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DETERMINATION OF HOST-SELECTIVE PHYTOTOXINS FROM *ALTERNARIA ALTERNATA* F. SP. *LYCOPERSICI* AS THEIR MALEYL DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, fully reversible, and highly reproducible procedure for separation and quantitative detection of disease-determining phytotoxic metabolites of *Alternaria alternata* f. sp. *lycopersici* as their maleyl amide derivatives by reversed-phase high-performance liquid chromatography is described. Maleyl-derivatized toxin, prepared by reaction with maleic anhydride, with an absorbance maximum at 250 nm, was detected for 0.5–10 nmoles following chromatography on a C_{18} bonded-phase column using binary gradient systems. Toxin samples, maleylated at pH 9.2 and then demaleylated at pH 3.0, showed no detectable loss of biological activity after the chromatographic separation. Quantification of the procedure was based on a trinitrophenol–aspartic acid standard. The procedure is currently being used to study kinetics of toxin production in culture and qualitative detection of phytotoxin in diseased host tissue.

INTRODUCTION

The fungal pathogen, *Alternaria alternata* f. sp. *lycopersici* has been shown to cause a stem canker disease on tomato[1]. Two fractions have been isolated from cell-free culture filtrates of the fungus which are phytotoxic and reproduce the macroscopic disease symptoms only on pathogen-susceptible tomatoes in concentrations less than 10 ng/ml. Such toxins are termed "host-selective" and are of interest as primary determinants of disease. One of these fractions (TA), characterized by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR), is reported to consist of two esters (at C-13 and C-14) of propane-1,2,3-tricarboxylic acid and 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol[2]. Preliminary evidence indicates that the second fraction (TB) also consists of two components with the same carbon skeleton as TA but which lack the C-5 hydroxyl and differ in stereochemistry at one or more of the chiral centers from C-11 to C-15[2].

Precise definition of the role of these host-selective toxins as the molecular basis of disease stress requires a quantitative procedure to detect the phytotoxic components in culture filtrates of the pathogen and diseased tomato tissue with concom-

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itant recovery of biological activity. Preparation of a toxin chromophore is required because of the lack of significant absorbance by the native toxins at wavelengths above 210 nm.

Maleic anhydride has been used for reversible modification of amino groups in proteins and peptides for enzymatic degradation, binding studies, and to increase peptide solubility[3–5]. This report describes the use of maleic anhydride as a fully reversible derivatizing reagent for aliphatic amines, and for quantitative detection of the aforementioned phytotoxins.

EXPERIMENTAL

Apparatus

The liquid chromatography system consisted of a Varian (Palo Alto, CA, U.S.A.) Model 5060 liquid chromatograph with ternary solvent delivery coupled to a Valco air-actuated injection valve fitted with a 10-μl sample loop. Column effluent was passed through a Varian UV-50 variable-wavelength detector with a 7.9-μl flow cell, connected to a Varian Model 9176 strip chart recorder. A Micro Pak MCH-10 column (30 cm × 4 mm I.D.), consisting of a monomeric C_{18} bonded onto 10-μm silica gel, with *ca.* 6000 theoretical plates was used for the reversed-phase separations. A guard column with disposable cartridges packed with the same material as the analytical column was installed between the injection valve and the analytical column.

Reagents and solvents

All reagents were analytical reagent grade. Maleic anhydride was recrystallized twice from chloroform before use. Methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and had a UV cut-off of 203 nm. Water was obtained from a Millipore (Bedford, MA, U.S.A.) Milli Q water purification system. The mobile-phase buffer was 0.05 *M* potassium phosphate adjusted to pH 3.5 or 2.7 with 0.1 *M* HCl by measurement with a pH meter, and filtered through a Gelman (Ann Arbor, MI, U.S.A.) 0.2-μm filter before use. All mobile-phase solvents were degassed before use.

