ISOLATION OF A NEW PEPTIDE ANTIBIOTIC COMPLEX, B-43

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XV[1])

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A new peptide antibiotic complex, B-43, active against Gram-positive and Gram-negative bacteria, was isolated from a strain of *Bacillus circulans*. This antibiotic contains aspartic acid, valine, isoleucine, leucine, phenylalanine and 2,4-diaminobutyric acid. It seems to be related to polypeptin and antibiotic complex 4205, but differs in that it contains aspartic acid residue.

In the course of our screening program for new antibiotics from the genus *Bacillus*[1], a strain numbered B-43 and identified as *B. circulans*, was found to produce an antibiotic substance active against Gram-positive and Gram-negative bacteria.

The antibiotic substance, tentatively named B-43, was isolated from the culture broth and proved to be a complex of homologous peptides which are difficult to separate from each other. From the constituents, it seems to be related to antibiotic polypeptin[2,3] and antibiotic complex 4205[4].

This paper presents the characteristics of the producing strain as well as the production, isolation and preliminary characterization of antibiotic B-43.

Taxonomic Characterization of the Producing Strain

The characteristics of strain B-43 are described below.

1. Morphology

(1) Vegetative cells (28°C, 2 days): Gram-positive rods on nutrient agar are 0.5~0.8 μ by 3.0~7.0 μ with rounded ends. They occur singly or in mass and are motile.

(2) Spores and Sporangia (28°C, 2 days): Spores on nutrient agar are mostly 1.2 by 2.2 microns, easily stainable, oval, central. The sporangia are definitely swollen.

2. Cultural Characteristics

(1) Colony on No. 172 agar medium* (28°C, 3 days): Circular (2~5 mm in diameter), convex, entire, smooth, shining, translucent, slimy to gummy.

(2) Nutrient agar slant (28°C, 2~12 days): Growth slowly, filiform, viscid to gummy, translucent changing to opaque with age. Diffusible and non-diffusible pigment are not observed.

(3) Nutrient broth (28°C, 2~5 days): Slow, uniform growth is observed with some sediments. A ring formation is observed at late stage.

3. Physiological Characters

(1) Relation to oxygen (28°C, 1~6 days): O-F test on GPYB-agar** stab is aerobic to slightly facultative anaerobic. Acid but no gas production is observed from glucose.

(2) Temperature relation (Gly-IM agar***, 1 day): Optimum is approximately 37°C. It does

* No. 172 Agar: Soluble starch 2.0%, glucose 1.0%, Casamino acids 0.5%, yeast extract 0.5%, $CaCO_3$ 0.1%, agar 1.2% (w/v), pH 6.8.

** GPYB-Agar: Glucose 1.0%, peptone 0.5%, yeast extract 0.2%, beef extract 0.3%, agar 0.4% (w/v), pH 6.6.

*** Gly-IM Agar: Glycerol 0.5%, peptone 0.25%, beef extract 0.25%, yeast extract 0.25%, Bacto soytone 0.25%, NaCl 0.3%, agar 1.25% (w/v), pH 6.8.

not grow at 50°C.

(3) Citrate utilization (28°C, 2 days): No growth on KOSER's synthetic medium.
(4) Starch hydrolysis (28°C, 2 days): Positive.
(5) Gelatin stab (28°C, 1~30 days): Slowly liquefied.
(6) Litmus milk (28°C, 2~30 days): No peptonization; slowly coagulated.
(7) Nitrate reduction to nitrite (28°C, 2 days): Positive.
(8) Acetylmethylcarbinol production (28°C, 1~6 days): Negative.
(9) H_2S formation (28°C, 1~6 days, Difco peptone-iron agar): Negative.
(10) Urease activity (28°C, 1~7 days): Weakly positive.
(11) NaCl broth (28°C, 1~4 days): No growth in 5, 7 and 10% NaCl broth.
(12) Carbohydrates cleavage (28°C, 1~11 days): Acid but no gas from D-glucose, D-fructose, D-ribose and maltose. No acid and gas from L-arabinose, L-rhamnose, D-mannose, D-galactose, sucrose, lactose, raffinose, dextrin, starch, glycogen, inulin, glycerol, inositol, mannitol, sorbitol, salicin and α-methylglucoside.

4. Speciation

The above descriptions indicate that this bacterium should be classified as *B. circulans* or *B. filicolonicus*[5,6]. But *B. filicolonicus* differs from B-43 by growth on 10% NaCl broth. The descriptions of *B. circulans*[5] are very similar to that of B-43 and there is no significant taxonomic difference between them. Therefore, we concluded that B-43 is a strain of *B. circulans*.

