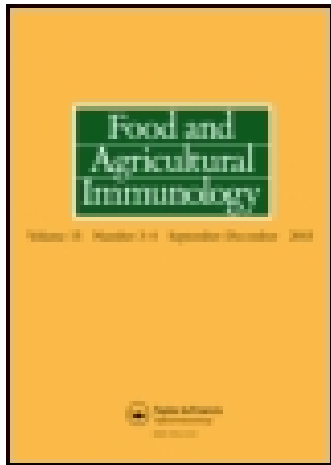




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Development of Solid-phase Immunoassays for the Detection and Quantification of *Aspergillus ochraceus* in Wheat Grain

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Aspergillus ochraceus Wilhelm is a common contaminant of stored foods, especially cereal grains, which produces the mycotoxin, ochratoxin A (OA). Solid-phase immunoassays (enzyme-linked immunosorbent assay [ELISA] and immunoblotting), utilizing antibodies raised in rabbits to *A. ochraceus* exoantigens (ExAgs, a mixture of extracellular macromolecules), were evaluated for their ability to provide an index of the degree of *A. ochraceus* contamination in cereals. *A. ochraceus* could be readily detected with a minimal degree of interference or cross-reactivity from the wheat matrix or from other fungal species naturally present in wheat. The ELISA was sensitive, with the limits of detection for ExAgs being as low as 50 ng ml⁻¹. The amounts of *A. ochraceus* ExAgs detected in wheat that was naturally molded and inoculated with *A. ochraceus* correlated favorably with other parameters indicative of the presence of the fungus. These parameters included the amount of OA ($r = 0.93$, $P < 0.05$), the percentage of *A. ochraceus* infection ($r = 0.89$, $P < 0.05$), the number of colony-forming units ($r = 0.68$, $P < 0.05$) and the glucosamine content ($r = 0.64$, $P < 0.05$). Immunoblotting patterns of ExAgs extracted from liquid and wheat cultures of *A. ochraceus* demonstrated that ExAgs consisted of several antigenic components, with the immunodominant ones having molecular weights of approximately 20 or 30 kDa. The concentration of particular components appeared to be influenced by the media on which *A. ochraceus* was cultivated. The data suggest that the immunoassays developed for *A. ochraceus* ExAgs can be used for the identification and quantitation of this fungus in grain.

Keywords: *Aspergillus ochraceus*, ochratoxin A, ELISA, wheat

INTRODUCTION

Aspergillus ochraceus Wilhelm is economically important as it is a common storage fungus and produces ochratoxin A (OA). This mycotoxin is nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunosuppressive (Prior & Sisodia, 1982; Roschenthaler *et al.*, 1984; Stein *et al.*, 1985; Marquardt & Frohlich, 1992) and has been reported to be present in a wide range of foods including cereal grains and animal products (Golinski *et al.*, 1985; Pitt & Hocking, 1985; Marquardt *et al.*, 1988). The contamination of food with *A. ochraceus* is therefore of concern. A reliable and sensitive method should be developed for *A. ochraceus* in food and feed, to detect its presence and provide a measure of quantification. Traditional methods such as direct plating and propagule counting are time-consuming to carry out and lack reliability and sensitivity (Pitt & Samson, 1990). Chemical analyses for compounds such as ergosterol and chitin suffer from being non-specific and require the use of expensive equipment (Jarvis *et al.*, 1983). Immunochemical methods have been used increasingly for the detection of fungi in foods because of their high sensitivity and specificity (Notermans & Heuvelman, 1985; Lin *et al.*, 1986; Tsai & Cousin, 1990). Kaufman and Standard (1987) have demonstrated that fungi produce soluble macromolecules (exoantigens, ExAgs) which appear to be unique to particular genera or even species of fungi. The development of an assay that will quantitatively detect *A. ochraceus* may be useful for indicating the level of contamination of food by this fungus or the corresponding presence of OA.

Only a few of the immunochemical techniques available have utilized the enzyme-linked immunosorbent assay (ELISA) for the detection of fungi in cereal grains. The ELISA developed by Notermans *et al.* (1986, 1987, 1988) and Kamphuis *et al.* (1989, 1992) was based on the detection of extracellular polysaccharides (EPS). It was genus-specific, but cross-reactivities occurred between closely related *Aspergillus* and *Penicillium* genera. Although antibodies raised against acid-hydrolyzed EPS were more specific in comparison with the antibodies against native EPS, they still were not species-specific (Kamphuis *et al.*, 1992).

Dewey *et al.* (1990) developed a monoclonal antibody-based ELISA for detecting *P. islandicum* Sopp in rice grains which indicated that it was feasible to develop such an assay for the specific monitoring of mold contamination. Until now, an ELISA that can detect and differentiate *A. ochraceus* from other *Penicillium* and *Aspergillus* species has not been described. In a previous study, however, rabbit antibodies against *A. ochraceus* ExAgs were raised and were shown to recognize predominantly *A. ochraceus* but not other *Aspergilli* or fungi belonging to other genera (Lu *et al.*, 1995). The purpose of the present investigation was to develop an indirect competitive ELISA using these antibodies which could be suitable for the detection and quantification of *A. ochraceus* in grain, in the presence of other fungi. The results demonstrated that *A. ochraceus* could be quantitatively monitored in: (i) samples containing different combinations of ExAgs from *A. ochraceus* and another common storage fungus, *P. aurantiogriseum* Dierckx; (ii) *A. ochraceus*- and *P. aurantiogriseum*-inoculated wheat samples; and (iii) naturally molded wheat samples inoculated with *A. ochraceus*.

MATERIALS AND METHODS

Materials

Potato dextrose agar (PDA) and yeast extract were from BBL (Microbiology System, Becton Dickinson and Co., Cockeysville, MD, USA). Microtiter plates (Falcon 3911 Microtest III Flexible Assay Plates) were from Becton Dickinson and Co. (Oxnard, CA, USA). Reagents for preparing phosphate-buffered saline (PBS) were obtained from Mallinckrodt Canada Inc. (Pointe-Claire, PQ, Canada). D-glucosamine-HCl was from Calbiochem (San Diego, CA, USA). Whatman No. 1 filter paper was from Whatman

(Clifton, NJ, USA). Ultra-pure reagents for electrophoresis and immunoblotting were obtained from ICN (Schwarz/Mann Biotech, Division of ICN Biomedical Inc., Cleveland, OH, USA) and Bio-Rad Laboratories (Hercules, CA, USA). Other reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). All solvents and reagents were of analytical grade or of better quality. The microplate reader (model 450), electrophoresis tanks (Mini-PROTEAN II), electrotransfer device (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell) and pre-stained broad-range protein standards were from Bio-Rad Laboratories (Richmond, CA, USA). The Cyclotec mill was from Tecator AB (Höganäs, Sweden), the wrist-action shaker, model 75, was from Bunell Corporation (Pittsburgh, PA, USA) and the C-18 reversed-phase (C18 RP) column was from Beckman (San Ramon, CA, USA).