Toxin purification

Phytotoxins were isolated as previously described[1,2] from cell-free culture filtrates of *A. alternaria* f. sp. *lycopersici* by treatment with barium acetate to a final concentration of 0.4 *M*, centrifugation, butanol extraction of the supernatant, exchange into water and concentration on a rotovap at 37°C, followed by gel permeation chromatography on polyacrylamide Biogel P-2 (Bio-Rad Labs, Richmond, CA, U.S.A.), which partially separates TA and TB. TA- and TB-rich fractions were detected by separation on analytical thin-layer chromatography (TLC) plates, 0.25 mm silica gel without gypsum (Sybron/Brinkmann, Westbury, NY, U.S.A.) developed in ethyl acetate–glacial acetic acid–water (6:3:1), and subsequently sprayed with ninhydrin[6]. Semi-preparative TLC with the same solvent system was used to purify TA and TB, which were located on the plates by spraying with water, extracted from the silica gel with butanol, exchanged back into water on the rotovap with separation confirmed by additional TLC and then used as HPLC standards. Equivalent prepa-

rations were characterized by NMR and HRMS as mixtures of the two isomers of TA and TB respectively[2]. Biological activity (gene-selective phytotoxin activity) of both TA and TB was confirmed by bioassay on excised tomato leaves as previously described[1]. Toxin concentration was quantified on the basis of μmoles/ml of amino group by reaction with trinitrobenzenesulfonic acid (TNBS), measurement of absorption at 340 nm, and comparison with a standard curve prepared with trinitrophenol (TNP)–aspartic acid[7].

Maleylation

Solutions containing 0.1–2 μmole/ml of toxin were treated with maleic anhydride by a method similar to that used for chemical modification of amino acids in proteins and peptides[3,4]. A 1 *M* solution of maleic anhydride in dioxane was prepared immediately before use, and 10-μl aliquots were added to a solution containing toxin in 0.1 *M* sodium carbonate, pH 9.2, at room temperature. The progress of the reaction was monitored by loss of ninhydrin positive reaction through spot testing on a silica gel TLC plate[6]. The pH was maintained above 9.0 by dropwise addition of 0.1 *M* sodium hydroxide, and was more than 90% complete in less than 5 min. Alternatively, maleic anhydride crystals were added slowly over a period of 5 min to a solution containing toxin buffered at pH 9.2 with 0.1 *M* sodium carbonate. At least a 10:1 molar excess of maleic anhydride to toxin was used in both cases to ensure complete derivatization. The maleylation reaction is specific for amino groups under the conditions used, and more extensive treatment with maleic anhydride is required for maleylation of hydroxyl groups[3,4]. Before HPLC analysis, the maleylation mixtures were adjusted to pH 6–7 by addition of hydrochloric acid or dilution with the mobile-phase buffer. HPLC analysis confirmed that derivatized TA, TB, or a mixture of the two were stable for at least a month at pH 6–7 when stored at 4°C.

HPLC analysis

HPLC analysis was performed at 24–26°C and the detector wavelength was 250 nm. The molar extinction coefficient for the maleylamino group has been reported to be 3360 at 250 nm[4]. Fractions for bioassay or rechromatography were collected directly from the detector flow-cell exit line in correspondence with the detector response. The methanol was removed by evaporation under a stream of gaseous nitrogen, and the maleyl groups removed by incubation of an aqueous solution of maleylated toxin at pH 3.0–3.5 for 20–24 h at 37°C. Demaleylation also was achieved by incubation of maleylated toxin for 20–24 h at 37°C in the methanol–phosphate buffer mixture in which the peaks eluted from the column. The half-life for maleylated amines has been reported[3] to be 11 h at 37°C at pH 3.5.

