ISOLATION OF A NEW PEPTIDE ANTIBIOTIC, PERMETIN A, FROM *BACILLUS CIRCULANS*

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Permetin A was purified from the culture filtrate of *Bacillus circulans* AJ 3902 by extraction with *n*-butanol, precipitation with sodium helianthate, CM-cellulose column chromatography and Sephadex LH-20 column chromatography. The compound was found to be a new peptide antibiotic containing 2,4-diaminobutyric acid (Dab), leucine, isoleucine, phenylalanine, valine, serine (in a molar ratio of 3: 2: 1: 1: 1: 1) and a fatty acid. This antibiotic showed activity *in vitro* against Gram-negative, Gram-positive and some anaerobic bacteria.

The outer membrane of Gram-negative bacteria usually prevents the passage of antibiotics or lytic enzymes to their target sites inside the bacteria. *Bacillus subtilis* YT-25, isolated as a lytic enzyme producer, simultaneously produces a substance altering the structure of the outer membrane. This bacterium, therefore, can synergistically lyse the cells of Gram-negative bacteria. The new substance was designated NLF-I.[1,2] We also found another substance altering the outer membrane produced by soil bacterium AJ 3902; this substance was designated NLF-II.[2]

Further studies of these compounds revealed that NLF-I was identical to antibiotic EM-49 which is similar to polymyxin and that NLF-II was a new peptide antibiotic containing Dab.

We have found many additional bacteria capable of lysing cells of Gram-negative bacteria. All belong to *Bacillus* and produce peptide antibiotics containing Dab. It is possible that many bacilli make use of the lytic activity of peptide antibiotics in cooperation with lytic enzymes to lyse Gram-negative bacterial cells.

NLF-II was named permetin. Production, isolation and some properties of permetin A, a main component of permetin, are reported in this paper.

Taxonomic Characterization of the Producing Microorganism

The taxonomic studies were carried out according to 'The Manual of Microbiological Methods'[3] and 'The Genus *Bacillus*'[4]. The strain was classified according to 'BERGEY's Manual of Determinative Bacteriology, 8th Ed.'[5].

Taxonomic characteristics of strain AJ 3902 are summarized in Tables 1 and 2. From the results, strain AJ 3902 is classified as *Bacillus circulans*.

Production and Isolation

Strain AJ 3902 was cultured at 30°C for 2 days on a reciprocal shaker in 500-ml SAKAGUCHI flasks

Table 1. Physiological properties of strain AJ 3902

Reduction of nitrate	positive	Temperature for growth	18~45°C
Denitrification	negative	pH for growth	5~9
Methyl red test	positive	Aerobiosis	aerobic
Reduction of 2,6 dichlorophenol-indophenol	negative	HUGH-LEIFSON	oxidation and
Production of indole	negative		fermentation
Production of H_2S	positive	Hydrolysis of casein	positive
Hydrolysis of starch	positive	Maximum concentration of NaCl allowing growth	0.5%
Utilization of ammonium salts	positive		
Utilization of nitrate	positive	Utilization of propionic acid	negative
Catalase activity	positive	Deamination of phenylalanine	negative
Urease activity	positive	Decomposition of tyrosine	negative
Oxidase activity	positive		

Table 2. Morphological and cultural properties of strain AJ 3902

1.	Vegetative cells	Rods, not in chains, motile by means of peritrichous flagella. $0.5\sim0.7\times1.8\sim3.0\ \mu$
2.	Endospore	Spores, $0.7\sim0.8\times1.2\sim1.5\ \mu$, ellipsoidal to cylindrical, paracentral or terminal.
3.	Sporangia	Spindle shape, definitely swollen
4.	Staining characteristics	Gram-positive, acid fast negative.
5.	Nutrient agar slants (24~48 hours at 30°C, Difco medium)	Growth moderate, echinulate, viscid, glistening, porcelaneous or primrose
6.	Nutrient agar plates (24~48 hours at 30°C, Difco medium)	Growth moderate, circular. Surface smooth, verrucose; Edge undulate. Elevation of growth, pulvinate to umbonate. Glistening, porcelaneous to primrose.
7.	Gelatin stab	Growth, best at top. Liquefaction, crateriform to stratiform
8.	Growth in 7% NaCl broth	Negative
9.	B.C.P. milk	Acid, peptonized
10.	Litmus milk	No reduction, peptonized
11.	King B agar	Moderate growth, no production of water-soluble pigment.

