Studies of the biosynthesis of tentoxin by *Alternaria alternata*

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Biosynthesis of the phytotoxin, tentoxin, its regulation and the enzymic synthesis steps were studied *in vivo* and *in vitro*. The physiology of biosynthesis of tentoxin *in vivo* was investigated by using sections of mycelial mats incubated in buffer. Differentiated mycelia could be studied under defined conditions. The *de novo* synthesis of tentoxin was measured by incorporation of [U-¹⁴C]leucine into tentoxin. The investigation system was stable for 10 h. Biosynthesis and the growth of biomass started before day 5 of culture, with the maximum between days 9 and 12. After this, biosynthesis quickly declined. pH values about 7 were optimal, and pH values above and below this led to an increased release of tentoxin stored in the cells. The formation of tentoxin by older mycelia was not regulated by acetate, phosphate or glucose, which was not utilized. Precursor amino acids, applied at the start of the culture, slightly activated the synthesis of tentoxin. Older mycelia were inhibited. Substances from the host plant (*Brassica chinensis*) reduced the *de novo* synthesis of tentoxin. Enzyme separation studies suggested that biosynthesis of tentoxin involves a multienzyme (≥ 400 kDa), which is a polyfunctional protein without subunits. Experiments suggested that the synthetase contains active SH-groups and an integrated activity of methyltransferase. The precursor amino acids are activated by ATP and bound at the enzyme. *N*-Methylation occurs with the enzyme-bound amino acids or during the elongation of the growing peptide chain. Methionine is the primary donor of the methyl groups, but the immediate methylation reaction needs *S*-adenosyl methionine (SAM). The methylation is essential for the continuation of biosynthesis. The elongation proceeds either stepwise from glycine by binding alanine/methylalanine, phenylalanine/methylphenylalanine and leucine or by formation and linkage of two dipeptides glycine-alanine/methylalanine and phenylalanine/methylphenylalanine-leucine. At the end of this process dihydrotentoxin, the direct precursor of tentoxin, is released from the synthetase probably by cyclization. Independent of this first enzyme, dihydrotentoxin is transformed into tentoxin. This last reaction step is reversible. The rate of transformation of dihydrotentoxin to tentoxin is higher, but in this direction the native turnover is relatively low. The synthesis of tentoxin probably occurs in a manner similar to other well-known cyclic peptides via a 'thiotemplate mechanism'; the highest enzyme activity *in vitro* occurs between days 9 and 11 of culture at a pH value of 7.

Keywords: *Alternaria alternata*, tentoxin biosynthesis, regulation, tentoxin synthetase

INTRODUCTION

The cyclic tetrapeptide tentoxin [cyclo-(L-leucyl-*N*-methyl-dehydrophenylalanyl-glycyl-*N*-methylalanyl): Meyer *et al.*, 1971; Koncewicz *et al.*, 1973; Grable *et al.*, 1966;

Meyer *et al.*, 1971; Templeton, 1972; Woodhead *et al.*, 1975; Liebermann & Oertel, 1983] is produced by the deuteromycete *Alternaria alternata*. It causes chlorosis in many sensitive plants (Durbin & Uchytel, 1977), probably by inhibiting the development of chloroplasts (Schadler *et al.*, 1976). Tentoxin also damages the CF1 subunit of the chloroplast-associated ATPase (Steele *et al.*, 1976). The

Abbreviations: DTE, dithioerythritol; SAM, *S*-adenosyl methionine.

last action is probably not directly connected with the chlorotic symptoms. In addition *A. alternata* excretes dihydrotentoxin, the precursor of tentoxin, which contains *N*-methyl-L-phenylalanine, and other related compounds, including a cyclic dipeptide of *N*-methyl-L-phenylalanine and leucine (Liebermann *et al.*, 1988), non-peptide compounds and conjugates between tentoxin and the non-peptides (B. Liebermann unpublished). These tentoxin-conjugates are produced by the fungus, but they can also be formed to some extent during the isolation of the substances (Liebermann, 1989).

A high level of production of cyclopeptides can be achieved by surface cultivation. The biosynthesis of tentoxin and dihydrotentoxin has so far been investigated only in connection with the optimization of peptide formation under these culture conditions in standing culture flasks. Exogenous phosphate inhibited the synthesis of tentoxin when it was added at the start of cultivation (Brückner *et al.*, 1983; Hänel, 1985). In contrast, acetate stimulated the production of tentoxin (Hänel *et al.*, 1985). The glyoxylate cycle is not activated by acetate but the utilization of glucose is enhanced during early stages of cultivation. Slowly utilized carbon sources do not support the formation of toxin (Oertel, 1983). Based on these results a culture medium for maximum production of tentoxin was developed. Studies of the biosynthesis of tentoxin using the standing culture flasks and selected media (Hänel *et al.*, 1985) were found to be unsuitable for investigations using differentiated mycelia: the culture medium contained tentoxin already released at some time after inoculation and this system was complex and ill-defined. Thus it was not possible to vary one factor selectively. For this reason a new and simple system has been developed which gives detailed conclusions after a short incubation time and with a simple isolation of tentoxin.