Procedures for the taxonomic study were in accordance with those described in the Manual of Microbiological Method[7] and Identification Method for Microbiologist[8] except where indicated otherwise.

Fermentation and Isolation

Preparation of B-43 was carried out several times by slightly different manners, but the following one example is described here.

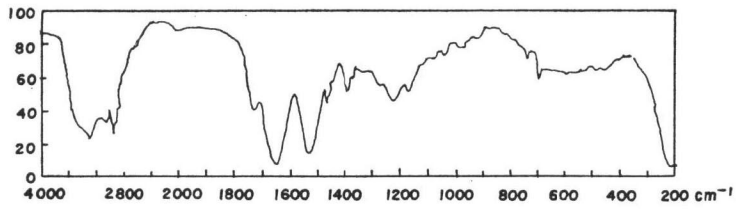
Spores of the strain B-43 were inoculated into 120 ml of a medium composed of peptone 1.0%, meat extract 0.5%, glucose 1.0% and sodium chloride 0.3% (pH 7.0) in a 500-ml shake flask and shake-cultured for 24 hours at 28°C. About 3 ml of the culture were then seeded in 120 ml of a medium made of glucose 1.0%, glycerin 0.25%, peptone 1.0%, meat extract 0.5%, sodium chloride 0.3% and calcium carbonate 0.3% (pH 7.0) in a flask, which was shake-cultured for 3 days at 28°C.

The culture broth (6 liters) was mixed with an equal volume of a mixture of *n*-butanol and methanol (1: 1) and filtered at pH 2.0. The filtrate was evaporated and extracted with *n*-butanol at pH 8.0. The *n*-butanol extract was then concentrated, and after addition of ethyl acetate, the antibiotic was transferred into water slightly acidified with hydrochloric acid. It was again extracted with *n*-butanol at pH 8.0, which was then washed with water, concentrated and mixed with ethyl acetate to precipitate crude material of the antibiotic (5.0 g).

The crude material (800 mg) was dissolved in methanol slightly acidified with hydrochloric acid. It was applied to four silica gel GF plates (20×100 cm, 750 μ), and developed with chloroform - ethanol - 14% ammoniacal water (4: 7: 2). A separated zone of the antibiotic was detected by a UV-lamp and ninhydrin reaction, and extracted with 50% aqueous methanol acidified with hydrochloric acid (pH 2.0). The extract was evaporated and extracted with *n*-butanol at pH 8.0, which was then washed with water, concentrated and mixed with ethyl acetate to give a colorless powder (495 mg) of B-43 free base.

The free base was dissolved in aqueous methanol acidified with hydrochloric acid (pH 2.0) and passed through a Sephadex LH-20 column with 20% aqueous methanol to remove a trace amount of ash-like materials. Lyophilization of the active fractions gave the hydrochloride of the antibiotic as a

Fig. 1. Infrared absorption spectrum of B-43 hydrochloride (KBr).



colorless powder.

The preparation gave a single spot on a Silica gel GF plate with the following solvent systems: Rf *ca.* 0.30 with chloroform - ethanol - 14% ammoniacal water (4: 7: 2) and Rf *ca.* 0.60 with chloroform - ethanol - 10% aqueous acetic acid (4: 7: 2), respectively.

Physico-Chemical Properties

B-43 hydrochloride is a colorless amorphous powder which decomposes at 240~245°C.

Anal. Found: C, 50.33; H, 7.95; N, 13.01; Cl, 8.17. $[\alpha]_D^{25}$ $-54.5\pm1.8°$ (*c* 0.523, water).

Ultraviolet absorption indicated the presence of a phenylalanine residue: λ_{max}^{MeOH}: 253 nm ($E_{1cm}^{1\%}$ 2), 258.5 nm ($E_{1cm}^{1\%}$ 2), 264 nm ($E_{1cm}^{1\%}$ 2). The i.r. spectrum shows this antibiotic to be a peptide possibly containing a lactone or ester linkage (Fig. 1).

The hydrochloride is soluble in water, methanol, aqueous ethanol and aqueous *n*-butanol, but slightly soluble or insoluble in acetone, ethyl acetate, chloroform and ethyl ether. It shows a positive reaction to ninhydrin, but not to potassium permanganate, ferric chloride, DRAGENDORFF's and SAKAGUCHI's reagents.

