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Biosynthesis of the Phytotoxin Tentoxin

II. Cell-Free Biosynthesis of Tentoxin— First Evidence on the Localization of Toxin Synthesis in *Alternaria alternata*

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ABSTRACT

In contrast to mechanical disruption of *Alternaria alternata* mycelia, disintegration of protoplasts by osmotic shock made it possible for the first time to develop a cell-free system that produces tentoxin without significant loss of enzyme activity. After separation of groups of cell organelles by fractional centrifugation, the main activity of tentoxin synthesis was detectable in the membrane/microsome fraction and in the plasma fraction. The proteins in the plasma seem **not** to be soluble. They are probably combined with lipids or small bits of membrane debris. For this reason, these proteins cannot be removed by ultracentrifugation. On the other hand, it is possible to precipitate the enzyme system with 2% NaCl. Disruption of cellular substructures (membranes?) by mechanical disintegration or by various detergents led to a rapid loss of enzyme activity. The tentoxin-synthesizing enzyme is probably localized at or in membranes. *De novo* synthesis of tentoxin in a cell-free system devoid of ribosomes from radioactively labeled amino acids shows that tentoxin is not a virus product.

Index Entries: *Alternaria alternata;* tentoxin; phytotoxin; biosynthesis; cell-free; localization.

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INTRODUCTION

Tentoxin is a phytotoxic cyclotetrapeptide containing methylamino acids. It is produced by the deuteromycete *Alternaria alternata* (Fries) Keissler. This toxin causes chlorosis in sensitive plants (*1*). Dihydrotentoxin is the hydrogenated precursor of tentoxin (*2*). Various papers have been published about the structure, isolation, and effects of these cyclopeptides (*3–12*). In contrast, the biosynthesis of these peptides is poorly understood. For the characterization of the synthesizing enzymes and to reveal biochemical details of the biosynthesis, we wanted to develop a cell-free synthesis of toxin.

Although Sheu and Talburt (*13*) demonstrated the synthesis of tentoxin in a system that inhibited protein synthesis, Lax and Shepherd (*14*) doubted the existence of a nonribosomal tentoxin synthesis. The latter authors detected a correlation between "virus RNA" contained in the fungus and the level of produced tentoxin. They suggested that early ribosomal formation of a large preprotein followed by limited proteolysis might account for a release of tentoxin in the absence of protein synthesis.

MATERIALS AND METHODS

Organism and Cultivation

The studies were carried out with the strain *Alternaria alternata* i 30/10, isolated in our laboratory from *Brassica chinesis*. The mycelia were grown in surface culture in phosphate-limited medium. Details are given by Hänel et al. (*15*).

Mechanical Disintegration of Mycellium

This was carried out with a cooled (−20°C) X-press (AB Biox, Stockholm, LKB). The frozen mycelium was pressed four times, and then 1 g was dissolved in 1 mL triethanolamine buffer A (20 m*M*, with 10 m*M* $MgCl_2$ and 0.25 m*M* dithioerythritol, pH 7.5). Twenty percent (w/v) glycerol was added.

By usage of a French press (LKB), the mycelium first pounded with pestle and mortar with small amounts of buffer A. Homogenated cell suspension was dissolved immediately in 5 mL buffer/g mycelium. All steps were carried out at 4°C. Protein crude extracts were prepared by stirring the homogenates for 20 min at 4°C and by centrifugation at 10,000*g* (Beckmann J2-MC, Palo Alto, CA) for 15 min (sediment was removed).

Preparation of Protoplasts

This was carried out with a mixture of lytic enzymes from *Helix pomatia, Trichoderma harzianum,* and commercial Novozym (details by

Ramm and Brückner, *see* part I of "Biosynthesis of Phytotoxin Tentoxin") from 5 g (wet wt) of mycelial mats from 9-d cultures. Disintegration of the prepared protoplasts was achieved by osmotic shock in distilled water for 15 min. Then all cells were disrupted. Subsequently, the solution was adjusted to 20 m*M* triethanolamine, pH 7.5 (10 m*M* $MgCl_2$; 0.25 m*M* dithioerythritol), with concentrated buffer solution to a final vol of 10 mL (20 μg/mL protein, Bradford analysis [*16*]).

Separation of Typical Groups of Cell Organelles (Crude Stage)

This was realized by common fractional centrifugation (*17*):

1. Nuclei and larger cell debris (10 mL of disintegrated protoplasts were centrifuged at 600*g*, 10 min, Beckmann J2-MC; sediment was dissolved in 1 mL triethanolamine buffer A);
2. Fraction of mitochondria (10 mL supernatant of 1) were centrifuged at 10,000*g*, 10 min, Beckmann J2-MC; sediment was dissolved in 1 mL triethanolamine buffer A;
3. Fraction of membranes and microsomes (10 mL supernatant of 2) were centrifuged at 100,000*g*, 90 min, Ultracentrifuge Beckmann L7-35, Palo Alto, CA; sediment was dissolved in 1 mL triethanolamine buffer; and
4. Plasma fraction (supernatant after ultracentrifugation, 10 mL).

Formation and Isolation of Tentoxin

By incubation of 1 mL of the cell organelle fractions with 20 μL radioactively labeled precursor amino acids (U-^{14}C-alanine, leucine, glycine, and phenylalanine each 1 n*M* and 3.2×10^4 Bq [from UVVR, Czechoslovakia]), 150 μL ATP (0.36 m*M*), and 30 μL SAM (1 m*M*), it was possible to detect the *de novo* synthesis of radioactively labeled tentoxin. The reaction was performed for 4 h at 28°C and was stopped by 7.2% TCA.

