ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1984, p. 939–940
0066-4804/84/120939-02$02.00/0

Vol. 26, No. 6

Mycocerein, a Novel Antifungal Peptide Antibiotic Produced by *Bacillus cereus*

SACHIO WAKAYAMA,[1] FUMIYASU ISHIKAWA,[2] AND KUNIO OISHI[2]*

Institute of Applied Microbiology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113,[2] and Kibun Company, Ltd., 14-13-7 Ginza, Chuo-ku, Tokyo 104,[1] Japan

Received 30 May 1984/Accepted 10 September 1984

A peptide was obtained from culture filtrates of a bacterium which was newly isolated and tentatively named *Bacillus cereus* SW. The peptide was composed of Asx, Ser, Glx, Leu, Tyr, Pro, and an unknown amino acid in a ratio of 2:1:1:1:1:1:1, but, unless hydrolyzed with HCl, it was ninhydrin reaction negative. The peptide effectively inhibited the growth of all fungi and yeasts so far examined, whereas it inhibited none of the bacteria tested.

Bacteria of the genus *Bacillus* produce a variety of peptide antibiotics (7, 12). Most of the peptides are antibacterial, and a few are known to be antifungal, antitumor, cytotoxic, fibrinolysis promoting (6), and immunosuppressive (2). All antifungal peptides so far known, including mycobacillin (9, 11), iturin A (5), bacillomycin (1), mycosubtilin (10), fungistatin (8), and subsporin (4), are cyclic ones and are produced by *Bacillus subtilis*. Their activities against fungi are not efficient and specific enough to allow their use for therapeutic purposes.

We have found a highly specific and sufficiently active antifungal peptide in the culture filtrate of a bacterium tentatively named *B. cereus* SW according to the description in *Bergey's Manual*, 8th ed. (3). This bacterium was newly isolated in our laboratory in 1983. It is a gram-positive rod, 1.3 by 3.5 μm, motile, sporeforming, aerobic, catalase positive, Voges-Proskauer reaction positive, intracellular protein crystal negative, and egg yolk reaction positive, suggesting that it is, at least, not *B. subtilis* but that it should be *B. cereus*.

B. cereus SW was cultured in a medium (pH 7.0) containing 3% peptone, 0.5% yeast extract, and 0.5% NaCl for 3 days at 27°C with shaking. Cells were removed by centrifugation, and $CaCl_2$ (final concentration, 1%) was added to the supernatant. The mixture stood at 4°C overnight. The resulting precipitates were collected by centrifugation, dissolved in 100 mM EDTA–0.05 M Tris-hydrochloride buffer (pH 8.0), and dialyzed extensively against 0.05 M phosphate buffer (pH 7.0). Ethanol (final concentration, 80%) was added to the dialysate, and the mixture was allowed to stand for 6 h at 4°C. Precipitates were removed by centrifugation, and the supernatant was dried in vacuo, dissolved in a minimal volume of water, and acidified with HCl to pH 3.0. Precipitates were collected by centrifugation, dissolved in 0.05 M $NaHCO_3$, and dialyzed against deionized water. The dialysate was passed through a Sephadex G-100 column. Active fractions were collected and freeze-dried.

The preparation thus obtained easily formed colorless prismatic crystals from an aqueous solution upon addition of ethanol and gave a single spot with concentrated H_2SO_4 on Merck Silica Gel 60 plates, using the following solvent systems: *t*-butanol–acetic acid–water (74:3:25), ethyl acetate-acetic acid-water (88:6:6), *n*-butanol–acetic acid–water (79:6:15), methanol-acetic acid-water (25:3:72), and acetone-acetic acid-water (20:6:74). This preparation consisted of C, H, O, and N (57.1, 7.38, 24.22, and 11.25%). P and S were not detected. Sugar and nucleic acid were not detected in this preparation by the phenol-H_2SO_4 method and UV absorption, respectively. The ninhydrin reaction was negative, but when the preparation was hydrolyzed with 6 N HCl at 110°C for 24 h, it became positive. The amino acid composition of the HCl hydrolysate was determined with an amino acid analyzer (Hitachi 835-65) to confirm that it was composed of aspartic acid, serine, glutamic acid, leucine, tyrosine, proline, and an unknown amino acid in a ratio of 2:1:1:1:1:1:1. From these results, it may be concluded that our preparation is an acidic cyclic peptide with a minimal molecular weight of about 1,000 and that the peptide is a novel compound in its amino acid composition.