The basic nature was indicated by paper electrophoresis carried out with acidic buffer solutions. However, the experiment with alkaline buffer solution could not be carried out because the antibiotic is insoluble in alkaline buffer solutions.

Repeated amino acid analyses with acid hydrolyzates of the antibiotic showed the following amino acids (μ moles of amino acid found per mg of antibiotic): aspartic acid (0.73), valine (1.39), isoleucine (0.51), leucine (0.92), phenylalanine (0.69), 2,4-diaminobutyric acid (2.29) and a trace amount of ammonia. The hydrolyzate was extracted with ethyl ether. When the ethereal extract was subjected to gas chromatographic analysis, without methylation, using a column packed with 15% diethylene glycol succinate polymer on Chromosorb W, nearly a single peak was observed as in the analysis of fatty acid esters. The chromatogram did not alter after that the ethereal extract was treated with methylation procedure by diazomethane. This suggested that the antibiotic contained a fatty component which is not a simple fatty acid. Elucidation of the fatty component remains in a future study.

Table 1. Antimicrobial spectrum of B-43.

Test organism	MIC (mcg/ml)
Bacillus subtilis PCI 219	6.25
Bacillus anthracis	12.5
Staphylococcus aureus FDA 209P JC-1	25
Staphylococcus aureus Smith	25
Streptococcus pneumoniae type I	50
Streptococcus pyogenes C-203	12.5
Escherichia coli NIHJ JC-2	25
Klebsiella pneumoniae	12.5
Salmonella typhimurium	25
Pseudomonas aeruginosa Ps-24	>50

Obtained by the usual agar dilution method.

When B-43 was dinitrophenylated by the usual way, a product which gave a predominant spot at Rf *ca.* 0.60 on a Silica gel GF plate with chloroform - methanol (4: 1) was obtained. It was isolated by the TLC and prepared as a yellow powder, m.p. 192~197°C.

Anal. Found: C, 52.81; H, 6.09; N, 15.28; MW (osmometry in methanol), 1692.

Biological Properties

B-43 is active against Gram-positive and Gram-negative bacteria as shown in Table 1. Administration of this antibiotic at a dose of 50 mg/kg *via* intraperitoneal route was lethal to mice.

Discussion

From the amino acid analytical data and the molecular weight determination with the dinitrophenyl derivative of antibiotic substance B-43, the molar contents of the amino acid residues appear to be as follows: aspartic acid (1), valine (2), isoleucine (0.7), leucine (1.3), phenylalanine (1) and 2,4-diaminobutyric acid (3).

The molar contents of the isoleucine and leucine residues are not integer values, but the sum of these is 2 moles, indicating that the antibiotic preparation is not homogeneous with respect to its isoleucine and leucine residues.

It is well known that some closely related amino acids can replace each other in certain homologous peptides produced by microorganisms and in some cases separation of the homologous peptides is quite difficult[9]. In a similar manner antibiotic substance B-43 is considered to be a complex of homologous peptides.

Antibiotic polypeptin (originally cited as circulin) was isolated from a strain of *Bacillus circulans* by McLeod[2] (1948). Later, Hausmann and Craig[3] (1952) showed that a major component of polypeptin is constructed with threonine (1), valine (1), isoleucine (1), leucine (2), phenylalanine (1), 2,4-diaminobutyric acid (3) and an acid ($C_8H_{18}O_2$). In 1966, Shaw *et al.*[4] isolated antibiotic complex 4205 from a nonsporulating bacillus resembling *Bacillus circulans*. They separated the complex into two components, A and B, each composed of serine, valine, isoleucine, leucine, phenylalanine and 2,4-diaminobutyric acid in proportions of 1: 1: 1: 2: 1: 4 and 2: 2: 1: 3: 1: 5, respectively, and showed the presence of a fatty acid-like substance in each component. Recently, a paper[10] reported the isolation of an antibiotic BN-7 whose constituents were very similar to those of polypeptin, the only clear difference being the content of 2,4-diaminobutyric acid.

In comparison with known antibiotics isolated from the genus *Bacillus*, antibiotic complex B-43 is somewhat related to the above three antibiotics in amino acid constituents. However, B-43 is unequivocally differentiated from them, as it contains an aspartic acid residue in place of the threonine or serine residue.

We have also isolated from other strains of *Bacillus* an antibiotic complex closely related to antibiotic complex 4205 in constituent amino acids, and found that it contains the same fatty component as that of B-43 (unpublished data).

It is of interest to assume that there occurs a group of antibiotics produced by *Bacillus* species including those mentioned above, whose structures are related to each other.

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