Isolates of *A. ochraceus* (NRRL 3174) were from the laboratory of R.R.M. Cultures of *P. aurantiogriseum* var. *aurantiogriseum* Chemotype I (3298) were provided by Dr G. Platford (Plant Pathology Laboratory, Manitoba Agricultural Services Complex, Winnipeg, MB, Canada). The fungus was subsequently renamed *P. aurantiogriseum* Dierckx (Frisvad & Filtenborg, 1983). Both fungi used in current work were subcultured from spores maintained on silica gel at 2°C. The subcultures were deposited in the Biosystematic Research Institute, National Identification Service, W. Saunders Building, Central Experimental Farm, Ottawa, ON, Canada K1A 0C6.

Preparation of ExAgs from the Liquid Culture and General Procedures

Exoantigens were prepared according to Standard *et al.* (1985) with some modifications. Fungal spores from *A. ochraceus* and *P. aurantiogriseum* were first propagated on PDA and then used to inoculate a sucrose–yeast–mineral (SYM) liquid medium. After 2 weeks of growth at 30°C, the mycelia were separated from the liquid medium by filtration through two layers of Whatman No. 1 filter paper. The filtrate was then freeze-dried and stored at –20°C. The freeze-dried material for immunization or the ELISA was redissolved and dialyzed against PBS (0.06 mol l⁻¹ Na₂HPO₄, 0.018 mol l⁻¹ NaH₂PO₄, 0.075 mol l⁻¹ NaCl, pH 7.2), and stored at –80°C. The antigen used for immunization was sterilized by passing it through a 0.22 µm sterile filter. The ExAgs from wheat were prepared as described below. Protein, glucosamine and galactosamine were quantified according to procedures outlined by Bradford (1976) and Rotter *et al.* (1989) respectively. Glucosamine or galactosamine levels, which are measures of the chitin content of the mold, were used to estimate fungal biomass.

Immunization, ELISA and Immunoblotting

Two female rabbits (Dutch Belted, 1–1.5 kg body weight) were subcutaneously injected (three sites) with *A. ochraceus* ExAgs at a dosage of 1 mg of protein at 3-week intervals. The antigen preparation was emulsified with Freund's complete (first injection) or incomplete (booster injections) adjuvant. When the desired antibody titer was achieved, blood was collected and the serum was harvested, divided into aliquots and stored at –80°C. Studies were carried out on the serum from one rabbit.

The ELISA procedures were as described by Harlow and Lane (1988). For the non-competitive assay, microtiter plates were coated with 0.1 µg/well (100 µl) of *A. ochraceus* ExAgs in PBS and incubated at 4°C overnight. After three washings with PBS containing 0.05% (v/v) Tween 20 (PBS-T), the plates were blocked with 5% (w/v) skim milk for 2 h at 37°C. Serial dilutions of pre-immunized or immunized sera (100 µl) were added to the plates and after 2 h of incubation at 37°C the plates were washed three times with PBS-T. The plates were then incubated for an additional 1.5 h with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000, 100 µl). *p*-Nitrophenyl phosphate (0.1%, w/v) in diethanolamine (DEA) buffer (10% DEA (w/v), pH 9.8, 100 µl) was added after six washings with PBS-T and the plates were read at 405 nm when the absorbance was between 1.5 and 2.0 absorbance units. Checkerboard titrations were used

to pre-determine the optimal coating antigen concentration and antiserum dilution. For the competitive assay, serially diluted ExAgs from the test fungi (50 μ l) were added to the plate before the addition of the rabbit antiserum (1:3000, 50 μ l). Dilutions of ExAgs and antiserum were made in PBS-T.

Samples of ExAgs were solubilized in 1.5% (w/v) sodium dodecyl sulfate (SDS), boiled for 5 min and separated by non-reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using 4–20% gradient gels. Pre-stained molecular weight standards were co-separated. The separated components were either visualized by silver stain or transferred onto a 0.22 μ m nitrocellulose membrane and immunoblotted. Semi-dry transfer was performed in Tris buffer (25 mmol l⁻¹ Tris, 192 mmol l⁻¹ glycine, pH 8.3) containing 20% (v/v) methanol and 0.1% (w/v) SDS (Erickson *et al.*, 1982). After transfer, the membrane was washed in Tris-buffered saline (TBS, 20 mmol l⁻¹ Tris, 500 mmol l⁻¹ NaCl, pH 7.5) containing 10% (v/v) methanol, blocked with 3% (w/v) bovine serum albumin (BSA) in TBS and incubated with rabbit antiserum followed by alkaline phosphatase-labelled goat-anti rabbit IgG. The antibodies were dissolved in TBS containing 0.05% (v/v) Tween 20 (TBS-T). After each incubation step, the membrane was washed three times for 5 min in TBS-T. Nitroblue tetrazolium combined with 5-bromo-4-chloro-3-indolyl phosphate was used as the chromogenic substrate (Blake *et al.*, 1984).

Experiment 1. Detection of *A. ochraceus* ExAgs in Solutions Spiked with ExAgs from *P. aurantiogriseum*

A. ochraceus ExAgs prepared as described above were pre-mixed, in various proportions, with similarly prepared ExAgs from *P. aurantiogriseum*. The samples were added to microtiter plates pre-coated with *A. ochraceus* ExAgs. Rabbit anti-*A. ochraceus* antibodies were then added and the competitive ELISA was carried out as described above. The amounts of fungal ExAgs added and recovered were calculated from standard inhibition curves obtained for *A. ochraceus* or *P. aurantiogriseum* ExAgs as described by Lu *et al.* (1995). The ELISA was carried out in duplicate and the experiment was repeated.

