Influence of Halogen Salts on the Production of the Ochratoxins by *Aspergillus ochraceus* Wilh.

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The first report of the biological production of bromo ochratoxin B by *Aspergillus ochraceus* Wilh. is presented as well as a study of the influence of potassium bromide, potassium iodide, potassium fluoride, and potassium chloride on the production of ochratoxin A and ochratoxin B. Potassium fluoride and potassium iodide inhibited the growth of the fungus, whereas potassium chloride substantially stimulated the production of ochratoxin A in shaken solid substrate fermentation on whole wheat or shredded wheat, generally giving a high yield of ochratoxins. Increasing levels of potassium bromide led to a decline in ochratoxin A production and an increase in bromo-ochratoxin B, ochratoxin B, and 4-hydroxy ochratoxin B. Nevertheless, *A. ochraceus* was much less versatile in the bromo analogues than other fungi, which produce metabolites containing chlorine. Analysis included aminopropyl solid-phase extraction column cleanup followed by quantitative analysis on reversed-phase HPLC using fluorescence detection and employing *N*-(5-chloro-2-hydroxybenzoyl)-phenylalanine as an internal standard.

Keywords: *Ochratoxin A; bromo-ochratoxin B; (4R)-hydroxy ochratoxin B; Aspergillus ochraceus Wilh.; solid-phase extraction*

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic, teratogenic, and immunotoxic mycotoxin, produced mainly by isolates of *Aspergillus ochraceus*, Wilh. and *Penicillium verrucosum* (Van der Merwe et al., 1965; Frisvad, 1989). OTA is a frequent contaminant in cereals, coffee (Pittet et al., 1996), wine (Majerus and Ottender, 1996), spices, and beer (Speijers and Van Egmond, 1993). OTA is the cause of Danish Porcine Nephropathy (Krogh et al., 1988) and is implicated as a cause of kidney disease among humans, viz Balkan Endemic Nephropathy in the Balkans and Chronic Interstitial Nephropathy in North Africa (Creppy et al., 1993). Ochratoxin B (OTB), the des-chloro analogue of OTA (see Figure 1), is approximately 10 times less toxic than OTA (Xiao et al., 1995) and is hydrolyzed 200 times faster than OTA by carboxypeptidase A (Doster and Sinnhuber, 1972). The halogen group is evidently important in the toxicity of the ochratoxins. Preliminary tests in kidney cells have indicated that bromo ochratoxin B is more toxic than OTA (Creppy, 1999). The question is whether the chlorine or bromine group plays a direct role in the toxicity or if it is the change in the compound's ability to chelate iron or to bind to DNA or the ability of carboxypeptidase A to cleave the toxin that causes the variation in the biological activity of the ochratoxins.

This conundrum has been investigated by our concerted efforts to produce the fluoro, bromo, and iodo analogues of OTA which could provide invaluable information on structure−function relationships and the mode of action of the ochratoxins.

The precedent of halogenation enzymes of, e.g., *Penicillium crustosum*, *Penicillium griseofulvum*, *Penicillium nigricans*, *Pseudomonas pyrrolnitrica*, and *Penicillium multicolor* readily accepting bromide ions to form bromo analogues of penitrem A (Mantle et al., 1983), griseofulvin (MacMillan, 1954), pyrrolnitrin (Ajisaka et al., 1969), or azaphilones (Matzuki et al., 1998) allowed for the expectation that *A. ochraceus* might be similarly versatile.

This paper relates the first report of the biological production of bromo ochratoxin B by South African and Australian isolates of *A. ochraceus* Wilh.and the effects of potassium bromide, potassium fluoride, potassium chloride, and potassium iodide on the dynamics of production of ochratoxins. In addition, a number of minor metabolites of the South African isolate, e.g., ochratoxin α, ochratoxin *β*, (4*R*) 4-hydroxy ochratoxin B, (4*R*)- and (4*S*)-4-hydroxy ochratoxin A, and citrinin, were identified. These metabolites were reported previously by Xiao et al. (1996). Methyl esters of OTA and OTB (esterified at the phenylalanine carboxylic acid) were recognized as significant minor metabolites of the Australian isolate.

MATERIALS AND METHODS—EXPERIMENTS DONE IN SOUTH AFRICA

Solid-Phase Extraction (SPE) Columns. Aminopropyl SPE columns, 500 mg (SUPELCO, 5-7014), were used in conjunction with a vacuum manifold.

