(containing colistin as substrate together with each enzyme) was chromatographed on filter paper, using the solvent system of *n*-butanol, pyridine, acetic acid and water (15:10:3:12), where only a few ninhydrin positive spots appeared. The spot of colistin (R_f 0.54) gradually became faint with the elapsing time and correspondingly new ninhydrin positive spots (R_f 0.75 and 0.45) appeared, indifferently of the enzymes used. In each case, the degradation rate for colistin estimated from the loss of its antimicrobial activity proceeded in parallelism with the disappearance of the former spot, and each paper chromatogram showing the degradative products other than colistin as substrate was practically the same, although the colistin degrading activity of the used enzymes, *i.e.* papain, ficin and bromelain, was different from each other.

2. Isolation of the enzymatic hydrolyzate of colistin by ficin

As, of the plant proteases used, the purified ficin preparation showed the most active degradation on colistin as substrate, this preparation was employed for the investigation of degradative products from colistin. For this purpose, 10 g of colistin sulfate dissolved in 1 liter of 0.1 M phosphate buffer, pH 7.0, was degraded by 1 g of the purified ficin preparation at 37°C for 24 hr in the presence of a few drops of toluene. The enzyme protein in the reacted solution was removed by boiling and the subsequent centrifugation, then acidified to pH 2.0 with 1 N HCl. This is the initial step for the isolation of degradative products from colistin, as indicated in Fig. 2. Along with this flowsheet, the isolation proceeded and fraction I was obtained by acidic butanol extraction while fraction II by the subsequent column chromatography on Amberlite IRA 410 (OH type), where amorphous pale yellowish powder of fraction I was crystallized in colorless needles with aqueous acetone. Yield of fraction I was 73 mg while that of fraction II 270 mg when purified from 500 mg of crude preparation. Counter current distribution method was

employed as described in MATERIALS AND METHODS, as detected with ninhydrin reagent, a main peak was located at the tube number 24, forming a narrow symmetrical shape in distribution. From this peak fraction, a white fluffy amorphous powder was obtained as purified fraction II after desalting with Biogel P-2 and subsequent lyophilization.

FIG. 4. Counter Current Distribution of Colistin Nonapeptide (Fraction II).

Five hundred milligrams of crude colistin nonapeptide was charged. Transfer number: 1100. At intervals of every four tube, 0.5 ml of aqueous layer was analyzed by colorimetric method with ninhydrin reagent.

3. Identification of fraction I as α -N-acyl Dab

Fraction I, which was obtained as colorless needles, showing mp 175~177°C and $[\alpha]_D^{25} -4.7^\circ$ ($c=0.5$, water), was assumed as monohydrochloride of α -N-acyl Dab in view of agreement with R_f value of an acylated Dab shown by Paulus and Gray⁷⁾ in polymyxin B degradation. When fraction I was hydrolyzed with 6 N HCl in a sealed tube at 110°C for 24 hr, oily substance floated on the surface of the hydrolyzate solution. This oily substance was extracted by ether and esterified with diazomethane for gas liquid chromatographical analysis.

Then, it was revealed that the oily substance comprised two fatty acid components, *i.e.* MOA and IOA, of which contents were distributed practically in the same ratio as in the

original colistin (Table II). Thus, fraction I was recognized as a mixture of two compounds acylated by MOA or IOA, each of which was certainly derived from the substrate colistin because of its mixed nature, a mixture of colistin A and B.

TABLE II. MOLAR RATIO OF IOA AND MOA CONTAINED IN α -N-ACYL DAB (FRACTION I) AND COLISTIN

Acid hydrolyzates of α -N-acyl Dab and colistin were extracted with ether, and fatty acid liberated was esterified with diazomethane, and then quantitatively estimated by gas liquid chromatography.

	Fatty acid	
	IOA	MOA
α -N-Acyl Dab	1.0	2.8
Colistin	1.0	2.4

Paper chromatography and amino acid analysis of the above acid hydrolyzate indicated that fraction I contained only one amino acid, Dab, which was then isolated by evaporation of acid hydrolyzates after defatted with ether as a white amorphous powder showing mp 232°C. Elemental analysis of this powder was C; 27.76, H; 7.05, N; 16.20% (Calcd. C; 27.83, H; 7.59, N; 16.23%) and its $[\alpha]_D^{25}$ was +11.2° ($c=0.54$, water), showing L form, as based upon dextro rotation of L-Dab in reference.¹²⁾ It is evident that the amino acid component of fraction I is L- α , γ -diaminobutyric acid.

