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RAPID PURIFICATION OF HOST-SPECIFIC PATHOTOXINS FROM *ALTERNARIA ALTERNATA* F. SP. *LYCOPERSICI* BY SOLID-PHASE ADSORPTION ON OCTADECYLSILANE

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SUMMARY

A simplified, rapid procedure for the purification of two phytotoxic metabolites (TA and TB) from cell-free culture filtrates of *Alternaria alternata* f. sp. *lycopersici* was developed using hydrophobic chromatography on macroporous, C_{18} bonded silica followed by gel filtration. Baseline separation of TA from TB was achieved in the final preparation and yields of 80% were obtained with retention of full biological activity in all assay systems tested. Toxin analysis by nuclear magnetic resonance and high-performance liquid chromatography indicated TA was at least 99% pure. The efficiency of the procedure allows rapid accumulation of pure TA and TB for studies on the molecular mode of action and host plant response to this novel host-specific phytotoxin.

INTRODUCTION

Phytotoxic metabolites (AAL-toxins) isolated from cell-free culture filtrates (CFCF) of *Alternaria alternata* f.sp. *lycopersici* have been shown to play an important role in the development of *Alternaria* stem canker disease on susceptible genotypes of tomato[1,2]. Structural characterization of one of these metabolites (TA) by high resolution mass spectrometry and nuclear magnetic resonance spectroscopy (NMR), yielded spectra consistent with 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol esterified at either C13 or C14 with 1,2,3-propanetricarboxylic acid[3,4]. Preliminary evidence also indicated that a second component (TB) shares the same carbon skeleton as TA but lacks the C5 hydroxyl and differs in stereochemistry at one or more chiral centers from C11 to C15[4]. Further, it was found that modification of the amino group of AAL-toxins by maleic anhydride[5] followed by separation with reversed-phase high-performance liquid chromatography (RP-HPLC), resulted in a reliable quantitative assay for TA and TB from either culture filtrates or necrotic leaves of *A. alternata* infected tomato plants[2].

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The reported method of extracting AAL-toxins from culture filtrates by barium acetate precipitation, butanol extraction, gel filtration and semi-preparative thin-layer chromatography (TLC) is reliable and produces chemically pure TA and TB but is laborious and time consuming[5]. The repeated extraction of toxin with water-saturated butanol and subsequent rotary evaporation requires continuous operator attention and several gel filtration runs are required per liter of culture filtrate. Adsorption of AAL-toxins directly from CFCF on disposable C_{18} bonded silica cartridges[2] was the starting point for a systematic investigation of the use of solid phase extraction in the purification of AAL-toxins. In this paper, development and evaluation of a rapid, simple and efficient purification scheme for TA and TB from cell-free culture filtrates of *A. alternata* using 40-μm particle-size octadecylsilane in conventional glass chromatography columns is described. The separation of TA from TB in the initial stage of purification and analysis of the purified toxin by TLC, HPLC, NMR and detached leaflet bioassay also are reported.

EXPERIMENTAL

Chemicals

L-Asparagine and L-malic acid were purchased from Sigma (St. Louis, MO, U.S.A.), yeast extract from DIFCO (Detroit, MI, U.S.A.), all other chemicals as analytical grade from Mallinkrodt (St. Louis, MO, U.S.A.). HPLC-grade methanol was from Burdick & Jackson (Muskegon, MI, U.S.A.). Water for all experiments was purified using a four cartridge (18 MΩ cm) Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Bio-Gel P-2 (Bio-Rad, Richmond, CA, U.S.A.) was prepared for use according to manufacturers' instructions.