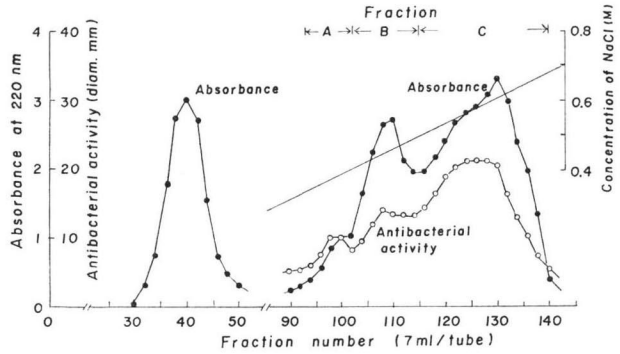
containing 100 ml of medium (2% Polypeptone, 1% meat extract and 0.3% NaCl, pH 6.0).

Seventeen liters of the culture filtrate were concentrated *in vacuo* to 2 liters and extracted three times with an equal volume of *n*-butanol. The butanol extract was evaporated *in vacuo*. The residue was dissolved in methanol and the insoluble material was removed. The methanolic layer was evaporated *in vacuo*. The residue was dissolved in 500 ml of water and the insoluble material was removed by centrifugation. A solution of 5 g of sodium helianthate in 375 ml of dimethylformamide (DMF) and 125 ml of water was added to the supernatant, resulting in the production of dark-red precipitate (the helianthate salt of permetin). The precipitate was collected by centrifugation, dissolved in 300 ml of DMF, and reprecipitated by the addition of 4,700 ml of water. The precipitate was collected and suspended in 300 ml of 0.36 N hydrochloric acid, resulting in the precipitation of helianthic acid and the liberation of the antibiotic into the solution. The insoluble material was removed and the supernatant was extracted three times with an equal volume of *n*-butanol. The butanol layer was evaporated *in vacuo*, then dissolved in 30 ml of methanol, and gel-filtered on a Sephadex LH-20 column (3×50 cm) equilibrated with methanol. The eluates active against *Escherichia coli* NIHJ JC-2 were combined and dried to give

13.1 g of crude permetin, which was further purified by CM-cellulose column chromatography. Two grams of crude permetin were dissolved in 10 ml of a mixture of 0.05 M formic acid-ammonia buffer (pH 7.2) and methanol (1 : 1) and then applied to a CM-cellulose column (2.4×37 cm) equilibrated with the methanolic buffer. The column was washed with 350 ml of the methanolic buffer, and permetin was eluted by a linear gradient of sodium chloride with a concentration from 0 to 1 M in the methanolic buffer. A typical chromatogram is shown in Fig. 1.

Fig. 1. Isolation of permetin A with a CM-cellulose column

Two grams of crude permetin were dissolved in 10 ml of a mixture of 0.05 M formic acid-ammonia buffer (pH 7.2) and methanol (1 : 1) and applied to a CM-cellulose column (2.4×37 cm) equilibrated with the methanolic buffer. The column was washed with 350 ml of the methanolic buffer and eluted with a linear gradient of NaCl from 0 to 1 M in the methanolic buffer. Antibacterial activity against *E. coli* NIHJ JC-2 (diffusion agar method with paper disk).



Fractions A, B and C showed antibacterial activities against *E. coli*. The most active fraction, fraction C, was collected and desalted with a Sephadex LH-20 column equilibrated with methanol. Fraction C was purified by passing it again through the same CM-cellulose and Sephadex LH-20 columns. Salt-free fraction C was dried to give a white powder, the hydrochloric acid salt of permetin A. About 5 g of permetin A were obtained from 17 liters of culture filtrate.

For analysis, a portion of the preparation was further purified by preparative high pressure liquid chromatography with a Nucleosil C-18 column (Chromato-Research Co., 8×300mm) equilibrated with a mixture of 0.01 N hydrochloric acid and methanol (3 : 7) at 50 kg/cm^2. Permetin A was determined by absorption at 220 nm.

