Characterization of Some *Claviceps* Strains Derived from Regenerated Protoplasts

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Z. Naturforsch. **42c**, 381–386 (1987); received November 6, 1986

Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Claviceps, Protoplasts Regeneration, Strain Development, Ergot Alkaloids, Enzymes

Three ergoline alkaloids producing *Claviceps* strains easily formed protoplasts when treated with snail digestive extract ex *Helix pomatia*. Colonies obtained after regeneration of protoplasts were monitored for alkaloid production. Among the regenerated progeny strains could be isolated which differ considerably in the quantity of ergolines and the composition of the alkaloid mixture compared with parental strains. Protoplast-derived strains were stable in morphology and alkaloid production for more than four years if the propagation was performed with hyphal fragments. Activities of some alkaloid-specific enzymes *e.g.* oxygenases and ergotamine synthetase were studied in various related strains.

Introduction

A number of ergot alkaloids and some semisynthetic ergolines are therapeutically important agents. For the production of ergot alkaloids fermentation procedures are in use since about two decades besides the parasitic cultivation of ergot on rye plants. The increasing biotechnological importance of some *Claviceps* species *e.g. Cl. purpurea* and *Cl. paspali* stimulated the search for efficient methods to obtain high-yielding strains. First experiments in this direction including mutagenesis followed by subsequent selection were described by Kobel *et al.* [1] and Kobel and Sanglier [2]. Another approach is the fusion of fungal protoplasts to obtain hybrid strains. Suitable techniques for preparing protoplasts from ergot fungi were at first described by Spalla *et al.* [3] and independently by Stahl *et al.* [4]. Methods for intra-specific and inter-specific fusions of protoplasts derived from alkaloid-producing ergot strains for strain improvement were introduced by Spalla and Marnati [5, 6].

We used protoplasts for physiological and biochemical studies with respect to ergot alkaloid formation [7, 8]. Furthermore during the past ten years several thousand of colonies which we have obtained after reversion of protoplasts were monitored for alkaloid synthesis. The present report describes the properties of some strains derived from *Claviceps* protoplasts without mutagenesis.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0400–0381 $ 01.30/0

Material and Methods

Organisms

The work was carried out with submerged cultivated mycelia. As parent strains were used: Pepty 695/S and JAP 471/I *(Claviceps purpurea)*, and SD 58/78 *(Claviceps fusiformis)*. The alkaloid spectra of the parent strains and their progeny are given in Fig. 1.

Culture technique

The stock cultures were maintained on asparagine/sucrose agar. Fermentation media: NL 614 ($g \cdot l^{-1}$); mannitol 50; sucrose 50; succinic acid 5.4; yeast extract (DIFCO) 3.0; KH_2PO_4 0.1; $MgSO_4 \cdot 7H_2O$ 0.3; $FeSO_4 \cdot 7H_2O$ 0.01; $ZnSO_4 \cdot 7H_2O$ 0.004; pH adjusted with ammonium hydroxid solution to 5.2–5.4.

NL 720 ($g \cdot l^{-1}$): sucrose 200; ammonium citrate 15; KH_2PO_4 0.25; $MgSO_4 \cdot 7H_2O$ 0.3; $FeSO_4 \cdot 7H_2O$ 0.001; $ZnSO_4 \cdot 7H_2O$ 0.03; pH adjusted to 5.2–5.4 with NH_4OH.

NL 833 ($g \cdot l^{-1}$): sucrose 300; ammonium citrate 20; $Ca(NO_3)_2$ 1.0; KH_2PO_4 0.25; $MgSO_4 \cdot 7H_2O$ 0.3; $FeSO_4 \cdot 7H_2O$ 0.01; $ZnSO_4 \cdot 7H_2O$ 0.03; pH adjusted to 5.2–5.4.

The strains were grown in submerged cultures at 24 °C at 240 rpm on a VEB Fanal rotary shaker. For preparing the inoculum mycelium of 4 weeks old agar slants were used. The 7 day old preculture mycelium was transferred into 500 ml flat bottom flasks containing 100 ml culture-broth. The inoculum rate amounted to 1:10. As preculture medium NL 720 was used except for strains JAP 471/I and MUT 170/I which were grown in a sucrose/skim milk medium NL

849 [9]. Alkaloid production was achieved with strains Pepty 695/S and SD 58/78 and their progeny in medium NL 720 and sometimes with SD 58/78 in NL 614. The strains JAP 471/I and MUT 170/I were cultivated in NL 833 for alkaloid synthesis.