Details of the biosynthesis of tentoxin are poorly understood. The investigations by Sheu & Talburt (1986) and Ramm *et al.* (1994a) showed that the synthesis of cyclopeptide was catalysed by enzymes. It was of interest to characterize these enzymes and to reveal biochemical details of the biosynthesis. We postulated an enzyme, similar to those for the synthesis of other cyclic peptides, which does not involve ribosomes (Kleinkauf & von Döhren, 1982). The synthesis probably proceeds without the appearance of free intermediates, because they have not been detected (Liebermann, 1989). Dihydrotentoxin is released first and can be detected in the culture medium (Liebermann & Ihn, 1988). The dehydrogenation to tentoxin probably is catalysed by a further enzyme that acts independently of the postulated synthetase.

METHODS

Organism and cultivation. Investigations were carried out with strain i30/10 of *A. alternata* (Fr.) Keissler isolated in our laboratory from *Brassica chinensis*. The strain was kept on agar plates at 4 °C. Conidia from the plates were inoculated in a liquid culture medium and cultivated for 48–60 h on a gyratory

shaker at 28 °C. The pellets (1%, w/v) were used as inoculum for surface culture in phosphate-limited medium incubated at 28 °C. Media and further details are given by Hänel (1985) and Hänel *et al.* (1985).

Treatment of mycelia. After a defined culture time the mycelium was removed and washed in distilled water. Pieces (1 × 2 cm) of mycelium (5 g) were cut from the mycelial mats and incubated without shaking in 20 ml triethanolamine buffer (20 mM with 10 mM MgCl₂, pH 7.5) at 28 °C for 8 h. To vary pH, 200 mM phosphate buffers with constant ion strength and the triethanolamine buffer were used. Tentoxin, newly formed during incubation, was measured by addition of the precursor amino acid [U-¹⁴C]leucine (19 kBq; UVVVR Prague; 8888 MBq mmol⁻¹) without addition of unlabelled leucine. After incubation the mycelial pieces were washed with 5 ml distilled water. Buffer and wash water were pooled. The experiments were repeated once more with similar results. Representative results are shown in the figures.

Preparation of protoplasts. Protoplasts were prepared with lytic enzymes from *Helix pomatia*, *Trichoderma harzianum* and commercial Novozyme (Ramm *et al.*, 1994a) from 5 g (wet weight) of 9-d-old mycelial mats. Disintegration of the protoplasts was achieved by osmotic shock in distilled water for 15 min. Subsequently, the solution was adjusted to 20 mM triethanolamine pH 7.5 [10 mM MgCl₂, 0.25 mM dithioerythritol (DTE)] with concentrated buffer solution to a final volume of 10 ml.

Disintegration of frozen mycelia. This was done with a X-Press or a French Press (LKB). The homogenates were suspended in buffer [50 mM Tris/HCl, pH 7.2, 50% (w/v) glycerol, 1 mM EDTA, 10 mM DTE] at 0–4 °C. Disintegrated mycelium was then stirred for 15 min and centrifuged at 500 g. The proteins of the supernatant were precipitated by 80% (w/v) ammonium sulfate and separated by centrifugation, solubilized in a small amount of buffer [50 mM Tris/HCl, pH 7.2, 10% (w/v) glycerol, 0.1 mM EDTA, 2 mM DTE] and dialysed against the same buffer for 30 min.

Host plant extracts. Extracts of host plant were prepared by homogenization of 15 g (wet weight) young leaves with 5 ml triethanolamine buffer (pH 7.5) and further filtration. A portion (20 ml) of this extract was used in the buffer system with mycelial pieces instead of the usual buffer.

