Detection of *Penicillium aurantiogriseum* by ELISA utilizing antibodies produced against its exoantigens

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An indirect competitive ELISA was developed using rabbit antiserum against the exoantigens (ExAgs) of *Penicillium aurantiogriseum*, and cross-reactivities were determined with 15 ExAgs from other species and genera of fungi and with the water-soluble extracts from four grains. The assay had good sensitivity to *P. aurantiogriseum* ExAgs (1 µg per ml serum), with generally low or no cross-reactivity with grain extracts and the other fungi, including five species of *Penicillium*, four species of *Aspergillus*, three species of *Fusarium*, two species of *Mucor* and one species of *Alternaria*. Immunoblotting analysis of the ExAgs from *P. aurantiogriseum* yielded at least 20 distinct antigenic bands, with about 3 or 4 being dark, 10–12 light and 5–8 faint in colour. Most of the bands had molecular masses of about 70–90 kDa. In contrast to *P. aurantiogriseum*, the ExAgs from the other fungi either did not react in the immunoblotting assay with the antiserum, or reacted only weakly with it. One antigenic band from *P. roqueforti*, however, reacted moderately strongly with the antiserum. The ELISA and immunoblotting analysis were used to detect *P. aurantiogriseum* in naturally contaminated wheat samples, and in wheat samples spiked with a second common storage fungus, *Aspergillus ochraceus*. The results demonstrated that the antiserum was able to detect *P. aurantiogriseum* in a background of other fungal species, and that these did not produce false positives. There was also a positive association between the concentration of *P. aurantiogriseum* ExAgs, as measured by ELISA, and the amount of mould as determined by the number of colony-forming units. Immunoblotting qualitatively confirmed the ELISA results obtained with fungi when cultured on liquid or on solid (wheat) media. The data suggest that the immunoassays developed for *P. aurantiogriseum* are useful for the detection and identification of *P. aurantiogriseum* on the basis of its ExAgs, with the advantages of being more efficient, simple and reliable than conventional techniques.

Keywords: *Penicillium aurantiogriseum*, ELISA, exoantigens, immunoblotting

INTRODUCTION

Mould contamination can not only cause deterioration of foods and feeds, but can adversely affect human and animal health since toxic metabolites, or mycotoxins, may be produced. The latter is of increasing concern as many different fungi have been shown to produce one or more

mycotoxin (CAST, 1989). The three major genera of mycotoxin-producing fungi are *Aspergillus*, *Penicillium* and *Fusarium*. Fusaria invade food and feed supplies mainly during production and processing, and are defined as field fungi. Aspergilli and penicillia, in contrast, mainly grow on foods and feeds during storage, and are referred to as storage fungi (CAST, 1989). These two genera include most of the economically important fungi as they are ubiquitous, can cause food spoilage and bio-deterioration, and are capable of producing many different mycotoxins (Pitt & Hocking, 1985; Pitt & Samson, 1990). *Penicillium* spp. are particularly abundant and have

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Abbreviations: ExAg, exoantigen; OA, ochratoxin A.

been shown to produce secondary metabolites belonging to five metabolite families (Lund & Frisvad, 1994). It is therefore necessary, not only to quantitatively monitor for mould contamination in foods and feeds, but also to accurately and reliably identify the fungi, as specific moulds are associated with the production of specific families of mycotoxins, each having different properties (Frisvad, 1988, 1989). Traditional fungal taxonomy, based primarily on morphology and growth characteristics, is generally considered to be tedious and time-consuming, and can lead to erroneous identification, even for experienced workers. This problem is serious, particularly with penicillia, as the large number of species, and their similar morphology, make identification especially difficult (Pitt & Samson, 1990; Samson & Frisvad, 1991). Conventional mould detection techniques include the use of culture methods, electrical measurements, microscopy and analyses for enzymes, chitin or other fungal components. These techniques suffer from low sensitivity and lack of specificity (Jarvis *et al.*, 1983; Notermans & Heuvelman, 1985; Jarvis & Williams, 1987).