Experiment 2. Detection of *A. ochraceus* ExAgs in Autoclaved Wheat Inoculated with One of Two Fungi

Wheat (100 g) was placed into 500 ml Erlenmeyer flasks, moisturized by the addition of 50 ml of distilled water, conditioned overnight at 4°C and autoclaved at 121°C for 20 min. The samples were aseptically inoculated with the spores of either *A. ochraceus* or *P. aurantiogriseum* (approximately 10⁶ spores ml⁻¹) and the flasks were sealed with a sterile sponge plug. The inoculated and un-inoculated, control wheat samples were incubated for 12 days at 30°C. Fungal growth was visually scored (none, slight or considerable). Three flasks were then taken from each inoculation group on days 0, 2, 4, 6, 8, 10 and 12, and from the un-inoculated controls on days 0, 6 and 12. Their contents were dried at ambient temperature in a fume hood and then ground in a Cyclotec mill equipped with a 1 mm screen and stored at -20°C.

The ExAgs were extracted from 10 g of the ground grain with 40 ml of PBS (0.1 mol l⁻¹, pH 7.2), the samples were then shaken vigorously for 1 h, centrifuged at 20 000 $\times$ g for 45 min and the supernatant collected, filtered, divided into aliquots and stored at -20°C. The concentration of *A. ochraceus* ExAgs was determined by the indirect competitive ELISA from a standard inhibition curve obtained for ExAgs originated from the liquid culture. Neat samples of extracted ExAgs were solubilized in SDS and analyzed by immunoblotting as described above. *A. ochraceus* ExAgs, obtained from the liquid culture, were co-separated (125 ng of protein/lane). Glucosamine and galactosamine analyses were carried out on 150 mg of each triplicate sample.

Experiment 3. Detection of *A. ochraceus* ExAgs in Naturally Contaminated Wheat

Hard spring, non-autoclaved wheat was used. The moisture content of 50 g of wheat was adjusted to 22% with sterile distilled water and the wheat was conditioned in 500 ml Erlenmeyer flasks for 24 h at 4°C. The grain had been naturally exposed to indigenous fungal spores as it became moldy under high moisture conditions. The moisturized grain was inoculated with *A. ochraceus* spores (0, 10^2 or 10^5 g⁻¹), sealed with a sterile sponge plug and incubated at 30°C for 0, 7 and 30 days. Three to six replicates for each group were prepared. The non-autoclaved, non-moisturized grain served as a control.

The number of fungal propagules was determined according to standard procedures (ISO, 1983a,b). In brief, 10 g of wheat were added to 90 ml of sterile diluent (0.1% peptone saline, pH 7.0), the sample was shaken for 30 min on a wrist-action shaker, and 1 ml was transferred into 9 ml of sterile diluent and mixed. Each sample was subjected to 10-fold serial dilutions and each dilution (1 ml) was poured on to four malt-extract agar (MEA; 2% malt extract, pH 6.25, containing 0.0006% (w/v) tetracycline) plates and incubated upright at room temperature (20–25°C) for 5 days. Plates with 5–50 colonies (occasionally up to 100) were counted. The type of mycoflora in each sample was determined following standard morphological and microscopical procedures (Mills & Wallace, 1979). The amount of OA in each sample was determined by high pressure liquid chromatography (HPLC) as described by Clarke *et al.* (1993). In brief, 1 g of dried, finely ground wheat was extracted with 15 ml of an 80:20 (v:v) mixture of methanol and H₂O (pH 2.1, acidified with H₃PO₄) with vigorous shaking for 30 min, and the samples were centrifuged at $10\,000 \times g$ for 15 min. The supernatant was collected, transferred into optical clear vials and 10 µl (pre-diluted if necessary) were injected on to a C18 RP column. OA was separated at a flow rate of 1.5 ml min⁻¹. The mobile phase was composed of a 60:7:33 (v:v) mixture of methanol, isopropanol and H₂O (pH 2.1, acidified with H₃PO₄). Exoantigens were extracted as in experiment 2 except that the proportion of sample to PBS was changed from 1:4 to 1:5. The ELISA, immunoblotting and glucosamine analyses were carried out as described above.

RESULTS

A typical standard inhibition curve for *A. ochraceus* ExAgs from the liquid culture is shown in Figure 1. The ability of the assay to detect the ExAgs from *A. ochraceus* in the presence of *P. aurantiogriseum* ExAgs was determined in experiment 1 and is illustrated in Table 1. The results demonstrated that the degree of cross-reactivity of *P. aurantiogriseum* with the anti-*A. ochraceus* serum was relatively insignificant (less than 1%) for all concentrations of *P. aurantiogriseum* ExAgs including samples that contained 100 µg ml⁻¹ of these ExAgs. The assay, however, became limiting for the detection of *A. ochraceus* ExAgs at concentrations above 50 µg ml⁻¹. This could be corrected by the appropriate dilution of the samples (data not shown).

Results from experiment 2 demonstrated that mycelial growth, evaluated either by visual inspection or by the concentration of glucosamine, was obvious on day 2 in sterilized wheat that was inoculated with either species of fungi. Extensive and progressively more growth occurred afterwards in all of the samples (Figure 2). There was a parallel increase (over 100-fold) in the concentration of *A. ochraceus* ExAgs produced in wheat inoculated with *A. ochraceus* ($r = 0.78$, $P < 0.05$) but not on wheat inoculated with *P. aurantiogriseum*. There was also a time-dependent increase in the content of galactosamine on *A. ochraceus* inoculated wheat, whereas galactosamine was not detected in any of the wheat samples inoculated with *P. aurantiogriseum*. No ExAgs, glucosamine or galactosamine were detected in the control wheat samples (autoclaved, un-inoculated and incubated wheat). Immunoblotting results showed that the *A. ochraceus* ExAgs were detectable in neither the autoclaved, un-inoculated wheat nor in the wheat inoculated with *A. ochraceus* at day 0 of incubation (Figure 3, lane 2) but they were present in the samples incubated for 6 (Figure

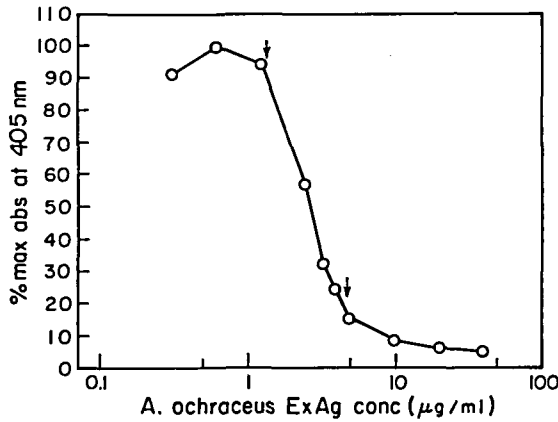


FIG. 1. Standard inhibition curve of *A. ochraceus* ExAgs prepared from liquid culture. The line between the arrows indicates the linear region which was used for determining the concentration of *A. ochraceus* ExAgs in all samples. Values represent means of duplicate analyses of two replicates with intra-assay coefficient of variability (CV) for the linear region of the assay being 8.4% and inter-assay CV being 21.1%.