RESULTS AND DISCUSSION

The choice of derivatizing reagent to permit efficient detection of the toxins was limited because of the need to study the biological activity of the phytotoxins after HPLC separation. The following were considered essential requirements: mild reaction conditions during derivatization with respect to temperature and pH in order to prevent cleavage of the ester bond in the toxin molecules; relatively inexpensive mobile-phase solvents which could easily be removed or which would not interfere

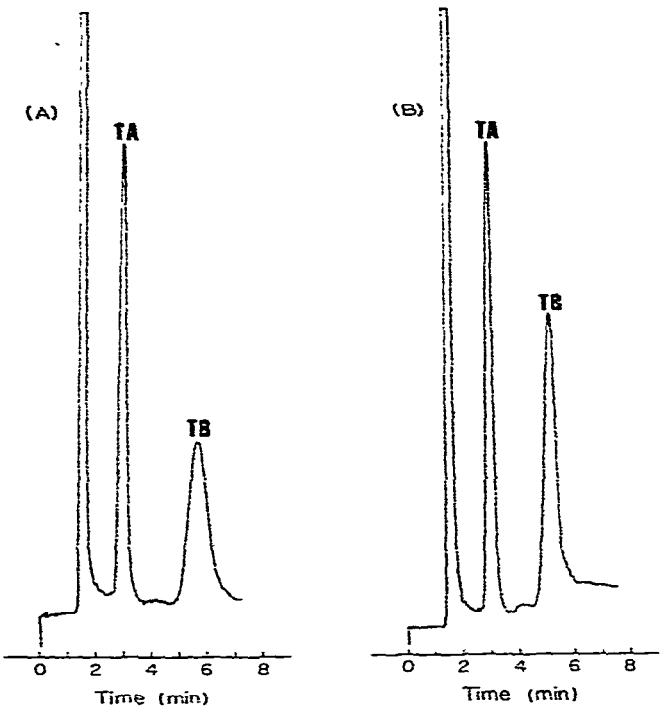

Fig. 1. HPLC separation of phytotoxic fractions TA and TB isolated from *A. alternata* f. sp. *lycopersici*. Conditions: mobile phase, (A) methanol–0.05 *M* potassium phosphate buffer (pH 3.5) (60:40) isocratic, (B) gradient (10 min) from 60:40 to 70:30 methanol–0.05 *M* potassium phosphate buffer pH 3.5; flow-rate 2 ml/min; UV detection at 250 nm, 0.05 a.u.f.s.; sample size 10 μl, 8 nmole of toxin injected.

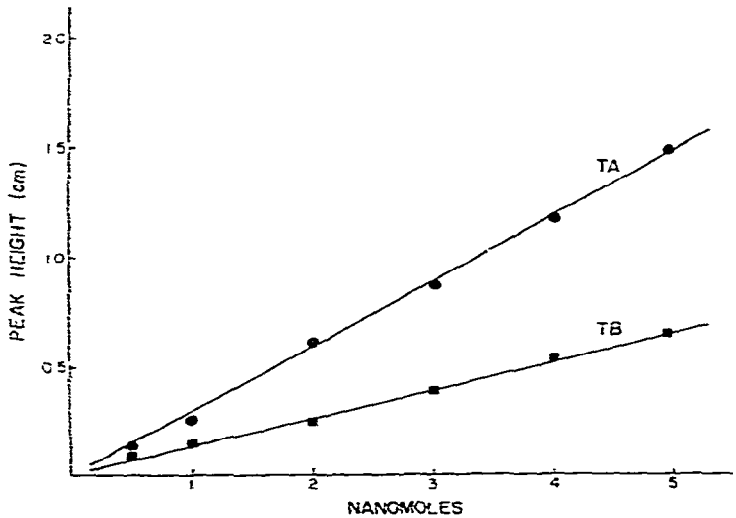

Fig. 2. Standard curves for amount (nmoles) of TA or TB maleylated and injected *vs.* peak height (measured peak height × attenuation factor). HPLC conditions were those described in Fig. 1B. The correlation coefficients for the curves are 0.999 (TA) and 0.998 (TB).

