JOLIPEPTIN, A NEW PEPTIDE ANTIBIOTIC

I. ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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A new antibiotic, jolipeptin, was isolated from the cells of *Bacillus polymyxa* var. *colistinus* KOYAMA, a colistin-producing strain. Jolipeptin is a polypeptide antibiotic containing glycine, alanine, serine, valine, glutamic acid and α,γ-diaminobutyric acid. It contains no fatty acids as does colistin. It inhibits growth of gram-positive and gram-negative bacteria.

The strain used for jolipeptin production, *Bacillus polymyxa* var. *colistinus* KOYAMA is well known as a colistin-producing strain[1]. Colistin which belongs to the polymyxin group, is produced extracellularly by *B. polymyxa* var. *colistinus*. It is composed of colistins A,B and C[2]; the chemical structures of colistins A and B were determined by SUZUKI *et al*[3,4,5].

Colistins A and B are biosynthesized in a supernatant fraction, ultracentrifuged at 105,000×*g* without participation of the ribosomal fraction, differently from protein biosynthesis[6,7]. During a search for active substances within the cells, a new antimicrobial substance localized in the ribosomal fractions precipitated by ultracentrifugation at 105,000×*g*, was observed. The new substance was not excreted into fermented broth except under special cultural conditions. The new antimicrobial substance, a peptide antibiotic, was named 'jolipeptin'.

In this paper, production, isolation, physico-chemical and biological properties of jolipeptin are described.

Materials and Methods

Microorganisms: *B. polymyxa* var. *colistinus* was used as the jolipeptin-producing strain throughout these experiments. Media and cultural conditions were described in a previous paper[8].

Bioassay of jolipeptin: Antimicrobial activity was assayed by a paper disk method using *Escherichia coli* NIHJ as a test organism. Crystalline jolipeptin was used as standard.

Identification of jolipeptin separately from colistin: The mixture of jolipeptin and the colistin group was applied to Toyo-Roshi No. 50 and the paper was developed with a solvent system of *n*-butanol-acetic acid-water (I; 4:

Table 1. Paper chromatography of jolypeptin and colistins

Solvent system *n*-Butanol-acetic acid-water	Rf value			
	Jolipeptin	Colistin A	Colistin B	Colistin C
I(4 : 1 : 2, by vol.)	0.7	0.45	0.45	0.45
II(3 : 1 : 1, by vol.)	0.67	0.42	0.24	0.06

1 : 2 or II ; 3 : 1 : 1, by volume). After the development, bioautography was carried out on a plate of *E. coli* NIHJ.

A comparison of Rf values of jolipeptin and colistins on two solvent systems is shown in Table 1. Jolipeptin shows a single spot at Rf values of 0.7 and 0.67, while Rf values of colistins A,B and C are 0.42, 0.24 and 0.06 respectively in solvent system II, and 0.45 in solvent system I. Through these experimental procedures, jolipeptin could be identified, in the presence of the colistin group.

Production and Isolation

When *B. polymyxa* var. *colistinus* was cultured in synthetic medium, production of colistin paralleled cell growth and maximum activity was reached in 24 hours. Activity of intracellular jolipeptin was simultaneously assayed at the various growth phases.

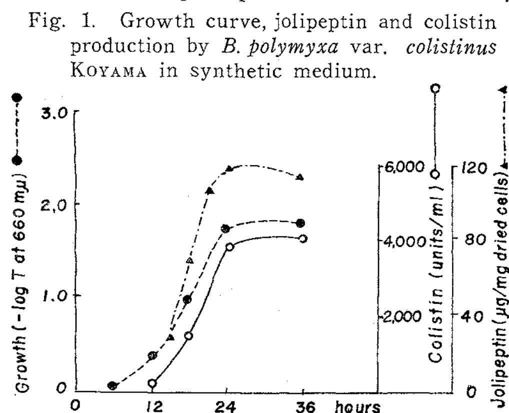
Cells harvested at various times were washed twice with physiological saline and then the washed cells were suspended in saline. The cell suspension was disrupted with a sonic oscillator at 10 KC for 10 minutes. The distrupted cells were adjusted to pH 2.0 with dil. HCl. Antimicrobial substances were extracted three times with *n*-butanol saturated with acidic water. The butanol extracts were combined and washed three times with acidic water (pH 2.0). By this procedure, colistin activity was completely eliminated from the butanol extract.

The butanol extract free of colistin was adjusted to pH 7.0, adjusted to a constant volume, and subjected to bioassay for jolipeptin activity. Activity of jolipeptin within the cells paralleled cell growth, similarly to colistin and reached a maximum at 24 hours as shown in Fig. 1.

