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SHORT COMMUNICATION

THE EXTRACTION OF A SEXUAL HORMONE FROM THE MYCELIUM OF *MUCOR MUCEDO*

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Abstract—A hormone capable of inducing the production of zygophores in unmated cultures of *Mucor mucedo* has been extracted from mated mycelium of the same fungus. Evidence is given that the mycelium is a richer source of the hormone than the medium. Techniques are described for the extraction and purification of the active substance.

INTRODUCTION

SINCE the work of Burgeff,[1] the induction of zygophores, the sexual hyphae of the Mucorales, has been ascribed to hormones produced in the presence of both mating types. Plempel[2, 3] has extracted and partially characterized hormones from the cell-free broth of a mated culture of *Mucor mucedo* grown supported on a loofah in vigorously aerated liquid medium. His experiments pointed to the presence of two substances in the extract, one active on the (+) strain and one active on the (—) strain. More recently, Ende[4] has obtained a substance that is active on cultures of *M. mucedo* from the culture filtrate of mated cultures of *Blakeslea trispora* grown submerged in nutrient medium. In Ende's work there is no evidence for the presence of two hormones.

In the present work a hormone capable of inducing zygophores in both mating types of *M. mucedo* has been extracted from the mycelium of mated cultures of this fungus grown on nutrient agar. Its properties encountered during purification are described, and comparisons drawn with the work of Plempel and Ende. Biological aspects of the production and action of the hormone are described elsewhere.[5] In the present work no evidence has been obtained for the presence of two complementary hormones, as described by Plempel.

RESULTS AND DISCUSSION

The hormone can be extracted from mated cultures of *Mucor mucedo* grown on any solidified nutrient medium capable of supporting zygospore production. As described elsewhere,[5] activity has been obtained from submerged cultures of *M. mucedo* only when

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[1] H. BURGEFF, *Bot. Abh.* **4**, 5 (1924).
[2] M. PLEMPEL, *Naturwissenschaften* **50**, 226 (1963).
[3] M. PLEMPEL, *Planta* **59**, 492 (1963).
[4] H. VAN DEN ENDE, *Nature* **215**, 211 (1967).
[5] G. W. GOODAY, *New Phytol.*, in press (1968).

these have been aerated with oxygen. Comparative bio-assays of extracts of mycelium and medium from cultures grown under three conditions showed that most of the hormone that could be extracted from a culture was in the mycelium and not in the medium (Table 1).

TABLE 1. PROPORTION OF TOTAL EXTRACTABLE ACTIVITY OBTAINED FROM THE MYCELIUM IN THREE CULTURAL CONDITIONS

Cultural conditions	% of total activity in mycelial extract
Grown on sand	85
Grown on polyacrylamide gel	83
Submerged culture aerated with O_2	93

The active substance is adsorbed by activated charcoal from aqueous solutions, and use was made of this in the extraction procedure. It was eluted from the charcoal by the use of ethanol. In common with the substances described by Plempel[2, 3] and Ende[4] the activity was extracted from acidified aqueous solutions by diethyl ether.

Considerable purification was obtained by chromatography on DEAE-cellulose with a stepwise increase in phosphate buffer, pH 7·8, followed by re-chromatography on the same column with a gradient elution. This is similar to the behaviour of the active substance as extracted by Ende. Further purification with clear separation of impurities (as detected by u.v. spectra) was obtained by chromatography on Ecteola-cellulose, first with a stepwise elution and then re-chromatography with a gradient elution. The most active samples from this procedure showed u.v. maxima at 235 and 305 nm. However, these samples showed a steady loss of biological activity, and after re-chromatography the peak showing biological activity was always just preceded and overlapped by inactive material showing u.v. maxima at 230 and 300 nm. A similar effect of instability was noticed by Ende during paper chromatography. In the present work, very low yields (about 5 per cent) were obtained from paper and thin-layer chromatography.[6] Column electrophoresis and gel filtration on Sephadex G-10 and Sephadex LH-20 failed to separate the inactive material. The active substance and the inactive accompanying material were reversibly adsorbed on the gel filtration materials, being eluted after low molecular weight marker compounds.

Further work on purification of the hormone must take into account the instability of the molecule. Techniques standard in work with other unstable natural products, such as using an atmosphere of nitrogen, might give better results.