Preparation of cell-free culture filtrates

The single conidial isolate of *A. alternata* f.sp. *lycopersici* (AS27-3) used for toxin accumulation was obtained from a naturally infected tomato plant in San Diego County, CA, U.S.A. as previously described[6]. A volume of 1 ml of a suspension containing *ca.* 10^5 AS27-3 conidia/ml was used to inoculate 300 ml of sterile medium in low-form Pyrex culture flasks. The medium used for AAL-toxin accumulation contained: 5.0 m*M* L-malic acid, 8.0 m*M* L-asparagine, 1.7 m*M* sodium chloride, 4.4 m*M* dipotassium hydrogen phosphate, 2.0 m*M* magnesium sulfate, 8.8 m*M* calcium chloride, 0.05% yeast extract and 0.12 *M* glucose. The medium was adjusted to pH 6.0 prior to autoclaving for 20 min at 120 p.s.i. Glucose solutions (10 ×) were autoclaved separately from the remainder of the medium to prevent carmelization. Stock solutions of L-malic acid (0.1 g/ml) were sterilized by passing through 0.22-μm filters (Millipore) and added aseptically to cooled, autoclaved media. Liquid cultures were grown on a laboratory shelf for 18 to 21 days at room temperature (22–25°C) under cool-white fluorescent lighting. Cultures were harvested by sequential filtration through Miracloth (Cal Biochem, San Diego, CA, U.S.A.), Whatman No. 1, Whatman GF/C glass fiber 0.45- and 0.22-μm cellulose acetate filters (Millipore). The cell-free culture filtrates (average pH 5.33 ± 0.37) were frozen at −12°C and kept for up to two years without apparent loss of toxin activity (determined by detached leaflet bioassay and peak height in reversed-phase HPLC).

Purification of AAL-toxins on RP Trap columns

A 10 × 1 cm (O.D.) C_{18} RP Trap column (Regis Chemical, Morton Grove, IL, U.S.A.) was pre-washed sequentially with 25 ml methanol and 50 ml water. Methanol was applied through a 12-ml plastic syringe fitted with a rigid polyethylene connector (methanol resistant); water and culture filtrates were applied through a peristaltic pump fitted with Tygon tubing. Freshly thawed CFCF (100 ml) was filtered through a 0.45-μm Millipore filter and loaded on the RP Trap column at 90 ml/h followed by 25 ml water at the same flow-rate. The application effluent and water wash were assayed for toxin activity and the remainder concentrated ten-fold on a rotary evaporator for TLC and HPLC analysis.

The column was developed sequentially with 9 ml each of 45, 55, 65 and 100% methanol. The first 3 ml of each rinse was pooled with the previous rinse to account for 3 ml void volume. Fractions (3 ml) were collected and aliquots (5–20 μl) spotted on 0.25-mm analytical silica gel TLC plates and developed as described previously[5]. Fractions containing TA were pooled, diluted with water and flash-evaporated to remove methanol and concentrated to *ca.* 5 ml. The entire sample (less 100 μl for assay) was loaded on a Bio-Gel P-2 gel filtration column (85 × 2.6 cm I.D.) equilibrated with 0.05 *M* sodium acetate, pH 5.0. Toxin was eluted at a flow-rate of 30 ml/h and 2 ml fractions were collected after 182 ml void volume. Every fourth fraction was spot-tested for ninhydrin reaction and positive fractions further analyzed by TLC as above. Fractions co-chromatographing with authentic TA were pooled and lyophilized. Methanol fractions from the RP column containing TB were treated in an identical way (but separately from TA).

Preparative purification of AAL toxins

For solid phase adsorption of toxin from culture filtrates, 26 g of 40-μm octadecylsilane silica gel (RP Trap column packing material, Regis Chemical) was slurried in 100 ml methanol (100%) and degassed under vacuum. The slurry was poured (using a funnel attachment giving 60 cm pressure head) into a 45 × 1.6 cm I.D. glass column fitted with solvent resistant endplates and flow-adaptor (Spectrum, Houston, TX, U.S.A.) and washed with 400 ml 100% methanol. A Marriotte flask fitted with rigid polyethylene tubing and a glass/PTFE stopcock was used for all methanol rinses (methanol releases plasticizers from soft plastic tubing; hence polypropylene connectors immediately downstream from the stopcock allowed rapid transfer of the line to a peristaltic pump for aqueous washes). Alternatively, a solvent-resistant metering pump with inert tubing could be used. The column was then washed with 250 ml water and loaded at 2 ml/min with approximately 900 ml CFCF (freshly thawed and filtered through a 0.45-μm Millipore filter). After completion of loading (7–8 h), the column was washed sequentially with 250 ml water and 200 ml degassed methanol–water (40:60). The column was then developed isocratically with 400 ml degassed methanol–water (55:45) at a flow-rate of *ca.* 30 ml/h. Fractions, collected by time every 8 min, were analyzed for TA and TB by TLC as described above, pooled and flash evaporated to 10 ml. The final column wash was with 100% methanol at a flow-rate of 6 ml/min, and 2-min fractions were collected until colored material had been eluted. The column was stored in 100% methanol.