Cultured broth was harvested at the end of the exponential growth phase, autolyzed at 4°C for 24 hours and filtered. The broth filtrate (10 liters) was adjusted to pH 8.6 with dil. NaOH and 1.5 % of benzaldehyde was added with vigorous stirring to the broth filtrate. The precipitate was collected by continuous centrifugation. Jolipeptin activity was extracted by 3×300 ml of *n*-butanol saturated with water at pH 1～2 from the collected precipitate.

The butanol extracts were combined and washed with 200 ml of acidic water (pH 1～2) until colistin activity was completely eliminated. A three-fold volume of ethyl ether was added to the washed *n*-butanol extract. The precipitate was collected by centrifugation at 3,000 rpm for 10 minutes, and then dissolved in methanol. The insoluble materials were removed by centrifugation. A three-fold volume of ethyl ether was added to the methanol solution. The precipitate was collected by centrifugation and dried *in vacuo* to yield 150 mg of crude powder. Further purification was done by column chromatography using Sephadex LH-20 (1.8× 45 cm) and methanol as the developing

Fig. 1. Growth curve, jolipeptin and colistin production by *B. polymyxa* var. *colistinus* Koyama in synthetic medium.



solvent. Jolipeptin-containing fractions were combined and ethyl ether was added. A colorless crystalline powder was obtained and used for the various experiments described.

Physical and Chemical Properties

Jolipeptin is soluble in methanol, *n*-butanol and isopropanol, but insoluble in acetone, methyl acetate, butyl acetate, ethyl ether, petroleum ether, benzene and chloroform. It does not decompose at 300°C. It gives a positive ninhydrin reaction.

The ultraviolet absorption spectrum of jolipeptin is shown in Fig. 2. The infrared spectrtm of jolipeptin in a KBr tablet is shown in Fig. 3. The optical rotation is $[\alpha]_D^{22°}+14°$ (*c* 1, ethyleneglycol). The elementary analysis gave the following composition: C 49.66, H 7.22, N 13.10.

The nitrogen content, the ninhydrin reaction and the amide band in the infrared spectrum indicated that jolipeptin is a peptide antibiotic. Consequently, jolipeptin was hydrolyzed in 6 N HCl at 105°C for 24 hours in a sealed tube. The resulting solution was evaporated to dryness *in vacuo* and the mixture of amino acids was analyzed by the automatic amino acid analyzer, Yanagimoto Model LC-2. The amino acid composition of the hydrolyzate was two moles of α,γ-diaminobutyric acid, serine, alanine and valine, for each mole of glycine and glutamic acid. The optical configuration of these amino acids has not yet been determined. The results are summarized and compared to colistin in Table 2.

Fig. 2. Ultraviolet absorption spectum of jolipeptin in methanol..

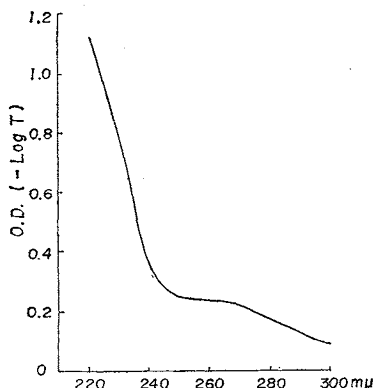
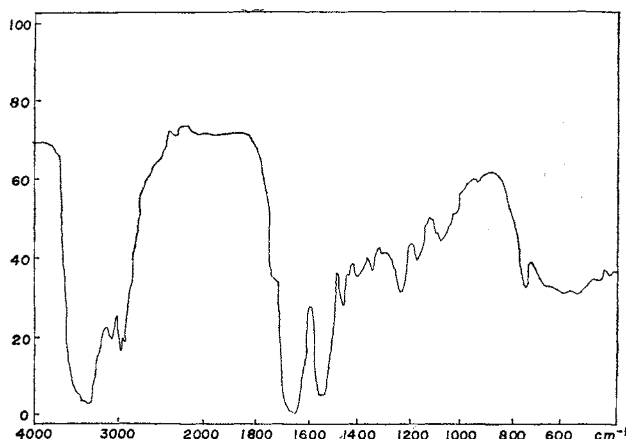


Fig. 3. Infrared absorption spectrum of jolipeptin (KBr)



For the detection of fatty acids in the jolipeptin molecule, gas chromatography was carried out on the petroleum ether extract of the hydrolyzate,

Table 2. Amino acid compositin of jolipeptin and colistin

Amino acid	Molar ratio		
	Jolipeptin	Colistin A	Colistin B
Serine	2. 18	0	0
Alanine	1. 73	0	0
Valine	1. 85	0	0
Glycine	1. 0	0	0
Glutamic acid	1. 19	0	0
α,γ-Diaminobutyric acid	2. 01	3. 15	3. 20
Threonine	0	0. 93	1. 01
Leucine	0	1. 0	1. 0

Molar ratio of colistins A and B was estimated by SUZUKI *et al*[3]

Table 3. Stability of jolipeptin solution at various pH levels at 100°C

pH	Activity remained (μg/ml)	Inactivation (%)
Control aq. solution	1, 400	
pH 2	1, 480	0
pH 5	1, 400	0
pH 7	1, 480	0
pH 9	1. 500	0
pH 11	1, 480	0

Each tube was boiled in water bath for 10 minutes except for control aqueous solution.