The antifungal activities of this compound are shown in Table 1. A volume of suspensions of fungal or yeast spores or vegetative cells in 0.05 M phosphate buffer (pH 7.0) containing 1% lactose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract was mixed with an equivalent volume of the serially diluted peptide solution on a microtiter plate and incubated at 27°C for 3 to 7 days. A bacterial suspension

TABLE 1. Antimicrobial spectra of mycocerein

Strain	MIC (μg/ml) of mycocerein
Fungi/yeasts	
Conidiobolus lamprauges 454	19.5
Conidiobolus lamprauges ATCC 28997	19.5
Fusarium oxysporum IAM 5009	39
Aspergillus nidulans IAM 2006	39
Eurotium chevalieri IFO 4928	19.5
Mucor rouxianus IAM 6131	78
Penicillium chrysogenum IAM 7106	39
Cryptococcus luteolus IAM 12207	19.5
Hansenula wingei IAM 4983	19.5
Saccharamyces cerevisiae IAM 4125	39
Bacteria	
Staphylococcus aureus NIHJ 2099	>625
Bacillus subtilis IAM 1026	>625
Escherichia coli IAM 1268	>625
Serratia marcescens IAM 12142	>625
Proteus vulgaris IAM 1025	>625

* Corresponding author.

in a medium (pH 7.2) consisting of 1% meat extract, 1% peptone, and 0.5% NaCl was also mixed with the peptide solution in the same manner and incubated at 30°C for 1 to 3 days. Growth inhibition was determined by the turbidity of the mixture. Table 1 shows that our compound potently inhibited the growth of all fungi and yeasts examined, whereas it inhibited none of the bacteria. The activities of most of the known so-called antifungal peptide antibiotics are not as strong or as wide ranging and are not fungus and yeast specific. In this respect, the peptide may be regarded as a novel compound with novel properties. We propose to name this peptide compound mycocerein. Its detailed properties are now under investigation.

We thank A. Kakinuma of Takeda Chemical Industries, Ltd., Osaka, Japan, for his kind advice and for supplying the surfactin sample. We are also indebted to K. Yamasato of our institute for his advice on the taxonomic diagnosis of the *Bacillus* sp. and H. Hino and I. Matsui of our institute for performing the elementary analysis and amino acid analysis, respectively.

LITERATURE CITED

1. **Besson, F., F. Peypoux, G. Michel, and L. Delcambe.** 1977. The structure of Bacillomycin L, an antibiotic from *Bacillus subtilis*. Eur. J. Biochem. **77**:61–67
2. **Borel, J. F.** 1976. Comparative study of *in vitro* and *in vivo* drug effects on cell-mediated cytotoxicity. Immunology **31**:631–641.
3. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
4. **Ebata, M., K. Miyazaki, and Y. Takahashi.** 1969. Isolation and characterization of Subsporins A, B, and C. J. Antibiot. **22**:467–472.
5. **Isogai, A., S. Takayama, S. Murakoshi, and A. Suzuki.** 1982. Structures of β-amino acids in antibiotics Iturin A. Tetrahedron Lett. **23**:3065–3068.
6. **Kakinuma, A., G. Tamura, and K. Arima.** 1968. Wetting of fibrin plate and apparent promotion of fibrinolysis by Surfactin, a new bacterial peptidelipid surfactant. Experientia **24**:1120–1121.
7. **Katz, E., and A. L. Demain.** 1977. The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. Bacteriol. Rev. **41**:449–474.
8. **Korzybski, T., Z. K. Gindifer, and W. Kurylowicz.** 1978. Fungistatin, p. 1612–1613. *In* Antibiotics. Origin, nature, and properties. American Society for Microbiology, Washington, D.C.
9. **Majumdar, S. K., and S. K. Bose.** 1958. Mycobacillin, a new antifungal antibiotic produced by *Bacillus subtilis*. Nature (London) **181**:134–135.
10. **Peypoux, F., G. Michel, and L. Delcambe.** 1976. The structure of Mycosubtilin, an antibiotic isolated from *Bacillus subtilis*. Eur. J. Biochem. **63**:391–398.
11. **SenGupta, S., A. B. Banerjee, and S. K. Bose.** 1971. γ-Glutamyl and D- or L-peptide linkages in Mycobacillin, a cyclic peptide antibiotic. Biochem. J. **121**:839–846.
12. **Shoji, J.** 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Adv. Appl. Microbiol. **24**:187–214.