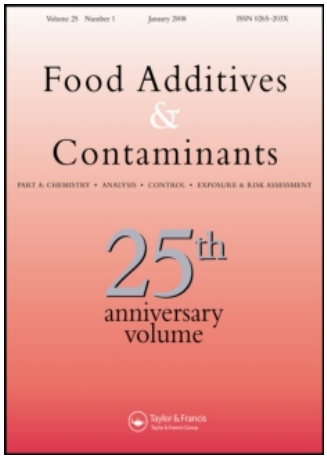

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Suppression of ochratoxin biosynthesis by naturally occurring alkaloids

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Suppression of ochratoxin biosynthesis by naturally occurring alkaloids

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Abstract

The effects of four alkaloids on the biosynthesis of ochratoxin A (OTA), ochratoxin B (OTB) and citrinin were examined on four OTA-producing aspergilli: *Aspergillus auricomus*, *A. sclerotiorum* and two isolates of *A. alliaceus*. Piperine and piperlongumine, natural alkaloids of *Piper longum*, significantly inhibited OTA production at 0.001% (w/v) for all aspergilli examined. Piperine and piperlongumine affected the polyketide synthesis step of OTA production and inhibited production of citrinin. Curcumin, a constituent of tumeric, completely inhibited mycelial growth of *A. alliaceus* isolate 791 at 0.1% (w/v) and decreased OTA production by ~70% at 0.01% (w/v). Sesamin, a constituent of sesame oil, inhibited OTA and OTB production by 60 and 45%, respectively, at 0.1% (w/v), showing its effect was on chloroperoxidase and polyketide synthase activity. The potential advantage of these natural products to reduce ochratoxin contamination of agricultural commodities is discussed.

Keywords: *Curcumin, sesamin, piperine, piperlongumine, ochratoxin A, ochratoxin B*

Introduction

Ochratoxin A (OTA) is a nephrotoxic and carcinogenic mycotoxin produced by certain species of *Aspergillus* and *Penicillium* (Wei et al. 1989; Harris and Mantle 2001). This mycotoxin can contaminate agricultural products, including cereals, coffee, dried fruits, wine and pork. Action threshold levels of OTA in agricultural foods are strictly regulated (van Egmond and Dekker 1997; Codex Alimentarius Commission 1998). These threshold levels significantly affect profitability in both domestic and foreign markets. The US Food and Drug Administration (FDA) has set guideline threshold levels for OTA in foods. For domestic consumption, these levels are $5\,\mu g\,kg^{-1}$ (5 ppb) for raw cereal grains, $3\,\mu g\,kg^{-1}$ for products derived from cereals and $10\,\mu g\,kg^{-1}$ for dried vine fruits, such as raisins or figs (van Egmond and Dekker 1997). The European Union has set OTA detection limits lower than $5\,\mu g\,kg^{-1}$ for all commodities, making OTA contamination a major export–import issue for a number of agricultural products (Codex Alimentarius Commission 1998).

OTA is a chlorinated dihydroisocoumarin covalently bound to l-phenylalanine via an amide bond. The steps in the biosynthesis of OTA have been studied using radioactively labelled precursors (Ferreira and Pitout 1969; de Jesus et al. 1980; Abell et al. 1982, 1983; Varga et al. 2001). In summary, these studies show the first steps involve the pentaketide pathway and chloroperoxidase to form ochratoxin alpha (Otα) from a putative intermediate, 7-methoxymellein, not isolated to date. Methoxymellein is converted to OTA by OTA synthetase. Based on a study using *A. ochraceus*, mellein (ochracin), a well-known precursor of OTA, is not always a required intermediate in OTA

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biosynthesis (Harris and Mantle 2001). This same study demonstrated that chlorination of OTα preceded biotransformation of OTα to OTA, indicating chlorination is a penultimate biosynthetic step in OTA biosynthesis (Harris and Mantle 2001). Ochratoxin (OTB), the dechloro-analogue of OTA, may be produced by the same biosynthetic pathways without addition of chlorination reaction by chloroperoxidase.

