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Biosynthesis of the Phytotoxin Tentoxin

I. Synthesis by Protoplasts of *Alternaria alternata*

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ABSTRACT

Alternaria alternata is well known as a producer of tentoxin as well as some other phytotoxic substances. A new method to prepare protoplasts from *Alternaria alternata,* suitable for many purposes, was developed. By use of a mixture of lytic enzymes from *Helix pomatia,* and *Trichoderma harzianum* with the commercial preparation "novozym," it was possible to prepare protoplasts from all stages of fungal development, including the tentoxin production phase. Optimal incubation conditions led to the conversion of 1 g (wet wt) mycelial cells into 2.3–2.5 × 10^7 protoplasts within 3–6 h. Submerged as well as surface-grown mycelia were suitable. Optimal stabilization of protoplasts was obtained in 0.8*M* KCl. The protoplasts were used for both mutagenic treatment and physiological studies. UV irradiation of protoplasts resulted in formation of hyperproducing mutants. Protoplasts were able to form tentoxin. The biosynthetic activity of protoplasts from surface-grown mycelium was 40% that of intact mycelia. Although intact submerged myclia did not synthesize tentoxin, protoplasts of both types of mycelia produced this toxin, indeed protoplasts from submerged mycelia were even more active than those from surface mycelia. Neither oxygen tension nor mechanical stress during the shaking culture is the reason for the lack of tentoxin production by intact submerged mycelial pellets. Since tentoxin-synthesizing enzymes were apparently present in both mycelial types, it is probable that metabolites or lytic products in the pellets inhibit tentoxin-forming enzymes under submerged conditions.

Index Entries: Phytotoxin tentoxin, synthesis of; protoplasts; *Alternaria alternata.*

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INTRODUCTION

The deuteromycete *Alternaria alternata* (Fr.) Keissler produces the cyclic tetrapeptide tentoxin. The phytotoxicity of tentoxin to many mono- and dicotyl plants is well known (*1–6*). Tentoxin is a typically secondary metabolite, whose biosynthesis is controlled by several regulatory mechanisms (*7–10*). Optimization of culture conditions and mutagenesis are suitable methods to increase the productivity of *Alternaria alternata*. For mutagenesis, the multicellular conidia cannot be used. Therefore, we tried to irradiate protoplasts, which contain less nuclei than spores. On the other hand, protoplasts can be used for physiological studies of tentoxin biosynthesis.

When *Alternaria alternata* is grown in shaken culture, the mycelial productivity is minimal in comparison to surface grown mycelia. Hänel (*11*) and Liebermann (*12*) discussed the possibilities that a limitation of oxygen tension, mechanical stress, or a reduced synthesis of tentoxin-synthesizing enzymes may be responsible for the lower level of tentoxin formation. We felt that study of the capacity for product formation by mycelia grown under different conditions could best be done by studying the biosynthetic activity of protoplasts. Similar investigations were summarized by Bennett (*13*). Protoplasts are normally prepared after a few hours or a few days of submerged culture. In our case, we wanted to work with mycelia from surface and submerged cultures of 5–9 d of age, which corresponds with the phase tentoxin production. Thus, it was important to develop a method for protoplast preparation from mycelia grown under different conditions up to 9 d of age.

MATERIALS AND METHODS

Organism and Cultivation

The studies were carried out with strain i 30/10 of *Alternaria alternata* isolated in our laboratory from *Brassica chinensis*. Cultivation was carried out in phosphate-limited medium in surface as well as submerged culture. Details of the strain and the cultivation conditions are given by Hänel et al. (*10*).

Treatment of Intact Mycelia, Buffer System

After a defined culture time in growth medium surface-grown mycelia were removed, washed in distilled water, and sections of the mycelia mats (1 × 2 cm) were transferred into a buffer-system in Erlenmeyer flasks (20 mL of triethanolamine buffer 20 m*M* with 10 m*M* $MgCl_2$, pH

7.5). Pellets from submerged cultures were separated by filtration from culture medium, washed in distilled water, and pressed on a filter by vacuo into a dense mycelial "mat" that could be handled like the mycelial sections above. This mycelium in buffer was incubated up to 10 h at 28°C (nonshaking, semidark light).