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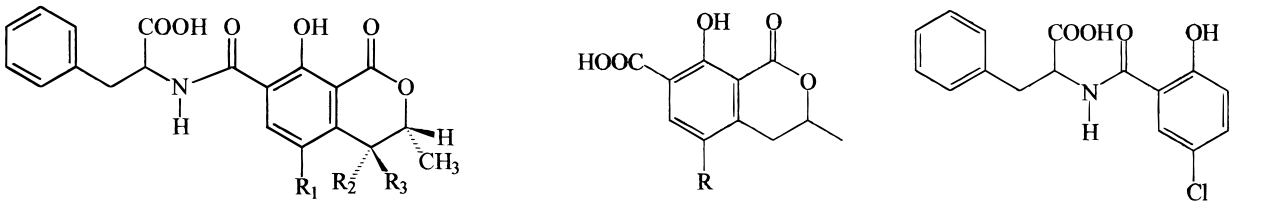

Ochratoxin A: R_1 = Cl, R_2, R_3 = H
Ochratoxin B: R_1 = H, R_2, R_3 = H
(4*R*)-4-Hydroxy-ochratoxin A: R_1 = Cl, R_2 = OH, R_3 = H
(4*S*)-4-Hydroxy-ochratoxin A: R_1 = Cl, R_2 = H, R_3 = OH
(4*R*)-4-Hydroxy-ochratoxin B: R_1 = H, R_2 = OH, R_3 = H
Bromo-ochratoxin B: R_1 = Br, R_2 = H, R_3 = H

Ochratoxin α: R = Cl
Ochratoxin β: R = H

N-(5-chloro-2-hydroxybenzoyl)-phenylalanine

Figure 1. Structures of the ochratoxins.

Thin Layer (TLC) and Column Chromatography. A mobile phase of toluene/acetic acid (4:1) was used on Silica gel 60 F_{254} TLC plates (Merck). The ochratoxins display very strong fluorescence upon UV illumination. Preparative TLC was done on Silica gel 60 F_{254} 2 mm plates (Merck).

Column chromatography was done with either Silica gel 60 (230−400 mesh ASTM), Merck, or Sephadex LH20.

Instrumentation. A Hewlett-Packard 1090 HPLC system fitted with a diode array (HP 1090) and fluorescence detector (HP 1100), autosampler, and ChemStation software was used. Separations were achieved using a 4.6 mm × 150 mm, 5 μm, C_{18} analytical column (Discovery C_{18}, SUPELCO) fitted with a C18 guard cartridge (Spherisorb ODS-2, SUPELCO) and a mobile phase of water/methanol/acetic acid (50:60:2). The injection volume was 5 μL, and flow rates of 1 mL/min were used. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 454 nm.

LC-ES-MS. A VG Quattro II HPLC 1090 system was used with positive ionization.

Safety. The ochratoxins are potent nephrotoxic compounds and should be handled with care.

Analysis of the Halogen Content of Durum Wheat. Durum wheat was digested according to the method of Havlin and Soltanpour (1980) to determine its halogen content. The chloride content of the Durum wheat was determined by the method of Weiss (1986), the fluoride content by the method of McQuaker and Gurney (1977), and the bromide and iodide contents by ICP/MS analysis.

Preparation of the Standards. OTA and OTB were obtained by cultivating *A. ochraceus* (MRC 10582) on wet sterilized Durum wheat (2.1 kg) at 25 °C for 14 days on a rotary shaker in 25 Erlenmeyer 500 mL flasks. The wheat was soaked with chloroform/methanol (1:1, 200 mL per flask containing 80 g of wheat) and left at ca. 20 °C for 12 h. This was subsequently homogenized by blending at 3000 rpm for 10 min and filtered, and the residues were thoroughly washed with chloroform/methanol (1:1, 20 mL). The filtrate was evaporated under vacuum to dryness. The crude extracts (121.5 g) were combined, resuspended in methanol/water (95:5, 3.5 l), and washed 4 times with hexane (4 × 1.5 L). The methanol layer was evaporated to dryness and partitioned between 1 M sodium bicarbonate (3.5 L) and chloroform (2.5 L). The aqueous layer containing the ochratoxins was acidified to pH 1 with 6 M hydrochloric acid and extracted 3 times with chloroform (1 L). The combined chloroform extracts were washed with water (2 × 0.5 L), dried over anhydrous sodium sulfate, and evaporated to dryness. The ochratoxin-containing fraction (54.7 g) was separated on silica gel (1.5 kg, 70−230 mesh) on a column (1 m × 50 mm) with chloroform/acetic acid (97:3) as the mobile phase. The OTA- and OTB-containing fractions were combined, and the solvent was evaporated under reduced pressure; the solid was dissolved in chloroform, extracted twice with water, and dried over anhydrous sodium sulfate. The chloroform was removed under reduced pressure, and OTA (3.433 g, mp 91 °C, lit. 90 °C, van der Merwe et al., 1965) and OTB (1.466 g, mp 219 °C, lit. 221 °C, van der Merwe et al., 1965) were recrystallized from benzene and methanol, respectively.

Bromo ochratoxin B and *N*-(5-chloro-2-hydroxybenzoyl)-phenylalanine were synthesized by Steyn and Payne (1999).

N-(5-chloro-2-hydroxybenzoyl)phenylalanine was used as the internal standard for HPLC analysis. It is a very stable compound with UV and fluorescence characteristics similar to those of the ochratoxins.

