SYNTHESIS OF ERGOT ALKALOIDS BY PROTOPLASTS OF *CLAVICEPS* SPECIES

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Investigations of the biosynthesis, physiology, and metabolism of secondary metabolites in fungi have been impeded by the fungal cell wall which acts as a permeability barrier and prevents access to the cytoplasmic enzymes. To some degree this problem has been circumvented by the use of protoplasts. For example Fawcett *et al.* (1), using antibiotic producing strains of *Penicillium chrysogenum* and *Cephalosporium acremonium*, prepared protoplasts with metabolic activity, including antibiotic production, which was similar to that of control mycelium. Dutton and Anderson (2) demonstrated aflatoxin synthesis in protoplasts derived from *Aspergillus flavus*. These protoplasts incorporated ^{14}C-acetate and ^{14}C-versicolorin A into aflatoxin B_1.

In our studies on the end-product regulation of ergot alkaloid biosynthesis, cell wall permeability barriers prevented movement of alkaloids into the cell. Since Stahl *et al.* (3) developed a method for the preparation of protoplasts from *Claviceps*, we utilized protoplasts to investigate end-product regulation in ergot alkaloid biosynthesis. An important prerequisite for our work was that the protoplasts retain the ability to synthesize alkaloids. We are making the first report of the synthesis of clavine and peptide-type ergot alkaloids by protoplasts of *Claviceps* species.

EXPERIMENTAL

ORGANISM AND CULTURE CONDITIONS.—The strain of ergot used for this study was *Claviceps* species, strain SD 58 (ATCC 26019), originally isolated from sclerotia obtained from the host *Pennisetum typhoideum* Richard.

The organism was maintained on Czapek-Dox agar slants and grown in shake cultures at 25° and 180 rpm on a New Brunswick model G54 rotary shaker. Cultivation of the strain was achieved by inoculation of 500-ml Erlenmeyer flasks containing 100 ml of culture medium NL-406 (4) with a portion of the mycelium from an agar slant.

After ten days, 2 ml of the shake culture was transferred to fresh NL-406 medium. This was repeated (usually four times) until the culture was homogeneous in appearance.

PROTOPLAST FORMATION.—Using the method of Stahl *et al.* (3), mycelium (5 g wet weight) from a 5-day-old shake culture (alkaloid titer 8 mg/culture) was harvested on a coarse sintered glass funnel and washed three times with a small volume of 0.7 M KCl solution. After being suspended in 60 ml of 0.7 M KCl stabilizing solution plus 6.7 ml liquid β-glucuronidase/arylsulfatase obtained from *Helix pomatia*, (Boehringer Mannheim Biochemicals) for a total volume of 66.7 ml, the mycelium was incubated with shaking (100 rpm) at 30° for 4 h at which time a drop of the incubation mixture was observed under the microscope for protoplasts. The resulting protoplast solution was centrifuged at 3,000 rpm for 10 min. The residue was washed twice with 0.7 M KCl solution and was centrifuged each time at 3,000 rpm for 10 min to recover the protoplasts and was finally resuspended in 10 ml 0.7 M KCl solution and passed through glass wool. The total protein in the protoplast residue was determined by the method of Bramhall *et al.* (5).

ALKALOID SYNTHESIS IN PROTOPLASTS.—After observing the protoplasts with a Zeiss model RA 38 microscope at 1000 X, the protoplast suspension was equally divided and transferred to two 250-ml Erlenmeyer flasks, designated A and B, each containing 60 ml 0.7 M KCl solution and 2 μCi of L-[methylene-^{14}C]tryptophan (52 mCi/mmole, Amersham Corp.). The protoplast suspensions were incubated for 24 h at 22° with shaking at 180 rpm and then centrifuged at 12,000 rpm for 10 min. The alkaloid titer of the supernatant was determined before it was extracted for alkaloids.

ALKALOID ISOLATION AND ASSAY.—The supernatant was made alkaline to pH 10

with NH_4OH, and the total alkaloids were extracted three times with chloroform. The alkaloidal extract was further extracted three times with 2% succinic acid aqueous solution. The acid was made alkaline to pH 10 with NH_4OH, and the alkaloids were extracted into chloroform three times. The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under vacuum. After dissolving the extracted alkaloids in 4 ml of absolute methanol, 0.2 ml was used to determine alkaloid titer, 0.2 ml was taken for radioactivity determination in a scintillation counter, and 0.8 ml was used for tlc on 5 x 20-cm silica gel, precoated, glass plates (Merck). The plates were developed in chloroform-methanol-NH_4OH (400:100:1) and the radioactivity in the separated alkaloids was determined on a radiochromatogram scanner (Packard). Agroclavine, chanoclavine I, and elymoclavine were identified by cochromatography with known alkaloids.

Alkaloids were quantitated in micrograms by the modified Van Urk method (4, 6, 7).

Indole alkaloids on chromatograms were visualized by spraying with Ehrlich's reagent (1.0 g of *p*-dimethylaminobenzaldehyde, 10 ml of water, and 20 ml of concentrated HCl). Blue-purple colored spots indicate the presence of indole compounds.