Preparation of Protoplasts

Submerged grown pellets were collected on a glass fritted filter and rinsed with water. Mycelial mats from the surface culture were also washed with water and gently homogenized. Hyphae (0.5 g) were incubated with 7 mL of lytic enzyme solution in a 25 mL Erlenmeyer flask with gentle shaking at 28°C for 3–6 h. The residual hyphae were removed on fine glass fritted filter afterward. The protoplasts were centrifuged (600*g*) and washed. After stabilizing with 0.8*M* NaCl, the protoplasts were counted using a Thoma chamber. The lytic enzyme solution contained the following three components:

1. *Helix pomatia* enzyme at 2% (w/v) (Boehringer-Mannhaim, Mannheim, Germany);
2. *Trichoderma harzianum* enzyme adapted to the cell wall composition of *Alternaria alternata* at 2% (w/v). (The fungus was grown on cell walls of *Alternaria alternata* in culture flasks. The culture medium containing the lytic enzyme was lyophilized and used as crude enzyme.)
3. Mycelium of the fungus *Alternaria alternata* was disrupted by a French press (LKB) and cell wall material was isolated after manyfold centrifugation steps for separation of proteins. Cell wall material (2% wet wt) was used as the sole carbon source for cultivation of the fungus *Trichoderma harzianum* in liquid Czapek-Dox medium. After 5 d the culture fluid was separated from the mycelium by filtration, lyophilized, and used for protoplast isolation from *Alternaria alternata*.
3. Novozym 234 at 0.3% (w/v) (Novo, Nordisk).

The enzymes were dissolved in 0.8*M* NaCl solution.

Mutagenic Treatment

The protoplast suspension ($10^6 \times mL^{-1}$) was irradiated in a sterile Petri dish (9 cm diameter) under an ultraviolet lamp (30 W, 254 nm, 30 cm distance). After different periods (10, 20, 30, 40, 50 and 60 s) of treatment, the protoplast suspension was diluted in liquified soft agar, spread on regeneration medium (*14*), and incubated in 28°C in darkness for 72 h. The survival rate was defined as the ratio between the number of colonies regenerating from the protoplasts after and before mutagenesis.

Determination of Number of Nuclei in Protoplasts

The numbers of nuclei per cell were determined by fluorescence microscopy. The protoplasts were fixed with increasing concentrations of glutaraldehyde (1.25, 2.5, and 5%) and stained overnight with 0.2 mg × mL^{-1} DAPI (4′, 6′-diamidino-2-phenylindole) at 4°C.

Formation and Isolation of Tentoxin

After incubation of both intact mycelia (4.5 g) in the buffer system for 4 and 10 h (*see above;* nonshaken) and protoplasts (from 1 g of each hyphae; gently shaken) in stabilizing 0.8*M* NaCl solution for 4 and 16 h with the precursor U-^{14}C-leucine (4 × 10^4 Bq (8888 × 10^6 Bq/mmol), Amersham, Arlington Heights, IL), the *de novo* synthesis of tentoxin was measured. For the isolation of produced tentoxin, mycelium and protoplasts were removed. After addition of 50 μg of nonradioactive tentoxin (for better isolation), the aqueous incubation mixture was extracted three times with a double volume of ether. The ether was evaporated, the residue dissolved in a small amount of methanol, and chromatographed by TLC on silica gel in methanol:ethylacetate:water, 100:4:1. The tentoxin spot (quenching of fluorescence) was eluted and separated again in *n*-butanol:acetic acid:water, 12:5:3. The distribution of the radioactivity on the chromatogram was measured in dioxan scintillator fluid with the Liquid Scintillator Counter (Wallac S 1410, Pharmacia, Uppsala, Sweden). At this isolation level there were no radioactive labeled impurities detectable.

Tentoxin Assay by HPLC

After a one-step extraction of the culture filtrate with ethyl acetate, tentoxin was estimated by HPLC analysis according to Liebermann et al. (*15*) with modification.

We used a Shimadzu instrument (LC-7A, SPD-7A, C-R4-AD), column: Li Chrosorb RP18, 5 μm, 4 × 250 mm, guard-column: Li Chrosorb RP18, 7 μm, 4 × 30 mm; conditions: 80% methanol, isocratic, 0.8 mL × min^{-1}; detection: 286 nm.

RESULTS AND DISCUSSION

Isolation and Regeneration of Protoplasts

For the preparation of protoplasts from physiologically older tentoxin producing cultures of *Alternaria alternata*, it was necessary to develop a new and efficient method. The mixture of lytic enzymes from *Helix pomatia, Trichoderma harzianum,* which was induced on cell walls of *Alternaria alternata* with the commercial preparation "novozyme" proved to be suitable. With the optimized method described herein, it was possible to