Isolation of tentoxin/dihydrotentoxin. For experiments *in vivo*, 10 ml samples of filtered surface culture medium or buffer solution from the incubations with mycelium pieces were passed through 50 mg Porapak Q (Serva). The column (7 × 10 mm) was washed with 10 ml distilled water and the bound cyclopeptides were eluted with 1 ml methanol (Liebermann *et al.*, 1992). The eluate was separated by TLC on silica gel G (Merck) in ethyl acetate/methanol/water (100:4:1, by vol.). Tentoxin/dihydrotentoxin was detectable by fluorescence quenching under UV light (*R_F* = 0.32). The spot was separated and the cyclopeptides were eluted. The peptide concentration was measured by UV absorption at 286 nm (maximum of absorption of tentoxin, Specord Carl Zeiss Jena) and 222 nm (maximum of absorption of dihydrotentoxin). Newly synthesized toxin was determined by measuring the radioactivity of the tentoxin spot separated from radioactively labelled leucine (LSC, Wallac S 1410, Pharmacia). These steps did not separate tentoxin and dihydrotentoxin. Estimates of relative amounts were possible by comparison of the absorption maxima for the two compounds. Also they could be separated by gel filtration on

Sephadex G15 (Liebermann & Ihn, 1988). By this method we detected *in vivo* no or only very small amounts of dihydro-tentoxin and no other products. Thus tentoxin was assumed to be the main component of the radioactively labelled material.

More dihydro-tentoxin was formed during experiments *in vitro* after cell disruption. The two cyclopeptides were measured together. After addition of 50 µg non-radioactive tentoxin/dihydro-tentoxin for better extraction efficiency the proteins precipitated by TCA were separated and the incubation mixture was passed through Porapak Q. After separation by TLC as before, the spot was eluted and tentoxin was rechromatographed in *n*-butanol/acetic acid/water (12:3:5, by vol.). The distribution of radioactivity on the second chromatogram was measured in a dioxan scintillation fluid.

The preparation of precursors of the cyclic peptides separated from the enzyme *in vitro* was carried out in an analogous way. The linear tetrapeptide chain was separated with the first solvent system on the origin of the chromatogram. A repeated separation in *n*-butanol/acetic acid/water (63:10:27, by vol.) liberated this metabolite from traces of free amino acids. Identification was done by the analysis of hydrolysate (dansylation) and by comparison with a linear tetrapeptide prepared from tentoxin (Konciewicz *et al.*, 1973).

For investigation of the conversion of tentoxin into dihydro-tentoxin both substances were separated by gel filtration on Sephadex G 15 (Liebermann & Ihn, 1988).

Isolation of enzyme-bound intermediates. Crude protein extract (1 ml) from protoplasts (20 µg ml protein⁻¹) was incubated with 50 µl ATP-mix, 20 µl [U-¹⁴C]amino acids (each 1 nM with 1.6×10^4 Bq), 20 µl SAM (0.7 mM) for 0, 5 and 15 min at 28 °C. The reaction was terminated by TCA and the protein was separated on membrane filters. Unbound amino acids were washed out with 14% (w/v) TCA and distilled water. The protein was dissolved in 1 ml water and the solution was adjusted to pH 9 with 1 M NaOH. The enzyme-intermediate complexes labile in alkali were hydrolysed by heating at 100 °C for 5 min. After neutralization with TCA 3 ml methanol was added and the filter and protein precipitate were separated by centrifugation. The intermediates remaining in the supernatant were separated by TLC in *n*-butanol/acetic acid/water (63:10:27, by vol.). The resulting three radioactively labelled peaks were eluted and separated in phenol/water [72:25 (v/v); 3% NH₃-atmosphere] after hydrolysis (6 M HCl, 24 h, 110 °C).

[U-¹⁴C]Amino acids. These were from UVVVR Prague (specific activities: alanine 4440 MBq mmol⁻¹; glycine 2960 MBq mmol⁻¹; leucine 8888 MBq mmol⁻¹; phenylalanine 13320 MBq mmol⁻¹). Also used were ³H-SAM and ³²P-pyrophosphate (PP_i) (Amersham).

Enrichment of tentoxin synthetase. Protein crude extracts were treated by the following steps (all at 4 °C): (a) gel filtration on Ultrogel AcA 34 (Serva) and Sephadex G-200 (Pharmacia) [buffer 50 mM Tris/HCl, pH 7.2 with 10% (v/v) glycerol, 0.1 mM EDTA, 2 mM DTE; 2.6 × 40 cm column, 3 mg protein, 4.5 ml fractions; 5 × 100 cm column, 25 mg protein, 13 ml fractions, respectively], absorbance at 280 nm was measured; (b) three-phase extraction of 6.7% disintegrated cells with 11% PEG 8000 (Serva), 5% Dextran 251 (Sigma), 8% Ficoll (Pharmacia) all w/v in buffer [50 mM Tris/HCl, pH 7.2 with 10% (v/v) glycerol, 0.1 mM EDTA, 2 mM DTE] by stirring for 30 min at 20 °C followed by centrifugation at 10000 *g* for

15 min and separation of the phases; (c) ultracentrifugation of protein in a density gradient of 0–30% (w/v) sucrose for 24 h at 100000 *g* followed by collection of 1 ml fractions from the gradient starting at the bottom of the centrifugation tube.

