Isolation of Altenuisol and Altertoxins I and II, Minor Mycotoxins Elaborated by *Alternaria*

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ABSTRACT

A method for the isolation of milligram quantities of 3 minor *Alternaria* mycotoxins, altenuisol (AS), altertoxin I (AT-I) and altertoxin II (AT-II) was developed. Crude toxin preparation was first subjected to a preparative high pressure liquid chromatography step using PrepLC/System 500 and a silica gel column. The 3 minor toxins were eluted from the column with 25% ethyl acetate. Further purification of these toxins was achieved by passing the partially purified toxins through a Sephadex LH-20 column (2.5 X 25 cm) twice. In a solvent system of hexane/methylene chloride/methanol (1:1:1, v/v/v), AT-I was eluted from the Sephadex column first, followed by AT-II and AS. The distribution of various *Alternaria* mycotoxins in different fractions obtained from the Sephadex step and some of the physicochemical properties of AT-I and AT-II are described.

Among 7 mycotoxins elaborated by *Alternaria* spp., altenuisol (AS), altertoxin I (AT-I) and altertoxin II (AT-II) amount to less than 0.1% of the crude extract compared to the major mycotoxins alternariol (AOH), alternariol methyl ether (AME) and tenuozonic acid (TA) which are usually in the 5-20% range (1). Because these toxins are produced in such small quantities by most *A. alternata* isolates, and because they are not well separated in most chromatographic systems, purification of these minor toxins has been extremely difficult. In earlier studies, small amounts of these toxins were obtained by repeated chromatography on silica gel (1,2) or by a combination of silica gel chromatography and preparative thin layer chromatography (TLC) (3).

Due to the limited availability of these toxins, their biological and toxicological properties have not been well characterized. Furthermore, the structures of AT-I and AT-II are unknown at present. Although the impact of these compounds on human and animal health is unknown, they have all been found to be toxic to bacterial and HeLa cell cultures (4). More recently, AT-II has been found to be mutagenic in *Salmonella* systems (3). Therefore, it is necessary to develop a more effective method to isolate large quantities of these toxins for subsequent studies. In this study, an efficient method involving the combination of preparative or semipreparative high pressure liquid chromatography (HPLC) and Sephadex LH-20 gel filtration was developed for isolation of these 3 compounds. Details of this method are presented in this paper.

EXPERIMENTAL

Preparation of Partially Purified AS, AT-I and AT-II

Partially purified AS, AT-I and AT-II can be prepared by several approaches, including adsorption chromatography on Silica Gel G (1,4) and the preparative or semipreparative method developed in our laboratory (5). For the present study, we selected the preparative HPLC method which involves the chromatography of 15-30 g of crude *Alternaria* toxin extracts on 1 or 2 PrepPak-500 silica gel cartridges in a Waters' PrepLC/System 500 preparative HPLC (Waters/Associates Inc., Milford, MA). After elution of AME from the column with 10% ethyl acetate (EtOAc) in hexane, the column was developed with 25% EtOAc in hexane. Alternariol was then eluted, followed by AT-II, AT-I and AS in that order. Careful pooling of these fractions resulted in 3 pooled fractions rich in AT-II, AT-II + AT-I, and AT-I + AS, respectively. Because large amounts of AOH were present in the crude extract, these fractions were always contaminated with AOH, as well as other impurities. Details of the preparation of these partially purified altertoxins and altenuisol are described elsewhere (5).

Preliminary Sephadex LH-20 Gel Filtration

Preliminary treatment of the samples obtained from the preparative HPLC was done in a short, small column (2.5 X 25 cm) packed with Sephadex LH-20 (Pharmacia Fine Chemicals, Pharmacia, Inc., Piscataway, NJ). The Sephadex column was equilibrated with hexane/methylene chloride/methanol (1:1:1, v/v/v; solvent system I) before use. This solvent system was also used in the subsequent elution. Generally, the concentrated samples from the different HPLC fractions were dissolved in 2 mL of methanol and then applied to the column. The column was eluted with solvent system I until all the AOH was removed. A final methanol (150 mL) rinse was used to remove any AOH left in the column. For the next use, the column was reequilibrated with ca. 150 mL of solvent system I. Fractions of 4 mL each were collected and analyzed by TLC. The fractions containing AT-I, AT-II and AS were pooled separately.

Final Sephadex LH-20 Gel Filtration

Final preparation of the minor toxins was done in a Sephadex LH-20 column under the conditions just described except that a larger column (3.5 X 37 cm) was used. Fractions containing the partially purified AS, AT-I or AT-II from the first Sephadex LH-20 step were dissolved in 5 mL of methanol and subjected to the gel filtration separately. After chromatography, fractions containing pure AS, AT-I or AT-II were pooled and evaporated to dryness; they were then redissolved in a small amount of ethyl acetate and crystallized in an ethyl acetate/hexane mixture. Fractions containing partially purified toxins were saved for further Sephadex LH-20 chromatography.