The epidemiological and biological mechanisms through which OTA exerts its toxicity have been extensively reviewed (Marquardt and Frohlich 1992). Toxicity of OTA has mainly been studied with rat models. Such studies reveal OTA inhibits mitochondrial succinate dehydrogenase and cytochrome *c* oxidase (Meisner and Chan 1974), modifies mitochondrial membrane transport and inner membrane ATPase (Meisner and Chan 1974), and inhibits enzymes involved in phenylalanine metabolism (Creppy et al. 1990). It has also been proposed that OTA competitively inhibits phenylalanyl-tRNA synthase (Creppy et al. 1983). Neurotoxic effects of OTA and its derivatives, such as OTα and mellein, have also been documented (Bruinink et al. 1998).

Wide commercial use of fungicides to control mycotoxin-producing fungi in agricultural commodities is not cost effective nor is it environmentally acceptable (Munimbazi et al. 1997). In view of the impracticality of fungicides and low action threshold levels for OTA in foods, an effective method to reduce or eliminate OTA contamination during pre- or post-harvest processing of agricultural products is needed. In a search for alternate approaches, a number of studies have focused on identifying natural products as inhibitors of mycotoxin biosynthesis, with only one reporting natural spices as potential inhibitors of OTA biosynthesis (Basilico and Basilico 1999).

In this report, we identify several natural products from edible sources that inhibit OTA biosynthesis. These products are the natural alkaloids, piperine and piperlongumine from *Piper longum*, sesamin from sesame oil and curcumin from tumeric. Additionally, the effects of piperine and piperlongumine on production of intermediates in the OTA biosynthethic pathway were examined to gain insight on their mode(s) of action.

A. alliaceus has recently been shown to be an important ochratoxin-producing species in California and may produce higher levels of ochratoxin than the more widely recognized *A. ochraceus* (Bayman et al. 2002). *Aspergillus sclerotiorum* and *A. auricomus* have been found on tree nuts and figs in California (Bayman et al. 2002), and can also produce OTA (Ciegler 1972; Tsubouchi et al. 1985). Since these species may play a greater role than *A. ochraceus* in ochratoxin contamination of foods in North America and, since production of OTB and citrinin has not been reported for these species, we selected these taxa for our study.

Materials and methods

Molds and media

Three ochratoxin-producing species were used: *Aspergillus alliaceus* (isolate 791, originally isolated from a California fig, and isolate 7–1, from soil in a Georgia peanut field), *A. auricomus* (isolate C18, from green Brazilian coffee beans) and *A. sclerotiorum* (isolate 01-4, from green Costa Rican coffee beans).

Fungi were cultured in liquid yeast extract–sucrose medium (YES; 15% sucrose, 2% Difco yeast extract), which supports high levels of ochratoxin production (Varga et al. 1996; Bayman et al. 2002). To test inhibitory effects on OTA production, ground, dried infructescences (fruit) of *P. longum* and natural products (piperlongumine, piperine, sesamin and curcumin) were added to media at concentrations ranging from 0.001 to 0.1%. Ground *P. longum* was added to media prior to autoclaving. Natural products were dissolved in acetone and added to media after autoclaving. Control cultures received acetone only. All cultures were grown in 50 ml of media in 125–250 ml flasks. Flasks were inoculated with 0.1 g of macerated agar culture of the respective fungus. After inoculation, flasks were incubated for 10 days in darkness at 28°C without agitation. Treated and control cultures were replicated at least three times. After incubation, the contents of each flask were quantitatively analysed for citrinin, OTA and ochratoxin B (OTB), as described below.

Chemicals

Whole dried infructescences of *P. longum* were purchased from Kyoung-dong traditional market, Seoul, South Korea. Curcumin, piperine and piperlongumine were purchased from Sigma (St. Louis, MO, USA). Sesamin was isolated and purified from sesame oil according to defined protocols (Fukuda et al. 1986). Purity of sesamin was determined by comparison of mass spectra to spectra supplied by Professor N. G. Lewis, Washington State University (Pullman, WA, USA). Mycotoxins, citrinin, OTB and OTA were also purchased from Sigma.