The active fractions were subjected to further purification as follows: ion exchange chromatography on QAE-Sephacel and DEAE-Cellulose (buffer 50 mM Tris/HCl, pH 7.2 with 10% (v/v) glycerol, 0.1 mM EDTA, 2 mM DTE, 2 × 15 cm column, 4.5 ml fractions). Elution was with a linear 0–1 M NaCl concentration gradient. Hydrophobic interaction chromatography and affinity chromatography were carried out on 3 ml Phenylsepharose CL-4B (Pharmacia), and the protein was eluted by steps, each of 4 ml, of 0–1 M NaCl followed by 50–100% (v/v) ethyleneglycol.

Measurement of tentoxin synthetase activity. (1) To detect activation of amino acids. (a) ATP/PP_i-exchange. The standard incubation mixture contained 100–150 µl enzyme solution, 5 µl of the respective amino acid (alanine, glycine, leucine, phenylalanine, 5 mM each), 50 µl ATP/³²P-PP_i-mix (ATP, PP_i, 1.2 mM each). The control tube contained buffer instead of amino acid. After 45–60 min incubation at 28 °C the reaction was terminated by addition of 0.5 ml of a solution of perchloric acid and charcoal [42.8 g Na₄P₂O₇ · 10H₂O, 43.2 ml HClO₄ l⁻¹ with 1.2% (w/v) Norit A] and the radioactivity bound to the charcoal was separated on paper filters (Schleicher & Schuell, 0.8 µm; Gevers *et al.*, 1969). After washing and drying the filters (15 min at 110 °C) the radioactivity was measured in a liquid scintillation counter. (b) The binding of precursor amino acids to tentoxin synthetase. The standard incubation mixture contained 100–150 µl enzyme solution, 200 µl [U-¹⁴C]amino acid (alanine, glycine, leucine, phenylalanine without addition of non-radioactive amino acids), 20 µl ATP-mix (ATP 0.5 mM). The control tube contained no ATP. The reaction (1 h, 28 °C) was terminated by the addition of TCA. After 30 min at 0 °C the precipitated protein was separated, washed on membrane filters (Sartorius, cellulose acetate, 0.45 µm) and the bound radioactivity was measured.

(2) To measure the complete biosynthesis of tentoxin and dihydro-tentoxin. The standard incubation mixture contained 1 ml enzyme solution, 150 µl ATP-mix (ATP, 0.35 mM), 30 µl SAM (1 mM), 20 µl of all amino acids (each 1 nM, only alanine, glycine, leucine were radioactively labelled). Incubation was carried out for 2.5 or 4 h at 28 °C and was terminated by the addition of 110 µl 72% (w/v) TCA. The ATP-mix contained 50 mM Tris/HCl, pH 7.2, 5 mM ATP, 50 mM MgCl₂, 5 mM EDTA and 5 mM DTE.

SAM-labelling. To 100 ml of crude enzyme 5 µl (5 µCi; 185 kBq) ³H-SAM was added and the mixture was exposed to UV-light (260 nm, 10 cm distance) for 10 min. The radioactively labelled SAM become bound to the catalytic centre of the methyl-transferase activity (H. von Döhren, personal communication).

Analytical methods. PAGE was carried out as described by Laemmli (1970) with 7.5% or, in most cases, 5% (w/v) gels. Gradient gels contained 3–15% (w/v) acrylamide. Protein staining was carried out with Coomassie brilliant blue R-250 and by silver staining (protein silver staining kit, National Diagnostics). Western blot analysis and ELISA were carried out each with lamb anti-tyrocidine synthetase 1 antibodies (given by H. von Döhren, Technical University, Berlin) and anti-lamb IgG-phosphatase conjugate. Protein determination was done using the method of Bradford (1976). Determination of chloride ions was carried out by the method of Mohr (1958). Detection

Fig. 1. Tentoxin formation by *Alternaria alternata*. ○, Total concentration of tentoxin in µg per incubation assay (each in 20 ml buffer); ■, newly formed tentoxin expressed as radioactivity per incubation assay.

of amino acids on chromatograms after TLC was done by (a) spraying with 0.3% ninhydrin in *n*-butanol and 3% acetic acid, or (b) spraying with solution described by Russell (1960), each then being heated at 110 °C. Dansylation was by the method of Neuhoff (1973).