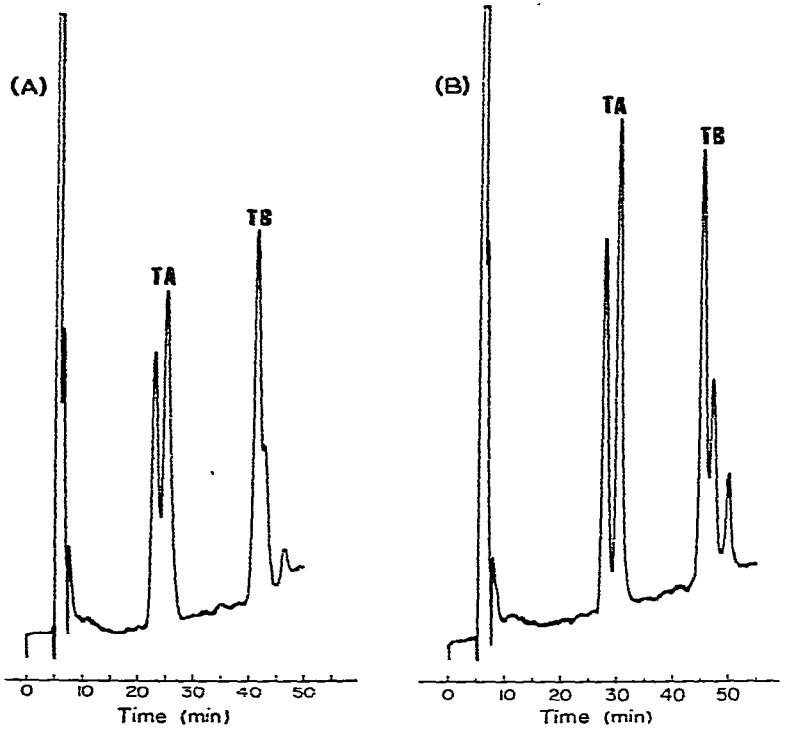

Fig. 3. HPLC of phytotoxins isolated from *A. alternata* f. sp. *lycopersici*, showing separation of TA and TB into their components. (A) Conditions: mobile phase, 60-min gradient from 50:50 to 70:30 methanol–potassium phosphate buffer (pH 3.5); flow-rate, 0.5 ml/min; UV detection at 250 nm, 0.05 a.u.f.s.; sample size 10 μl. Conditions for (B) were identical to those for (A), except that the potassium phosphate was buffered at pH 2.7. The sample was identical in both cases and contained 12 nmole of toxin. The small peak eluting after the TB peaks is a contaminant often found in the fungal extracts.

with biological assays; the ability to remove non-destructively the derivatizing group from the toxin molecules before biological assay. Maleic anhydride as a derivatizing reagent met all of these criteria since elevated temperatures were not required, the reaction took place in aqueous solution, and high pH was maintained for only a very short time. Excess maleic acid and dioxane in the reaction mixture eluted close to the solvent peak and did not interfere with chromatography of the phytotoxins. Toxin samples which were maleylated at pH 9.2 and then demaleylated at pH 3.0 as described above showed no detectable loss in biological activity.

Due to the presence of three carboxylic acid groups on the maleylated toxin molecules, ion suppression HPLC was achieved by including 0.05 *M* phosphate buffer with a pH of 2.7–3.5 in the mobile phase. The net charge on the maleylated toxin molecules under these conditions would be close to zero, for maximal selectivity in the reversed-phase mode during chromatography.

Baseline separation of maleylated TA and TB was achieved using an isocratic solvent system with 60% methanol–phosphate buffer (pH 3.5) as the mobile phase and a flow-rate of 2 ml/min (Fig. 1A). In contrast, Fig. 1B shows the effect on peak