On removal of the solvent, the most active extracts obtained in the present work gave a yellow acidic oil with a characteristic "clinging" sweet smell, and u.v. maxima in aqueous solutions at 235 and 305 nm.

MATERIALS AND METHODS

Cultures

The strains of *Mucor mucedo* Brefeld are kept in the Department of Botany, University of Bristol, as Z43 (−) and Z46 (+). Both cultures originally came from C.B.S., Baarn. The hormone was extracted from mated mycelium grown in glass dishes on a medium containing per litre: glucose 20 g, malt extract 20 g, peptone 5 g, solidified with agar 20 g. The dishes

[6] G. W. GOODAY, Ph.D. Thesis, Univ. of Bristol (1967).

were inoculated with a dense mated spore suspension and incubated at 20° for 3 days. The mats of fungus were readily harvested as they could be peeled cleanly away from the medium. 10 l. of medium gave about 1 kg fresh weight of mycelium.

Three experiments were carried out to determine the amount of hormone that could be extracted from the medium and to compare this with the amount that could be extracted from the fungus. One culture was grown on sand with the glucose, malt, peptone medium. After 3 weeks' growth the mycelial mat was harvested, washed and the hormone extracted with acetone. The sand was washed through with water to provide the culture filtrate. Both extracts were concentrated by the use of activated charcoal and then assayed for hormone activity. A second culture was grown on the same medium solidified with Bio-gel P-300 polyacrylamide gel. The mycelium was supported on the gel on a cellophane membrane and harvested after 5 days' growth. Mycelium and medium were extracted as above. A third culture was grown in liquid medium of the same composition in a culture vessel with a sintered glass base aerated with a stream of oxygen. The culture was harvested after 3 days' growth and the hormone extracted and assayed from both medium and mycelium as before.

The bio-assay for the hormone is described elsewhere.[5]

Extraction and Purification of the Hormone

The crude extract was made by homogenizing the mycelium in a blender (M.S.E. Ato-Mix) with 20 times its own volume of acetone and re-extracting once with 95 per cent acetone. After evaporation of the acetone the active aqueous extract was filtered and passed through a bed of activated charcoal (B.D.H.) in a sintered funnel and washed well with water. The active substance was then eluted through with ethanol. The active yellow oil obtained by evaporating the ethanol was dissolved in 100 ml 0·2 M phosphate buffer, pH 7·8. This solution was treated with 1 g DEAE-cellulose and filtered. The aqueous solution was made to pH 1 with hydrochloric acid and extracted with three successive volumes of diethyl ether. The ethereal extract was dried (Na_2SO_4) and the ether evaporated to give a yellow oil. This was dissolved in 1 ml 0·001 M phosphate buffer, pH 7·8, and the solution chromatographed on a column (30 × 1·7 cm) of DEAE-cellulose (Whatman DE22) equilibrated in the 0·001 M buffer with a stepwise increase in buffer concentration to 0·06 M in 300 ml. The most active tubes were bulked, extracted with ether at pH 1, transferred to 1 ml 0·001 M buffer and re-chromatographed on the same column with a gradient elution between 0·001 M and 0·04 M phosphate buffer. The most active tubes from this elution were chromatographed on a column (25 × 1·1 cm) of Ecteola cellulose (Whatman ET11) with a stepwise elution from 0·001 M to 0·04 M phosphate buffer, pH 7·8. The active material was re-chromatographed on the same column with a gradient elution of 160 ml from 0·001 M to 0·015 M buffer.

Other Methods

Electrophoresis was on a column (32 × 1·5 cm) of Sephadex G-25 in 0·1 M veronal buffer, pH 9·8, at 1000 V, 7 mA for 5 hr, and in 0·05 M phosphate buffer, pH 7·8, for 32 hr. A 1 ml sample was used in both cases and electrophoresis was followed by elution with the buffer. Gel filtration was on Sephadex G-10 in a column (100 × 1·3 cm) equilibrated with 0·05 M phosphate buffer, pH 7·8, with a 0·5 ml sample and 1 ml fractions were collected. A column of Sephadex LH-20 in ethanol was also used, eluting through with ethanol from a 1 ml sample.

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