RESULTS AND DISCUSSION

Physiology of tentoxin biosynthesis – *in vivo* experiments

Tentoxin and dihydrotentoxin are very similar in both their structure and properties. For this reason it was not possible to separate them by measuring the *de novo* synthesis from [U-¹⁴C]leucine. However, it is not important to separate the peptides to get information about the biosynthesis of tentoxin because dihydrotentoxin is the direct precursor of tentoxin. On the other hand, the measurement of total tentoxin at 286 nm is specific, because dihydrotentoxin does not absorb at this wavelength. Moreover, in the buffer system with washed pieces of mycelium only very small amounts of dihydrotentoxin were formed in contrast to the surface culture medium in standing flasks.

Mycelium from *Alternaria alternata* produced tentoxin by day 5 after inoculation (Fig. 1). Synthesis quickly increased after day 5 and reached a maximum between days 9 and 12 of culture. Between days 14 and 18 the synthesis declined to the level of day 5. Dihydrotentoxin was not detected in the buffer during this experiment.

It was possible to distinguish between newly formed tentoxin/dihydrotentoxin and the tentoxin released. By measuring the absorbance at 286 nm and 222 nm we could determine the sum of both. By incorporation of [U-¹⁴C]leucine the *de novo* synthesis of cyclopeptides could be detected selectively. While pH < 6 and > 8 led to a higher release of accumulated tentoxin, pH 6–8 stimulated *de novo* synthesis. Therefore culture at pH 7 and increasing the pH at the end of the cultivation enhanced the tentoxin yield.

A. alternata produces only traces of tentoxin in submerged cultures (Hänel, 1985; Liebermann, 1989). Under sub-

merged conditions NH₃ was formed earlier and in greater amounts than in surface cultivation. Therefore the pH increased more quickly to a suboptimal level. This might be one reason for the minimal yield of tentoxin in submerged culture.

During the high production of tentoxin, glucose was not taken up within 10 h and was not utilized in either primary or secondary metabolism. The toxin formation rate was not changed by addition of glucose (Fig. 2). In contrast, glucose did not regulate the biosynthesis as an external effector. Evidence of the function of glucose was given by Oertel (1983). When glucose was added at the start of the cultivation it was readily utilized compared to slowly usable carbon sources and led to an optimal peptide synthesis. Carbon catabolite repression was not observed, and glucose did not inhibit the expression of the tentoxin synthesizing enzymes, in contrast to the situation with β -lactam antibiotics (Martin & Demain, 1980; Zhang *et al.*, 1989). Tentoxin is produced at a glucose concentration of 20 g l⁻¹ (Hänel, 1985).

Hänel *et al.* (1985) and Liebermann (1989) described the positive regulatory effect of acetate on the toxin synthesis at the start of cultivation. Acetate probably acts as an inducer for the expression of synthesizing enzymes. Metabolic effects of acetate on differentiated mycelia were not detected in our work. Addition of acetate to older mycelia did not activate the uptake or the utilization of glucose in contrast to young mycelia. Acetate together with glucose led to steady formation of tentoxin in the mycelium-section system after 6–8 hours (Fig. 2). These results with the mycelial pieces on buffer agree with data from surface cultures (Liebermann *et al.*, 1991).

Our results suggest a channelling by the amino acid pools of the fungal cells. Therefore it was possible to supply only traces of [U-¹⁴C]leucine into the pool of leucine leading to a high incorporation rate into tentoxin.

Phosphate inhibited tentoxin synthesis when added at the start of cultivation (Brückner *et al.*, 1983; Edel *et al.*, 1985; Hänel, 1985). In this study, *de novo* biosynthesis of tentoxin

Fig. 2. Production of tentoxin by mycelial sections. ○, Under standard conditions without glucose (0–10% of cyclopeptides as dihydrotentoxin); ▼, after added glucose (5 g l⁻¹) (0–30% of cyclopeptides as dihydrotentoxin); □, after addition of glucose and acetate (3.7 g l⁻¹) (no cyclopeptide as dihydrotentoxin).

Table 1. Regulation of the synthesis of tentoxin by precursor amino acids

Amino acids were added at a concentration of 4 g l⁻¹ at the start of surface culture in liquid culture medium or at 5 g l⁻¹ to mycelium at day 10 of cultivation. No dihydrotentoxin was included. Values are expressed as a percentage of the control without amino acids and are means of two experiments.

Amino acid	Tentoxin	
	Start of culture	Day 10
Alanine	105	95
Glycine	85	70
Leucine	130	55
Phenylalanine	100	53
Methionine	3	70
All amino acids together	15	30

by differentiated, toxin-forming mycelium was not affected by adding phosphate up to 200 mM. Mycelial pieces at day 10 of culture in standing flasks produced 230 µg tentoxin on 20 ml triethanolamine buffer corresponding to 200 µg tentoxin on 20 ml phosphate buffer.