Mycotoxin analysis

Three ml of medium from each flask were extracted with 3 ml chloroform. The chloroform fraction was removed and evaporated under nitrogen and the

residue redissolved in methanol. Mycelium remaining in the aqueous fraction was extracted with 50 ml methanol and sonicated for 20 min. Samples were quantitatively analysed for mycotoxins by reverse-phase HPLC using a Hewlett-Packard (CA, USA) 1100 HPLC work station. Analysis consisted of isocratic elution with methanol/water/acetic acid (60:50:2, v/v) at 1.0 ml min^{-1} through a Supelco LC-18 column (4.6 × 250 mm, 5 μm I.D.; PA, USA). Mycotoxins were detected by fluorescence with excitation at 280 nm and emission at 454 nm. In this system, citrinin, OTB and OTA had retention times of 12.2, 12.8 and 26.7 min, respectively. Statistical differences of mycotoxin levels among cultures were tested for significance by ANOVA, with significant differences set at $P < 0.05$ (SAS 1995).

Determination of fungal growth

After methanol extraction (see above), the remaining mycelial mats were collected on dried, preweighed Whatman no. 1 filter paper. The filter paper was then washed with distilled water, dried at 80°C overnight and weighed.

Results

Dried fruits of *P. longum* were potent inhibitors of citrinin, OTA and OTB biosynthesis in *A. alliaceus* (Table I). Inclusion of *P. longum* at 0.1% reduced production of OTA, OTB and citrinin by 73.7, 36.0 and 54.8%, respectively, compared to controls. However, fungal growth was not significantly affected by incorporation of fruit at 0.1% (w/w) into the medium. At 0.5% *P. longum*, production of OTA and citrinin was reduced by 84.8 and 92.8%, respectively, compared to controls. At 0.5%, fungal growth was also significantly reduced by 26.4% compared to controls. However, OTB production was not significantly different between the 0.1 and 0.5% treatments.

The effects of a 0.1 and 0.01% treatment of piperine and piperlongumine, sesamin and curcumin on mycelial growth and mycotoxin production in *A. alliaceus* are shown in Table II. At 0.1%, all compounds tested significantly reduced fungal growth and production of mycotoxins, with the exception of sesamin on production of citrinin. Piperlongumine practically eliminated and piperine significantly reduced production of all three mycotoxins. Sesamin significantly inhibited production of OTA and OTB, but not citrinin. At 0.1%, curcumin completely inhibited fungal growth and, hence, production of any mycotoxins. When tested at 0.01%, piperlongumine showed greater inhibitory activity than curcumin or piperine, though the difference was not significant for OTB. Since sesamin was the least inhibitory of the alkaloids at the higher treatment level of 0.1%, it was not tested at 0.01%. A notable result was the effect of the 0.01% treatment of curcumin, where fungal growth was inhibited by 50% but OTB production actually increased almost three-fold over controls.

Effects of alkaloids of *P. longum* were tested at 0.001% on three fungal isolates in addition to *A. alliaceus* (791) (Table III). Piperine and piperlongumine significantly inhibited growth of *A. alliaceus* 791 and 7–1 and *A. sclerotiorum*, but not of *A. auricomus*. The effects of piperlongumine and piperine on OTA production varied considerably among fungi. Piperlongumine decreased OTA production by about 50% in the two strains of *A. alliaceus* but by almost 66% in *A. auricomus* and *A. sclerotiorum*. However, piperine decreased OTA production by 70 and 80% in *A. sclerotiorum* and *A. alliaceus* (791), respectively, but only by 40% in *A. alliaceus* (7–1). Piperine had no significant effect on OTA production by *A. auricomus*.

The effects of 0.001% piperine and piperlongumine on production of the OTA biosynthetic intermediates, OTB and citrinin, were examined to gain insight as to possible modes of action (Table III). Both compounds significantly decreased OTB production in *A. alliaceus* (791) and *A. sclerotiorum*; only piperlongumine decreased OTB production in *A. auricomus*. Conversely, piperine appeared to promote production of OTB

Table I. Effect of dried fruit of long pepper, *Piper longum* L., on mycelial growth and production of ochratoxin A (OTA), ochratoxin B (OTB) and citrinin by *Aspergillus alliaceus* (791).