The four hydroxylated ochratoxins (4*R*)- and (4*S*)-4-hydroxy ochratoxin A, 10-hydroxy ochratoxin A, and (4*R*)-4-hydroxy ochratoxin B were supplied by Prof. R. R. Marquardt, Department of Animal Science, University of Manitoba, Canada.

Citrinin was kindly provided by Prof. F. C. Størmer, National Institute of Public Health, Oslo, Norway.

Ochratoxin α and ochratoxin *β* were produced by hydrolyzing OTA and OTB respectively, under reflux in excess 6 M hydrochloric acid for 60 h.

Cultivation of *A. ochraceus* at Different Levels of Halogens. A lyophilized culture of *A. ochraceus* Wilh. (MRC 10582) kept at −70 °C, obtained from the CSIR Culture Collection, was plated onto potato dextrose agar plates. The Petri dishes were incubated at 25 °C for 3 days in the dark. Twelve different concentrations, ranging from 0 to 2000 mg of potassium bromide, potassium iodide, potassium chloride, and potassium fluoride per 40 g of whole Durum wheat kernels, were prepared by adding the salt, wheat, and 25 mL of distilled water together in an Erlenmeyer flask (500 mL) for each concentration. A second set identical to the first was also prepared to give a total of 84 flasks. These were subsequently incubated for 16 h at 25 °C on a rotary shaker at 350 rpm, autoclaved for 30 min, and cooled to room temperature. The wheat was then inoculated with a 2 mL spore suspension of 3-day old cultures of *A. ochraceus*. The flasks were replaced on the rotary shaker for 14 days, after which they were harvested for analysis. The content of the flasks was quantitatively transferred to beakers; methanol/chloroform (1:1, 200 mL) was added and milled for 10 min at 3000 rpm, sealed, and left for 24 h. The wheat extracts were then vacuum filtered through a Büchner funnel containing Whatman No. 1 filter paper. The flask and filter pad were rinsed with 40 mL of methanol/chloroform (1:1) followed by another filtering step using microfiber filter paper. The combined extracts from each separate experiment were then transferred to volumetric flasks (250 mL) and filled with methanol/chloroform (1:1). The recovery of the extraction step was 85%. This was determined by repeating the extraction until no more OTA could be observed by HPLC. The total OTA, as determined in all the extraction steps, was used as the total OTA in the wheat.

Purification of the Ochratoxins by the SPE Columns. The SPE columns were conditioned with 2.5 mL of methanol/chloroform (1:1); 2 mL of the above extracts were subsequently placed on the columns and allowed to flow at ±2 drops per second. The SPE columns were washed with 2.7 mL of chloroform and allowed to run completely dry. The ochratoxins were eluted from the columns with 2.5 mL of methanol/acetic acid (4:1) and collected into test tubes. A stock solution of internal standard was prepared by dissolving *N*-(5-chloro-2-hydroxybenzoyl)phenylalanine (100 mg) in methanol (100 mL) and diluting it 10 times. The eluant (1.5 mL) of the SPE

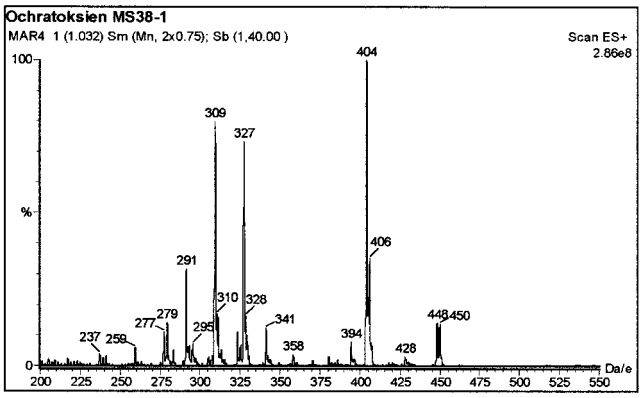

Figure 2. ES-MS spectrum of extract 1 from cultivated wheat supplemented with potassium bromide containing ochratoxin A (M + 1, *m*/*z* 404, 406) and bromo-ochratoxin B (M + 1, *m*/*z* 448, 450).

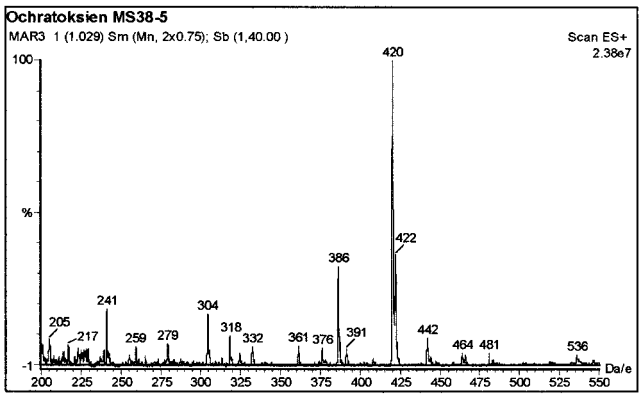

Figure 3. ES-MS spectrum of extract 5 from wheat cultivated with the South African isolate of *A. ochraceus*, supplemented with potassium bromide containing 4-hydroxy ochratoxin A (M + 1, *m*/*z* 420, 422).