REGENERATION OF INTACT CELLS FROM PROTOPLASTS.—The regeneration of intact cells was carried out by plating protoplasts onto agar plates containing a medium composed of 5% beerwort (courtesy of Anheuser-Busch Inc., St. Louis, MO), 0.3% yeast extract (Difco), 2.5% agar at a pH of 5.5 (3). After 3 to 5 days the regenerated cells were transferred to NL-406 medium and grown in shake cultures.

RESULTS AND DISCUSSION

Table 1 shows the results of L-[methylene-^{14}C]tryptophan incorporation into alkaloid by the ergot protoplasts. These results compare favorably with those that have been reported by Floss *et al.* (8) in which L-[indol-^{3}H]tryptophan was incorporated at the rate of 35.6% into elymoclavine by cultures of the same strain, SD 58, which we used to prepare protoplasts.

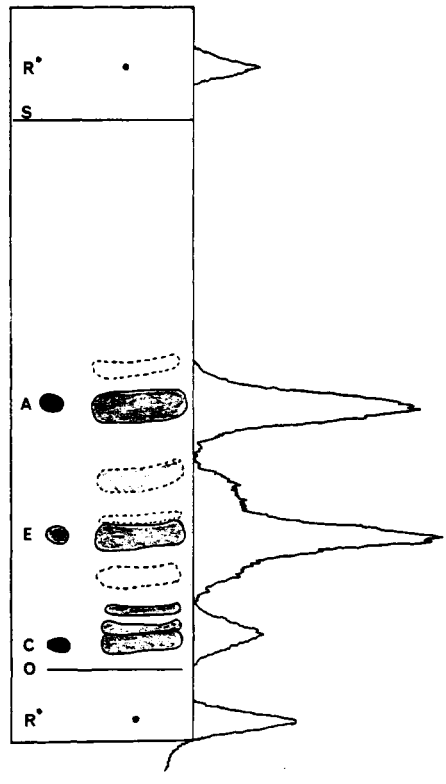

FIG. 1. An illustration of a thin-layer chromatogram of the alkaloidal extract obtained from protoplasts of *Claviceps*, strain SD 58 which had been incubated for 24 h with 2 μCi of L-[methylene-^{14}C]tryptophan. The chromatogram which was developed in $CHCl_3$-methanol-NH_4OH (400:100:1) and sprayed with Ehrlich's reagent to visualize alkaloids (dark spots) is superimposed over a tracing obtained on a radiochromatogram scanner which illustrates radioactive labeling of the separated alkaloids. Dotted line indicates u.v. fluorescence; S=solvent front; O=origin; A=agroclavine; E=elymoclavine; C=chanoclavine I; and R*=radioactive standard marker.

TABLE 1. Incorporation of L-[methylene-^{14}C]tryptophan into clavine alkaloids by protoplasts of *Claviceps* species, strain SD 58.

Expt.	Radioactivity fed	Alkaloid formed, μg	Radioactivity of alkaloid	% Incorporation
A	3.90 x 10^6 dpm ^{14}C	952	1.50 x 10^6 dpm ^{14}C	38.4
B	4.11 x 10^6 dpm ^{14}C	770	1.44 x 10^6 dpm ^{14}C	35.0

Radiochromatogram scanning of the developed chromatograms (fig. 1) demonstrated that the radioactivity resided principally in the three alkaloids, agroclavine, chanoclavine I, and elymoclavine with greatest activity divided equally between agroclavine and elymoclavine and chanoclavine I containing approximately one-fifth the activity of each of the other two alkaloids. It was determined that the protoplasts which were formed from 5 g of mycelium represented 110 mg of total protein, a value comparable to the protein content of the intact mycelium (9), indicating that the recovery of protoplasts from mycelium is nearly quantitative.

Evidence that we were indeed working with protoplasts rather than intact cells or cell-free enzyme preparations in which not only the cell wall but also the cell membrane had been destroyed, was obtained by examining the protoplast suspension under the microscope. The suspension consisted of spheres that ranged from 3 to 5 μm in diameter and careful examination revealed no intact cells. Other evidence comes from the fact that when elymoclavine is added to the protoplast incubation mixture, alkaloid synthesis is inhibited. This effect is not observed with intact cells, since apparently the cell wall prevents the uptake of elymoclavine into the cell (L.-j. Cheng, J. E. Robbers and H. G. Floss submitted for publication). In addition, intact cells which had regained the full alkaloid-producing ability and morphology of the parent strain could be regenerated from the protoplasts indicating that we were not working with a cell-free enzyme preparation. Also, the regeneration of cells from protoplasts resulted in the appearance at approximately the same time of an extensive lawn of colonies on agar plates. If a small number of intact cells were present in the protoplast suspension, one would expect that these cells would cause the appearance of a few isolated colonies prior to the appearance of colonies arising from protoplasts. This was not the case.

Preliminary studies utilizing protoplasts prepared from an ergotamine producing strain of *Claviceps purpurea* PCCEI, a selection from strain FI 32/17 (ATCC 20102) also indicate peptide alkaloid synthesis in protoplasts based on the incorporation of L-[U-^{14}C]proline into ergotamine, albeit at much lower levels than the incorporation of tryptophan in the protoplasts of *Claviceps* strain SD 58.

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