Pitt & Samson (1990) have reviewed new approaches for *Aspergillus* and *Penicillium* systematics, and consider none of them to be sufficiently accurate when used alone for the detection and identification of storage fungi. Kaufman & Standard (1987) reported that the ExAgs, a soluble mixture of extracellular fungal products, could be used as indicators of the presence of fungal species or genera. They produced antibodies against the ExAgs of medically important fungi and assayed them by immunodiffusion. This method, however, is relatively insensitive and requires a large quantity of antibody. Immunoassays (ELISAs) have been developed for detecting and identifying several species of moulds but with variable success (Notermans & Heuvelman, 1985; Lin *et al.*, 1986; Notermans *et al.*, 1986; Dewey *et al.*, 1990; Fuhrmann *et al.*, 1990, 1992; Tsai & Cousin, 1990; Banks *et al.*, 1993). Currently only a few ELISAs have been developed that can specifically and quantitatively detect the presence of the common storage fungi (Dewey *et al.*, 1990). Therefore, there is a need for immunoassays that are not only capable of identifying the presence of the most common *Penicillium* species, but also of estimating their content in grain. *P. aurantiogriseum*, being the most common *Penicillium* species, was selected as a model fungus for determining whether ExAgs could be utilized in an ELISA. ExAgs of *P. aurantiogriseum* were prepared and tested by indirect competitive ELISA and SDS-PAGE, followed by Western blotting. A rapid, specific and sensitive assay for detecting and identifying *P. aurantiogriseum* was developed utilizing these ExAgs. The assay was capable of detecting *P. aurantiogriseum* in grain.

METHODS

Sources of fungi and grain. Isolates of *Penicillium aurantiogriseum* Dierckx, nos 798, 832 and 3298, were provided by Dr G. Platford, Plant Pathology Laboratory, Manitoba Agricultural Services Complex, Winnipeg MB. Isolate no. 3298 was used for all studies and isolate IBT 3471, obtained from Dr J. Frisvad, Department of Biotechnology, The Technical Uni-

versity of Denmark, DK-2800, Lyngby, Denmark, was the standard reference strain. Cultures of *Aspergillus ochraceus* Wilhelm (NRRL 3174) and *A. versicolor* (Vuill.) Tiraboschi (NRRL 573) were pure cultures maintained in our laboratory. Isolates of *Fusarium poae* (Peck) Wollenw., *F. avenaceum* (Fr.) Sacc., *F. equiseti* (Corda) Sacc., *Mucor* species and *Alternaria alternata* (Fr.) Keissler were provided by Mr R. M. Clear, Canadian Grain Commission, Grain Research Laboratory, Winnipeg MB. Cultures of *Aspergillus candidus* Link (ATCC 44054), *A. flavus* var. *columnaris* Link (ATCC 44310), *Penicillium chrysogenum* Thom (IBT 3359), *P. commune* Thom (IBT 10501) and *P. roqueforti* var. *roqueforti* Thom (IBT 5229) were provided by Dr J. T. Mills, Agriculture Canada, Winnipeg Research Centre. The identities of *Penicillium* species were confirmed using procedures described by Frisvad & Filtenborg (1989) and Mills *et al.* (1994). Grains and soybean were from local sources and did not contain any visible fungal biomass.

Materials. The following were purchased: potato dextrose agar and yeast extract from Becton Dickinson; microtitre plates (Falcon 3911 Microtest III Flexible Assay Plates; Becton Dickinson); ammonium sulfate from Fisher Scientific Chemical; Whatman no. 1 filter paper from Whatman; sterile filters from Nalgene, Nalge; Minicon CS-15 spinal fluid concentrator from Amicon; reagents for preparing PBS from Mallinckrodt Canada; and the ultra-pure reagents for electrophoresis and immunoblotting from ICN, Schwarz/Mann Biotech and Bio-Rad. Other reagents were from Sigma. All solvents and reagents were of analytical grade. The microplate reader (model 450), electrophoresis tank (Mini-PROTEAN II) and electroblotting device (Transblot SD Semi-Dry Transfer Cell) were from Bio-Rad.