Addition of precursors often leads to an increased formation of products. The production of cyclosporin was increased after addition of valine and leucine (Lee & Agathos, 1989). Thus it was possible that provision of precursor amino acids might influence the synthesis of tentoxin. Methionine as a donor of methyl groups for the methylation of alanine and phenylalanine was also thought to be necessary for a high yield of tentoxin (Liebermann & Ramm, 1991).

Amino acids added at the start of the cultivation or after 10 d to the surface culture in standing flasks did not increase the yield of toxin. Only alanine and leucine had a small positive influence (Table 1). Addition of methionine probably led to a channelling of the formation of secondary product to other compounds and the synthesis of tentoxin was inhibited. The changed spectrum of products points to competitive reactions (Fig. 3). Methionine might regulate the alternariol-*o*-methyltransferase which forms alternariol monomethyl ether. Alternariol and alternariol monomethyl ether synthesis are inhibited by 12 mM NaNO₃ (Orvehed *et al.*, 1988). Because the medium used for tentoxin formation contains 70 mM NaNO₃ this effect was exploited for toxin synthesis. Methionine might abolish this influence by a strong activation or induction of this enzymic step towards alternariol monomethyl ether. Thus, the formation of alternariol monomethyl ether increased and the synthesis of tentoxin declined.

Extracts of the host plant *Brassica chinensis* inhibited the synthesis of tentoxin in the mycelium–buffer system.

Fig. 3. Effect of addition of amino acids on spectrum of products synthesized, as shown by separation of TLC in ethylacetate/methanol/water (100:4:1, by vol). (a) Tentoxin/dihydrotentoxin; (b), (c) alternariol and alternariol monomethyl ether-like products, respectively.

Mycelium incubated with plant extract formed only 37 % tentoxin compared to the control on buffer. The production of secondary compounds was displaced to other substances perhaps similar to alternariol and alternariol monomethyl ether.

Enzymic biosynthesis of tentoxin – *in vitro* investigations

The multienzyme responsible for tentoxin biosynthesis is likely to be a protein with molecular mass ≥ 200 –250 kDa (Kleinkauf & von Döhren, 1982). The search for such proteins showed that the crude protein extract contained large proteins, which ran as single bands in the polyacrylamide gel under reducing conditions (Fig. 4a, left). There were also proteins which showed cross reaction with anti-tyrocidine synthetase antibodies [tyrocidine synthetase also has a binding locus for ATP and for phenylalanine (Roskoski *et al.*, 1970) (Fig. 4a, right)]. The SAM-labelling in such large proteins [high-molecular-mass proteins (arrows in Fig. 4b, lanes 3 and 4 correlated with radioactive labelling on lane 7)] suggested integration of methyltransferase in the multienzyme as in the case of actinomycin synthetase (Keller & Kleinkauf, 1977). Results correlating tentoxin synthetase activity and

Fig. 4. High-molecular-mass proteins of tentoxin-producer *A. alternata* strain i30/10. (a) Coomassie blue-stained 5% polyacrylamide gel (left); Western blot (right). (b) Gradient-PAGE with 3–15% acrylamide with crude protein from days 8 (lane 2); 11 (lane 3); 12 (lane 4); 14 (lane 5) of mycelial culture, silver-stained and autoradiographed for 3 weeks after SAM-labelling. Lanes 1 and 6, protein markers; lane 7, autoradiograph on Kodak X-Omat S.

possible SAM-labelling during enzyme enrichment experiments support this hypothesis.

The highest content of proteins above 200 kDa was detected in mycelia from the day 11/day 12 of culture (Fig. 4b, lanes 3 and 4). It correlated with the highest biosynthetic ability of mycelium. The danger of enzyme degradation by proteases is relatively low during this time, because there was low activity of proteases in the crude protein extract as assayed with casein agar (results not shown).

Investigations in enrichment of tentoxin synthetase gave

Fig. 5. Activation of amino acids after precipitation with ammonium sulphate and separation of 25–30 mg protein on Ultrogel AcA 34. —, Control without amino acid, showing unspecific ATP-PP_i exchange; ▲, alanine; ●, glycine; □, leucine; ■, phenylalanine; ---, A₂₈₀.

proteins which activated all four amino acids and formed tentoxin/dihydrotentoxin in part. The active proteins ran in the void volume during gel filtration on Ultrogel AcA 34 (Fig. 5) and showed a little cross reaction with anti-tentoxin synthetase 1 antibodies in the ELISA. Fractions containing tentoxin synthetase eluted from DEAE-cellulose at 0.2–0.3 M NaCl (Fig. 6). During three-phase extraction the enzyme was concentrated in the Ficoll phase. The enzyme eluted at 0.4 M NaCl from Phenylsepharose CL-4B.