Table 4. Inactivation of jolipeptin by Nagarse and colistinase

Enzyme	Residual activity (μg/ml)	Jolipeptin inactivated (μg/ml)	Inactivation (%)
None	1, 800		
Nagarse (125 PUN)*	1. 600	95	6. 3
Colistinase (25,000 u)*	1, 650	60	3. 6

Incubation was carried out at pH 9.0 for 3 hours at 37°C.

* The concentration of these enzymes completely inactivated 1,500 μg of colistin under the same experimental conditions[9].

with and without esterification. No fatty acids could be detected in the hydrolyzate of jolipeptin.

On paper electrophoresis at 2 mAmp/cm and 20 volts/cm for 5 hours in M/15 phosphate buffer (pH 7.0, μ= 0.1), jolipeptin did not move from the origin line, but colistin moved 1.5 cm toward the cathode.

The stability of jolipeptin was measured by antibacterial activity. Aqueous jolipeptin solutions were prepared at pH 2, 5, 7, 9 and 11, and boiled at 100°C for 10 minutes. After cooling, each solution was adjusted to pH 7.0, and assayed by a paper disk method against a non-heated control. As shown in Table 3, jolipeptin was stable under these experimental conditions.

Colistin was inactivated by bacterial proteinase, Nagarse and colistinase isolated from the fermented broth of *B. polymyxa* var. *colistinus*[9].

The enzymatic inactivation of jolipeptin was investigated in comparison with colistin. As shown in Table 4, jolipeptin was neither inactivated by Nagarse, nor by colistinase prepared from the colistin- and jolipeptin-producing broth.

Table 5. Antimacrobial spectra of jolipeptin

Test organisms	Minimum inhibitory concentration (μg/ml)
Escherichia coli NIHJ	0. 312
Escherichia coli B	0. 312
Pseudomonas aeruginosa IFO 3901	10
Pseudomonas aeruginosa IFO 3924	5
Pseudomonas desmolytica IFO 12570	10
Pseudomonas fluorescens IFO 3081	10
Pseudomonas fluorescens IFO 3903	2. 5
Proteus mirabilis IFO 3849	40
Proteus vulgaris IFO 3045	20
Proteus vulgaris IFO 3167	20
Proteus morganii IFO 3848	>40
Staphylococcus aureus FDA 209 P	1. 25
Bacillus subtilis PCI 219	0. 625
Sarcina lutea	2. 5
Micrococcus lysodeikticus	0. 625
Mycobacterium tuberculosis 607	10
Candida albicans	>40
Candida krusei	>40
Saccharomyces cerevisiae	40
Saccharomyces rosei	40
Fusarium lini	>40
Fusarium roseum	>40
Aspergillus oryzae	>40
Penicillium expunsum	>40
Absidia butleri	>40
Gibberella sanbinettii	>40

Minimum inhibitory concentration was determined by the liquid dilution method using nutrient broth for bacteria and YAMANE's modified medium for *Mycobacterium tuberculosis* 607. Activities of jolipeptin against yeasts and fungi were determined by agar dilution method using 1% glucose nutrient broth and CZAPEK medium, respectively.

Biological Properties

The antimicrobial activity of jolipeptin against various microorganisms was studied by the dilution method. The minimal inhibitory concentrations observed are listed

in Table 5. Jolipeptin inhibits *E. coli* B, *E. coli* NIHJ, *Staphylococcus aureus* FDA 209 P, *Bacillus subtilis* PCI 219, *Micrococcus lysodeikticus* at 0.312~1.25 μg/ml, but is inactive against yeast and fungi even at 40 μg/ml.

Acute toxicity studies were carried out by BEHRENS-KÄRBER's method in *dd* mice weighing 18~20 g with 3-day observation period. The LD_{50} for mice was 5.21 mg/kg intravenously and 62.5 mg/kg intrapertoneally.

Discussion

From the results obtained jolipeptin was easily distinguishable from the polymyxin group including colistin and circulin.

Polypeptide antibiotics produced by bacteria such as bacitracin, gramicidin, tyrocidin and polymyxin are mixtures of antimicrobial substances. However, the individual materials in the mixture are usually similar in amino acid composition with substitution in one or two amino acid residues. However *B. polymyxa* var. *colistinus* produces simultaneously jolipeptin and colistin with different amino acid composition except for α,γ-diaminobutyric acid.

It is assumed that jolipeptin is a cyclic polypeptide, based on the evidence that it is not inactivated by Nagarse and colistinase. Studies on the amino acid sequence of jolipeptin are in progress.

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