3, lane 3) or 12 (Figure 3, lane 4) days. The ExAgs that were produced on the wheat matrix were similar to those produced in liquid medium. A total of three or four major ExAgs bands and as many as nine minor bands were detected in samples prepared from the liquid medium (Figure 3, lane 1), with a similar number being obtained in samples extracted from the grain (Figure 3, lanes 3 and 4). The most intensely staining bands in both preparations were those having a molecular weight (M_r) of approximately 30 kDa and, to a slightly lesser degree, those with a M_r of approximately 20 kDa.

The objective of experiment 3 was to determine if the ELISA could be used to detect the presence of *A. ochraceus* in wheat that was naturally contaminated with fungi. Figure 4 shows that the naturally contaminated grain had a relatively low rate of infection with *A. ochraceus* on day zero (<10%) which decreased further, to near zero, on days 7 and 30 of incubation; infection with *Penicillium* species was 20% on day 0 but increased to 100% on days 7 and 30 of incubation (Figure 4). Grain inoculated with a high concentration of *A. ochraceus* spores had an initial infection with *A. ochraceus* that was much higher than that observed in the un-inoculated grain while the corresponding degree of infection with the *Penicillium* species was comparable. Incubation of the grain, however, resulted in a

TABLE 1. Recovery of *A. ochraceus* ExAgs from samples spiked with different amounts of *A. ochraceus* and *P. aurantiogriseum* ExAgs as determined by an indirect competitive ELISA using rabbit antibodies to *A. ochraceus* (experiment 1)

Sample number	Concentration of ExAgs added ($\mu\text{g ml}^{-1}$)		Concentration of <i>A. ochraceus</i> ExAgs detected ^a ($\mu\text{g ml}^{-1}$)
	<i>A. ochraceus</i>	<i>P. aurantiogriseum</i>	
1	100	0	35 ± 15
2	50	0	35 ± 15
3	1	0.05	1 ± 0
4	0.2	0.2	0.1 ± 0.05
5	0.05	1	0.45 ± 0.05
6	0	50	0.7 ± 0.1
7	0	100	0.55 ± 0.05

^aValues represent means ± SD of two replicate analyses. Values for samples 1 and 2 are low because samples were not re-assayed at higher dilutions.

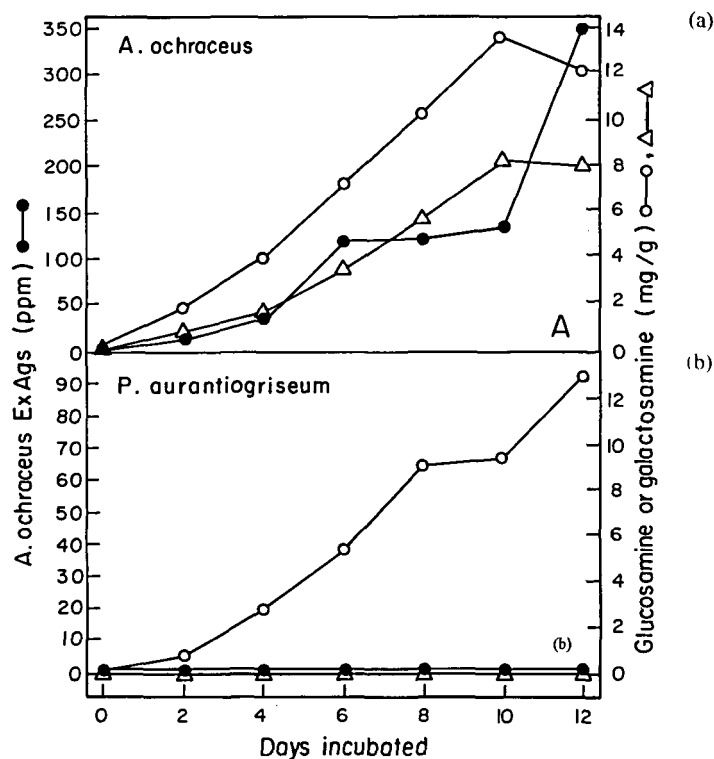


FIG. 2. Kinetics of the *A. ochraceus* ExAgs, and glucosamine and galactosamine production in autoclaved and moisturized wheat inoculated with *A. ochraceus* (a) or *P. aurantiogriseum* (b). The ExAgs were extracted from wheat samples and assayed by an indirect competitive ELISA (experiment 2). Visual score of fungal growth were zero on day 0, slight on day 2 and highly positive for days 4–12. Values represent an average of three replicate samples with the average CV being 45.2% for *A. ochraceus* ExAgs, 15.4% for glucosamine and 16.9% for galactosamine (a) and 3.9% for *A. ochraceus* ExAgs and 27.3% for glucosamine (b).

dramatic increase in the percentage infection with *A. ochraceus* (100% infection) while there was a progressive decrease in percentage infection with *Penicillium* species with the values at 30 days being zero. Inoculation of the grain with 10^2 *A. ochraceus* spores/g yielded a pattern of response that was intermediate to the un-inoculated grain and that inoculated with 10^5 *A. ochraceus* spores g^{-1} . These data demonstrated that the composition of the grain mycoflora was influenced by spore load and incubation period.