Table 1. Fractions Obtained after Chromatography on Silica Gel of Different Ochratoxins Present in 120 g of Wheat Supplemented with 5500 mg of Potassium Bromide and Cultivated with *A. ochraceus*

extract	fractions	amount (mg)	ochratoxins
1	10−70	324	OTA, Br-OTB
2	81−135	427	OTB, OTβ
3	157−183	66	OTβ, OTα
4	229−251	23	(4*R*)-OH-OTA
5	253−318	42	(4*S*)-OH-OTA and/or 10-OH-OTA
6	329−349	208	(4*R*)-OH-OTB

extraction was transferred to autosampler vials, and 100 μL of the internal standard was added to each vial for HPLC analysis.

Isolation of the Different Ochratoxins in the Potassium Bromide Supplemented Wheat. Three of the above Erlenmeyer flasks containing extracts of cultured wheat (3 × 40 g), each supplemented with 1500 and 2000 mg of potassium bromide, were used to isolate the ochratoxins produced in wheat cultivated with *A. ochraceus* and supplemented with high levels of potassium bromide. The contents of the flasks were combined and evaporated to dryness under vacuum. The extract (15 g) was redissolved in methanol/water (95:5, 200 mL), hexane (250 mL) was added (to remove excess oils), and the two layers were separated. The methanol layer was concentrated under vacuum and redissolved in chloroform (350 mL), and the ochratoxins were extracted with 1 M sodium bicarbonate (2 × 200 mL). The aqueous layer was carefully acidified with 6 M hydrochloric acid and re-extracted 3 times with chloroform (3 × 150 mL). The chloroform extract was evaporated to dryness, and the residue (2.5 g) was transferred to a glass column (1 m × 50 mm) packed with silica (200 g) in chloroform. Initially chloroform/acetic acid (98:2, 1 l) was used to elute most of the lipids still present in the extract. The first 180 fractions (10 mL each) containing ochratoxins were eluted with chloroform/acetic acid (92:8, 2 L) followed by 100 smaller fractions (5 mL) which eluted with chloroform/acetic acid (90:10, 500 mL) and 80 fractions which eluted with chloroform/acetic acid/methanol (90:12:3, 500 mL). These fractions were analyzed by TLC, and similar fractions were combined and evaporated to dryness to yield six ochratoxin-containing extracts. The six extracts were compared with the reference standards by using HPLC and TLC (see Table 1).

The two ochratoxins (OTA and Br-OTB) present in extract 1 proved to be inseparable by using TLC or column chromatography; their identity was confirmed by comparison of their retention time on HPLC and the very distinctive molecular ion pattern of chlorine (M + 1, *m*/*z* 404, 406, ratio 3:1) and bromine (M + 1, *m*/*z* 448, 450, ratio1:1) as present in OTA and Br-OTB, respectively (see Figure 2).

The ochratoxins (OTB and OTβ) present in extract 2 (427 mg) were separated on a glass column (1 m × 15 mm), containing Sephadex LH20 (10 g), using methanol as the mobile phase. The substances were unambiguously identified as OTB and OTβ by comparison of their TLC and HPLC retention and ES-MS characteristics.

The two ochratoxins (OTβ and OTα) present in fraction 3 (66 mg) were separated by preparative TLC using five plates and chloroform/acetic acid (96:4) as the mobile phase, and their identities were confirmed by ES-MS, showing molecular ions at *m*/*z* 223 and 257 (M + 1), respectively.

Extract 4 (23 mg) contained mostly one compound, a hydroxy ochratoxin A which was cleaned by preparative TLC using two preparative TLC plates and chloroform/acetic acid (98:2) as the mobile phase. Its presence was confirmed as (4*R*)-OH-OTA by retention time comparison on HPLC and ES-MS (M + 1, *m*/*z* 420).

Extract 5 (42 mg) was first purified on a glass column (1 m × 15 mm, containing 5 g of Sephadex LH 20) employing methanol as the mobile phase; the ochratoxin-containing fractions were evaporated to dryness, followed by preparative TLC using eight analytical TLC plates and toluene/acetic acid (5:1) as the mobile phase. The presence of (4*R*)-OH-OTA and (4*S*)-OH-OTA was confirmed by retention time comparison on HPLC and ES-MS (see Figure 3).

Extract 6 (208 mg) contained only one ochratoxin-type compound as well as unidentified brown material. It was cleaned on a glass column (1 m × 15 mm, containing 8 g of Sephadex LH20) and using methanol as the mobile phase. It yielded crystals (150 mg, mp 237−238 °C) and was identified to be (4*R*)-4-hydroxy ochratoxin B by NMR (Xiao et al., 1996) and ES-MS.