Volumes of 5–10 ml of TA or TB (dark yellow concentrate from flash evaporator) were loaded onto the Bio-Gel P-2 column and eluted as described above.

Alternatively, a 90 × 1.6 cm I.D. column of Fractogel HW-40S (Alltech, Deerfield, IL, U.S.A.) was used for gel filtration with 3-ml sample size. Toxin fractions from gel filtration (determined by TLC) were concentrated and the acetate removed by repurification on a 10 × 1 cm O.D. Regis RP trap column developed as described above except that 9 ml 40% methanol replaced the 45% step and 9 ml 70% methanol replaced both the 55 and 65% methanol steps. Pure toxin was identified by spot-testing with ninhydrin, exchanged into water by flash evaporation and lyophilized. Purified toxin was stored desiccated at −12°C. A semi-preparative method which eliminates the need for a solvent resistant column can be constructed by attaching three RP Trap columns in series with short lengths of polyethylene tubing. Such a column has a capacity of 300 ml culture filtrate. The volume and flow-rates of other washes are adjusted accordingly.

Toxin assays

Cultivars of tomato (*Lycopersicon esculentum* Mill.) susceptible (Earlypak-7) and resistant (Ace 55-VFN) to *A. alternata* f.sp. *lycopersici* were maintained in a heated greenhouse (23–29°C) in standard UC soil mix[7] under natural lighting. Leaflets of 4- to 8-week old plants were excised as described previously[1] and placed in glass petri plates on 9-cm disks of Whatman No. 1 filter paper saturated with 3 ml of test solution. The covered plates were transferred to an airtight clear-plastic box, arranged to minimize shading and incubated in a growth chamber under previously described conditions[2].

Quantification of the amino group of purified toxin was performed by reacting toxin with trinitrobenzene sulfonic acid (TNBS) for 2 h in the dark at 40°C and pH 8.5[8]. Absorbance at 340 nm was compared to a standard curve generated by diluting 10^{-3} *M* aspartic acid with water ($r = 0.999$, average of ten triplicate points).

Toxin analysis

Samples from each stage of the purification scheme were analyzed by RP-HPLC and TLC. HPLC instrumentation, columns and mobile phases have been described in detail elsewhere[2,5]. Samples were prepared for HPLC analysis by derivatizing the amine with maleic anhydride[5]. Aliquots were diluted as necessary to a final volume of 1.5 ml and mixed with an equal volume of 1 *M* sodium carbonate, pH 9.2. Approximately 100 mg of maleic anhydride crystals were then added slowly with stirring, while maintaining the pH above 9.0 with 1 *M* sodium hydroxide. After completion of the reaction (ca. 5 min) the pH was lowered to 6.5 with 1 *M* hydrochloric acid and the mixture passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) previously equilibrated with 5 ml 100% methanol, followed by 5 ml water. The cartridge was developed by washing sequentially with 2 ml water and 2.5 ml 100% methanol. Toxin was eluted in 1 ml of the methanol wash after discarding the first 0.5 ml as void volume. The toxin fraction was then diluted with 1.0 ml water, filtered through a Millex-HV4 HPLC filter (Millipore) and 10–50 μl injected onto the analytical, RP-HPLC column. TLC analysis was performed as previously described[5]. ^{1}H NMR spectra were obtained from A. Bottini and W. Swensen, UC Davis Chemistry Department, using previously published methods[3,4].

RESULTS

RP Trap columns

Initial experiments in which toxin was eluted from RP Trap columns by 100% methanol were hampered by formation of a yellowish-brown, waxy material which precipitated during flash evaporation prior to loading on Bio-Gel P-2. The majority of the yellow material could be separated from toxin by an initial wash with 45% methanol. TLC analysis of methanol fractions from an RP Trap column showed elution with 45% methanol removed two significant contaminants with R_F values of 0.90 for the yellow compound and 0.14 for the ninhydrin-positive compound, neither of which had toxin activity.