Table 2 shows that on day 0 the number of colony-forming units (cfu) and the contents of glucosamine, OA or *A. ochraceus* ExAgs were similar in naturally contaminated wheat and in wheat inoculated with 10^2 or 10^5 *A. ochraceus* spores. Generally, these values were much lower than those obtained from wheat samples that were incubated for 7 or 30 days. The number of fungal propagules increased equally in all three inoculation groups in a near-logarithmic manner from approximately 10^4 to 10^{11} cfu during the 30-day incubation period, suggesting that the total number of fungal propagules was not affected by inoculation of wheat with *A. ochraceus*. The concentration of glucosamine also increased over time with the most pronounced increase occurring between days 7 and 30. The total increase in glucosamine was greater in the grain inoculated with *A. ochraceus* than in the un-inoculated grain, with the maximal change being 14-fold for grain inoculated with the highest number of spores (0.5 versus 7.0 mg g^{-1}). The pattern of response of OA was different to that observed with the number of fungal propagules and glucosamine as there

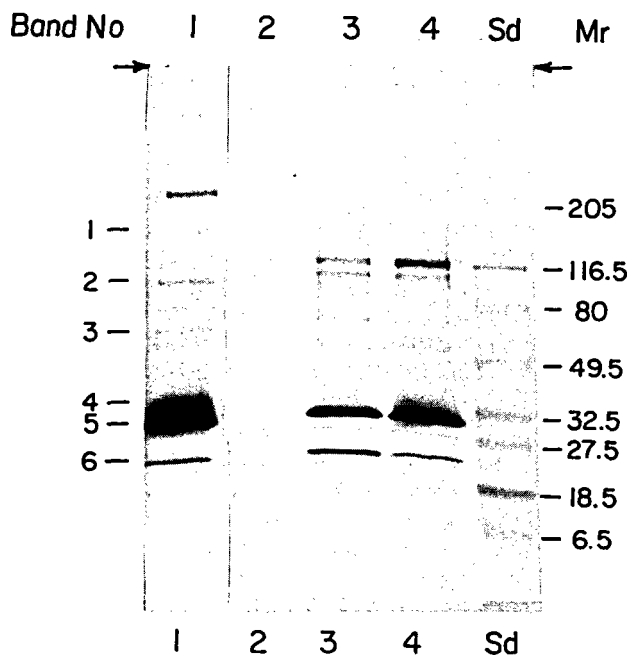


FIG. 3. Detection of *A. ochraceus* ExAgs by immunoblotting using rabbit anti-*A. ochraceus*. *A. ochraceus* ExAgs from liquid culture (lane 1) or from autoclaved wheat inoculated with *A. ochraceus* and incubated for different time periods (day 0 (lane 2), day 6 (lane 3) and day 12 (lane 4)) were solubilized by boiling in 1.5% SDS and separated on 4–15% gradient gels. The molecular weights (M_r) of the bands (kDa) were estimated using pre-stained standards (Sd). Arrows represent points of application of samples. The concentration of *A. ochraceus* ExAgs applied to the gel was 125 ng/lane.

was no detectable OA in the naturally contaminated grain. There was, however, a dramatic linear increase in the production of OA in samples inoculated with *A. ochraceus*, particularly in those inoculated with the high number of *A. ochraceus* spores. The concentration of *A. ochraceus* ExAgs followed a pattern similar to that observed for the production of OA. The content of ExAgs detected after 30 days of incubation was approximately 10-fold greater in the inoculated than in the un-inoculated wheat.

Figure 5 illustrates the immunoblotting patterns of *A. ochraceus* ExAgs of four groups of naturally contaminated wheat sampled at different incubation times. The controls (wheat that was non-autoclaved, un-inoculated and incubated for zero days (lane 1) or 7 days (lane 2)) contained only trace amount of *A. ochraceus* ExAgs. However, wheat that was inoculated with low (lane 3) or high (lane 4) numbers of *A. ochraceus* spores and incubated for 7 days exhibited additional bands, including a predominant band having a M_r of approximately 20 kDa. The ExAgs patterns obtained from wheat that was inoculated with either 10^2 (lane 6) or 10^5 (lane 7) *A. ochraceus* spores and incubated for 30 days were similar to each other except for the band intensity that was much greater for ExAgs obtained from wheat that was inoculated with a higher number of *A. ochraceus* spores. The pattern of bands exhibited by the ExAgs from the inoculated wheat was different to that obtained with the ExAgs from liquid culture. One noticeable difference was that the predominant component of ExAgs from liquid culture (M_r approximately 30 kDa) appeared to be considerably more abundant than that found in the ExAgs from wheat samples. The band with a lower molecular weight (with apparent M_r about 20 kDa) and many other higher molecular weight bands, in contrast, were more intense for the ExAgs obtained from the inoculated wheat samples compared with those obtained from liquid culture.

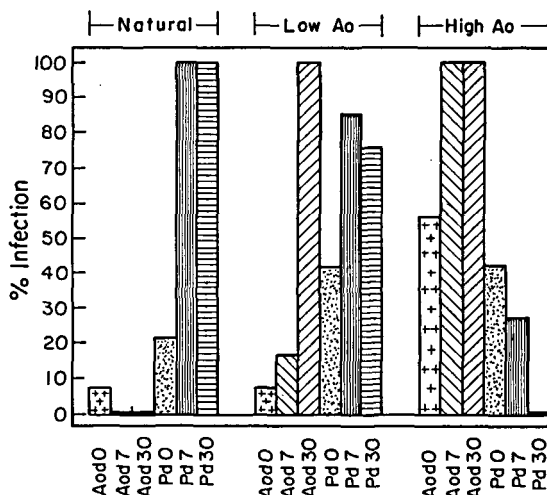


FIG. 4. The composition of mycoflora in wheat inoculated with various numbers of *A. ochraceus* spores g^{-1} of wheat (0, natural; 10^2 , low; 10^5 , high). The moisture content of the wheat was 22% (w/w). The percentages of *A. ochraceus* (Ao) and *Penicillium* (P) infection were determined after incubation for 0 (d0), 7 (d7) and 30 (d30) days. The average CV among samples was 38%.

The relationships between various quantitative parameters that were used for evaluation of the contamination of wheat with *A. ochraceus* are shown in Table 3. There was a positive correlation between the amount of *A. ochraceus* ExAgs and the amount of OA produced ($r=0.93$, $P<0.05$), the percentage of *A. ochraceus* infection ($r=0.89$, $P<0.05$), the number of fungal propagules ($r=0.68$, $P<0.05$) or the amount of glucosamine ($r=0.64$, $P<0.05$). The amount of *A. ochraceus* ExAgs weakly and inversely correlated with the percentage of *Penicillium* infection ($r=-0.32$, $P>0.05$).