Protoplast formation and regeneration

For preparing protoplasts the method of Stahl [4] was employed. Mycelium from 3- to 4-day-old shake cultures was harvested on a coarse sintered glass funnel and washed several times with a small volume of 0.7 M KCl solution. Following the incubation with a self-prepared snail digestive extract the mixture of protoplasts and mycelial fragments was centrifuged at 3000 rpm for 15 min. The pellet was washed twice with 0.7 M KCl solution and finally taken up with the salt solution and filtered through cotton. The protoplast suspension was immediately used for plating.

To carry out the regeneration of intact cells, protoplasts were plated onto agar plates containing a medium composed of ($g \cdot l^{-1}$): sucrose 100; ammonium citrate 10; $Ca(NO_3)_2$ 1; $MgSO_4 \cdot 7H_2O$ 0.3; KH_2PO_4 0.250; KCl 0.1; agar 30 at a pH of 5.5.

Enzyme assays

Lyophilized mycelia were used to prepare the enzyme extracts. Chanoclavine-I-cyclase: Lyophilized mycelium from a 4-day-old culture was ruptured by grinding with dry ice in a mortar and suspended in 0.1 M Tris-HCl buffer (pH 7.8) containing 10% glycerol, 2 mM EDTA and 2 mM mercaptoethanol. The suspension was centrifuged at $20000 \times g$ for 30 min. The supernatant was used as crude enzyme extract. The reaction mixture in a total volume of 2 ml contained: 0.250 mg chanoclavine-I; 1.25 μmol NADPH; 10 μmol ATP; 20 μmol Mg^{2+}; 200 μl Tris-HCl buffer and 0.5 ml enzyme solution. The mixture was incubated for 3 h at 32 °C. The extraction and purification of the substrate and the reaction product (agroclavine) was done as described [10].

Agroclavine 17-monooxygenase and elymoclavine 17-monooxygenase: Lyophilized mycelium was ruptured by grinding with dry ice and suspended in 0.1 M Tris-HCl buffer (pH 7.8) containing 20% glycerol and 10 mM dithioerythritol. The suspension was centrifuged at $20000 \times g$ for 30 min. The supernatant was removed and centrifuged at $100000 \times g$ for 1 h. The pellet (microsomal fraction) was resuspended in 0.1 M Tris-HCl buffer and gently homogenized at 0 °C.

The activities of the oxygenases were assayed in an incubation medium consisting of 1 μmol NADPH, 1 mg protein, 15 μg [^{14}C]agroclavine (1×10^5 dpm) or 20 μg [^{14}C]elymoclavine (2.2×10^4 dpm) and 0.1 M Tris-HCl buffer (pH 7.8) in a final volume of 250 μl. Incubations were carried out for 2 h at 27 °C with shaking at 240 rpm. The reaction products were purified by TLC. In the case of "agroclavine hydroxylase" non-labelled elymoclavine was added as carrier and the alkaloids separated on silica gel PF_{254} (Merck). Solvent systems, I: $CHCl_3$/MeOH (8:2, v/v), II: $CHCl_3$/MeOH/AcOH (7:3:0.1, v/v). Aliquots of the twice purified elymoclavine were used for quantitation and counting of radioactivity. The reaction product of "elymoclavine hydroxylase" was separated after adding of non-labelled lysergic acid in solvent III: $CHCl_3$/MeOH/NH_4OH conc (7:3:0.5, v/v). After elution with 1% KOH in MeOH the radioactivity was counted. The counts of controls containing boiled protein extracts and incubated without NADPH were substracted. The enzyme activities were expressed as nmol elymoclavine or lysergic acid per mg protein per 2 h.

Ergotamine synthetase: The preparation of cell-free extracts and purification of ergotamine synthetase were performed as described previously [11]. The cell-free extract was fractionated by ammonium sulfate precipitation. The precipitate was chromatographed on a Sepharose 4B column, and the active fractions subsequently applied onto a DEAE-Sepharose CL-6B column. After elution with a linear potassium phosphate gradient were the active fractions pooled and used for the enzyme assay.

The reaction mixture contained in 0.7 ml: 41.2 pmol of L-[ring 2,6-^{3}H] phenylalanine (2 μCi), 0.1 μmol of each L-alanine and L-proline, 0.05 μmol of each agroclavine and elymoclavine, 1 μmol of ATP (pH 7.8), 7 μmol Mg^{2+}, 300 μl Tris-HCl buffer (pH 7.8) and enzyme solution containing 100–200 μg protein. The mixture was incubated for 1 h at 27 °C on a shaker at 240 rpm. The enzymatically formed ergotamine was assayed according to [8, 11].

