Short Communication

Arthrospore-inducing Substances
from *Acremonium chrysogenum*
Which Stimulate Cephalosporin
C Production

Masahiro NATSUME and Shingo MARUMO

Department of Agricultural Chemistry,
Nagoya University,
Chikusa-ku, Nagoya 464, Japan

Received September 30, 1983

During our chemical research on fungal morphogenic substances (asexual spore-inducing substances^{1,2)}), we have been interested in the possible roles of morphogenic substances which may control the production of such important secondary metabolites as antibiotics. *Acremonium chrysogenum* (THIRUMALACHAR *et al.* SUKAPURE) W. Gams³⁾ is a fungus extensively used for the production of cephalosporin C in the fermentation industry. Nash and Huber⁴⁾ and Matsumura *et al.*⁵⁾ reported that there is a parallel correlation between the formation of arthrospores and the production of cephalosporin C in *A. chrysogenum*; strains bearing large numbers of arthrospores tended to produce more antibiotic. We have assumed that an arthrospore-inducing substance(s) is produced when hyphae differentiate into arthrospores and, if the active substance could be isolated, it then could be used to stimulate the production of cephalosporin C. Here, we wish to describe purification of two arthrospore-inducing substances named Factors A and B and their stimulating effect on cephalosporin C production.

A. chrysogenum ATCC 14553 was cultured by shaking in Nash's medium⁴⁾ without methionine, in which most hyphae differentiated into arthrospores. In a preliminary experiment, the culture filtrate was mixed with two parts of acetone, filtered and evaporated to dryness, and addition of the residue to non-

sporulating Bhuyan's medium⁶⁾ produced dramatic arthrospore formation. Active substances contained in the culture filtrate were purified by sequential column chromatographies, on active carbon (elution with 10% acetone in water), DEAE-Sephadex A-25 (OH⁻ form, 0.1 N AcOH) and Sephadex G-10 (water). This last column fractionated the sample into ten portions. Surprisingly, none of the fractions alone showed activity, but the recombination of all ten fractions restored the original activity. Of all the possible combinations of these fractions, only the combination of fractions A and B had activity comparable to that of the sample before chromatography, indicating the necessity of these two components for induction of the activity.

An arthrospore-inducing substance contained in fraction B (Factor B) was purified further by column chromatography on DEAE-Sephadex A-25 (AcO⁻ form, two times repeated elution with water) followed by HPLC on LiChrosorb RP-18 (two times repeated elution with 0.05% NH₄OAc), Nucleosil 7C₆H₅ (0.05% NH₄OAc) and Develosil ODS-5 (5% CH₃CN in water). Factor B was finally isolated as a single peak in the last two chromatograms (yield *ca.* 200 µg from 200 liters of culture filtrate). Chromatographic results of two experiments are shown in Fig. 1. The peak detected by UV absorption (210 nm) coincided with the eluted portion that had arthrospore-inducing activity. Purified Factor B induced arthrospore formation at 0.03 µg/ml in hyphae grown in nonsporulating Bhuyan's medium in the presence of Factor A, the amount of which to be added depended upon the degree of purification. A preliminary physicochemical investigation revealed that Factor B is a polar substance with a MW of 242 as determined from EI mass spectra of its tetrakis(trimethylsilyl) derivative (*m/z* 440 (M⁺ - TMSOH)) and triacetate (*m/z* 368 (M⁺)), and UV absorptions at λ_{max}^{pH 6} (ε) 302 (7260), 251 (8930) and 216 (16,300).

The active substance in fraction A, Factor A, was purified on Sephadex G-10 (0.05 M

FIG. 1. Chromatograms of Active Fraction B (Factor B) from Two Chromatographic Columns.

(A) The 1st column of LiChrosorb RP-18. (B) The last of Develosil ODS-5. Arthrospore-inducing activity (▨) was determined microscopically. Cephalosporin C production was analyzed quantitatively by the HPLC method (LiChrosorb RP-18, 4×250 mm, 0.05% NH_4OAc -MeOH (98:2); retention time of cephalosporin C, 14.6 min). The yield of cephalosporin C in the control experiments was $1 \sim 1.5$ µg/ml. This low yield may be due to the use of Bhuyan's medium, in which the fungus hardly sporulates; thus the difficulty in producing the antibiotic.

NH_4OAc), but still was a mixture of several components. This crude Factor A at 12 µg/ml induced considerable arthrospore formation in the presence of Factor B.

Arthrospores induced by pure Factor B together with crude Factor A are shown in Fig. 2B. The swollen, thick and highly septate hyphal fragments (arthrospores) are well differentiated, whereas only normal slender hyphae (Fig. 2A) were produced without the addition of Factors A and B.

Detailed examination revealed that Factor A may be the major active substance because it induced arthrospores by itself at a high concentration (five-fold the concentration required for the induction in the presence of Factor B), and this induction was enhanced effectively by the addition of Factor B. Thus, arthrospore formation in Nash's medium seems to be the result of the combined action of Factors A and B, both compounds being produced in small amounts in the culture medium.

We also investigated whether the production of cephalosporin C is, or is not, enhanced by

arthrospore-inducing substances. The stimulating effect of the spore-inducing substances on antibiotic production was analyzed quantitatively by the HPLC method (see Fig. 1) for every purification step. As a result, we found that antibiotic production was enhanced during every step by the addition of arthrospore-inducing factors. Quantitative assay results for two HPLC (Fig. 1) show that only the peak with arthrospore-inducing activity stimulated antibiotic production. The yield of antibiotic was enhanced six- to twenty-fold those of the controls which had no added factors and the yields of the non-active fractions from chromatography.

Our study showed that arthrospore formation in *A. chrysogenum* is stimulated by two active substances, Factors A and B, which are produced in the culture broth and that a combination of these factors also enhances the production of cephalosporin C, as first assumed. Hyphal differentiation into arthrospores seems to be a prerequisite growth condition in order for the fungus to produce the antibiotic. The exact role of Factors A and B

FIG. 2. Arthrospore-inducing Activity of Factors A and B in *A. chrysogenum*: (A) Normal Hyphae Grown in Nonsporulating Bhuyan's Medium; (B) Arthrospores Induced by the Combined Addition of Crude Factor A (12 µg/ml) and Pure Factor B (0.03 µg/ml) in Bhuyan's Medium ($\times 400$).

in cell differentiation, as well as in cephalosporin C production, will be reported elsewhere. The possibility that the two factors are biosynthetic precursors of cephalosporin C was ruled out by the large production of antibiotic when only a small amount of the two compounds was added to the culture. The isolation of Factor A and the determination of the structures of the two active compounds as well as their physiological function(s) are now in progress.

REFERENCES

- 1) S. Marumo, M. Nukina, S. Kondo and K. Tomiyama, *Agric. Biol. Chem.*, **46**, 2399 (1982).
- 2) M. Katayama, S. Marumo and H. Hattori, *Tetrahedron Lett.*, **24**, 1703 (1983).
- 3) This specific name was recently given to those fungi formerly called *Cephalosporium acremonium* Corda. (American Type Culture Collection, Catalogue of strains 1. Fifteenth edition, 1982).
- 4) C. H. Nash and F. M. Huber, *Appl. Microbiol.*, **22**, 6 (1971).
- 5) M. Matsumura, T. Imanaka, T. Yoshida and H. Taguchi, *J. Ferment. Technol.*, **58**, 197 (1980).
- 6) B. K. Bhuyan, J. Mohberg and M. J. Johnson, *J. Bacteriol.*, **76**, 393 (1958).