TABLE 2. The content of *A. ochraceus* ExAgs, OA, glucosamine and the number of cfus in non-autoclaved, moisturized wheat samples that were inoculated with different numbers of *A. ochraceus* spores and incubated for different periods of time (experiment 3)

Parameters assayed	Days of incubation	Number of <i>A. ochraceus</i> spores used for inoculation ^a		
		0	10^2 g^{-1}	10^5 g^{-1}
cfu ($\times 10^3 \text{ g}^{-1}$) ($\times 10^6 \text{ g}^{-1}$) ($\times 10^{11} \text{ g}^{-1}$)	0	15.6 ± 5.7	7.7 ± 10.6	16.6 ± 10.7
	7	1.4 ± 1.2	5.3 ± 7.8	2.0 ± 2.8
	30	4.2 ± 6.9	1.0 ± 1.0	2.0 ± 1.2
Glucosamine (mg g^{-1})	0	0.5 ± 0.05	0.4 ± 0.1	0.5 ± 0.1
	7	0.6 ± 0.05	0.7 ± 0.1	1.0 ± 0.1
	30	3.7 ± 1	3.5 ± 1	7.0 ± 3
OA ($\mu\text{g g}^{-1}$)	0	0.01 ± 0.01	0	0
	7	0.02 ± 0.02	12 ± 1	53 ± 9
	30	0.1 ± 0.1	100 ± 15	130 ± 17
<i>A. ochraceus</i> ExAgs ($\mu\text{g g}^{-1}$)	0	2.5 ± 0.3	9 ± 3.5	6 ± 2.5
	7	10 ± 5	55 ± 30	310 ± 250
	30	30 ± 28	345 ± 330	315 ± 135

^aValues represent means $\pm$ SD of 3–6 replicate samples.

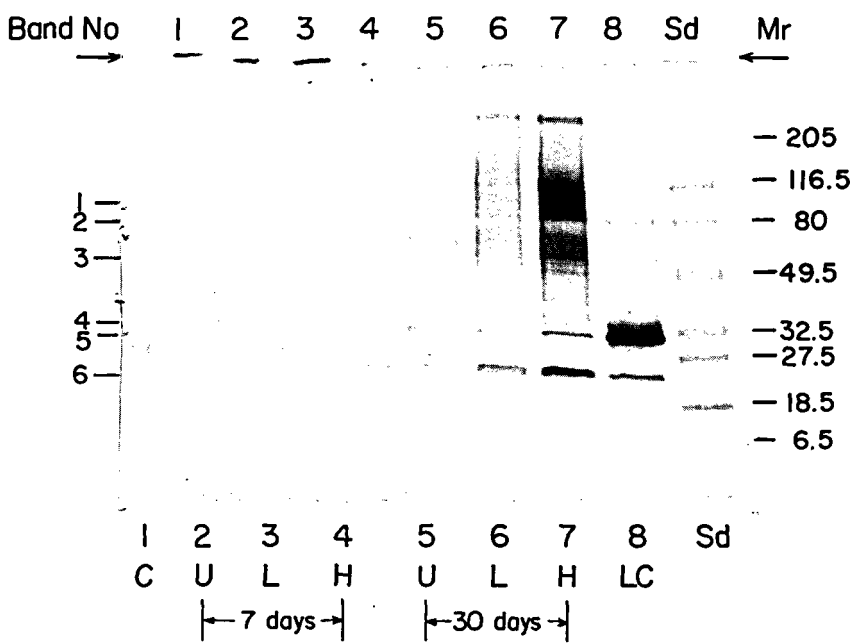


FIG. 5. Immunoblotting patterns of *A. ochraceus* ExAgs obtained from different cultures of *A. ochraceus* which were sampled at various incubation time. The ExAgs from wheat that was either un-incubated (C, lane 1), or incubated for 7 (lanes 2, 3, 4) or 30 (lanes 5, 6, 7) days, and was either un-inoculated (U, lanes 2 and 5) or inoculated with 10^2 (L, lanes 3 and 6) or 10^5 (H, lanes 4 and 7) of *A. ochraceus* spores g^{-1} of wheat were solubilized by boiling in 1.5% SDS and separated on 4–15% gradient gels. ExAgs from the liquid culture (LC, lane 8) were co-separated. The separated components were transferred on to nitrocellulose and immunostained with rabbit anti-*A. ochraceus* antibodies. The molecular weights (M_r) of the bands were estimated using pre-stained standards (Sd). The arrows indicate points where samples were applied.

DISCUSSION

The results of this study and a previous study (Lu *et al.*, 1995) demonstrated that the ELISA which used antibodies raised against the ExAgs from *A. ochraceus* could detect this fungus in the presence of other fungi that naturally occur on grain, including *P. aurantiogriseum*. *P. aurantiogriseum* is well recognized as the most common storage fungus (Williams & Bialkowska, 1985). There was also a high correlation between the concentration of ExAgs detected and the concentration of the secondary metabolite (OA) that was produced by this fungus. The assay was highly sensitive as it could detect as little as 50 ng ExAgs/ml of

TABLE 3. Correlation between the concentration of *A. ochraceus* ExAgs and other parameters used for estimating *A. ochraceus* contamination in wheat samples (experiment 3)

X variable	Y variable	Correlation coefficient (<i>r</i>)	Confidence level (<i>P</i>)
<i>A. ochraceus</i> ExAgs	OA content	0.93	<0.05
	Percentage <i>A. ochraceus</i> infection ^a	0.89	<0.05
	Glucosamine content	0.64	<0.05
	Number of cfu	0.68	<0.05
	Percentage <i>Penicillium</i> infection ^a	−0.32	>0.05

^aPercentage infection is the percentage of the kernels that were infected with *A. ochraceus* or various *Penicillium* species.

sample (i.e. 5 ng/assay). The detection level for fungi, however, would be considerably lower as the ExAgs presumably represent only a small portion of the total fungal biomass. The ELISA for *A. ochraceus* ExAgs is a much better and more direct predictor of the amount of *A. ochraceus* in the grain than the glucosamine assay as it specifically detects the presence of *A. ochraceus*, whereas the glucosamine assay only provides an estimate of the total amount of mold but not the amount of *A. ochraceus* (Donald & Mirocha, 1977). In addition, the glucosamine assay is not only considerably more difficult to conduct than the ELISA but it can also yield false positives due to insect contamination. One interesting observation was that both galactosamine and glucosamine were present in *A. ochraceus*-inoculated grain, whereas only glucosamine was present in the grain inoculated with *P. aurantiogriseum*. This difference, if shown to be general, could be used as a marker to distinguish between species or genera of fungi.

