Production of Peptide Ergot Alkaloids in Submerged Culture by Three Isolates of *Claviceps purpurea*

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Three strains of *Claviceps purpurea* (Fr.) Tul., isolated from sclerotia grown on rye, produce under submerged conditions ergocryptine and ergotamine, ergocornine and ergosine, and ergocristine, respectively. All of the strains either lacked the ability to produce conidia or formed them sparingly, but they accumulated large quantities of lipids and sterols. The fermentations are typically divided into two phases. The first is characterized by the rapid utilization and exhaustion of the phosphate contained in the medium, rapid uptake of ammonium nitrogen and of citric acid, rapid growth, and low alkaloid production; the second phase is characterized by slower growth and by a marked accumulation of lipids, sterols, and alkaloids.

High yields of lysergic acid alkaloids in submerged culture were first obtained in 1961 by Arcamone et al. (6), who described the production of α-hydroxyethyl-lysergamide by a strain of *Claviceps paspali* (Fr.) Tul. Subsequently, other authors (8, 13) obtained the same compound with different strains of *C. paspali*. Later, Kobel et al. (11) described the production of 6-methyl-ergol-8-ene-8-carboxylic acid by different strains of the same species. In 1966, Amici et al. (2) described the production in high yield of ergotamine by a submerged culture of a strain of *C. purpurea*. Studies on the physiology, genetics, and metabolism of the same strain were also published (3-5).

In 1967, we isolated three strains of *C. purpurea* that produce large amounts of ergocryptine and ergotamine, ergocornine and ergosine, and ergocristine in submerged culture. This paper deals with a description of these strains and of their production processes.

**MATERIAL AND METHODS**

Culture media. Medium CZ 4M contained (g per liter): glucose, 40; Vegedor (a vegetable extract produced by Liebig, Italy), 1; (NH₄)₂HPO₄, 5; KH₂PO₄, 1; MgSO₄·7H₂O, 2.5; KCl, 0.5; FeSO₄·7H₂O, 0.01; ZnSO₄·7H₂O, 0.01; agar, 18; and distilled water to a volume of 1,000 ml. The pH was not adjusted and sterilization was achieved by heating at 110 C for 20 min.

Medium TS5 contained (g per liter): sucrose, 100; asparagine, 5; KH₂PO₄, 0.25; MgSO₄·7H₂O, 0.15; yeast extract, 0.05; agar, 18; and distilled water to a volume of 1,000 ml. The pH was adjusted to 5.2 with NaOH; sterilization was achieved by heating at 110 C for 20 min.

Medium Pep3 (peptone-sucrose) contained (g per liter): sucrose, 300; peptone, 10; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.007; ZnSO₄·7H₂O, 0.006; agar, 18; and tap water to a volume of 1,000 ml. The pH was not adjusted and sterilization was achieved by heating at 110 C for 20 min.

Medium TS contained (g per liter): sucrose, 100; asparagine, 10; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.3; FeSO₄·7H₂O, 0.007; ZnSO₄·7H₂O, 0.006; yeast extract, 0.1; and distilled water to a volume of 1,000 ml. The pH was adjusted to 5.2 with NaOH, and sterilization was achieved by heating at 110 C for 20 min.

Medium TG contained (g per liter): glucose, 100; citric acid, 10; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.3; FeSO₄·7H₂O, 0.007; ZnSO₄·7H₂O, 0.006; yeast extract, 0.1; and distilled water to a volume of 1,000 ml. The pH was adjusted to 5.2 with aqueous ammonia; sterilization was achieved by heating at 120 C for 20 min.

Medium TV contained (g per liter): sucrose, 100; Vegedor, 10; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.3; FeSO₄·7H₂O, 0.007; ZnSO₄·7H₂O, 0.006; and distilled water to a volume of 1,000 ml. The pH was not adjusted; sterilization was achieved by heating at 120 C for 20 min.

Medium T25 contained (g per liter): sucrose, 300; citric acid, 15; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.5; KCl, 0.12; FeSO₄·7H₂O, 0.007; ZnSO₄·7H₂O, 0.006; yeast extract, 0.1; and tap water to a volume of 1,000 ml. The pH was adjusted to 5.2 with aqueous ammonia; sterilization was achieved by heating at 120 C for 20 min.