Analyses

Fractions obtained from the Sephadex column were analyzed for individual mycotoxins by TLC using Silica Gel GF plate (250 μ; Analtech, Inc., Newark, DE) and developed with toluene/ethyl acetate/formic acid (5:4:1) according to Seitz et al. (6). The final preparations were analyzed by both TLC and an HPLC method described elsewhere (5). Ultraviolet (UV) spectra were determined in a Beckman Model 25 spectrophotometer with a 1-cm light path. Mass spectra were obtained on a mass spectrometer (Hewlett-Packard Model 5980, Palo Alto, CA).

RESULTS AND DISCUSSION

The results of a typical experiment for the separation of a fraction containing partially purified AT-II in the small Sephadex LH-20 column are shown in Figure 1. Altertoxins were concentrated between tubes 23 and 29 whereas other

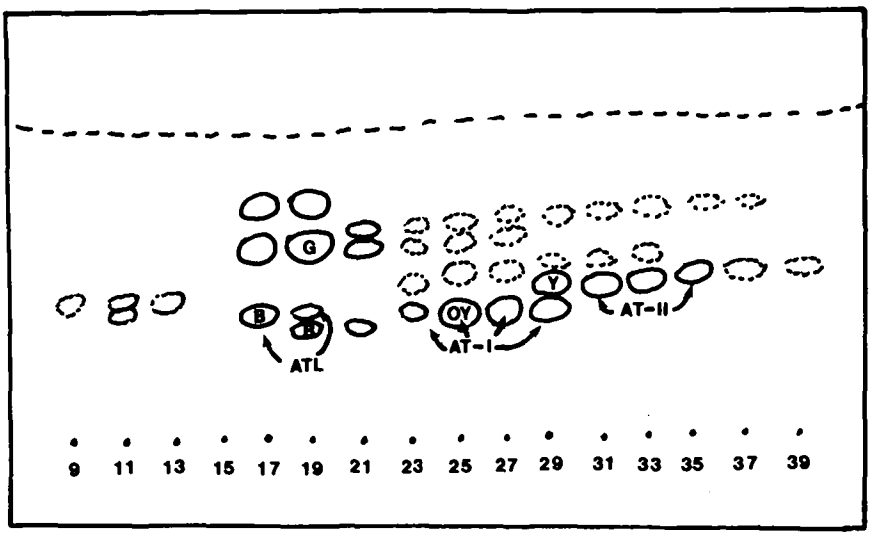

FIG. 1. Thin layer chromatography of minor alternaria mycotoxins obtained from different fractions after Sephadex LH-20 gel filtration. The abbreviations B, G, O and Y represent blue, green, orange and yellow fluorescent spots under long wavelength UV, respectively. The dotted line around the spot indicates a weak fluorescent spot or minor component. The starting materials of this experiment were obtained from a preparative HPLC experiment and contained primarily AT-II. The numbers shown at the bottom of the figure are tube numbers. Each tube contained 4 mL of sample from which 5 μL were applied to the TLC plate. Before fraction 9 (1-8) a total 45-mL of sample was collected.

toxins and metabolites were found in other fractions. Similar results were obtained when fractions containing partially purified AT-I or AS were applied to the column. Altenuisol eluted from the column later than AT-I and AT-II. The distribution of various *Alternaria* toxins in the pooled fractions obtained in a number of experiments are summarized in Table I. The overall results indicate that, although complete separation of AT-I, AT-II and AS was not possible, fractions rich in one or another of these toxins were obtained. In addition, most pigments and contaminants were removed after this simple and quick chromatographic step.

Further purification of these 3 minor *Alternaria* toxins was achieved when the fractions obtained from the last step were passed through the larger Sephadex LH-20 column. The results shown in Table II indicate that, while the elution sequence of different *Alternaria* toxins in the large Sephadex column was qualitatively similar to that of the short column, the resolution for AT-I, AT-II and AS was much better. Various minor *Alternaria* toxins were concentrated in certain defined fractions. Highly purified AT-I, AT-II and AS were obtained when materials from the last step were subjected to a second Sephadex LH-20 gel filtration. For example, as much as 200 mg of pure AT-I was obtained by this method in one experiment. The materials prepared in this way were shown to be pure as analyzed by TLC and HPLC. In some instances, small amounts of one of the other toxins was present; however, rechromatography of those preparations in the large Sephadex column always improved the purity of the products. Due to the capacity of the Sephadex LH-20 column, as well as its inability to separate AME and ALT, it cannot be used in the preparation of the major *Alternaria* toxins unless a pretreatment such as silica gel chromatography (1) or HPLC separation (5) is used.