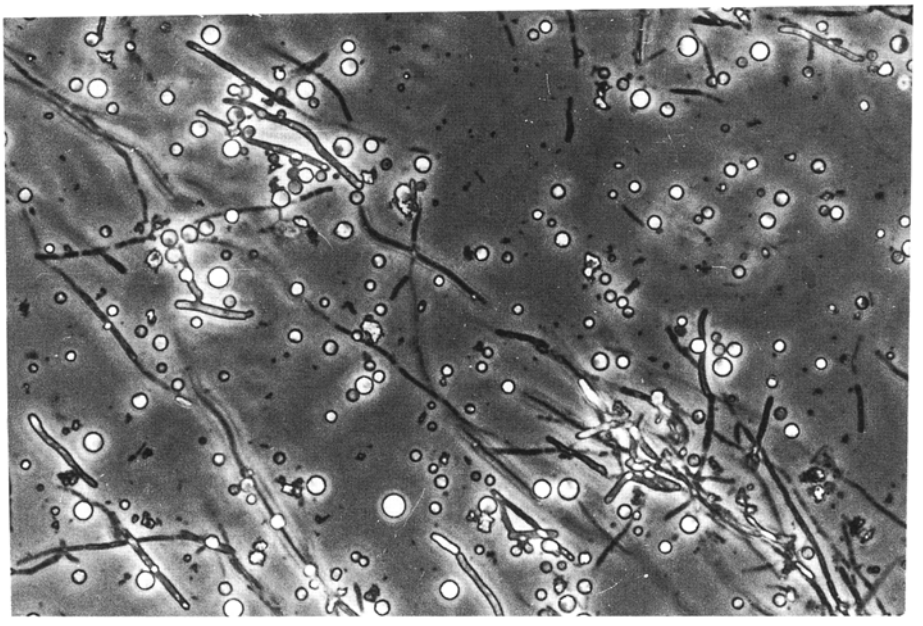

Fig. 1. Protoplasts of *Alternaria alternata*.

prepare protoplasts from mycelia of several days of age in a high yield. The conversion of 1 g (wet wt) mycelial cells into 25 × 10^6 protoplasts was possible within 3–6 h. Reduction of the concentration of enzymes by 50% led to the same high yields. Mycelia from the 5th d of culture had to be incubated for 3 h, whereas mycelia from the 9th d required 6 h. The best results were achieved with 500 mg (fresh wt) of mycelium in 10 mL of enzyme solution. Of the stabilizers tested, 0.8*M* NaCl or KCl were found to be the best for stabilizing protoplasts (tested 0.5–1.0*M*) (*see* Fig. 1).

In contrast to our results, many other workers found a strong negative correlation between the yield of protoplasts and the age of the culture from which the mycelium was obtained. Therefore, only very young cultures had been used for protoplast preparation (*14,16*). Cary and Stovall (*18*) described a method to produce protoplasts from *Alternaria alternata* using a mixture of hydrolytic enzymes different from ours. Their yield of released protoplasts was poor when they used cultures older than 20 h. With cultures of 40 h, almost no protoplasts were observed. They used pellets from submerged culture only. We were able to prepare high yields of protoplasts from submerged and surface-grown mycelium as well. Therefore, it was possible to compare intact mycelia as well as protoplasts from the two types of culture for their productivity of toxin.

Mutagenesis of *Alternaria* Protoplasts

Alternaria alternata is an ascomycete-producing multicellular conidia. Strain improvement by mutagenic treatment of these spores seems to be

Table 1
The Survival Rates of UV Irradiated Protoplasts from *Alternaria alternata*, %

Irradiation times, s	10	20	30	40	50	60
Survival rate, %	72	42	25	12	7	2

hopeless. Therefore, we used protoplasts for UV irradiation experiments in order to get mutants producing more tentoxin. Protoplasts were found to contain 0–4 nuclei/cell, with an average of 1.05.

The surviving cells were counted as colonies and compared to the number of untreated cells on regenerating medium after 3 d incubation. As expected, the survival rate of UV irradiated protoplasts decreased with increasing doses of UV light (Table 1).

Most of the regenerated colonies from UV-irradiated protoplasts showed the typical *Alternaria* black color of colonies. However, approx 3% of colonies produced brown or white spores instead. Twenty-five of the colonies were inoculated into liquid culture medium (*10*), and their tentoxin formation was compared to that of the original strain, *Alternaria alternata* i30. The results are shown in Table 2. It is apparent that tentoxin titer was increased in some mutants and decreased in others. Mutant 13 was the most active isolate, producing four times as much tentoxin as did the wild type strain. The productivity of mycelium is four times higher, too.

There are some other examples of mutagenesis of fungal protoplasts. Junwai and Schnyun (*19*) isolated several mutants, quite different from the original strain in spore color and cellulase activity, after UV irradiation of *Aspergillus niger* protoplasts. Keller (*20*) reported the isolation of auxotrophic mutants and strains with higher levels of alkaloid production after ethyl methanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment of *Claviceps purpurea* protoplasts. Therefore, mutagenic treatment of fungal protoplasts seems to be an efficient method of strain improvement.