Preparation of ExAgs. ExAgs from all the fungal species listed above were prepared according to Standard *et al.* (1985) with some modifications. Briefly, fungal spores from different genera that had been propagated on potato dextrose agar were used to inoculate sucrose-yeast-mineral (SYM) liquid medium. After two 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. Filtrates were freeze-dried and stored at -20 °C. The freeze-dried material that was used for immunization or ELISA was redissolved in 0.1 M PBS (0.1 M sodium phosphate, 0.145 M NaCl, pH 7.2), dialysed for 24 h against five changes of PBS and concentrated in a Minicon. Particulate matter was removed by centrifuging at 20000 g for 30 min at 4 °C. The antigen preparation used for the ELISA was aliquoted and stored at 4 °C with the addition of 0.01 % (w/v) Thimerosal (sodium ethylmercurithiosalicylate). Thimerosal-free antigens used for immunization were sterilized by passing through a 0.22 µm filter. These preparations were referred to as ExAgs. The protein concentration of these and other samples was determined using the procedure of Bradford *et al.* (1976).

Extracellular protein and extracellular polysaccharide antigens were prepared from ExAgs by ammonium sulfate precipitation (Tsai & Cousin, 1990). Briefly, the freeze-dried ExAgs of *P. aurantiogriseum* were redissolved in PBS and the proteins were precipitated by adding ammonium sulfate to 80 % saturation. The precipitate was redissolved in PBS. This fraction, and the supernatant containing polysaccharides, were dialysed against saline and concentrated. Sugar quantification was performed according to the method of Dubois *et al.* (1956).

Preparation of water extracts of grain. Clean wheat, barley, maize and soybean samples were ground using a Cyclotec sample mill equipped with a 1 mm screen (Tecator); 50 g of the ground sample was mixed with 100 ml SYM liquid medium and shaken vigorously for 1 h on a wrist action shaker (model 75, Burrell).

The suspensions were centrifuged at 20 000 *g* at 4 °C for 30 min, and the supernatants were carefully removed, concentrated and aliquoted. All stock antigen preparations were stored at -20 °C; the working solutions were stored at 4 °C.

Immunization and preparation of antibody. Two rabbits (Dutch Belted, 1–1.5 kg body weight) were injected subcutaneously (three sites) with sterile ExAgs of *P. aurantiogriseum* isolate 3298. The antigen preparation was emulsified with complete Freund's adjuvant (first injection), or incomplete Freund's adjuvant (booster injections), and administered at a dosage of 1 mg protein at 3-week intervals. Rabbits were bled at weekly intervals, starting one week after the third injection, and serum antibody response was assayed by immunodiffusion (Standard *et al.*, 1985). The rabbit of choice was killed and exsanguinated when the desired antibody response was obtained, and serum was harvested, aliquoted and stored at -80 °C.

ELISA titre assay. The protocols were those of Harlow & Lane (1988). Briefly, microtitre plates were coated with 0.1 µg per well of *P. aurantiogriseum* ExAgs in 100 µl PBS and incubated overnight at 4 °C. After three washings with PBS containing 0.05 % (v/v) Tween 20 (PBST), the plates were blocked with 5 % (w/v) skimmed milk in PBS for 2 h at 37 °C. Serial dilutions of the antiserum (100 µl per well) together with the negative control serum (before immunization) were added to the plates, and after 2 h incubation at 37 °C the plates were washed three times with PBST. The plates were incubated for an additional 1.5 h with goat anti-rabbit IgG conjugated to alkaline phosphatase. *p*-Nitrophenyl phosphate solution (100 µl) was added after six washings with PBST, and the plates were read at 405 nm. The optimal antigen and antibody concentrations for the competitive inhibition assay were determined using a checker-board titration assay.