Analytical procedures

Total alkaloid determination was carried out colorimetrically [12] using van Urk's reagent. The optical density was determined at 580 nm. The quantity

of alkaloids were calculated according to a standard curve using elymoclavine for clavine-producing strains or the appropriate peptide alkaloids for ergopeptine producers.

For identification the alkaloids were chromatographed or rechromatographed using TLC (silica gel PF_{254}) in different solvent systems [13, 14]. The chromatographically pure alkaloids were used for recording mass spectra. Furthermore ergopeptines were identified by comparing HPLC retention times with authentic samples. This was carried out with a HP 1090 Liquid Chromatograph (Hewlett Packard) on a Separon SIX C-18 column. Alkaloids were eluted with methanol/3% triethylamine (1:1, v/v) and detected by UV absorption at 312 nm.

The protein content of enzyme preparation was estimated by the method of Bradford [15].

Results and Discussion

The genealogy of various *Claviceps* strains is given in Fig. 1. During a screening program we tested single colonies derived from hyphal fragments of the *Claviceps* purpurea strain Pepty 695/S [16]. One isolate showed no changes in morphology and pigmentation compared with the parent strain but failed to produce lysergic acid derivatives: strain Pepty 695/ch accumulates only secoclavines (Fig. 2). An isolate with a similar alkaloid pattern as Pepty 695/ch was obtained from regenerated protoplasts of the *Claviceps* fusiformis strain SD 58/78, which synthesizes agroclavine and elymoclavine but never lysergic acid derivatives.

After reversion of protoplasts of the secoclavine producer Pepty 695/ch to the normal cell form an isolate was found which differed remarkably from the parent. The total alkaloid yield was about six

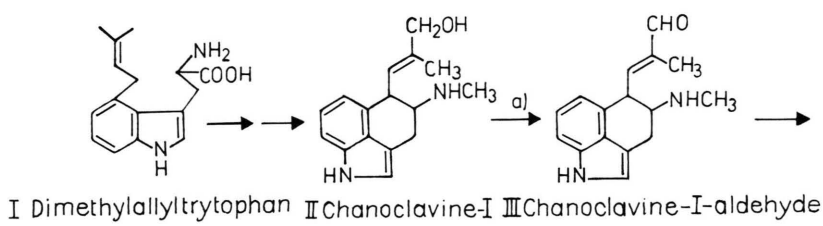
I. Pepty 695/S
(1–1.2 mg·ml^{-1}, of it 60% ergocornine plus ergokryptine)
↓ a)
Pepty 695/ch
(300–350 µg·ml^{-1} chanoclavine-I and the corresponding aldehyde)
↓ b)
Pepty 695/ch–I
(1.5–2 mg·ml^{-1}, of it 90% agroclavine plus elymoclavine)

II. Pepty 695/S
↓ b)
Pepty 695/ch-I/3
(2.5–3 mg·ml^{-1}, 95% a mixture of agroclavine plus elymoclavine)
↓ b)
Pepty 695/ch-I/3R
(500–600 µg·ml^{-1}, of it 50–100 µg·ml^{-1} ergotoxine)

III. JAP 471/I
(700–800 µg·ml^{-1}, of it 50–70% ergotamine 10% ergokryptine)
↓ b)
MUT 170/I
(800 µg – 1 mg·ml^{-1}, of it 50% ergosine)

IV. SD 58/78
(1.2–1.5 mg·l^{-1}, of it 75% agroclavine plus elymoclavine)
↓ b)
SD 58/78/ch
(300–350 µg·ml^{-1} chanoclavine-I and the corresponding aldehyde)

Fig. 1. Genealogy of ergot strains. Alkaloid yields and main components of the alkaloid spectrum in parentheses. Selection methods: a) single colonies derived from hyphal fragments; b) single colonies derived from regenerated protoplasts.