		Mycotoxin production (μg per 50 ml media)		
Treatment	Mycelial weight (g)	OTA	OTB	Citrinin
Control	1.82 ± 0.04^{a}	3855.0 ± 481.8^{a}	21.1 ± 1.03^{a}	1051.0 ± 49.8^{a}
0.1% *Piper longum*	1.81 ± 0.04^{a}	1007.0 ± 58.9^{b}	13.5 ± 0.51^{b}	475.0 ± 17.6^{b}
0.5% *Piper longum*	1.34 ± 0.05^{b}	587.4 ± 38.6^{c}	10.7 ± 0.50^{b}	75.6 ± 8.9^{c}

Values followed by different superscript letters within a column are significantly different ($P < 0.05$). Numbers are means ± one standard error ($n = 4$).

Table II. Effects of acetone (control), piperine, piperlongumine, sesamin and curcumin at 0.1 and 0.01% on fungal growth and production of mycotoxins, ochratoxin A (OTA), ochratoxin B (OTB) and citrinin, by *Aspergillus alliaceus* (791).

			Mycotoxin production (μg per 50 ml media)					
	Mycelial weight (g)		OTA		OTB		Citrinin	
Treatment	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%
Acetone	0.90 ± 0.12^{a}	1.26 ± 0.20^{a}	10850 ± 4490^{a}	15385 ± 4285^{a}	29.00 ± 10.8^{a}	22.6 ± 5.43^{b}	1687.0 ± 167.9^{a}	2197 ± 193.7^{a}
Piperine	0.50 ± 0.16^{b}	0.88 ± 0.08^{b}	1930 ± 325.5^{b}	2805 ± 374.9^{b}	5.21 ± 0.20^{c}	14.2 ± 3.04^{c}	346.7 ± 101.3b	1113 ± 93.1^{b}
Piperlongumine	0.51 ± 0.06^{b}	0.76 ± 0.10^{bc}	5.6 ± 0.7^{c}	549.7 ± 294.6^{c}	0^{d}	13.6 ± 1.60^{c}	ND^{c}	37.3 ± 22.8^{d}
Sesamin	0.66 ± 0.15^{b}	NT	4473 ± 752.7^{b}	NT	16.10 ± 1.85^{b}	NT	1460.0 ± 330.1^{a}	NT
Curcumin	ND^{c}	0.58 ± 0.18^{c}	ND^{d}	4313 ± 616.7^{b}	0^{d}	73.4 ± 1.68^{a}	ND^{c}	241 ± 117.4^{c}

Values followed by different superscript letters within a column are significantly different ($P < 0.05$). Numbers are means ± one standard error (0.1%, $n = 4$; 0.01%, $n = 3$). ND = not detected; NT = not tested.

Table III. Effect of treatment with acetone (control), 0.001% piperine and 0.001% piperlongumine on mycelial growth and production of mycotoxins, ochratoxin A (OTA), ochratoxin B (OTB) and citrinin, by four ochratoxin-producing aspergilli.