Competitive inhibition assay. The procedures were essentially the same as those of the titre assay except that 50 µl of a single fixed dilution of the antiserum (1:3000) was used after the addition of 50 µl of fungal ExAgs. The protein concentration of the competing antigen was usually 0.5 mg ml⁻¹ for *P. aurantiogriseum*, and up to 10 mg ml⁻¹ for antigens from other species. The concentration of ExAgs, expressed as concentration of proteins which inhibited 50 % of the antibody binding, was calculated from the competitive-inhibition curve and referred to as the sensitivity (Tsai & Cousin, 1990). The degree of reactivity (expressed as percentage cross-reactivity) of different fungal ExAgs to anti-*P. aurantiogriseum* serum was calculated by comparing the sensitivity of the fungal ExAgs with that of *P. aurantiogriseum* (scale set at 100). The assays were done twice in duplicate and the results were averaged.

Extracellular protein and extracellular polysaccharide antigens of *P. aurantiogriseum* were also tested as inhibiting antigens by applying them at known concentrations. Unfractionated *P. aurantiogriseum* ExAgs were used as the control. The sensitivity and reactivities were calculated on the basis of the protein and sugar concentrations of these fractions.

SDS-PAGE and immunoblotting analysis. ExAgs were solubilized in 1.5 % (w/v) SDS at 100 °C for 5 min, and separated by SDS-PAGE according to the method of Laemmli (1970) using a 4–20 % (w/v) T gradient gel (Mini-PROTEAN II, Ready Gel, Bio-Rad). The pre-stained standard proteins (Bio-Rad) were myosin, β -galactosidase, phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin. Their respective molecular masses were 205, 116.5, 106, 80, 49.5, 32.5, 27.5, 18.5 and 6.5 kDa. The separated components were either visualized by silver staining

or transferred onto nitrocellulose membranes (0.22 µm, Bio-Rad) in a semi-dry transblot cell using 25 mM Tris, 192 mM glycine buffer (pH 8.3) containing 20 % (v/v) methanol and 1.3 mM SDS (0.00375 %). The membrane was washed for 1 h with TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 10 % methanol and blocked for 2 h with 3 % (w/v) BSA. After washing with TBS containing 0.05 % (v/v) Tween 20 (TBST), the membrane was incubated for 1.5 h with the antiserum (1:5000) diluted in TBST containing 1 % (v/v) gelatin (antibody buffer). The membrane was then incubated for 1 h in goat anti-rabbit IgG conjugated with alkaline phosphatase diluted 1:8000 in antibody buffer. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, *p*-toluidine salt) were used as the substrate (Blake *et al.*, 1984). All the incubations were at room temperature with agitation unless otherwise specified. Electrophoresis and electroblotting were performed following the manufacturer's instructions (Bio-Rad).

Detection of *P. aurantiogriseum* ExAgs in wheat. Wheat samples were moisturized with distilled water to a final water content of 22 % (w/w), inoculated with 0, 10² or 10⁵ *A. ochraceus* spores g⁻¹, and incubated for up to 30 d at 30 °C. The number of replicates in each group was 15. Five samples were taken from each group on days 0, 7 and 30, and were dried and ground as described above. The same samples were analysed for *P. aurantiogriseum* ExAgs using the ELISA and immunoblotting procedures, and for glucosamine (Rotter *et al.*, 1989), ochratoxin A (OA) (Clarke *et al.*, 1993), the number of c.f.u. (International Organization for Standardization, 1983a, b) and for type of mycoflora (Mills & Wallace, 1979). The procedure for detecting *P. aurantiogriseum* ExAgs in wheat samples using the ELISA was the same as for the competitive inhibition assay, except that mouldy wheat extracts were used as the source of antigen. The wheat extracts were prepared by mixing 5 g of the wheat sample with 25 ml PBS followed by vigorous shaking for 1 h and centrifuging at 20 000 *g* for 45 min. The supernatant was filtered (Whatman no. 1 filter paper) and used for the competitive inhibition assay along with appropriately diluted rabbit anti-*P. aurantiogriseum* serum. The concentration of ExAgs present in the grain extracts was calculated from the standard inhibition curves obtained during the same assay. Selected PBS extracts were also solubilized and analysed using the immunoblotting SDS-PAGE described for fungal ExAgs.