Chemical Properties

Permetin A hydrochloride was obtained as a colorless amorphous powder which melted at 210~220°C with decomposition. It was freely soluble in water and methanol and sparingly soluble to insoluble in acetone, ethylacetate, chloroform and ether.

Thin-layer chromatographic mobilities of permetin A hydrochloride in a number of solvent systems are listed in Table 3. The antibiotic was detected by bioautography against *E. coli* and by ninhydrin.

The ultraviolet absorption spectrum of its hydrochloride in methanol are shown in Fig. 2. A series of very week maxima between 245 and 270 nm were detected.

The infrared absorption spectrum of its hydrochloride is illustrated in Fig. 3. Absorption at 1530 and 1650 cm^{-1} indicated the presence of peptide bonds in this substance. Absorption due to an ester

Table 3. Thin-layer chromatography of permetin A hydrochloride

Solvent system*	Rf
1. *n*-Butanol, acetic acid, water (2: 1: 1)	0.60
2. *n*-Butanol, acetic acid, water (4: 1: 5, upper)	0.33
3. *n*-Propanol, pyridine, acetic acid, water (15: 10: 3: 8)	0.75
4. *n*-Butanol, chloroform, water (4: 1: 1)	0.33
5. Chloroform, methanol, 17% ammonia (2: 1: 1, lower)	0.19

* Pre-coated Silica Gel F-254 plates (Merck) were used.

Table 4. Amino acid analysis of permetin A hydrochloride

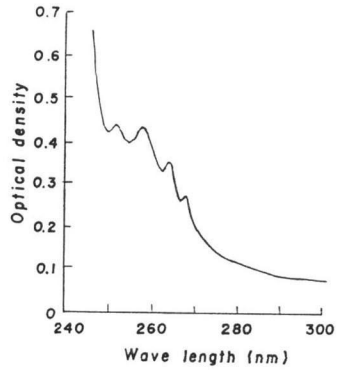
Permetin A hydrochloride was hydrolyzed in 6 N HCl at 110°C for 24, 48 or 72 hours. The maximum contents of amino acids (except serine) from these hydrolysates are shown. The content of serine was calculated by extrapolation to zero time.

Amino acids	Amino acid residues (%)	Molar ratio*
2,4-Diaminobutyric acid	38.3	3.0
Leucine	17.4	2.1
Phenylalanine	10.5	1
Isoleucine	7.9	1.0
Valine	7.4	1.0
Serine	5.8	1.0
Total	87.3	

* phe=1

Fig. 2. Ultraviolet absorption spectrum of permetin A

U. V. absorption of permetin A hydrochloride in methanol (3.275 mg/ml) was determined.



bond was detected at 1740 cm^{-1}.

The results of amino acid analysis of permetin A hydrochloride are summarized in Table 4. The antibiotic contains 2,4-diaminobutyric acid, leucine, isoleucine, phenylalanine, valine and serine in a molar ratio of 3: 2: 1: 1: 1: 1. In addition, this antibiotic contains a fatty acid. The structure of the fatty acid will be discussed in detail in a subsequent paper.

Biological Properties

Permetin (NLF-II) has an activity promoting the enzymatic lysis of Gram-negative bacteria[2]. Further, permetin A shows broad-spectrum antibacterial activity *in vitro* and inhibits some anaerobic as shown in Table 5.