			Mycotoxin production (μg per 50 ml media)		
Aspergillus isolate	Treatment	Mycelial weight (g)	OTA	OTB	Citrinin
A. alliaceus (791)	Acetone	0.86 ± 0.032^{a}	4681 ± 257^{a}	56.4 ± 2.4^{a}	1274 ± 46.12^{a}
	Piperine	0.60 ± 0.038^{c}	928 ± 50.6^{b}	12.4 ± 0.6^{bc}	480.7 ± 41.08^{b}
	Piperlongumine	0.74 ± 0.095^{b}	2079 ± 64.0^{b}	34.3 ± 1.8^{b}	1085 ± 28.00^{a}
A. alliaceus (7–1)	Acetone	1.03 ± 0.025^{a}	2370 ± 27.9^{a}	16.9 ± 0.77^{a}	596.4 ± 28.20^{a}
	Piperine	0.75 ± 0.023^{b}	1556 ± 63.6^{b}	23.3 ± 0.68^{b}	512.9 ± 14.00^{a}
	Piperlongumine	0.55 ± 0.023^{c}	1314 ± 62.3^{c}	21.0 ± 0.50^{ab}	447.0 ± 12.08^{a}
A. auricomus	Acetone	0.54 ± 0.052^{a}	305.1 ± 17.5^{a}	100.6 ± 4.62^{a}	ND
	Piperine	0.73 ± 0.072^{a}	407.0 ± 40.9^{a}	78.3 ± 11.45^{a}	ND
	Piperlongumine	0.67 ± 0.065^{a}	96.1 ± 8.2^{b}	34.4 ± 4.15^{b}	ND
A. sclerotiorum	Acetone	0.79 ± 0.073^{a}	2885 ± 271.3^{a}	643.6 ± 31.2^{a}	ND
	Piperine	0.46 ± 0.060^{b}	826.7 ± 54.6^{b}	247.4 ± 34.4^{c}	ND
	Piperlongumine	0.55 ± 0.073^{b}	1057 ± 77.6^{c}	262.4 ± 34.9^{c}	ND

Values within the same *Aspergillus* isolate followed by different superscript letters are significantly different ($P < 0.05$). Numbers are means ± one standard error ($n = 6$). ND means not detected.

in *A. alliaceus* (7–1). Piperine significantly inhibited production of citrinin only in *A. alliaceus* 791. Piperlongumine, on the other hand, did not significantly inhibit production of citrinin in either of the two strains of *A. alliaceus*. *Aspergillus auricomus* and *A. sclerotiorum*, although capable of synthesizing OTA, did not produce citrinin.

Discussion

Fruits of some species of *Piper* are the source of black and white table-pepper. Some fruits also possess insecticidal and antifungal properties (Miyakado et al. 1979; Su and Horvat 1981; Tyagi et al. 1993). Among natural products identified in these fruits, unsaturated amides constitute the major group. These amides are toxic to houseflies (*Musca domestica* L.), rice weevils (*Sitophilus oryzae* L.) and cowpea weevils (*Callosobruchus maculatus* F.) (Su 1977; Su and Horvat 1981). There was 100% mortality of adult *Sitophilus zeamais* within 6 days of applying ground black-pepper at 600 mg kg^{-1} to maize (Javier and Morallo-Rejesus 1986). A crude ethanol extract of fruits of *P. nigrum*, applied at 250 mg kg^{-1}, caused 98% adult mortality of rice weevils within 6 days after treatment (Javier and Morallo-Rejesus 1986). The essential oil of pepper admixed with wheat at 200 mg kg^{-1} caused 100% mortality in adult *S. oryzae* and *Ryzopertha dominica* (F.), a beetle which is also a storage pest of grains (Sighamony et al. 1986). Other insecticidal amides, such as pipericide, (*E*,*E*)-*N*-(2-methylpropyl)-2,4, 12-tridecadienamide and (*E*,*E*,*E*)–11-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)-2,4,10-undecatrien-amide, have also been isolated from *P. nigrum* (Miyakado et al.1979; Su and Horvat 1981).

Fruits of black pepper are also known to inhibit growth of *A. flavus* (Bartine and Tantaouri-Elaraki 1997). Ground seeds of black pepper incorporated at 10% (w/v) into a media of rice powder or corn steep liquor prevented production of aflatoxin B_1 by *A. flavus* after an incubation period of 6 days (Mabrouk and El-Shayeb 1980). Piperine was also shown to inhibit aflatoxin B_1 production (Madhyastha and Bhat 1984). Piperlongumine, pipernonaline and piperoctadecalidine were previously isolated from *P. longum* and *P. retrofractum*, but their biological activities were not determined (Chartterjee and Dutta 1967; Tabuneng et al. 1983; Ahn et al. 1992). These compounds, in addition to piperine, were later found to be potent inhibitors of aflatoxin B_1 biosynthesis (Lee et al. 2002). However, the mode of action of inhibition was not determined. Piperine and piperlongumine are moderately toxic to mice (Shah et al. 1998).