RESULTS

Confirmation of the identity of *P. aurantiogriseum*

P. aurantiogriseum isolates 798, 832 and 3298 had similar morphological and chemical characteristics to isolate IBT 3471 identified as *P. aurantiogriseum* Dierckx (Lund & Frisvad, 1994). Agar plugs of isolates 798, 832 and 3298, when placed on TLC plates, exhibited light blue and light brown bands of aurantiamine and verrucosidin, respectively, in the same positions as those obtained with aurantiamine and verrucosidin standards and IBT 3471. Isolates 798, 832 and 3298 were therefore confirmed to be *P. aurantiogriseum* Dierckx (Mills *et al.*, 1994).

Optimization of ELISA

The checker-board titration assay showed that the optimal concentration of the coating antigens was 0.05–0.5 µg protein per well. Optimal sensitivities were obtained when the antiserum dilutions were between 1:3000 and 1:6000 (data not shown).

Fig. 1. Typical competitive inhibition curves of *P. aurantiogriseum* isolate 3298 (●) and *P. roqueforti* (◇) with rabbit antiserum raised against *P. aurantiogriseum* ExAgs. Arrows indicate the antigen concentration which caused a 50% reduction in antibody binding, and is the value used to denote sensitivity. Values represent mean $\pm$ SD of duplicate assays. See Methods for further detail.

Detection of specific ExAgs of *P. aurantiogriseum* by competitive ELISA

Typical competitive inhibition curves obtained with rabbit serum against *P. aurantiogriseum* isolate 3298, and the ExAgs from two different species of *Penicillium* are illustrated in Fig. 1. A total of 16 ExAgs from five genera of fungi were used to test the specificity of the antibody (Table 1). Included were six species of *Penicillium*, four of *Aspergillus*, three of *Fusarium*, two of *Mucor* and one of

Alternaria, and three extracts from cereals and one from soybeans. The results demonstrated that the antiserum directed against *P. aurantiogriseum* ExAgs was highly reactive to the ExAgs of *P. aurantiogriseum*, reacted to a negligible degree with the ExAgs of the other fungal species (< 5% cross-reactivity), and did not react with extracts from grain or soybeans. The sensitivity of the assay, for three duplicate assays, against *P. aurantiogriseum* ExAgs, as measured by the concentration of protein which inhibited antibody binding by 50%, was approximately $1 \mu\text{g ml}^{-1}$, with the intra-assay coefficient of variation being 10% and that for inter-assay variation being 30%. The lower limit of detection, which was calculated as the mean $\pm$ 2 SD (i.e. 20% coefficient of variation), was approximately 50 ng ml^{-1} . The detection limits for the fungus itself would be approximately 4000-fold higher ($200 \mu\text{g ml}^{-1}$) as the ExAgs comprised only a small portion of total fungal mass [i.e. $0.26 \pm 0.05 \text{ mg ExAgs (g fungal dry mass)}^{-1}$].

Composition of ExAgs as determined by silver staining and immunoblotting

Silver staining profiles of the ExAgs from different fungal species yielded more than 20 bands (data not shown). Immunoblotting analysis of the ExAgs from three different isolates of *P. aurantiogriseum* revealed several distinct bands (Fig. 2). The immunoblotting and the silver staining (data not shown) profiles of the three isolates were similar. The molecular masses of these bands varied from less than 18.5 kDa to more than 106 kDa, with approximately 3 or 4 yielding dark bands, 10–12 light bands and 5–8 faint bands. Most of the immunodominant bands were between about 70 and 90 kDa. Individual band intensity varied somewhat among the different isolates of *P. aurantiogriseum*. In contrast to the above results, the reaction of the antiserum with the ExAgs from

Table 1. Cross-reactivities of different ExAgs with rabbit antiserum raised against the ExAgs of *P. aurantiogriseum* isolate 3298

Cross-reactivity was calculated from the competitive inhibition curve by comparing the sensitivity (antigen concentration that caused 50% reduction in antibody binding) of different fungal antigens with that of *P. aurantiogriseum* (taken as 100%). Values represent mean $\pm$ SD of two assays carried out in duplicate. See Methods for further detail.