Separation by ultracentrifugation in a sucrose density gradient led to active fractions in the sediment but also in a region of less density, correlating with the report that some tentoxin synthetase is localized in the membrane (Ramm *et al.*, 1994b). All enzyme fractions active by synthesis contained a protein of about 400 kDa shown in PAGE under reducing conditions (markers: Merck VIII, ferritin, catalase).

The activation of the precursor amino acids, the first step of biosynthesis, was dependent on ATP; the reaction products PP_i and AMP were released. These results are similar to those from other multifunctional enzymes which synthesize cyclic peptides or peptide derivatives.

Fig. 6. Activation of amino acids after separation by ion exchange chromatography on DEAE-cellulose of peaks B and C from the gel filtration shown in Fig. 5. —, Control; ▲, alanine; ●, glycine; □, leucine; ■, phenylalanine; ---, NaCl gradient; - · -, protein.

ADP was not used and AMP inhibited the reaction (Table 2). Similar properties were found by Gevers *et al.* (1968) and Roskoski *et al.* (1970) during the investigation of gramicidin synthetase.

Experiments suggested that the tentoxin synthetase contains active SH-groups. The enzyme needed the protection of SH-groups by DTT or DTE. A short time separation of the thiolprotector from the enzyme (compare control with 3 mM DTT and probe with 3 mM DTT in Table 3) led to a loss of enzyme activity. The optimum was 1 mM DTT; above this concentration the thiolprotector started to competitively inhibit the enzyme (Table 3). Similar results were found with ACV-synthetase (van Liempt, 1988). These effects could be seen both in activation of amino acids and in translocation for peptide elongation, so that SH-groups are important in both processes. Thioester binding of the amino acids by the enzyme was suggested by the finding that activated and protein-bound amino acid (leucine) could be separated by gentle alkaline hydrolysis (pH 9) up to 60% (compare Gevers *et al.*, 1969).

Isolation and identification of enzyme-bound intermediates gave evidence for the process of biosynthesis. Three main spots, detected by measuring radioactivity of intermediate products, could be obtained for 0, 5 and 15 min of incubation after the first selection step. At 0 min spot 1 was the major compound. At 5 min spots 2 and 3 were the major compounds; after 15 minutes spot 3 was somewhat decreased. Analysis of the compounds 1–3 (5 min and 15 min incubation time) was done by hydrolysis and separation of the radioactively-labelled amino acids. Hydrolysate of compound 1 contained nearly all amino acids (methylated and unmethylated) in the same total radioactivity. The compound from spot 2 contained highly labelled glycine and alanine/methylalanine and less

Table 2. Dependence of tentoxin synthesis on ATP

Added radioactivity 2.4×10^4 Bq, incubation 2.5 h. The radioactivity shown (d.p.m.) is after subtraction of reference.

ATP (mM)	ADP (mM)	AMP (mM)	Products (d.p.m.)		
			Tentoxin/ dihydrotentoxin	Linear tetrapeptide, precursor of cyclopeptides	Total
0	0	0	0	0	0
0.5	0	0	1320	2440	2065
1	0	0	2881	2821	4287
3.1	0	0	4201	5261	6352
0	3.1	0	412	ND	ND
3.1	0	3.1	1510	ND	ND

ND, Not determined.

Table 3. Dependence of tentoxin synthesis on thiolprotectors, correlation between enzyme activity and dithiothreitol (DTT) concentration

Radioactivity added was 3.2×10^4 Bq in each case, incubation 2 h, references with denaturated protein were subtracted. With the exception of the control, DTT was separated by gel filtration before the experiment was started.

DTT concn (mM)	Tentoxin (d.p.m.)	Activated amino acids (d.p.m.)
0	0	360
0.3	1250	2580
1	1820	1230
3	824	3690
3 (control)	2370	16200

radioactivity in phenylalanine and only traces of leucine. In the hydrolysate of compound 3 leucine and phenylalanine/methylphenylalanine were labelled. Radioactivity in glycine and alanine was low.