MATERIALS AND METHODS—EXPERIMENTS MAINLY ON THE AUSTRALIAN ISOLATE

The principal differences from experimentation in South Africa concerned the fungus which was of Australian origin (Tapia and Seawright, 1984), the wheat substrates either in the form of whole UK wheat or as a processed food (shredded wheat; Cereal Partners UK), and the basic optimized solid substrate fermentation process for shredded wheat involving addition of aqueous spore suspension in dilute Tween 80 and sterile distilled water (total volume 16 mL) to sterile substrate (40 g) in a 500 mL Erlenmeyer flask. Incubation was at 29 °C on a rotary shaker at 200 rpm and 10 cm eccentric throw (Harris, 1996). This system has been found to be particularly suitable for expression of the potential for production of OTA by *A. ochraceus* (Mantle and Chow, 2000). Analysis of ochratoxins involved optimized (after Nesheim et al., 1992) extraction with ethyl acetate:0.01 M H_3PO_4 (9:1), partition into 3% $NaHCO_3$, acidification with HCl, partition into ethyl acetate, and evaporation to dryness. A standard solution of the residue was made in methanol and analyzed (20 μL) by reversed-phase HPLC in acetonitrile:H_2O:acetic acid (59:39:1) with diode array detection, facilitating monitoring of UV spectra of eluted

Table 2. Identification of Ochratoxins Produced on Wheat Inoculated with *A. ochraceus*

number on Figure 4	retention time	TLC R_f value	ochratoxin	technique used
1	3.0	0.23	ochratoxin α	*a, b, c, d*
2	4.7	0.19	ochratoxin *β*	*a, b, c*
3	6.3	0.098	(4*R*)-4-hydroxy ochratoxin B	*a, b, c, d*
4	7.8	0.16(5)	(4*S*)-4-hydroxy ochratoxin A	*a, b, c*
5	9.1	0.19	(4*R*)−4-hydroxy ochratoxin A	*a, b, c*
6	9.4	0.46	citrinin	*a, c*
7	10.0	0.35	ochratoxin B	*a, b, c, d*
8	10.7	0.17(0)	10-hydroxy ochratoxin A	*a, b, c, d*
9	15.6		*N*-(5-chloro-2-hydroxybenzoyl)phenylalanine	
10	21.0	0.50	ochratoxin A	*a, b, c*
11	24.0	0.50	bromo-ochratoxin B	*a, b*

[a] HPLC retention time comparison with standard compound. [b] ES-MS analysis. [c] TLC retention time comparison. [d] NMR.

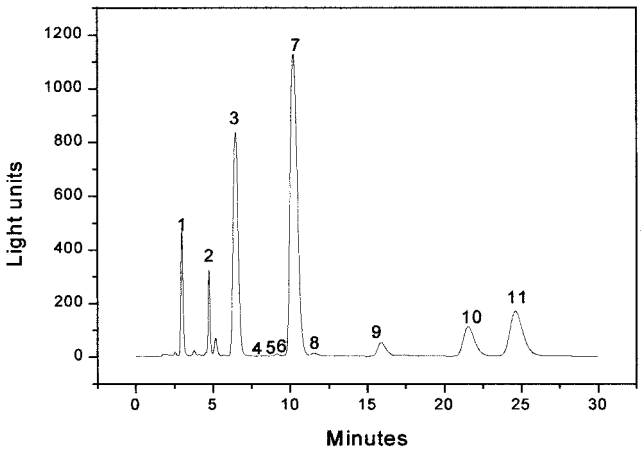

Figure 4. HPLC chromatogram depicting the distribution of the ochratoxins produced by the South African isolate of *A. ochraceus* at a concentration of 1.5 g of potassium bromide per 40 g of Durum wheat. For the identity of components 1−11, see Table 2.

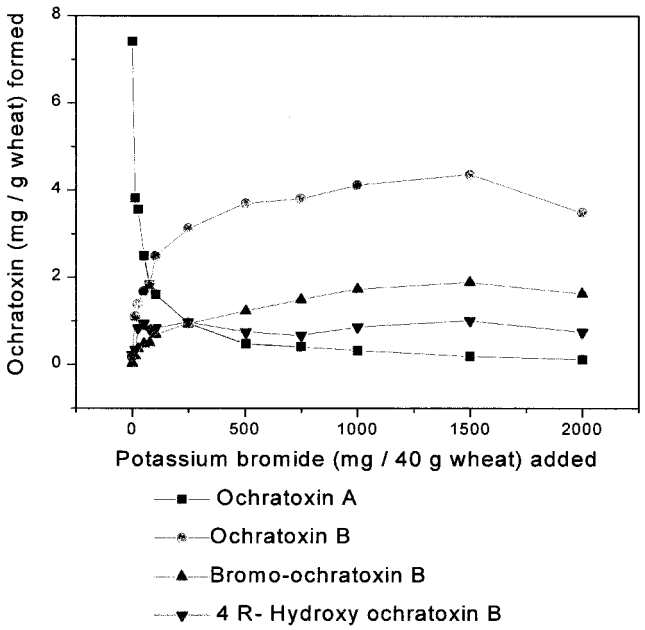

Figure 5. Production of OTA, OTB, Br-OTB, and (4*R*)-4-hydroxy ochratoxin B at different concentrations of potassium bromide by the South African isolate of *A. ochraceus*.

compounds. Satisfactory validity of analytical methodology between London and South Africa was confirmed in representative samples.