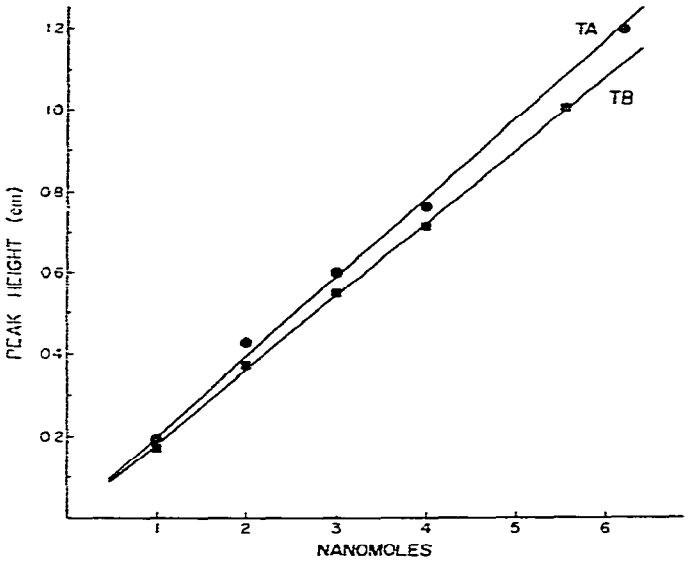

Fig. 4. Standard curves for amount (nmoles) of TA or TB maleylated and injected *vs.* peak height (measured peak height × attenuation factor). HPLC conditions were those described in Fig. 3A. The correlation coefficients for the curves are 0.999 (TA) and 0.999 (TB).

sharpening of a 60:40 to 70:30 (10 min) gradient of methanol–phosphate buffer (pH 3.5) with flow-rate of 2 ml/min.

Standard curves (Fig. 2) for quantitative analysis of the toxins were prepared by maleylation of known amounts of TA and TB, which were then analyzed by HPLC separately and together under the conditions described for Fig. 1B. Indistinguishable retention times and peak heights for TA and for TB were obtained when the toxins were maleylated and analyzed together or separately. These results show that both the recorder response and the maleylation reaction are linear within the limits of amounts of toxin used. The toxins were quantified on the basis of nanomoles of amino groups by reactions with TNBS as described in methods.

Maleylated TA and TB were further resolved into two components each with a 60-min gradient from 50:50 to 70:30 methanol–potassium phosphate buffer, (pH 3.5) and a flow-rate of 0.5 ml/min (Fig. 3A). Standard curves for this system at pH 3.5 were prepared as described above except that the sum of the peak heights of the two components of TA and TB, respectively, were plotted against the amount of TA or TB maleylated and injected (Fig. 4). The effect of the pH of the mobile-phase buffer on maleylated toxin separation was investigated by lowering the pH of the phosphate buffer to 2.7. A better resolution of the four toxin components with this solvent system was achieved at pH 2.7 (Fig. 3B). The ratio of components of TA and TB was constant at both pH 2.7 and 3.5 for a given sample, but varied in different fungal extracts. The peak areas for each of the toxin components were identical when the same sample was chromatographed at pH 2.7 or 3.5 (calculated as peak height times width at half height) although peak heights varied. Standard curves prepared with the pH 2.7 system were also linear.

The identities of the peaks corresponding to TA and TB for both gradient

systems were confirmed by spiking extracts with purified TA and TB as well as by collection of the HPLC eluate fractions corresponding to the TA and TB peaks from several HPLC runs, de-maleylation of the toxin as described above, concentration, and TLC analysis. In all cases the HPLC analysis indicated that the toxin peaks initially ascribed to TA and TB were duplicated with spiking and rechromatography by HPLC. The TLC R_F values of the demaleylated toxins agreed with those for authentic TA and TB (0.32 and 0.44, respectively), and no other ninhydrin positive compounds were detected. Following demaleylation, biological activity was detected in fractions corresponding to TA and TB. The relative standard deviations were typically less than 0.7% for retention times and 2–4% for peak heights.

The HPLC procedure described in this report has been useful both as an analytical method for detection and quantification of toxins in fungal extracts and infected plant tissue and as a semi-preparative method for TA and TB and their two components, since the use of a reversible derivatizing reagent allows us to obtain small amounts of the phytotoxins in a purified state for further chemical and biological studies.

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