Piperine and piperlongumine from *P. longum* significantly inhibited growth and production of OTA in *A. alliaceus* and *A. sclerotiorum* (Tables II and III). The almost complete inhibition of OTA and OTB production suggests piperine and piperlongumine function by inhibiting the polyketide pathway. In general, piperlongumine has a greater inhibitory effect on OTA production than piperine. However, at 0.001%, piperine showed fairly significant inhibition of OTA production of *A. alliaceus* (791) and *A. sclerotiorum*, only moderate inhibition of *A. alliceus* (7–1) and no inhibition of *A. auricomus*. Alternatively, at 0.001%, piperlongumine had its greatest inhibitory activity against OTA production in *A. alliaceus* (7–1) and *A. auricomus*, rather than *A. alliaceus* (791) and *A. sclerotiorum*. The complementary activities of these compounds in the different taxa of *Aspergillus* tested are worrying.

Even though piperine and piperlongumine putatively have similar modes of inhibitory activity against OTA production, their different potencies among the *Aspergillus* taxa may result from interactions involving chemical structure and particular biological characteristics of the fungi. Such factors could include different rates of absorption or metabolism of the compounds by the various fungi examined. Some of the effects observed may be dependent on concentration; some compounds that inhibit aflatoxin biosynthesis at one concentration may stimulate it at another. Further studies are needed to determine factors explaining the different activities amongst the taxa.

Curcumin from *Curcuma longa* L. and sesamin from *Sesamum indicum* L. also inhibited OTA production (Table II). Curcumin inhibited fungal growth, as well. In *A. alliaceus* (791), 0.01% curcumin inhibited OTA production and fungal growth but appeared to stimulate OTB production. These findings suggest the mode of action of curcumin is inhibition of chloroperoxidase and, thus, chlorination of OTB to OTA. Additionally, curcumin inhibited citrinin production. Citrinin is produced through the polyketide pathway (Barber et al.1987; Steyn 1992; Hajjaj et al. 1999). Thus, it appears curcumin inhibits the polyketide pathways involved in production of citrinin and OTA. Sesamin, at 0.1%, only moderately inhibited OTA production, and weakly inhibited OTB and citrinin production and fungal growth. It may be that sesamin has some mild anti-chloroperoxidase activity, inhibiting conversion of OTB into OTA or other chlorinated precursors of OTA.

It is currently believed that contamination by OTA is mainly caused by *A. ochraceus* in coffee beans, *A. carbonarius* in grapes and *Penicillium verrucosum* in cereals (Pitt 2000). A recent study, however, found that not all strains of these species produced OTA (Bayman et al. 2002). Surprisingly, this study found that none of the strains of *A. ochraceus* or *A. melleus*, cultured from soil and tree nuts in California, produced OTA.

An important step towards protecting crops from OTA contamination is identification of fungal taxa that are ochratoxigenic (Bayman et al. 2002). This paper is the first report of OTB production in *A. alliaceus*, *A. auricomus* and *A. sclerotiorum*, and of citrinin in *A. alliaceus*. *A. auricomus* and *A. sclerotiorum* did not produce citrinin. Differences between OTA-producing fungi in the production of OTB and citrinin, and differential responses of the fungi to inhibitory compounds, suggests that prevention of contamination of crops in a given situation will be more effective if the responsible fungi are identified. Past studies on OTA production by *Aspergillus* usually focused on *A. ochraceus* (Bayman et al. 2002). We suggest studies on OTA production should include *A. alliaceus* and *A. auricomus* (Table III).

Our study suggests natural products, specifically those of piperaceous plants, may be effective in curtailing ochratoxin contamination in agricultural commodities, either pre- or post-harvest. The fact that these natural products are toxic to grain storage insects (as cited above) may make them doubly effective; there is generally a correlation between insect damage and mycotoxin contamination in grains and seeds (Cotty and Lee 1989). Treating commodities with black pepper extracts may, therefore, provide double protection against ochratoxin contamination: it could reduce both insect damage and mycotoxin biosynthesis.

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