The [^{14}C]amino acids used had the same absolute radioactivities but not the same specific radioactivities. For this reason we expressed the detected radioactivity in the amino acids relative to the number of carbon atoms and the specific radioactivity of each amino acid to compare their molar relationship (Table 4). The first compound contained all four amino acids including the methyl amino acids and was probably the linear tetrapeptide. The high content of alanine and glycine appeared to be contaminations with traces of free amino acids which had the same R_F value as spot 1 under these separation conditions. The compound from spot 2 contained only alanine/methylalanine and glycine and so

was a dipeptide. Spot 3 contained mainly leucine and phenylalanine/methylphenylalanine but also traces of alanine and glycine. Because there was no contamination of spots 2 and 3 by free amino acids, two interpretations are possible for compound 3: either it was a dipeptide of the first two amino acids or a tetrapeptide (perhaps cyclic?). The biosynthesis of tentoxin via dihydrotentoxin therefore starts either with glycine and alanine/methylalanine and proceeds to leucine and phenylalanine/methylphenylalanine in a linear process similar to the synthesis of gramicidin (Kleinkauf & von Döhren, 1982) or with the formation and linking of two dipeptides of glycine-alanine/methylalanine and phenylalanine/methylphenylalanine-leucine similar to the synthesis of actinomycin (Keller, 1987).

Methylation of the enzyme-bound amino acid intermediates occurred during their elongation, because both methylated and unmethylated forms were found as intermediates of the biosynthesis. This corresponds to the biosynthesis of enniatin (Zocher *et al.*, 1976). Synthesis of cyclosporin A differs by terminal methylation after completion of peptide synthesis with unmethylated amino acids and cyclization (Zocher *et al.*, 1986). The immediate methylation of alanine and phenylalanine occurs by SAM and is essential for continuing the biosynthesis. Without methylation the biosynthesis of tentoxin/dihydrotentoxin was strongly inhibited, as in the biosynthesis of enniatin, and no cyclopeptide could be detected. Free unmethylated intermediates could not be detected in culture medium or incubation solutions. The primary donor of the methyl groups is methionine (Liebermann & Ramm, 1991).

The optimum pH value *in vitro* lies between pH 6 and 7. Biosynthetic activity decreases slowly below pH 6 but rapidly above pH 7. This corresponds to the results of *in vivo* experiments.

Tentoxin synthetase activity in crude protein extract of protoplasts in Tris buffer (50 mM, pH 7.2) could be

Table 4. Ratios of radioactivity of single amino acids and composition of the intermediates of tentoxin synthesis after hydrolysis

The ratios shown are glycine:alanine/methylalanine:leucine:phenylalanine/methylphenylalanine.

Probe	Incubation time (min)	Amino acid ratio		
		Measured	Relative to C and specific radioactivity	Molar ratio
Intermediate 1	5	1:1.3:0.9:1.2	1:0.4:0.15:0.13	
	15	1:1.2:0.2:0.4	1:0.4:0.03:0.04	10:5:1:1
Intermediate 2	5	1:1.5:0.09:0.3	1:0.5:0.01:0.04	
	15	1:1.7:0.1:0.3	1:0.6:0.01:0.03	100:50:1:3
Intermediate 3	5	1:6.5:44:48	1:2:7:5.5	
	15	1:3:16:21	1:1.2:6:2.4	1:1:5:5

preserved for 3–4 weeks at -20°C without much loss of activity, but after that the activity decreased quickly.

The identification of the tentoxin/dihydrotentoxin fractions formed *in vitro* showed that dihydrotentoxin was the major product under these conditions. Also the cyclopeptides were partly connected with nonpeptides (Liebermann, 1989) to form 'tentoxin conjugates'. This reaction of the tentoxin probably resulted from loss of compartmentation during preparation. These products were characterized by extensive separations by TLC in several solvent systems (compared with standard substances). The compounds were also characterized by Sephadex G12 (comparison with known K_{av} values of standard substances; Liebermann & Oertel, 1983), by hydrolysis and identification of the reaction products, as done for the analysis of the enzyme-bound intermediates, and by dansylation.

The results correspond to those of other multienzymes or multienzyme complexes which work by the 'thiotemplate mechanism' (Kleinkauf & Koischwitz, 1978; Kleinkauf & van Döhren, 1982). Synthesis of tentoxin probably also occurs by a polyfunctional multienzyme with a molecular mass of about 400 kDa. Tentoxin synthetase is localized at or in membranes and is sensitive to changes in the surrounding environment (Ramm *et al.*, 1994b). The primary product of peptide synthesis is dihydrotentoxin, which is released probably by cyclization (Liebermann & Ihn, 1988). The further conversion to tentoxin, independent of the first synthetase but probably contiguous in the membrane, is relatively low in the intact mycelium. The turnover of added radioactively labelled dihydrotentoxin in the system with mycelium pieces into tentoxin was detectable but only at 0.07% (Liebermann *et al.*, 1992). Added labelled tentoxin was converted in dihydrotentoxin *in vivo* with a turnover rate of 0.03%. The second stage of tentoxin synthesis is therefore reversible.

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