In a previous study, it was shown that the degree of cross-reactivity between the antibodies developed against *A. ochraceus* ExAgs and three other species of *Aspergillus*, seven species of *Penicillium*, three species of *Fusarium*, two species of *Mucor* and one species of *Alternaria* was either zero or less than 1% for all but one species (*A. flavus*) which had a cross-reactivity of 2% (Lu *et al.*, 1995). The current study documents that the ELISA developed by using these antibodies, is more specific than immunoassays developed previously for *Aspergillus* and *Penicillium* species by other researchers (Notermans & Heuvelman, 1985; Tsai & Cousin, 1990). The lower specificity of the previous assays may be related to the nature of the antigens used, as these assays have utilized the EPS which occur in many species of fungi. The EPS contain galactofuranoside residues, the immunodominant polysaccharides in *Aspergillus* and *Penicillium* species (Notermans *et al.*, 1988; Kamphuis *et al.*, 1989). As a result, they cannot be used to distinguish between species from these two genera. The highly conserved EPS may also be present in other biological material, including food components (Notermans & Kamphuis, 1992; Van der Horst *et al.*, 1992), which would preclude their use as specific markers for the presence of any fungus.

Another important feature of the current assay was that the antiserum did not cross-react substantially with the naturally contaminated, non-autoclaved wheat matrix. The negligible cross-reactivities of the antiserum with non-autoclaved wheat and also corn and barley has been reported (Lu *et al.*, 1995). In contrast, other assays that were developed to detect the presence of fungi cross-reacted with the matrix (Notermans & Heuvelman, 1985). These authors, however, did not exclude the possibility that the cross-reactivity was caused by natural contamination of grains with fungi.

Immunoblotting studies demonstrated that the *A. ochraceus* ExAgs were absent from autoclaved wheat samples but were present in both liquid and solid cultures of this fungus and that the type of culture influenced their relative concentrations. These studies, therefore, indicate that environmental conditions may not only affect the total amount of ExAgs produced but also the amounts of individual components of ExAgs. This would reduce the accuracy for predicting mycelial dry weight on the basis of its content of ExAgs. These shortcomings, however, are probably no worse than those obtained with other mold assays that have been used as an index of fungal biomass, including the assays for chitin (glucosamine), ergosterol and number of fungal propagules (Pitt & Samson, 1990).

The immunogens used in this study were not purified and, as indicated by immunoblotting data, consisted of several different antigenic components, a few of which were immunodominant. Many of the antigens detected with the antiserum by immunoblotting had electrophoretic mobilities that were the same as those of some of the silver-stained bands, suggesting that they may be proteinaceous in nature. Previous studies with ExAgs from *P. aurantiogriseum* indicated that crude ExAgs, as prepared for the purpose of that and the current research, are proteins and/or glycoproteins (Lu *et al.*, 1995). Additional research is required to isolate and purify the ExAgs that are unique to *A. ochraceus*, to determine the nature of the isolated antigens and to produce antibodies against them. Utilizing these

refined reagents, the solid-phase immunoassays suitable for discriminative monitoring of *A. ochraceus* contaminations in food products may be developed. A more discriminative assay is required as high concentrations of a fungus such as *P. aurantiogriseum* could, nevertheless, result in false positives, even though the cross-reaction of the antiserum with this fungus was low (less than 1%). Nevertheless, the current assay developed using the crude ExAgs seems to be superior in many respects to other types of assays that have been developed for the detection of fungi as it is capable of specifically detecting and semi-quantitatively determining the amount of *A. ochraceus* in wheat in the presence of other fungi.

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REFERENCES

- BLAKE, M. S., JOHNSTONE, K. H., RUSSELL-JONES, G. J. & GOTSCHLICH, E. C. (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots, *Analytical Biochemistry*, **136**, 175–179.
- BRADFORD, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, **72**, 248–254.
- CLARKE, J. R., MARQUARDT, R. R., OOSTERVELD, A., FROHLICH, A. A., MADRID, F. J. & DAWOOD, M. (1993) Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibodies from the yolk of the laying hen, *Journal of Agricultural and Food Chemistry*, **41**, 1784–1789.
- DEWEY, F. M., MACDONALD, M. M., PHILLIPS, S. I. & PRIESTLEY, R. A. (1990) Development of monoclonal-antibody-ELISA and -DIP-STICK immunoassays for *Penicillium islandicum* in rice grains, *Journal of General Microbiology*, **136**, 753–760.
- DONALD, W. W. & MIROCHA, C. J. (1977) Chitin as a measure of fungal growth in stored corn and soybean seed, *Cereal Chemistry*, **54**, 466–474.
- ERICKSON, P. F., MINIER, L. N. & LASHER, R. S. (1982) Quantitative electrophoretic transfer of polypeptides from SDS polyacrylamide gels to nitrocellulose sheets: a method for their re-use in immunoradiographic detection of antigens, *Journal of Immunological Methods*, **51**, 241–249.
- FRISVAD, J. C. & FILTENBORG, O. (1983) Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites, *Applied and Environmental Microbiology*, **46**, 1301–1310.
- GOLINSKI, P., HULT, K., GRABARKIEWICZ-SZCZESNA, J., CHELKOWSKI, J. & SZEBIOTKO, K. (1985) Spontaneous occurrence of ochratoxin A in porcine kidney and serum samples in Poland, *Applied and Environmental Microbiology*, **49**, 1014–1015.
- HARLOW, E. & LANE, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbour Laboratory, New York, NY, USA.
- ISO (INTERNATIONAL ORGANIZATION FOR STANDARDIZATION) (1983a) 6887E Microbiology. *General Guidance for the Preparation of Dilutions for Microbiological Examination*, Geneva, Switzerland.
- ISO (INTERNATIONAL ORGANIZATION FOR STANDARDIZATION) (1983b) TC34/SC4/WG2. *Grain, Seeds and Derived Products—Microbiological Examination—Colony Count Technique*, Geneva, Switzerland.
- JARVIS, B., SEILER, D. A. L., OULD, A. J. L. & WILLIAMS, A. P. (1983) Observations on the enumerations of molds in food and feedingstuffs, *Journal of Applied Bacteriology*, **55**, 325–336.
- KAMPHUIS, H. J., VEENEMAN, G. H., VAN BOOM, J. H., NOTERMANS, S. & ROMBOUTS, F. M. (1989) Antibodies against synthetic oligosaccharide antigens reactive with extracellular polysaccharides produced by molds, *Food and Agricultural Immunology*, **1**, 235–242.
- KAMPHUIS, H. J., DE RUITER, G. A., VEENEMAN, G. H., VAN BOOM, J. H., ROMBOUTS, F. M. & NOTERMANS, S. H. W. (1992) Detection of *Aspergillus* and *Penicillium* extracellular polysaccharides (EPS) by ELISA: using antibodies raised against acid hydrolysed EPS, *Antonie van Leeuwenhoek*, **61**, 323–332.
- KAUFMAN, L. & STANDARD, P. G. (1987) Specific and rapid identification of medically important fungi by exoantigen detection, *Annual Review of Microbiology*, **41**, 209–225.