HPLC analysis was used to monitor AAL toxins at each stage of elution from the RP Trap column (Fig. 1) and the resulting chromatograms were compared with previously published data[5] for authentic TA (retention time of 3.2 min under given condition) and TB (retention time of 5.6 min). Capacity of the 10 × 1 cm RP Trap column exceeded 100 ml of CFCF since no AAL-toxin was recovered in the application effluent from this size column as determined by bioassay and HPLC analysis. A small quantity of TA eluted with 45% methanol (indicated by a minor peak at 3.2 min in Fig. 1a) however, it represented only 0.8% of the biological activity (determined by detached leaflet bioassay) of the major TA peak in the 55% methanol eluate (Fig. 1b). HPLC analysis (Fig. 1b and c) confirmed the baseline separation of TA from TB directly from CFCF by this single step. HPLC separation of the Bio-Gel P-2 purified 55% methanol fraction resulted in a single peak of absorbance at 250 nm. ^{1}H NMR of the purified TA at 360 or 500 MHertz (Fig. 2a) indicated TA was greater than 99% pure[9]. TB (Fig. 2b) was also highly pure but showed trace impurities not present in TA.

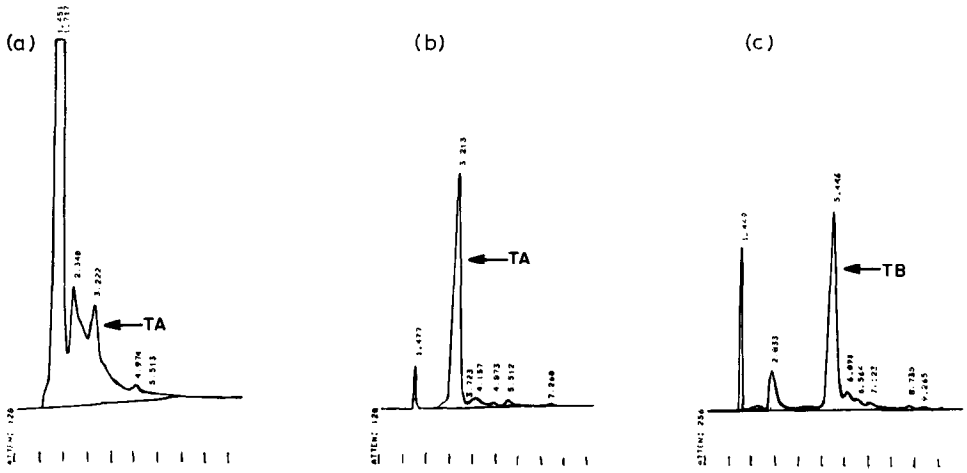

Fig. 1. HPLC analysis of fractions from an RP Trap column. Column: Micro-Pak MCH-10 (30 × 0.4 cm I.D.). Mobile phase: 10-min gradient (60:40) to (70:30) methanol–0.1 *M* sodium acetate, pH 3.0. Flow-rate: 2 ml/min. Sample size: 50 μl. Detection: 250 nm. (a) 45% methanol wash, (b) 55% methanol wash diluted 1:10, (c) 65% methanol wash.

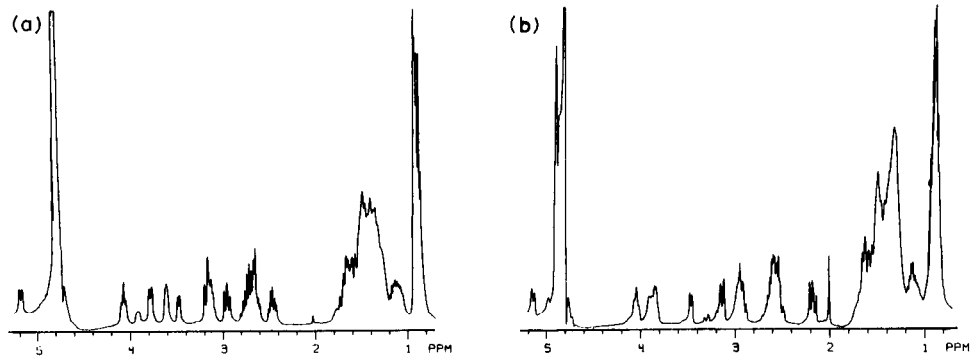

Fig. 2. ^{1}H NMR of RP Trap column and Bio-Gel P-2 purified AAL-toxins. Nicolet spectrometer, 500 MHertz. (a) Deuterated TA, (b) deuterated TB.

