Procedures for Crystallization and Further Purification of Tentoxin

Suzan H. Woodhead, G. E. Templeton, W. L. Meyer, and R. B. Lewis

Research Assistant, Professor of Plant Pathology, Professor of Chemistry, and Postdoctoral Research Associate respectively, Department of Plant Pathology and Department of Chemistry, University of Arkansas, Fayetteville 72701.

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ABSTRACT

Procedures for collecting, screening, and maintaining strains of *Alternaria tenuis* that produce high yields of tentoxin were refined. Tentoxin was crystallized as the benzene solvate after extraction from concentrated filtrates and column chromatography over silicic acid with chloroform:acetone then further purified by removal of the benzene of solvation. Yields ranged from 25-35 mg pure peptide per liter of original filtrate.

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Tentoxin is a cyclic tetrapeptide, produced in culture filtrates and mycelium of *Alternaria tenuis* Nees. It produces a striking variegated chlorosis in the cotyledons of cotton, many other dicotyledonous species and certain monocotyledonous species when applied to seeds or seedlings during germination (4, 8). That this peptide is functional during pathogenesis in cotton seedling chlorosis disease in the field may be assumed with reasonable certainty, because of its distinctive biological activity. These symptoms could result, however, from infiltration of tissue by the peptide during saprophytic growth of the fungus, rather than from its production and excretion during parasitic growth of the fungus. Whether or not tentoxin is functional during parasitism by the weakly parasitic *A. tenuis* or some more highly parasitic species of *Alternaria* is therefore, still an open question.

The stability, relatively small size of the molecule, the existing knowledge of its amino acid sequence, conformation and how conformation changes as analogues are formed (6, 7) make it an excellent model with which to relate structure and function at the molecular level (10). And whether or not it is functional during parasitism, an understanding of the mode of action of tentoxin would likely improve our understanding of the biochemical processes dealing with formation, maturation and/or function of the chloroplast, the apparent target organelle as well as provide some insight into the mode of action of other peptide-host specific toxins.

These studies have been initiated (1, 2). Unfortunately, many of these initial studies have been done either with crude preparations of this toxin or with the benzene solvate as originally crystallized by Grable *et al.* (5). This study was undertaken to refine and simplify the Grable's procedure for producing, crystallizing, and removing the benzene of solvation in the peptide that it might be more readily available for biochemical studies. Since isolates of *A. tenuis* vary in ability to produce tentoxin under laboratory conditions, and commonly lose their capacity for toxin production after repeated transfer, a necessary adjunct to this work was to develop a system for finding high-yielding strains of the fungus and storing them in such a way that they retain their ability to produce toxin.

Selection and maintenance of active isolates.—Plant residue from a wide variety of field sources was placed on moist filter paper in petri dishes overnight. *Alternaria* spores were then picked from the abundant conidia with a sterile needle at × 125 magnification and transferred to potato carrot agar (9) in petri dishes. After three days growth at room temperature, stock cultures were made from these plates by transfer of a 0.5-cm diameter disk to a 15 × 150 mm test tube containing 15 ml of silt loam soil at 25% water holding capacity that had been autoclaved at 1.035 bar for 30 minutes on two successive days. These inoculated soil tubes were held for one week at room temperature, then stored in the refrigerator at 8 C. At the same time, three flasks of modified Richard's solution (9) were inoculated with 0.5 cm disks from the original culture and incubated for 28 days at room temperature. Filtrates were collected and toxin production checked with the cucumber chlorosis bioassay (5). Stock cultures of active isolates have been maintained in soil for over 3 years without detectable changes in viability or toxin-producing capability.

Production of active filtrates.—Toxin was produced in still culture at room temperature in 500-ml Erlenmeyer flasks containing 250 ml of Richard's solution modified by substitution of sucrose for glucose and by addition of 0.5 g/liter of yeast extract and 1 ml/liter of Pratt's (3) microelement solution. These flasks were inoculated with 0.5 cm potato carrot agar disks cut from the leading edge of actively growing colonies of *A. tenuis* that had been started with a few grains of soil from the stock culture tubes.

Filtrates were harvested by vacuum filtration and combined in six liter batches after 28-30 days growth at room temperature. Each mycelial mat was squeezed by hand to remove spent medium and then discarded. The combined filtrates were sterilized at 1.035 bar for 30 minutes.

Crystallization of the benzene solvate.—The sterile filtrates were concentrated to one tenth their original volume in a flash evaporator under reduced pressure below 40 C and extracted with benzene for 24 hours in a Kutscher-Steudel extraction apparatus. The benzene solution was evaporated to a brown resinous material, taken up in chloroform, and placed on top of a 3.5 × 50 cm column of silic acid [Bio-Sil A 149-74 μm (100-200 mesh)] that had been equilibrated with chloroform. The

TABLE 1. Calculated and actual[a] analyses for tentoxin, $C_{22}H_{30}N_4O_4$

	Carbon(%)	Hydrogen(%)	Nitrogen(%)
Calculated	63.77	7.25	13.52
Actual	63.80	6.98	13.39

[a]Alfred Bernhardt, Mikroanalytishes Laboratorium, 5251 Elbach über Engelskirchen, Fritz-Pregl-Strasse 14-16, West Germany.

column was chromatographed with 100-ml aliquots of 100:0, 95:5, 90:10 mixtures of chloroform:acetone which were discarded, and the toxin was eluted with 300 ml of 75:25 of the same solvent. This fraction was evaporated with a flash evaporator under reduced pressure at room temperature to a light brown resinous material. The residue was dissolved in a minimum of benzene and placed in a refrigerator at 8 C for 2-4 weeks where it crystallized as the benzene solvate. The benzene solvate was separated from the mother liquor by centrifugation and recrystallized from benzene.

Further purification of the toxin.—The peptide was freed from benzene of crystallization by drying the crystals in a vacuum oven at 70 C at 762 mm Hg (30 in.) vacuum for 18 hours. This purification procedure yielded 25-35 mg toxin per liter of culture filtrate. Tentoxin is a colorless powder with molecular extinction coefficient of 17,500. Microanalysis showed the tentoxin to be of analytical purity.

The biological activity of pure tentoxin in the cucumber seedling bioassay appeared to be equivalent to that of the solvate when correction for weight of the contaminating benzene was made. The minimum detectable level with cut cotyledons steeped in toxin for 2 hours after 24 hours germination in the dark was 3.18×10^{-6}M, and complete chlorosis was obtained with concentrations greater than 1.59×10^{-5}M.

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