- LAEMMLI, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680–685.
- LIN, H. H., LISTER, R. M. & COUSIN, M. A. (1986) Enzyme-linked immunosorbent assay for detection of mold in tomato purée, *Journal of Food Science*, **51**, 180–192.
- LU, P., MARQUARDT, R. R., FROHLICH, A. A. & MILLS, J. T. (1994) Detection of *Penicillium aurantiogriseum* by ELISA utilizing antibodies produced against its exoantigens, *Microbiology*, **140**, 3267–3276.
- LU, P., MARQUARDT, R. R. & KIEREK-JASZCZUK, D. (1995) Immunochemical identification of fungi using polyclonal antibodies raised in rabbits to exoantigens from *Aspergillus ochraceus*, *Letters in Applied Microbiology*, **20** (in press).
- MARQUARDT, R. R. & FROHLICH, A. A. (1992) A review of recent advances in understanding ochratoxicosis, *Journal of Animal Science*, **70**, 3968–3988.
- MARQUARDT, R. R., FROHLICH, A. A., SREEMANNARAYANA, O., ABRAMSON, D. & BERNATSKY, A. (1988) Ochratoxin A in blood from slaughter pigs in western Canada, *Canadian Journal of Veterinary Research*, **52**, 186–190.
- MILLS, J. T. & WALLACE, H. A. H. (1979) Microflora and condition of cereal seeds after a wet harvest, *Canadian Journal of Plant Science*, **59**, 645–651.
- NOTERMANS, S. & HEUVELMAN, C. J. (1985) Immunological detection of molds in food by using the enzyme-linked immunosorbent assay (ELISA); preparation of antigens, *International Journal of Food Microbiology*, **2**, 247–258.
- NOTERMANS, S., HEUVELMAN, C. J., BEUMER, R. R. & MAAS, R. (1986) Immunological detection of moulds in food: relation between antigen production and growth, *International Journal of Food Microbiology*, **3**, 253–261.
- NOTERMANS, S., WIETEN, G., ENGEL, H. W. B., ROMBOUTS, F. M., HOOGERHOUT, P. & VAN BOOM, J. H. (1987) Purification and properties of extracellular polysaccharide (EPS) antigens produced by different mould species, *Journal of Applied Bacteriology*, **62**, 157–166.
- NOTERMANS, S., VEENEMAN, G. H., VAN ZUYLEN, C. W. E. M., HOOGERHOUT, P. & VAN BOOM, J. H. (1988) (1→5)-linked β -D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species, *Molecular Immunology*, **25**, 975–979.
- NOTERMANS, S. & KAMPHUIS, H. J. (1992) Detection of fungi in foods by latex agglutination: a collaborative study, in *Modern Methods in Food Mycology* (SAMSON, R. A., HOCKING, A. D., PITT, J. I. & KING, A. D., Eds), Elsevier, Amsterdam, pp. 205–212.
- PITT, J. I. & HOCKING, A. D. (1985) *Fungi and Food Spoilage*, Academic Press, Sydney, Australia.
- PITT, J. I. & SAMSON, R. A. (1990) Approaches to *Penicillium* and *Aspergillus* systematics, *Studies in Mycology*, **32**, 77–90.
- PRIOR, M. G. & SISODIA, C. S. (1982) The effects of ochratoxin A in the immune response of Swiss mice, *Canadian Journal of Comparative Medicine*, **46**, 91–96.
- ROSCHENTHALER, R., CREPPY, E. E. & DIRHEIMER, G. (1984) Ochratoxin A: on the mode of action of an ubiquitous mycotoxin, *Journal of Toxicology and Toxin Review*, **3**, 53–86.
- ROTTER, R. G., FROHLICH, A. A., MARQUARDT, R. R. & MILLS, P. A. (1989) Estimation of fungal contamination of cereal grains as determined by measuring glucosamine concentration, *Canadian Journal of Animal Science*, **69**, 235–245.
- STANDARD, P. G., KAUFMAN, L. & WHALEY, S. D. (1985) *Rapid Identification of Pathogenic Mould Isolates by Immunodiffusion*, CDC Laboratory Manual, Atlanta, GA, USA, US Department of Health Human Service, Public Health Service, Centers for Disease Control.
- STEIN, A. F., PHILLIPS, T. D., KUBENA, L. F. & HARVEY, R. B. (1985) Renal tubular secretion and reabsorption as factors in ochratoxicosis: effects of probenecid on nephrotoxicity, *Journal of Toxicology and Environmental Health*, **16**, 593–605.
- TSAI, G.-J. & COUSIN, M. A. (1990) Enzyme-linked immunosorbent assay for detection of molds in cheese and yogurt, *Journal of Dairy Science*, **73**, 3366–3378.
- VAN DER HORST, M., SAMSON, R. A. & KARMAN, H. (1992) Comparison of two commercial kits to detect molds by latex agglutination, in *Modern Methods in Food Mycology* (SAMSON, R. A., HOCKING, A. D., PITT, J. I. & KING, A. D., Eds), Elsevier, Amsterdam, pp. 241–245.
- WILLIAMS, A. P. & BIALKOWSKA, A. (1985) Molds in Mold Spoiled Foods and Food Products. Leatherhead Food Research Association Research Report No. 527.