Methods of growth. A portion (1 cm²) of the mycelial mat of each strain, grown on slants of a solid medium at 28 C for 8 days, was mashed with a sterile...
spatula and transferred to a 300-ml Erlenmeyer flask containing 50 ml of an inoculum medium. The flasks were incubated for 6 days at 24 C on a rotary shaker operating at 220 rev/min, and describing a circle 8 cm in diameter. Samples (5 ml) of the cultures thus obtained were employed as inocula for 300-ml flasks, each containing 50 ml of a production medium. These were incubated for 10 to 14 days, according to the strain, as described for the inoculum cultures.

Methods of analyses. Alkaloids, sterols, sugars, dry weight, lipids, protein nitrogen, and citric acid were determined by the methods of Amici et al. (3). All of the strains studied were found to retain more than half of the alkaloids produced in their mycelia.

Extractions of the alkaloids were carried out by adding to the culture broths the equivalent volume of an aqueous solution of 4% tartaric acid and two volumes of acetone. After a careful homogenization, the mixture was filtered and its pH was adjusted to 8.5 with saturated Na2CO3 solution; alkaloids were then extracted with chloroform.

The solvent was removed under vacuum at 30 C and the crude chloroform extract was passed through a silica-gel column. Elution was then carried out with increasing amounts of methanol to separate the alkaloids, which were later purified and crystallized in the proper solvent (10).

The alkaloids were identified by thin-layer chromatography by using silica-gel plates with the following solvents: ethyl acetate-N,N-dimethylformamide-ethyl alcohol [13:1.9:0.1 (12)] or chloroform-methanol-concentrated ammonia [80:20:0.2 (1)]. Identification was also made by paper chromatography by using Whatman No. 1 paper soaked with formamide in benzene-petroleum ether [6:4 (9)].

The identity of the alkaloids was further confirmed by comparing their rotatory powers (10), melting points (10), and mass spectrograms with those of authentic samples.

They were also subjected to acid and alkaline hydrolyses, and the degradation products obtained were identified and compared with the data reported in the literature (10). The results are summarized in Table 1.

RESULTS

Strain FI 32/17 of C. purpurea was isolated from a sclerotium collected from an ear of rye grown in a field near Brunico, South Tyrol, Italy. It sometimes produces conidia on certain solid-culture media. Normally, it is maintained on medium TS5 on which it never produces conidia. For alkaloid production with this strain, medium TG is employed for the inoculum phase and medium T25 is used for the production phase. Alkaloid production reaches about 1,800 µg/ml. The alkaloids consist of a mixture of practically equal amounts of ergocryptine and ergotamine. The course of a typical fermentation is shown in Fig. 1.

Strain FI 43/14 of C. purpurea was isolated from a sclerotium collected from an ear of rye grown near Usseglio, Piedmont, Italy, and was maintained on medium CZ4M. It does not normally form conidia on the usual solid-culture media. For alkaloid production, medium TS is employed for the inoculum phase and medium T25 is used for the production phase. The alkaloids reach a level of about 1,000 to 1,100 µg/ml within 12 to 14 days and consist of almost equal quantities of ergocornine and ergosine. The course of a typical fermentation is shown in Fig. 2.

Strain FI S40 of C. purpurea was isolated from a sclerotium collected from a ear of rye grown in
the Goms district of the Rhone Valley, Switzerland and was maintained on Pep3 medium. It does not generally form conidia on the common solid-culture media. For alkaloid production by this strain, medium TV is employed for the inoculum phase and medium TS is used for the production phase. About 1,000 to 1,100 µg of ergocristine per ml of culture is produced within 8 to 10 days. The course of a typical fermentation is shown in Fig. 3.

**DISCUSSION**

Of the peptide ergot alkaloids, only ergotamine (2–5) had been produced in large amounts under submerged conditions. Our data demonstrate that ergosine, ergocryptine, ergocornine, and ergocristine can also be formed under the same submerged conditions. It is possible, therefore, to produce large amounts of the alkaloids belonging to the two important groups of peptide alkaloids, the ergotamine group and the ergotoxine group, with *C. purpurea*.

A comparison between the main characteristics of the strains described in this paper and those of strain 275 Fl, the producer of ergotamine described in preceding papers (2–5), is summarized in Table 2. All of the strains are characterized by the fact that, under our conditions, they do not form conidia or, at best, that they produce very few of them. This behavior was investigated and discussed in previous studies (4, 14) in which it was also established that alkaloid production is correlated with the heterokaryotic condition of the producing strains. This condition is not normally compatible with the presence of mononucleate forms, such as conidia, since segregation of the single nuclei and consequent disjunction of the heterokaryon occurs during the sporulation.