RESULTS AND DISCUSSION

Studies on the South African Isolate of *A. ochraceus* Wilh. The use of the aminopropyl SPE columns proved to be very effective, with a percentage recovery of (98 ± 4)% (±RSD, $n = 6$). The SPE columns retain only compounds containing a free carboxylic acid group. The methyl esters of the ochratoxins were thus not retained (they were detected with TLC (toluene:acetic acid 5:1 $R_f = 0.66$ in the crude extract). To our knowledge, this is the first report of the use of this type of SPE column in ochratoxin analysis; a more detailed report on their use and effectiveness will follow shortly. A typical HPLC chromatogram of the supplementation of the *A. ochraceus* growth medium with potassium bromide is shown in Figure 4. The presence of the bromo ochratoxin B was confirmed by using retention time studies and electrospray mass spectroscopy (M + 1, *m/z* 448, 450) and comparison with synthetic bromo ochratoxin B (see Table 2).

The Durum wheat used in the South African experiments was found to contain much higher concentrations of chloride (827.5 mg/kg) than fluoride (34.3 mg/kg). Only trace amounts of bromide (<0.05 mg/kg) and iodide (<0.05 mg/kg) were found.

The results of the potassium bromide experiment are summarized in Figure 5. The amount of OTA decreased markedly with the increase of potassium bromide concentration. However, the accumulated amounts of OTB and bromo-ochratoxin B increased with an increase of potassium bromide concentration, but at high concentrations (e.g., 1.5 g of KBr/40 g of wheat), the yield of the total ochratoxins decreased due to the apparent poisoning of the microorganism. The results indicate that chloride is the preferred halogen for incorporation into the ochratoxin-type molecules, and the organism only accepts bromide at relatively high bromide concentrations, with a disproportionately weak influence of the high concentration of bromide. OTB is the likely biosynthetic precursor to Br-OTB.

The results obtained upon addition of potassium iodide and potassium fluoride to cultures of *A. ochraceus* on wheat are summarized in Figure 6. The yield of both OTA and OTB decreased markedly as the amount of potassium iodide and potassium fluoride was increased, the fungus evidently being very sensitive to fluoride and iodide. No iodo-ochratoxin B or fluoro-ochratoxin B was detected using HPLC, ES-MS, and FAB-MS (positive ionization).

The chloride supplementation experiment was conducted 2 months after the initial supplementation experiments when the overall production of OTA was less than that in previous experiments. However, a 5-fold increase in OTA production was observed (see Figure 7) when 1 g of potassium chloride was added.

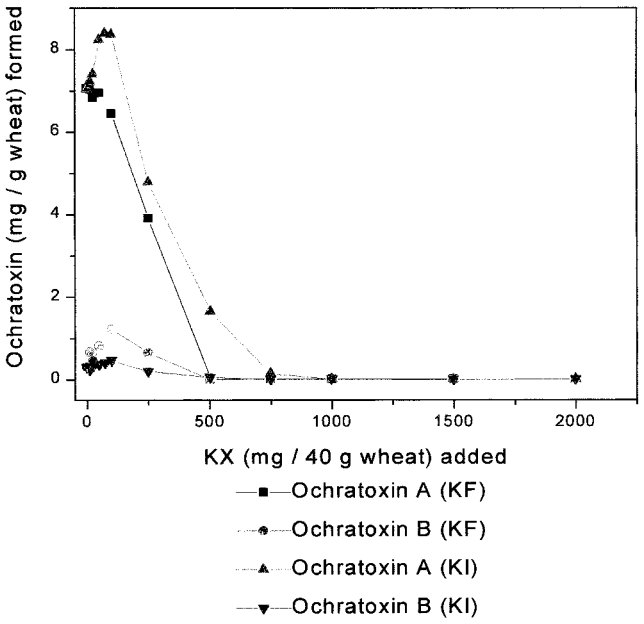

Figure 6. Influence of potassium fluoride and potassium iodide on the production of OTA and OTB in wheat: Concentration OTA and OTB produced by the South African isolate of *A. ochraceus* on wheat versus amount of potassium salt added to the wheat.