Molar ratio of fatty acid and L-Dab contained in fraction I was determined by weighing each of both components obtained from the above acid hydrolyzate, and was found to be in 1:1, as shown in Table III. Furthermore, in order to determine either of the α - and γ -amino group contained in Dab molecule as acylated by carboxyl group of fatty acid, fraction I was dinitrophenylated by usual method and hydrolyzed at 110°C for 24 hr with 6N HCl in a sealed tube. After ether extraction, the acid hydrolyzate was dried up *in vacuo*, then subjected to paper electrophoresis. As indicated in Fig. 5, only one spot

TABLE III. MOLAR RATIO OF FATTY ACID AND DAB IN α -N-ACYL DAB

Fatty acid and Dab were obtained from acid hydrolyzate of α -N-acyl Dab and weighed. On the basis of the following molecular weight, each molarity was determined: α -N-acyl Dab·HCl, 280.6; Octanoic acid, 144; Dab·2HCl, 191.

	α -N-Acyl Dab	Fatty acid	Dab
Weight (mg)	30.0	18.3	22.4
Molar ratio	1.0	1.19	1.10

FIG. 5. Paper Electrophoresis of Mono-DNP-Dab Contained in DNP-Colistin Nonapeptide.

In this experiment, paper strip (Toyo Roshi No. 50) was used in 1/30 M phosphate buffer, pH 8.0. Electric voltage between both electrodes was stabilized at 200 V per 15 cm width of the filter paper for 6 hr. 1. α -DNP-Dab. 2. γ -DNP-Dab. 3. mono-DNP-Dab fraction.

corresponding to the authentic γ -DNP-Dab as control migrated. Accordingly, the amino group linked to carboxyl group of fatty acid in the molecule of Dab as fraction I compound is ascribed to α -amino group of Dab. From the results obtained, it is concluded that fraction I is α -N-acylated L-Dab of which fatty acid component is a mixture of colistin fatty acids, MOA and IOA, derived from the original colistin preparation. In this connection, the optical rotation of the N-terminal Dab component in colistin or polymyxin B molecule was argued by several authors,^{5,7)} and, according to our results, dextro-rotatory Dab but not levo-rotatory Dab was found as N-terminous in ficin degradation of colistin.

The α -N-acylated Dab isolated in this case served to play a decisive role in the biosynthesis of colistin by cell-free preparation of *B. polymyxa* var. *colistinus*,¹³⁾ where each of free Dab and fatty acids given in association to the reaction system instead of N-acylated Dab were entirely inert as synthesizing material.

4. Identification of fraction II as colistin nonapeptide

The purified preparation of fraction II was obtained as hydrochloride, because of its basic nature, showing mp 234~238°C (decomp.), $[\alpha]_D^{25} -22.4^\circ$ ($c=0.57$, water) and pKa 6.9 and 9.6. Paper chromatography of acid hydrolyzate of this fraction gave three kinds of amino acid and, as determined by amino acid analyzer, amino acid composition was as follows: 5 moles of Dab, 2 moles of Leu and 2 moles of Thr (Table IV). For the

TABLE IV. AMINO ACID COMPOSITION OF COLISTIN NONAPEPTIDE (FRACTION II)

Amino acid	Molar ratio
Dab	4.8
Thr	1.8
Leu	2.0

determination of N-terminal amino acid of fraction II, it was dinitrophenylated, hydrolyzed with 6 N HCl at 110°C for 12 hr in a sealed tube, and the hydrolyzate was then subjected to thin-layer chromatography and paper electrophoresis. On the thin-layer chromatogram, there appeared three yellow spots of DNP-amino acids. When compared with the behaviors of variously dinitrophenylated authentic Dab and DNP-amino acids such as DNP-Thr and DNP-Leu on the thin-layer plate, the highest spot, though a very small one, corresponded to di-DNP-Dab while the middle spot to DNP-Thr and original spot without migration to mono-DNP-Dab, as shown in Fig. 6. As mono-DNP-Dab did not migrate at the thin-layer chromatography, the spot remaining at the original point was

FIG. 6. Thin-layer Chromatogram of Acid Hydrolyzate of DNP-Colistin Nonapeptide (Fraction II).

Thin layer plate: Silica gel H. Solvent system: Chloroform, methanol, acetic acid, 90: 5: 1.

