ISOLATION OF TWO NEW RELATED PEPTIDE ANTIBIOTICS, CEREXINS A AND B

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. I)

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Two new antibiotics cerexins A and B were isolated from different strains identified with *Bacillus cereus*. These two antibiotics are amphoteric in nature, soluble in particular solvents such as dimethylsulfoxide, dimethylformamide and alkaline water, and show typical infrared absorptions of peptide. These also have similar antimicrobial properties active against gram-positive bacteria.

In the past studies for searching new antibiotics, the most abundant source of new antibiotics was the order *Actinomycetales*, while the order *Eubacteria* was relatively poor as a source of antibiotics. From the genus *Bacillus*, some 65 members of well-characterized antibiotics could be seen in the reviews by KORZYBSKI *et al.*[1] and by SUMIKI[2], and some fifteen additional members have been reported up to the present time.

In our screening studies for new antibiotics from the genus *Bacillus*, an antibiotic named cerexin A active against gram-positive bacteria was isolated from several strains, a representative one of which, the strain 60-6, was identified with *Bacillus cereus* by routine taxonomic study based on BERGEY's System[3]. It has been deposited in the Fermentation Research Institute, Chiba, Japan, and the American Type Culture Collection, U.S.A. under the accession numbers, FERM-P No. 1896 and ATCC 21929, respectively.

At the same time, a closely related antibiotic, cerexin B was found to be produced by several strains, one of which, the strain Gp-3, was also identified with *Bacillus cereus* and deposited as the accession numbers, FERM-P No. 1895 and ATCC 21928.

In this paper, the production and isolation as well as the primary characterization of cerexins A and B are described.

Production and Isolation

Since the antibiotics cerexins A and B are closely similar, their production and isolation were carried out in the same manner. The following is one example.

The strain 60-6 was shake-cultured at 27°C for 24 hours in a medium consisting of glucose 1.0%, glycerin 0.25%, peptone 1.0%, meat extract 0.5% and sodium chloride 0.3% (pH 7.0).

Some 5 liters of the harvested broth was adjusted to pH 3.0 by hydrochloric acid and filtered with the aid of Celite (100 g). The filter cake was extracted with 80% aqueous acetone and followed by extraction with a mixture of chloroform, methanol and water (3:7:2). These extracts were combined and evaporated under reduced pressure to nearly aqueous solution,

and the antibiotic was transferred into *n*-butanol. The culture filtrate was also extracted with *n*-butanol. These two *n*-butanol extracts were combined and washed with dilute sodium bicarbonate, water, dilute hydrochloric acid and water, successively. Concentration of the *n*-butanol solution to a small volume resulted in the formation of a gel-like precipitate. To this ethanol was added and the mixture allowed to stand over-night at 4°C to complete the precipitation of the antibiotic. The precipitate was then collected by filtration and washed with ethanol and acetone.

The crude material thus obtained was repeatedly extracted with a mixture of chloroform, methanol and water (3:6:2) at 60°C to leave behind inactive residues. Concentration of the extract followed by addition of ethanol gave a colorless powder (1.2 g) whose antibiotic content was about 70%, as measured by paper-diffusion method on a *B. subtilis* assay plate.

Further purification was achieved by preparative thin-layer chramatography. Some 200 mg of the crude powder as described above was applied to a silica gel plate (Merck, Silica gel GF, thickness 750 μ, 20×100 cm) and developed with chloroform - ethanol - 14% aqueous ammonia (4:7:2). A separated zone of the antibiotic was detected by ultraviolet absorption and extracted with a mixture of chloroform, methanol and conc. ammoniacal water (2:2:1). The extract was evaporated, and the antibiotic was transferred into *n*-butanol. After washing with acidic and then neutralized water, the *n*-butanol solution was concentrated to give a colorless amorphous powder (160 mg) of the antibiotic cerexin A free form.

The same procedure as above with the strain GP-3 gave a similar result for the preparation of the antibiotic cerexin B.

Physical and Chemical Properties

The antibiotics cerexins A and B are closely similar in physical and chemical properties. Cerexin B exhibited somewhat faster mobility than cerexin A in TLC with some solvent systems indicated in Table 1.

Table 1. Rf values of cerexins A and B on thin-layer chromatography (Merck, Silica gel GF)

Solvent system	Rf value	
	Cerexin A	Cerexin B
n-Butanol - acetic acid - water (3:1:1)	0.51	0.59
Chloroform - ethanol - water (4:7:2)	0.50	0.55
Chloroform - ethanol - 14% ammonia (4:7:2)	0.37	0.41

The free forms of both antibiotics are obtained as colorless amorphous powders which decompose at above *ca.* 190°C. Amphoteric natures were shown by paper electrophoresis with buffer solutions of different pHs. Titration of cerexin A hydrochloride in aqueous dimethylsulfoxide with dilute sodium hydroxide gave two apparent pK's (*ca.* 6.5 and 10.5) and a neutralization equivalent of 1,517. Molecular weights of both the antibiotics were deduced to be approximately 1,500 from amino acid analytical data with their acid hydrolyzates.