Although the large Sephadex LH-20 column is an effective method to separate AT-I, AT-II and AS, the small Sephadex column chromatography is an essential step to achieve quick removal of AOH and some pigments which have very high affinity for Sephadex LH-20 gel. If the partially purified samples were directly applied to the large Sephadex LH-20 column, large amounts of AOH and pigments would be absorbed on the gel and a subsequent prolonged methanol wash would be necessary. Separation on the short column to remove the pigments and AOH eliminates such problems and saves considerable time and solvents.

Other methods, such as rechromatography of the partially purified toxins under the same conditions used in the preparative or semipreparative HPLC, have been tested in

TABLE I

Distribution of Different *Alternaria* Mycotoxins in Fractions Obtained from Short Sephadex LH-20 Columns

Fraction number	Elution volume (mL)	*Alternaria* mycotoxins
A	0- 49	Nothing
B	50- 72	Contaminated pigments (deep brown)
C	73- 81	Metabolites not identified, if present
D	82-105	AME, AT and contaminants, if present
E	106-129	AT-I and AT-II
F	130-160	AT-II (minor), AS (major) and AOH (minor)
G	161-250	AOH (major) and AS (minor)

TABLE II

Distribution of Different *Alternaria* Mycotoxins in Different Fractions Obtained from Large Sephadex LH-20 Columns

Fraction no.	Elution volume	*Alternaria* mycotoxins
A	0-160	–
B	161-195	Blue fluorescent spot[a]
C	196-272	–[b]
D	273-328	Yellow fluorescent spot[a]
E	329-419	AT-I (major)
F	420-503	AT-II and AS (minor)
G	504-594	AS (major) and AOH (minor)
H	595-850	AOH (major) and AS (minor)

[a]The substance was not characterized.

[b]AME and ATL are usually present in this fraction if the partially purified starting materials containing these toxins are used.

our laboratory. However, complete separation was not achieved and a final Sephadex gel filtration was still necessary.

The availability of the purified AT-I, AT-II and AS permitted study of the physicochemical properties of these toxins. When analyzed by TLC in the solvent system we described, AS, AT-II and AT-I have R_f values of 0.5, 0.48 and 0.43, respectively, compared to AME, AOH and AT which have R_f values of 0.74, 0.54 and 0.33. Under long wavelength UV light, AT-I fluoresces a bright yellow and AT-II an orange yellow. Altenuisol gave a blue fluorescent spot that faded after 10 min. Because the R_f values of AOH and AS are very close and both fluoresce blue, this characteristic could be used to distinguish these 2 toxins.

Mass spectral analysis revealed that AS has a distinct parent molecular ion peak at m/e (relative intensity) of 274 (100) with an empirical formula $C_{14}H_{10}O_6$. Other mass fragments for AS were found at m/e 258 (5.9), 245 (11.7), 205 (26.4), and 177 (20.0). Altertoxin-I has a parent molecular ion peak at 352 and other major fragments at m/e 334, 316, 308, 280, 262 and 238. For AT-II, the parent molecular ion peak was seen at 350 and other major fragments were found at 332, 314 and 288. The empirical formulas for AT-I and AT-II were found to be $C_{20}H_{16}O_6$ and $C_{20}H_{14}O_6$, respectively. Single ion monitoring at m/e 352 for AT-I over a period of 10 min revealed that this compound may have 3 isomers existing in a ratio of 3:2:1. Spectrophotometric analysis showed that AT-I absorbed maximally at 260 nm ($\epsilon = 2.67 \times 10^4$), 289 nm ($\epsilon = 1.88 \times 10^4$), and 364 nm ($\epsilon = 4.45 \times 10^3$). Altertoxin-II absorbed maximally at 257 nm ($\epsilon = 2.36 \times 10^4$), 285 nm ($\epsilon = 1.14 \times 10^4$), and 352 nm ($\epsilon = 3.87 \times 10^3$). The minimal absorptions were found at 278 nm ($\epsilon = 1.72 \times 10^4$) and 321 nm ($\epsilon = 1.51 \times 10^3$) for AT-I and 278 nm ($\epsilon = 1.03 \times 10^4$), 313 nm ($\epsilon = 2.25 \times 10^3$) for AT-II. These properties of the isolated products are similar to those reported in the literature (1-4).

Some functional group tests for AT-I and AT-II were done by spraying the reagents on the TLC plates. Both toxins gave purple-green spots after being sprayed with saturated ferric chloride in methanol. Both toxins also gave reddish-brown spots after being sprayed with 0.4% 2,4-dinitrophenyl chloride in ethanol. No reaction was observed after spraying with 0.04% bromocresol purple in 50% ethanol. These results suggest that altertoxins contain phenolic and carbonyl functions but contain no carboxylic group.

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