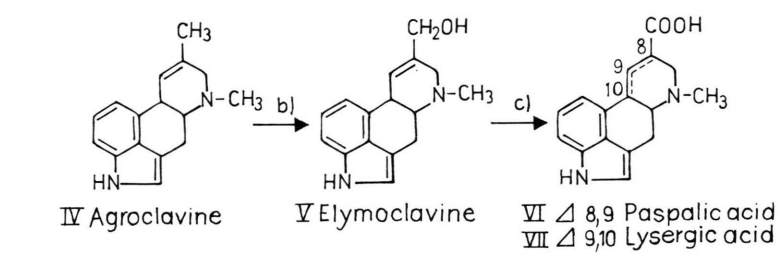


Fig. 2. Biosynthetic pathway of ergoline alkaloid formation. a) Chanoclavine-I-cyclase; b) agroclavine 17-monooxygenase; c) elymoclavine 17-monooxygenase.

times higher and as endproducts of the ergoline pathway tetracyclic clavines were accumulated instead of chanoclavine-I (strain Pepty 695/ch-I). Three isolates with similar properties regarding alkaloid formation were obtained after protoplasting and regeneration of the ergotoxine producer Pepty 695/S. After reversion of protoplasts of this particular strain one isolate was found with a reduced total alkaloid yield. Remarkably in low quantities ergotoxine alkaloids were present in the alkaloid mixture. Presumably this strain is a revertant and was designated as Pepty 695/ch-I/3R. An isolate of an ergopeptine strain JAP 470/I, which we obtained after protoplasting produced only ergosine instead of ergotamine. This means in the peptide portion of ergotamine phenylalanine was replaced by leucine in strain MUT 170/I.

It is noteworthy that all protoplast-derived strains were stable for more than 4 years with regard to alkaloid production and the alkaloid pattern if the transfers were done with hyphal fragments.

Strain Pepty 695/ch is an "alkaloid-blocked mutant". After feeding of appropriate intermediates (mutasynthesis) the mutant produces the normal alkaloid spectrum of the parent strain. Surprisingly after administration of agroclavine ergosine is additionally synthesized besides the ergotoxines [13]. It could be demonstrated that in this strain the activity of the enzyme converting II → IV is drastically reduced compared with the ergotoxine-producing strain Pepty 695/S [16]. This prompted us to look for several alkaloid-specific enzymes in the isolated ergot strains. In strain Pepty 695/ch-I which is derived from protoplasts of the secoclavines accumulating blocked mutant chanoclavine-I-cyclase activity is fully restored: the conversion rate of chanoclavine-I to agroclavine amounted to 50% like in other tetracyclic ergolines producing *Claviceps* strains.

Oxygenases are involved in the conversion of agroclavine to lysergic acid. Apparently the formation of lysergic acid proceeds *via* paspalic acid [17]. The time-course of two oxygenases of strain Pepty 695/S is shown in Fig. 3. In the microsomal fraction both oxygenases are present. Therefore a relative low amount of V is accumulated if agroclavine is used as substrate. The synthesized elymoclavine is in turn converted to lysergic acid as it is seen in the curve agroclavine → lysergic acid. We assume that the activities of both agroclavine 17-monooxygenase and elymoclavine 17-monooxygenase are roughly equal in peptide-alkaloids producing strains (Table).

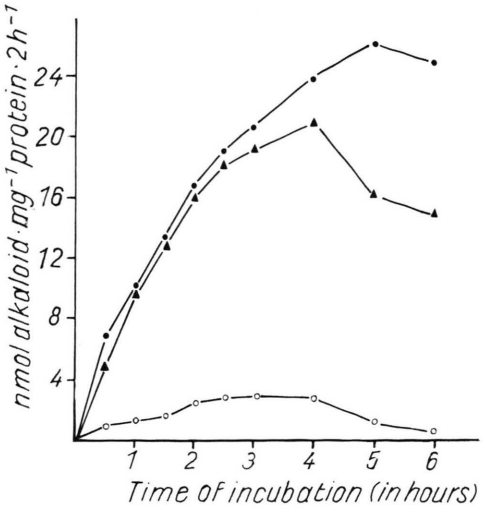


Fig. 3. Time course of the formation of elymoclavine and lysergic acid by a cell-free extract of ergot strain Pepty 695/S. ○——○ Agroclavine 17-monooxygenase; ●——● elymoclavine 17-monooxygenase; ▲——▲ conversion of agroclavine to lysergic acid.