1. DNP-Thr. 2. di-DNP-Dab. 3. α -DNP-Dab. 4. γ -DNP-Dab. 5. Acid hydrolyzate of DNP-colistin nonapeptide.

eluted with methanol and subjected to paper electrophoresis. Only one yellow spot migrating toward cathode showed a good agreement with the behavior of the authentic γ -DNP-Dab. The detection of γ -DNP-Dab and DNP-Thr in acid hydrolyzate of dinitrophenylated fraction II revealed that fraction II is a peptide, of which amino acid placed in N-terminus is Thr and that any molecule of Dab involved in fraction II is not terminated in the peptide bond of this fraction. In view of the minority of the spot of di-DNP-Dab, the isolation of such compound as dinitrophenylated in both amino groups of Dab molecule was considered to result from free Dab or a peptide containing Dab in N-terminus contaminated in fraction II as impurity, and thus this di-DNP-Dab was neglected. The molar ratio of DNP-Thr and γ -DNP-Dab contained in the eluates with methanol from the cut of each corresponding spot was further investigated. As indicated in Table V, the molar ratio of these dinitrophenylated amino acids was 4.00:0.88 (γ -DNP-Dab: DNP-Thr), corresponding to the theoretical ratio, 4.0: 1.0, if fraction II peptide was assumed to be colistin nonapeptide. The difference between the experimental and theoretical data in the molarity may be as-

TABLE V. MOLAR RATIO OF N-TERMINAL AMINO ACIDS IN DNP-COLISTIN NONAPEPTIDE (FRACTION II)

DNP-Amino acid	Molar ratio	
	Theoretical	Experimental
γ -DNP-Dab	4.00	4.00
DNP-Thr	1.00	0.88

cribed partially to the impurities contained in the fraction II preparation. In view of the results obtained on fractions I and II, it is concluded that the cleavage of colistin with ficin occurs at the linkage between α -N-acylated Dab and Thr in the straight tripeptide part of colistin (Fig. 1), thus producing α -N-acylated Dab (fraction I), and colistin nonapeptide (fraction II), like in the case of polymyxin B as reported by Paulus and Gray.⁷⁾ Such peptide derived from a partial hydrolysis of colistin as fraction II belongs to the so-called colistin nonapeptide. Antimicrobial activities of the above ficin hydrolyzates, α -N-acyl Dab and colistin nonapeptide, were determined by agar dilution method, using 17 strains of bacteria as test organisms. As summarized in Table VI, none of these hydrolyzates were active to each of test organisms at a given concentration of 800 mcg/ml, and further almost inactive features were observed with the addition of equal amount (400 mcg/ml) of each hydrolyzate. Nakajima *et al.*¹⁴⁾ have reported that peptide fragments, which contained the ring peptide moiety of colistin, maintained the growth inhibitory activity, though far less than the activity of colistin, towards *E. coli* in liquid medium. Thus they pointed out the necessity of the ring structure of colistin for the antimicrobial activity. On the other hand, colistin-like peptide of chain form containing lysine instead of Dab has been recently synthesized by Kurihara *et al.*¹⁵⁾ and was found to have the antibacterial activity as strong as colistin. Considering these contradictory results, the relation between chemical structure and antibacterial activity of colistin requires further precise investigations.

TABLE VI. ANTIMICROBIAL SPECTRUM OF α -N-ACYL DAB (FRACTION I) AND COLISTIN NONAPEPTIDE (FRACTION II)

Test organism	M.I.C. (mcg/ml)		
	Colistin nonapeptide	α -N-Acyl Dab	Colistin nonapeptide ^{a)} + α -N-acyl Dab
<i>M. lysodeikticus</i>	> 800	> 800	> 800
<i>St. aureus</i>	> 800	> 800	> 800
<i>S. lutea</i>	> 800	> 800	> 800
<i>B. subtilis</i>	> 800	> 800	> 800
<i>B. cereus</i>	> 800	> 800	> 800
<i>E. coli</i> NIHJ	> 800	> 800	> 800
<i>E. coli</i> K-12	> 800	> 800	> 800
<i>A. aerogenes</i>	> 800	> 800	> 800
<i>Ser. marcescens</i>	> 800	> 800	> 800
<i>Pr. vulgaris</i>	> 800	> 800	> 800
<i>Pr. mirabilis</i>	> 800	> 800	> 800
<i>Pr. morgani</i>	> 800	> 800	> 800
<i>Sal. enteritidis</i>	> 800	> 800	> 800
<i>Sal. infantis</i>	> 800	> 800	> 800
<i>Ps. aeruginosa</i>	800	> 800	800
<i>Ps. desmolytica</i>	200	> 800	400
<i>X. oryzae</i>	400	> 800	400

^{a)} 400 mg of colistin nonapeptide + 400 mg of α -N-acyl Dab.

In addition, the acute toxicity of the above colistin nonapeptide was examined by intravenous injection to mice, where LD₅₀ was 145 mg/kg, showing rather lower toxicity than that of colistin.