Biosynthesis of Tentoxin by Intact Mycelia and Protoplasts

Undependent on composition of culture medium, *Alternaria alternata* produces much more tentoxin in surface culture than in submerged culture (Table 3).

There are some possibilities for explanation of significant differences of mycelial productivity. Some authors discuss that a temporary change of oxygen tension in growing pellets or mechanical stress in shaken culture lead to a reduced synthesis of tentoxin (*11,12*). In this case, the tentoxin synthesizing enzymes would be produced but inhibited. On the

Table 2
Comparison of Tentoxin Formation Between Wild Type Strain *Alternaria alternata* i30 and its Mutants After 12 d

Strain	Tentoxin, μg/mL[a]	Percent of control	Productivity[b] $P \times 10^{-3}$
i30, control	13.5	100	1.2
2	25.8	191	2.3
7	14.5	107	1.5
10	15.5	114	1.4
13	56.7	420	5.2
15	33.3	250	3.0
16	33.9	251	3.2
18	13.1	97	1.2
22	28.3	209	3.4
25	31.6	234	2.7
26	14.0	102	1.5
28	6.1	45	0.7
33	7.9	59	0.9
42	5.7	42	0.5
45	13.6	100	1.3
46	3.7	27	0.4
50	5.0	37	0.6
59	13.8	101	1.4
64	21.6	160	2.0
66	13.2	98	1.0
69	14.0	102	1.1
73	14.2	104	1.3
80	3.5	26	0.3
82	13.3	99	0.9
89	4.2	31	0.8
94	21.6	160	2.1

[a]Values are means of 3 replications.
[b]P = (μg tentoxin / μg dry weight)

Table 3
Comparison of Tentoxin Formation Between Submerged and Surface Cultures of *Alternaria alternata* i30 in a Synthetic Medium (*11*)

	Submerged cultivation			Surface cultivation		
Time, d	Tentoxin, μg/mL	Dry wt, mg/mL	Productivity, $P \times 10^{-3}$	Tentoxin, μg/mL	Dry wt, mg/mL	Productivity, $P \times 10^{-3}$
3	0	0.6	0	1.9	1.8	1.05
5	0.3	2.0	0.15	4.6	4.2	1.10
7	0.3	3.5	0.08	9.8	9.5	1.03
10	0.4	8.1	0.05	26.7	11.2	2.4
12	0.2	8.0	0.025	28.3	10.8	2.6

Table 4
Production of Tentoxin by Preparations
of Mycelia Grown in Surface and Submerged Culture

Type of culture	Preparation	Tentoxin production	
		dpm / ($g_{mycelium}$ × h)	Percent
Surface	Mycelial sections	407.5	100
	Protoplasts[a]	164.7	40.4
Submerged	Pellets as mycelial mat	~0	0
	Protoplasts[a]	253.5	62.2

[a]Protoplasts isolated from 1 g (wet wt) of mycelium (between 2.3 and 2.5 × 10^7).

other hand, it is important to clarify whether a reduced *de novo* synthesis of enzymes is responsible for decreased level of tentoxin formation. In order to study the influence of oxygen limitation on tentoxin formation, 10-d-old pellets from submerged cultures of *Alternaria alternata* were pressed into a dense mycelial mat and incubated in the same way as intact surface grown mycelium on the surface of buffer without shaking for 4 and 10 h. There was no tentoxin in contrast to surface grown mycelium (Table 4). Since the oxygen tension here was comparable to that of naturally grown mycelial mats, an effect of the oxygen level on tentoxin synthesis is probably not the reason for the lack of production.

We noted that protoplasts from submerged-grown mycelia produced even higher amounts of the cyclopeptide than protoplasts from surface-grown mycelia measured by incorporation of radioactive labeled leucine into tentoxin during an incubation time of 16 h. Later experiments with 4 h incubation time and with fractions without ribosome could support this result and showed that synthesis of the tentoxin synthesizing enzymes during the incubation with radioactive labeled precursors could be excluded.

The results show that the tentoxin forming enzyme system exists in both cultures. In shaking culture, in consequence of the other physiological conditions, metabolites or degradation products may be formed by early lysis of inner areas of the pellets, localized between the strong connected hyphae and inhibit synthesis of tentoxin. Presumably, these substances were not removed by washing the pellets before incubating them with radioactive leucine. It is obvious that the lack of toxin production by submerged cultures is neither owing to the oxygen tension nor mechanical stress, but is a regulatory effect. Now we have to isolate and characterize the inhibiting substances and clarify whether they can be removed to produce tentoxin in the agitated fermenter.

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