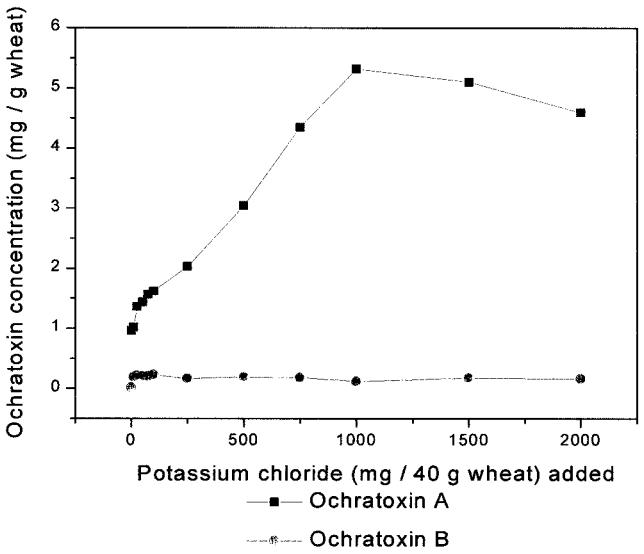

Figure 7. Influence of potassium chloride on the production of OTA and OTB in wheat by the South African isolate of *A. ochraceus*.

Increased potassium chloride must have allowed the corresponding increased OTB production, and amounts of residual OTB remained roughly constant. This supposition is based on the premise that OTA is derived from OTB, though there is experimental evidence that OTA is also derived from OTα (Harris, 1996). However, supplementary chloride was influential whether chlorination of the isocoumarin occurred before or after linkage of phenylalanine. Similar results were obtained by Babiker et al. (1999) on the effects of the chloride ion on the production of acutumine and dechloroacutumine by *Menispermum dauricum*.

Studies Mainly on the Australian Isolate. Comparison between a culture of the South African isolate and the Australian isolate on whole wheat in shaken culture conditions in London showed that the latter

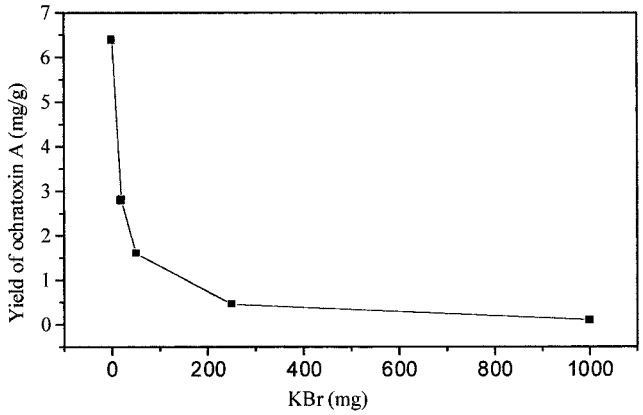

Figure 8. Effect of addition of batched potassium bromide on 17-day shaken shredded wheat fermentations ($n = 4$) of the Australian isolate of *A. ochraceus* concerning the mean yield of ochratoxin A.

yielded 3- to 4-fold more OTA than the former. On the shredded wheat substrate there was an even greater difference in OTA yield between the two fungi, but they both showed the same profound adverse effect on the total ochratoxin yield in response to increasing amounts of KBr (illustrated for the Australian isolate in Figure 8). In a further experiment with the Australian isolate only, which included analysis of ochratoxin methyl esters that have a relatively long residence time in HPLC, the marked decrease in OTA yield with increasing KBr was matched by increased occurrence of OTB, which becomes the dominant ochratoxin at the highest concentrations of KBr (Figure 9a). Correspondingly, the methyl esters of OTB and of OTA, identified by the mass spectral fragmentation pattern as esterified at the phenylalanine carboxyl rather than substitution at the isocoumarin hydroxyl, become notable metabolites with the addition of 50 mg of KBr to the wheat (40 g). They were still evident with 100 mg of KBr but were absent with 250 mg of KBr in 40 g of wheat. Consequently, although total chlorinated ochratoxins again declined with increasing KBr (Figure 9b), des-halo ochratoxins became the most abundant metabolites at 50 mg of KBr in 40 g of wheat, which also supported the highest total ochratoxins yield within which BrOTB was identified as a significant though very minor component (Figure 9a). Thus, both fungi were qualitatively similar in being slow to biosynthesize the bromo analogue of OTA and to have halogenation enzymes which were adversely sensitive to bromide by comparison with those of other fungi producing the chlorine-containing metabolites, penitrem A and griseofulvin.

Another experiment with the Australian isolate, using pure wheat substrate either in the form of whole grain or as a processed food product (shredded wheat), supported a high OTA:OTB ratio even at the higher overall yields obtained (illustrated for the shredded wheat substrate in Figure 10). However, a 50 mg KCl supplement further enhanced chlorination and OTA yield reflected through a higher OTA:OTB ratio. In the same experiment, this contrasted with confirmed typical changes in ochratoxins in response to the same amount of KBr. Addition of 50 mg of KCl to 40 g of shredded wheat approximately doubles the available chlorine (ca. 0.065%; Cereal Partners UK), and the highest yields of OTA in unsupplemented shredded wheat substrate in the present study apparently used all the available chlorine.