Of the four strains mentioned in Table 2, three produce alkaloids in medium T25; only one needs medium TS. All of the four strains elaborate high concentrations of lipids and sterols. In a previous study with ergotamine-producing strains, a positive correlation was found between the two characteristics described above and the alkaloid production (3). The problem was studied further by determining the lipid and sterol content of five strains of *C. purpurea*, freshly isolated from sclerotia and grown on medium T25 under submerged conditions, which failed to synthesize the alkaloids. The lipid content of the mycelium of these strains varied between 12.1 and 19.3%. (In the four alkaloid-producing strains, it ranged from 39 to 69%.) The sterol production varied between 48 and 166 µg/ml. (The four alkaloid-producing strains synthesized them in concentrations of 316 to 510 µg/ml). The correlation between alkaloid biosynthesis and the accumulation of lipids and sterols is evident in Table 2.
TABLE 2. Comparison among the main characteristics of the strains of 
Claviceps purpurea producing peptide 
alkaloids in shaken cultures

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strains</th>
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<tbody>
<tr>
<td></td>
<td>275 FI (2)</td>
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<tr>
<td></td>
<td>FI 32/17</td>
</tr>
<tr>
<td></td>
<td>FI 43/14</td>
</tr>
<tr>
<td></td>
<td>FI S40</td>
</tr>
<tr>
<td>Conidia</td>
<td>Absent</td>
</tr>
<tr>
<td>Alkaloids produced</td>
<td>Ergotamine</td>
</tr>
<tr>
<td>Alkaloids (µg/ml)</td>
<td>1,150</td>
</tr>
<tr>
<td>Production media</td>
<td>T25</td>
</tr>
<tr>
<td>Dry weight (mg/ml)</td>
<td>38.5</td>
</tr>
<tr>
<td>Lipids (mg/ml)</td>
<td>15</td>
</tr>
<tr>
<td>Lipids (per cent of dry weight)</td>
<td>39</td>
</tr>
<tr>
<td>Protein nitrogen (mg/100 ml)</td>
<td>92</td>
</tr>
<tr>
<td>Sterols (µg/ml)</td>
<td>316</td>
</tr>
<tr>
<td>Sucrose utilized (g/liter)</td>
<td>160</td>
</tr>
<tr>
<td>Citric acid utilized (g/liter)</td>
<td>14</td>
</tr>
<tr>
<td>Ammonium nitrogen utilized (mg/100 ml)</td>
<td>246</td>
</tr>
</tbody>
</table>

The exhaustion of one of the substances present in the medium (7). This seems plausible because it is known that P or Mg exhaustion causes an accumulation of nutrients present in the medium for the initial phase of production has been demonstrated by Taber and Vining (15) and by Arcamone et al. (5). The latter authors demonstrated that production of alkaloids is favored by growth-limiting phosphate concentrations and concluded that "during the second phase of growth the reduction of protein synthesis should make available the simple nitrogenous precursors (i.e. amino acids) for LAD (lysergic acid derivatives) synthesis." We believe this conclusion to be valid also for the strains described in this paper.

ACKNOWLEDGMENTS

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LITERATURE CITED

5. Arcamone, F., G. Cassinelli, G. Ferri, S. Penco, P. Pennella, and C. Pol. 1968. Ergotamine production and metabolism of sterols in C. purpurea seems, therefore, to be a general rule. For a more detailed examination of the course of the fermentations, strains FI 32/17 and FI 43/14 were considered apart from strain FI S40. The fermentation process in the first two strains could be separated into two phases. The first one is characterized by the rapid utilization and exhaustion of the phosphate contained in the medium, by the rapid uptake of ammonia nitrogen, citric acid, and (in strain FI 32/17) sucrose. In this phase, there is rapid growth and low production of alkaloids. The second phase, which begins between 4 and 6 days, is characterized by slower growth (particularly evident if the protein nitrogen is taken as an index of growth) and by a marked tendency to accumulate lipids, sterols, and alkaloids. An analogous pattern has been reported for strain 275 FI incubated in flasks (3) and in fermentors (5). In the latter case, a third phase, characterized by the absence of ammonia nitrogen in the medium, was also described during which there is only a slight increase in the dry weight and little utilization of carbohydrates; there is, however, further accumulation of alkaloids. This phase has not been taken into consideration in fermentations carried out in flasks because in these cases there is not a significant increase in alkaloids. Although the data for strain FI S40 are not as complete, a growth phase and an alkaloid production phase can be distinguished. The first phase is characterized by the rapid utilization and exhaustion of the phosphate contained in the medium.

In all of the strains, the passage from the first to the second phase is probably determined by