To estimate the recovery of TA and TB from 1 l of CFCF by the RP Trap column method, the number of moles of pure toxin from 10 × 100 ml RP Trap column runs was determined by the TNBS assay for amines. A value of $4.91 \cdot 10^{-4}$ moles of amine was determined and assumed to be entirely due to TA and TB since AAL toxins have only one amino group and HPLC and TLC analysis indicated no impurities with a free amino group. The dilution endpoint (d.e.) of a 52 mg/ml solution of pure toxin on susceptible tomato leaflets was $5 \cdot 10^6$ confirming the previously determined value of 10 ng/ml as d.e. for pure AAL toxins[2,3,5]. Since the CFCF for this experiment had a d.e. of $3.2 \cdot 10^4$, a value of $6 \cdot 10^4$ moles of AAL toxins per liter CFCF could be calculated assuming 10 ng/ml d.e. and 528 daltons as the average molecular weight (TA, 522 daltons, +TB, 540 daltons, in a 2:1 ratio). Therefore, the recovery was calculated as $5 \cdot 10^4$ moles/$6 \cdot 10^4$ = 80%.

Preparative purification of TA and TB

To further investigate the separation of TA from TB on C_{18} silica, three RP Trap columns were connected in series to increase the number of theoretical plates and a 55% methanol isocratic elution was used. Baseline separation of TA from TB also was achieved by this method (Fig. 3) which then led to the scaled-up preparative column purification using a high capacity C_{18} silica column followed by P-2 gel filtration as described in the Experimental section. The first methanol rinse was reduced to 40% to avoid loss of TA under heavy column loads. Also, it was found that flash evaporation of the 55% methanol TB fractions resulted in a very cloudy solution which could be cleared by a few drops of 1.0 *M* sodium hydroxide. Analysis by NMR, HPLC and TLC indicated that the AAL-toxin (TA) purified by the two step scaled-up method was indistinguishable from that described above for the RP Trap columns (Fig. 4). Gel filtration by either Bio-Gel P-2 or Fractogel HW-40S gave similar results as determined by NMR and HPLC. Bio-Gel P-2 was chosen for routine use because of its ease of preparation and elution.

Fifteen repetitions of the preparative procedure over an eighteen-month period gave essentially identical results. However, since silica slowly dissolves in the aqueous mobile phase, the column must be repoured with new C_{18} packing at regular intervals to prevent loss of resolution of TA and TB.

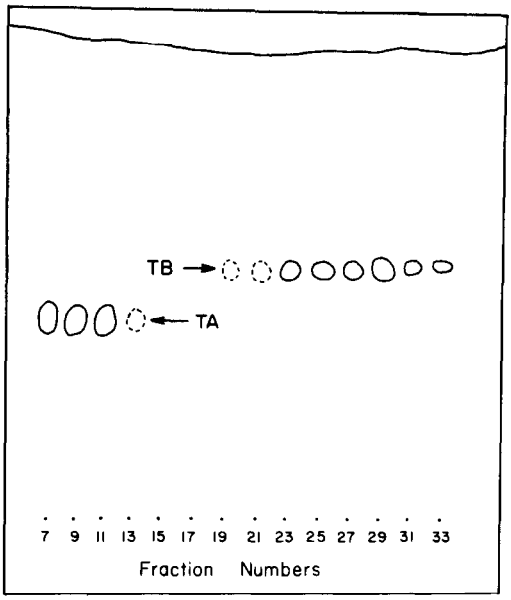

Fig. 3. TLC analysis of toxin eluted with 55% methanol from three interconnected RP Trap columns. Samples of 20 μl were spotted (2 ml fractions, 0.2 ml/min flow-rate) on silica gel plates and developed as in ref. 5.