The two antibiotics are soluble in dimethylsulfoxide, dimethylformamide and dilute alkaline water, slightly soluble in aqueous alcohols and aqueous acetone, but insolule in other

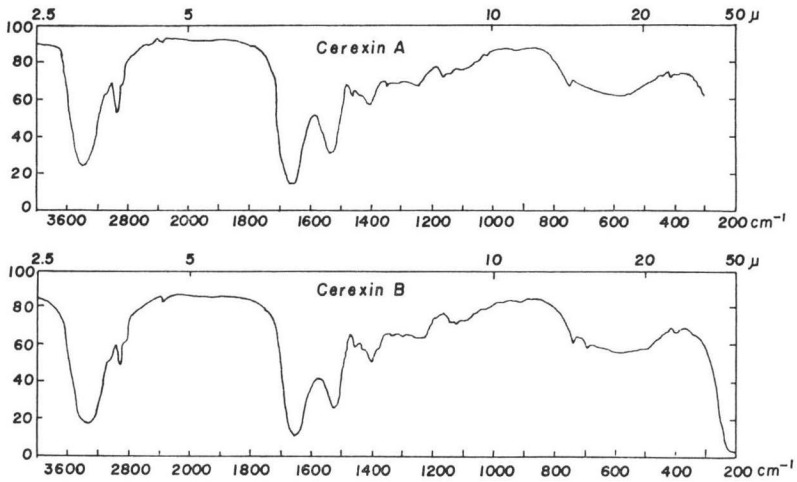
organic solvents. Positive reactions with ninhydrin, DRAGENDORFF's and EHRLICH's reagent are observed with the two antibiotics.

As listed in Table 2, the two antibiotics showed similar elemental analytical data, optical rotation and ultraviolet absorption. No sulfur and halogen are present. The infrared absorption spectra (Fig. 1) indicated that these antibiotics are peptides. The presence of tryptophan residues is indicated by the UV spectra.

Table 2. Physico-chemical properties of cerexins A and B

	Cerexin A (free form)	Cerexin B (free form)
Anal. Found:	C, 53.31; H, 7.72; N, 14.27	C, 55.36; H, 7.28; N, 14.39
$[\alpha]_D^{23°}$	19.5±3.50° (*c*, 0.172, DMF)	19.8±2.30° (*c*, 0.263, DMF)
λ_{max}^{MeOH}	275 mμ ($E_{1cm}^{1\%}$ 36)	275 mμ ($E_{1cm}^{1\%}$ 36)
	282 mμ ($E_{1cm}^{1\%}$ 38.5)	282.5 mμ ($E_{1cm}^{1\%}$ 38.5)
	290.5 mμ ($E_{1cm}^{1\%}$ 33.5)	290.5 mμ ($E_{1cm}^{1\%}$ 34)

Fig. 1. Infrared absorption spectra of cerexins A and B (KBr)



Acid hydrolysis revealed the presence of 10 amino acid residues and a variety of fatty acid residues in both the antibiotics. These data will be discussed in the next publication.[4]

Biological Properties

The antimicrobial spectra of cerexin A and B, obtained by the usual twofold agar dilution technique, are listed in Table 3. These antibiotics are similarly active against gram-positive bacteria but not against gram-negative bacteria, yeast and fungi. Though the activities are not so strong *in vitro*, these antibiotics exhibited chemotherapeutic effects when tested against mice infected with *Streptococcus pyogenes*, *Diplococcus pneumoniae* or *Staphylococcus aureus*. The ED_{50} values observed with cerexin A are listed in Table 4. Toxicities of these antibiotics to mice were shown to be relatively low. The LD_{50} values obtained with cerexin

Table 3. Antimicrobial spectra of cerexins A and B

Organism	MIC (mcg/ml)	
	Cerexin A	Cerexin B
Bacillus subtilis PCI 219	6.25	6.24
Bacillus anthracis	25	12.5
Staphylococcus aureus FDA 209P JC-1	50	50
Diplococcus pneumoniae type I	3.13	6.25
Streptococcus pyogenes C-203	6.25	6.25
Mycobacterium tuberculosis $H_{37}Rv$	>50	>50
Escherichia coli NIHJ JC-2	>50	>50
Klebsiella pneumoniae	>50	>50
Salmonella typhimurium	>50	>50
Pseudomonas aeruginosa PS-24	>50	>50

Obtained by the usual agar dilution method.

Table 4. Therapeutic effect of cerexin A administered subcutaneously to ICR mice infected with *Streptococcus pyogenes*, *Staphylococcus aureus* or *Diplococcus pneumoniae*

	S. pyogenes	*S. aureus*	*D. pneumoniae*
ED_{50}* (mg/kg×2)	4.4	4.7	3.9

* The ED_{50} is expressed as mg/kg in two subcutaneous doses, given 1 and 5 hours postinfection.

A sodium salt were as follows: 100~200 mg/kg (i.p.), 50~100 mg/kg (i.v.), >500 mg/kg (sc) and >500 mg/kg (p.o.).

References

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