In addition to protein crude extract supplied detergents and substances with detergent-like influence (on solubilization of membrane enzymes) in various incubations were: 50% glycerol, 5 m*M* EDTA, 75 parts by vol of *n*-butanol, 2*M* KSCN, 10% SDS, 2*M* urea, 3% triton, and 3% SPS (sucrose palmitate stearate). Addition of this substances was carried out for 20 min at 4°C before the incubation for tentoxin formation was started.

Tentoxin was isolated and determined together with its precursor dihydrotentoxin, because these compounds are very difficult to separate from each other. Moreover, a separation of dihydrotentoxin was not important in view of the circumstances studied. After addition of 50 μg nonradioactive tentoxin (for better extraction efficiency), the incubation mixture was extracted three times with a double volume of ether. The ether was removed *in vacuo*, and the residue was dissolved in a small volume of methanol and chromatographed by TLC on silica gel in methanol:ethylacetate:water, 100:4:1. The tentoxin spot was detected by UV

Table 1
De Novo Synthesis of Tentoxin/Dihydrotentoxin
from Radioactively Labeled Precursor Amino Acids

Fraction		Radioactivity in tentoxin/dihydrotentoxin in % of the whole preparation*
Nuclei and cell debris		6.1
Mitochondria		2.0
Membranes/microsomes		12.1
Plasma		79.8
	Sediment†	62.1
	Supernatant†	17.7

*100% = 21,874 dpm.
†Separate fractions of plasma fraction after treatment with 2% NaCl. In the sediment are localized proteins that are attached to lipids or membrane debris. The supernatant contains very solubled proteins.

absorption, and eluted and separated again in *n*-butanol:acetic acid:water, 12:3:5. At this stage, no impurities were detectable in the tentoxin spot. The distribution of radioactivity on the chromatogram was measured in dioxan scintillator fluid with a Liquid Scintillation Counter (Wallac S 1410, Pharmacia).

RESULTS AND DISCUSSION

Investigations with mechanical disintegration of the intact cells (X press, French press) always led to a high loss of tentoxin-synthesizing enzyme activity. We could show that protoplasts of *Alternaria alternata* are able to form tentoxin in easily detectable amounts of radioactively labeled precursor amino acids (*18*). The preparation and lysis of protoplasts proved to be a gentle enough disintegration method to allow an easily detectable biosynthesis, too.

A crude extract was prepared from one preparation of protoplasts (from 5 μg mycelium—1.2×10^8 protoplasts; 20 μg/mL protein) synthesized tentoxin/dihydrotentoxin with an incorporated radioactivity of 21,874 dpm from radioactively labeled precursor amino acids (each with 3.2×10^4 Bq, *see* Materials and Methods), and an incorporation of 1.14% into newly formed tentoxin. Thus, it was possible for the first time to demonstrate the *de novo* synthesis of tentoxin in vitro.

Synthesis was also detectable with each of the four fractions of cell organelles (Table 1). In contrast to this, mechanical disintegration of intact hyphae to prepare protein crude extracts (earlier experiments, here not shown) led only to incorporation rates of about 0.02%.

Table 2
Influence of Detergents on In Vitro Synthesis of Tentoxin

Detergent	Tentoxin/dihydrotentoxin in %
None	100
EDTA	63
n-Butanol	6.7
Glycerol	25.5
KSCN	93.6 (no real solubilization)
SDS, urea, triton, SPS	Near 0

Synthesis of tentoxin in the fractions of cell organelles was detected in the membrane/microsome fraction and the highest activity in the plasma fraction. Therefore, the main part of the synthesizing enzymes seemed to be soluble in the plasma, but Penefsky and Tzagoloff (*19*) warned of such conclusions. Many proteins attached to lipids or small membrane debris do not sediment during ultracentrifugation for several hours. For this reason, they only seem to be soluble. The authors recommended usage of 2% saline to distinguish between free and attached proteins. Lipoproteins, in contrast to solubilized proteins, precipitate and can be separated by low-speed centrifugation. In consideration of this information, 78% of active proteins in the plasma fraction seem to be associated with lipids or membranes, because after treatment with 2% NaCl followed by centrifugation, only 22% of the enzyme activity remained in the supernatant. Considering the enzyme activity of the membrane/microsome fraction, probably up to 75% of the enzyme system for tentoxin synthesis may be localized at or in the membranes.

The results shown in Table 2 demonstrate that the activity of the synthesizing enzymes is decreased by inclusion of some detergents in the incubation mixture. The content of protein did not change. In correspondence to the loss of enzyme activity by mechanical cell disruption described above (destruction of essential substructures), this supports our hypothesis about the role of membranes and an essential microenvironment, too.

We cannot exclude the possibility that the detergents or other protein-solubilizing substances themselves inhibit the enzymes, but in view of the variety of the applied substances, we suggest that the native conformation of the enzyme system is changed or essential neighboring proteins are lost, resulting in a decrease of enzyme activity. Usually after ultracentrifugation for 1.5 to 2 h, there are no ribosomes in the supernatant. Nevertheless, the toxin was synthesized. We could exclude the possibility that tentoxin is a viral product that is produced as a large preprotein by ribosomes (*20*), because the plasma fraction freed of ribosomes formed tentoxin *de novo* from the labeled amino acids. Together with the results of further in vitro investigations characterizing the enzyme system

(Ramm et al., in preparation), we assume an enzymatic biosynthesis for tentoxin analogous to the biosynthesis of similar peptides with modified amino acids and cyclic structures (*21*).

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