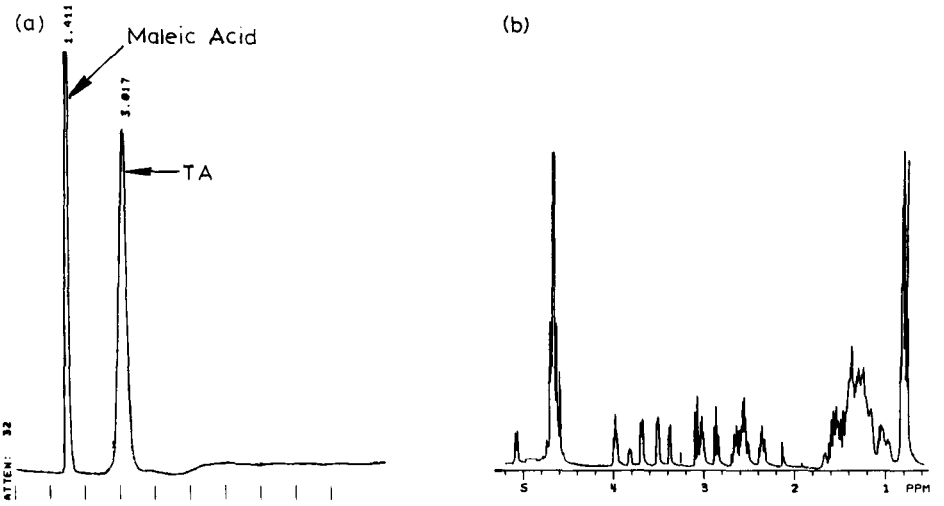

Fig. 4. (a) HPLC analysis of TA purified by preparative C_{18} chromatography and Bio-Gel P-2; conditions as in Fig. 1; (b) ^1H NMR of TA purified by C_{18} chromatography and Fractogel HW-40S; conditions as in Fig. 2.

DISCUSSION

A number of recent reports have discussed the advantages of solid phase adsorption on commercially available C_{18} silica columns for the purification of biological molecules. Wachob[10,11] replaced solvent extraction with C_{18} bonded extraction on disposable cartridges for isolation of water soluble vitamins prior to HPLC analysis. Meryn and Bauman[12] substituted Sep-Pak C_{18} cartridges for Sephadex G-50 in recovering radioactive pancreatic polypeptides for radioimmunoassay. The results presented here show that solid-phase extraction on C_{18} bonded silica is a reliable and efficient alternative to tedious and time consuming liquid extraction with water-saturated butanol for the purification of AAL-toxins.

Dramatic reduction in total time from CFCF to pure toxin, in hours of operator attention, and in quantities of organic solvent used are important advantages of the new procedure. A volume of 1 l of CFCF can be loaded on the preparative C_{18} column, washed and eluted in a 24-h period with less than 1 h of operator time. The baseline separation of TA from TB by C_{18} chromatography further increases the efficiency of the method; only two Bio-Gel P-2 runs per liter of CFCF (one for TA, the second for TB) are required. Formerly, seven P-2 runs per liter of butanol-extracted CFCF were normally required because of the inability of Bio-Gel P-2 to cleanly separate TA and TB (only the leading and trailing edges of the toxin peak contained pure TA or TB, respectively, the remainder being TA plus TB).

AAL-toxins purified by the new procedure showed nearly identical properties when compared to previously purified toxin with respect to NMR spectra at 360 and 500 MHertz, retention times and relative mobilities on RP-HPLC and TLC, and dilution endpoint in detached leaflet bioassay (10 ng/ml). Additionally, C_{18} purified toxin affected sensitive protoplasts and cultured cells of tomato in the same way as butanol extracted toxin[13].

The AAL-toxins, as host-specific primary determinants of the Alternaria stem canker disease of tomato, are of considerable importance in studies of the molecular regulation of this disease as well as tools for screening resistant genotypes of tomato in plant breeding programs. A complete understanding of the role of AAL-toxins in disease development requires identification of primary receptor site(s) in the host, kinetic studies of toxin–target interaction and elucidation of the causal sequence of events leading from the primary toxin–receptor interaction to disturbed host physiology, culminating in visible symptom expression[14]. The preliminary identification of aspartate carbamoyl transferase (ACTase) as an intracellular binding site for AAL-toxins is the basis for ongoing studies of host–toxin interaction[15]. Relatively large quantities of purified TA and TB will be required for a thorough kinetic analysis of the AAL-toxin–ACTase interaction. The successful development of the rapid purification scheme described in this paper will facilitate accumulation of AAL-toxins for continued analysis of the molecular basis of toxin action and host response.

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