5. Peptides obtained from enzymatic hydrolyzates of colistin by ficin, papain and bromelain

As already mentioned, ficin and papain showed almost the same activity while bromelain distinguishably weaker one in the hydrolysis of colistin. Considering together with the results obtained by Paulus and Gray,⁷⁾ this was assumed to be attributable not only to the hydrolyzing activity of each enzyme preparation, but also to different nature of each enzyme in colistin hydrolysis. Then, peptide fraction, *e.g.* the above fraction II in case of ficin hydrolysis, was isolated in each enzymatic hydrolysis and partially purified according to the same procedures as in the case of colistin hydrolyzate by ficin. These peptides were dinitrophenylated by usual method and

subjected to the acid hydrolysis to investigate their terminal amino acid. Table VII indicates the results obtained. Thin layer chromatography indicated that the molar ratio of γ -DNP-Dab to DNP-Thr in the hydrolyzate by papain (4.00:0.95) was almost the same as that in the hydrolyzate by ficin (4.00:1.00), in contrast to the different molar ratio shown in the hydrolyzate by bromelain. In the last hydrolyzate, no DNP-Thr but a considerable

available enzyme for obtaining colistin nonapeptide, which may serve as an important material for the preparation of modified colistin.

Acknowledgement. The authors wish to express their thanks to Teijiro Uemura, emeritus professor, the University of Tokyo for his valuable suggestions and Miss Isoko Haneda for her assistance in the microbiological analysis of antibiotics.

REFERENCES

TABLE VII. COMPARISON OF THE N-TERMINAL AMINO ACIDS IN CRUDE PEPTIDE PREPARATION OBTAINED HYDROLYZATES BY FICIN, PAPAIN AND BROMELAIN

	Molar ratio of DNP-amino acids		
	Ficin hydrolyzate	Papain hydrolyzate	Bromelain hydrolyzate
DNP-Thr	1.00	0.95	0
di-DNP-Dab	0.24	0.09	0.74
γ -DNP-Dab	4.00	4.00	3.00

amount of di-DNP-Dab was found, and the molar ratio of γ -DNP-Dab to a mole of DNP-colistin distributed in 5 moles was 3.0, less by one mole than in case of colistin hydrolyzate by ficin or papain. In view of the formation of di-DNP-Dab, Dab but not Thr was thus recognized as N-terminal amino acid of peptide fraction formed in the bromelain hydrolysis. Since no free fatty acids such as MOA or IOA in the colistin hydrolyzate by bromelain were detected by gas liquid chromatography, cleavage of the linkage between fatty acid moiety and peptide moiety in colistin molecule, *i.e.*, the functioning of colistin deacylase^{16~18)} in the bromelain hydrolysis, was not presumable.

As a conclusion, ficin is one of the most

- 1) T. Suzuki, H. Inouye, K. Fujikawa and Y. Suketa, *J. Biochem.*, **54**, 25 (1963).
- 2) T. Suzuki, H. Inouye, K. Fujikawa and S. Nagasawa, *ibid.*, **54**, 173 (1963).
- 3) T. Suzuki, K. Hayashi and K. Fujikawa, *ibid.*, **54**, 412 (1963).
- 4) T. Suzuki and K. Fujikawa, *ibid.*, **56**, 182 (1964).
- 5) K. Hayashi and T. Suzuki, *Bull. Inst. Chem. Res., Kyoto Univ.*, **43**, 259 (1965).
- 6) M. Ito, T. Aida and Y. Koyama, *Agr. Biol. Chem.*, **30**, 1112 (1966).
- 7) H. Paulus and E. Gray, *J. Biol. Chem.*, **239**, 865 (1964).
- 8) T. Kurihara, K. Suzuki and K. Chiba, *Tohoku Yakka Daigaku Kiyo*, **1**, 16 (1954).
- 9) K. Suzuki, *ibid.*, **4**, 127 (1957).
- 10) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
- 11) G. Kärber, *Arch. Exp. Path. Pharm.*, **162**, 480 (1931).
- 12) "Data for Biochemical Research," ed. by R. M. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, Oxford University Press, 1969, p. 20.
- 13) M. Ito, Y. Koyama, K. Aida and T. Uemura, *Biochim. Biophys. Acta*, **215**, 418 (1970).
- 14) K. Nakajima, *Chem. Pharm. Bull.*, **15**, 229 (1967).
- 15) T. Kurihara, H. Takeda and H. Ito, *Yakugaku Zasshi*, **92**, 129 (1972).
- 16) Y. Kimura, T. Tazuki and M. Numata, Abstracts of Papers, 21st Meeting of Pharmaceutical Society of Japan, Tokushima, October, 1965, p. 422.
- 17) Y. Kimura, T. Tazuki, M. Numata, T. Suzuki and M. Tuchiya, *ibid.*, 1965, p. 422.
- 18) Y. Kimura, T. Tazuki and H. Takeda, *ibid.*, 1965, p. 423.