The highest activity of agroclavine 17-monooxygenase is found in strain Pepty 695/ch-I/3 which accumulates the highest amount of elymoclavine among our clavine strains. Remarkable is the considerable activity of elymoclavine 17-monooxygenase in the blocked mutant Pepty 695/ch which accumulates under *in vivo* conditions secoclavines. The same enzyme exhibits a drastically reduced activity in strains Pepty 695/ch-I and Pepty 695/ch-I/3. Both strains are derived by protoplasting and stem from a parent with a high level of elymoclavine oxygenase. Interestingly

Table. Activities of oxygenases in various ergot strains. The mycelia were harvested after 3–4 days of cultivation.

Strain	Agroclavine 17-monooxygenase [nmol V · mg^{-1} protein · 2 h^{-1}]	Elymoclavine 17-monooxygenase [nmol VII · mg^{-1} protein · 2 h^{-1}]
Pepty 695/S	2.6	18.8
Pepty 695/ch	0.4	12.0
Pepty 695/ch-I	6.0	0.9
Pepty 695/ch-I/3	18.0	1.1
Pepty 695/ch-I/3R	n.d.	10.0
SD 58/78	7.3	0.8
JAP 471/I	2.8	17.0

n.d. = not determined

strain Pepty 695/ch-I/3R originates from Pepty 695/ch-I/3 and showed a ten-fold higher elymoclavine 17-monooxygenase activity as its progenitor. This strain produces in small amounts lysergic acid derivatives. This means the "cyclol-synthetase" responsible for the formation of the peptide is partially active compared with strain Pepty 695/S (Fig. 1). Furthermore one may conclude that the "cyclol synthetase", should be also present but "silent" in Pepty 695/ch-I/3 and Pepty 695/ch-I. Using the purification method [11] we could demonstrate the presence of ergotamine synthetase in cell free extracts of both clavines accumulating strains of the Pepty-series. The specific activity amounted to 2.1×10^6 dpm ergotamine $\cdot mg^{-1}$ protein $\cdot h^{-1}$. Furthermore we diluted the enzymatically formed ergotamine with non-labelled alkaloid and recrystallized the mixture to constant specific radioactivity. In control experiments using boiled enzyme extract no ergotamine was formed. It should be emphasized that ergotamine is not synthesized under normal fermentation conditions in strain Pepty 695/S. However it is well known that the cyclol synthetases in various *Claviceps* strains possess a rather broad specificity [13, 18, 19] and that the alkaloid spectrum can be changed by mutation [2]. The reason why the "cyclol synthetase" is not operative in strains Pepty 695/ch-I and Pepty 695/ch-I/3 is not clarified but might be due to the scarcity of the appropriate ergoline moiety *e.g.* lysergic acid within the cells.

Additionally we found also that an enzyme system catalyzing the incorporation of L-phenylalanine into ergotamine is present in strains Pepty 695/ch and Pepty 695/ch-I/3R.

At present there is no plausible explanation which mechanisms are responsible for suppression or restoration of enzyme activities involved in alkaloid synthesis in related ergot strains *e.g.* the Pepty series.

Genetic instabilities associated with protoplast techniques and their influence on secondary metabolite formation were first observed in *Streptomycetes*. Formation and regeneration of protoplasts caused the loss of plasmid DNA in different *Streptomyces* species [20, 21]. In *S. fradiae* protoplasting and regeneration was associated with deletion of tylosin biosynthetic genes and several antibiotic resistance genes [22, 23]. Furumai *et al.* [21] isolated a spontaneous mutant 18a from *S. kasugaensis* exhibiting a pleiotropic effect such as loss of aerial mycelium formation, aureothricin production and of citrullin biosynthesis. Regeneration of 18a protoplasts caused a reversion of pleiotropic mutation with high frequency. Ikeda *et al.* [24] observed besides variations in morphology that antibiotic productivities of the regenerated progeny of *S. fradiae* and *S. ambofaciens* were drastically changed. One strain from *S. fradiae* obtained after two rounds of protoplast regeneration produced about three times as much as the parent strain. Enzymes involved in antibiotic synthesis were not studied in *Streptomyces* strains obtained after protoplast regeneration. Keller [25] described an efficient method for mutation and selection by using *Claviceps purpurea* protoplasts. In controls without mutagenic treatment a high portion of colonies with altered morphology and a lowered level of alkaloid production were detected after protoplast regeneration. This event was never observed when hyphal fragments were streaked on solid medium. Interestingly plasmids have been also found in some wild strains of ergot [26]. Their role in ergoline biosynthesis has not yet been elucidated. Further research is necessary for a better understanding of the genetics of alkaloid formation and the metabolic changes associated with protoplast formation and regeneration in *Claviceps*.

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