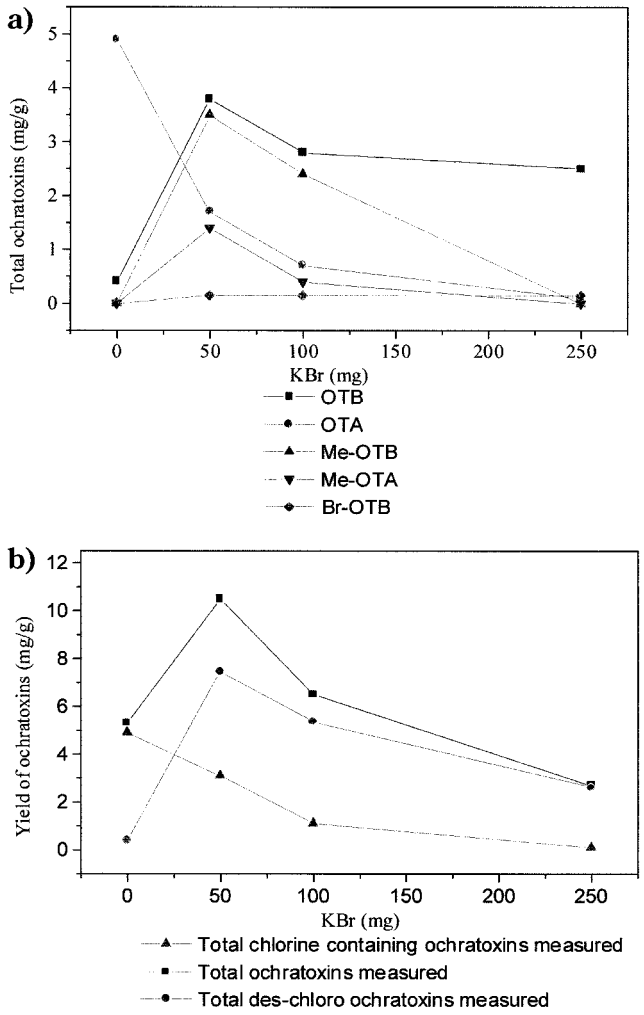

Figure 9. Effect of batched potassium bromide on 14-day shaken shredded wheat fermentation of the Australian isolate of *A. ochraceus* concerning the yield (a) of individual ochratoxins and (b) of groups of chloro, des-chloro, and total ochratoxins.

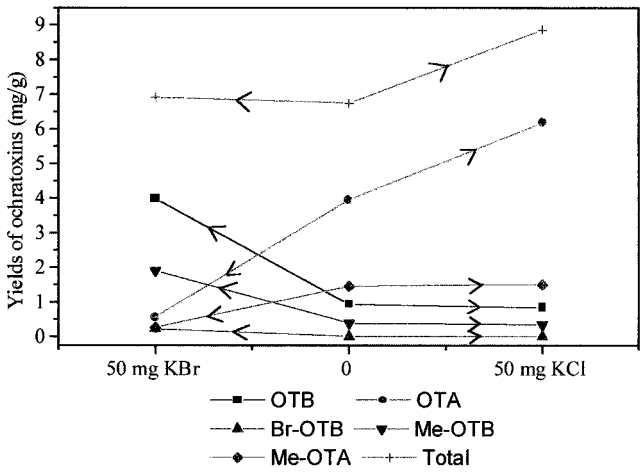

Figure 10. Direct comparison of the effects of addition of 50 mg of potassium bromide or potassium chloride per 40 g of shredded wheat to 17-day shaken shredded wheat fermentation of the Australian isolate *of A. ochraceus* on the mean yield ($n = 3$) of total and individual ochratoxins.

In conclusion, high levels of bromide in the wheat on which *A. ochraceus* was cultivated have a substantial influence on the yields and the formation of different ochratoxin metabolites. Bromo-ochratoxin B can be produced by *A. ochraceus* Wilh. in the presence of high bromide levels, although not in high yield.

An increase in the chloride concentration in wheat resulted in increased production of chloride-containing ochratoxins by *A. ochraceus*.

Iodide and fluoride are generally too toxic to support formation of ochratoxin analogues.

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Supporting Information Available: Tables of ^{1}H NMR data for ochratoxin B, ochratoxin A, ochratoxin α, and (4*R*)-4-hydroxy ochratoxin B and figures of the structures of ochratoxin B, ochratoxin A, and (4*R*)-4-hydroxy ochratoxin B and ^{1}H NMR spectrum of (4*R*)-4-hydroxy ochratoxin B (3 pages). This material is available free of charge via the Internet at http://pubs/acs.org.

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