Fig. 3. Infrared absorption spectrum of permetin A hydrochloride in KBr

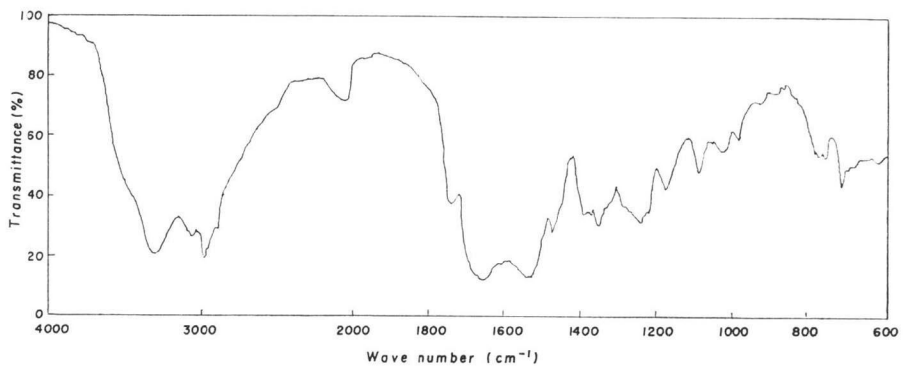


Table 5. Antibacterial spectrum of permetin A

Bacteria	Minimum inhibitory concentration (μg/ml)[a]		Cultural condition
	Permetin A hydrochloride	Colistin methanesulfonate	
Escherichia coli NIHJ JC-2	12.5	6.25	d
1236[b]	12.5	12.5	d
Salmonella typhimurium AJ 3224	12.5	12.5	d
Klebsiella sp. 29[b]	12.5	6.25	d
48[b]	12.5	6.25	d
Pseudomonas aeruginosa ATCC 10145	25.0	12.5	d
IFO 3445	12.5	12.5	d
Serratia sp. 32[b]	12.5	6.25	d
34[b]	12.5	12.5	d
Proteus vulgaris 2[b]	>50.0	>50.0	d
5[b]	>50.0	>50.0	d
Staphylococcus aureus FDA 209 P	6.25	6.25	d
Bacillus subtilis AJ 1234	3.13	25.0	d
Bacillus cereus AJ 1310	12.5	>50.0	d
Brevibacterium lactofermentum AJ 1511	6.25	>50.0	d
Bacteroides sp. ZSB 1[c]	25.0	>100.0	e
ZSB 12[c]	6.25	>100.0	e
Clostridium sp. ZSB 5[c]	6.25	>100.0	f
ZSB 13[c]	25.0	>100.0	f
Lactobacillus sp. ZSL 3[c]	100.0	>100.0	e
Peptostreptococcus sp. ZSP 1[c]	25.0	<0.78	e

a: Obtained by the agar dilution method
b: Clinically isolated strains from the Department of Microbiology, Toho University School of Medicine, Tokyo
c: Strains anaerobically isolated from the extrements of domestic animals by the Central Institute for Feed and Livestock Research Laboratory, Ibaraki
d: Aerobically cultured at 37°C for 12 hours on Heart Infusion Medium (Eiken)
e: Anaerobically cultured[8] with steel wool, in CO_2 at 37°C for 24 hours on *Brucella*-agar (BBL) containing 5% horse blood
f: Anaerobically cultured as in e. on TEP-Medium (Eiken)

Table 6. Amino acid composition of permetin A and similar antibiotics

Antibiotics	Amino acids (molar ratio)								Fatty acid	References
	Dab	Leu	Phe	Ile	Val	Ser	Thr	Asp		
Permetin A	3	2	1	1	1	1			a–C_7*	
Polypeptin	3	2	1	1	1		1		a–C_7 i–C_7	(7)
4205–A –C	4	2	1	1	1	1			+	(8)
B–43 complex	3	1.3	1	0.7	2			1	+	(9)

Dab: 2,4-Diaminobutyric acid. a–C_7: Anteisoheptanoic acid. i–C_7: Isoheptanoic acid
* This fatty acid will be discussed in a subsequent paper.
+: Unidentified fatty acid

The LD_{50} values in mice by intraperitoneal and oral routes were 36 mg/kg and 2,100 mg/kg, respectively.

Discussion

Permetin A contains 2,4-diaminobutyric acid, leucine, isoleucine, phenylalanine, valine and serine in a molar ratio of 3: 2: 1: 1: 1: 1. Many kinds of peptide antibiotics produced by *Bacillus* have been isolated. Permetin A is similar to such antibiotics as polypeptin[6], antibiotic 4205[7], and antibiotic B-43[8] but clearly differs from these antibiotics in amino acid composition as given in Table 6.

As for the antibacterial spectrum of permetin A, this antibiotic is active against not only aerobic Gram-negative and positive bacteria but also against some anaerobic bacteria. Other peptide antibiotics containing Dab, such as polymyxin, are usually active against aerobic Gram-negative bacteria but not against anaerobic bacteria.

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