ExAgs	Cross-reactivity (%)	ExAgs	Cross-reactivity (%)
<i>P. aurantiogriseum</i>	100	<i>F. avenaceum</i>	1.0 ± 0
<i>P. chrysogenum</i>	0.1 ± 0.03	<i>F. equiseti</i>	1.0 ± 0
<i>P. citrinum</i>	0.5 ± 0.4	<i>F. poae</i>	0.2 ± 0
<i>P. commune</i>	0.2 ± 0.01	<i>Mucor</i> sp. 1	1.0 ± 0
<i>P. roqueforti</i>	0.9 ± 0	<i>Mucor</i> sp. 2	1.0 ± 0
<i>P. verrucosum</i>	0 ± 0	<i>Al. alternata</i>	4.8 ± 1.9
<i>A. candidus</i>	0.7 ± 0.3	Barley extract	0 ± 0
<i>A. flavus</i>	1.0 ± 0	Maize extract	0 ± 0
<i>A. ochraceus</i>	4.5 ± 0.1	Soy extract	0 ± 0
<i>A. versicolor</i>	0.7 ± 0.3	Wheat extract	0 ± 0

Fig. 2. Immunoblotting fingerprints of different fungal ExAgs using rabbit anti-*P. aurantiogriseum* serum. The specific ExAgs are from *P. aurantiogriseum* isolates 798, 832 and 3298 (lanes 1, 2 and 3, respectively); *P. roqueforti* (lane 4); *A. ochraceus* (lane 5); *A. flavus* (lane 6); *F. poae* (lane 7). Lane 8 contains pre-stained molecular mass standards. See Methods for further detail. The amount of proteins applied on each lane was 1.7 µg. Replicate analysis gave identical patterns.

Table 2. Inhibition of *P. aurantiogriseum* isolate 3298 ExAg fractions containing different amounts of protein and carbohydrate by anti-*P. aurantiogriseum* serum

ExAgs fraction	Protein (µg µl ⁻¹)	Carbohydrate (µg µl ⁻¹)	Protein: carbohydrate ratio	Sensitivity* (µg µl ⁻¹)		Cross-reactivity† (%)	
				Protein	Carbohydrate	Protein	Carbohydrate
Crude extract	0.12	17	0.007	0.0002	0.001	100	100
Carbohydrate	0.03	11	0.003	ND	0.004	ND	25
Protein	0.12	0.5	0.24	0.0003	ND	67	ND

ND, Not done.

* Concentration of protein or carbohydrate required to cause 50 % inhibition of antibody binding. See Table 1 and Methods for further detail.

† See legend to Table 1.

the other species of fungi was negligible. Only one band from *P. roqueforti* (approximately 80 kDa, lane 4) reacted with the antiserum, with traces of the same band being observed with *A. ochraceus*, *A. flavus* and *F. poae* ExAgs (lanes 5–7). Two bands from *A. ochraceus* (lane 5) of approximately 32 and 34 kDa also reacted either moderately or weakly, respectively, with the antibody. One band from each of *A. flavus* (lane 6) and *F. poae* (lane 7) ExAgs, having respective molecular masses of approximately 14 kDa and 16 kDa, reacted slightly with the antibody.

Competitive properties of ammonium-sulfate-fractionated antigens

Ammonium-sulfate-precipitated ExAgs from *P. aurantiogriseum* had the same concentration of protein (0.12 µg

µl⁻¹) as that of the unfractionated sample, but the ExAg carbohydrate concentration of the precipitated fraction was 35-fold lower (0.5 vs 17 µg µl⁻¹). The supernatant fraction had a similar content of carbohydrates (11 vs 17 µg µl⁻¹) and fourfold reduced content of protein (0.03 vs 0.12 µg µl⁻¹). Removal of the majority of carbohydrates from the extract only slightly increased the concentration of extract required to achieve the same degree of inhibition (i.e. sensitivity, 0.0003 vs 0.0002 µg protein µl⁻¹, Table 2). In contrast, when the sensitivity of the carbohydrate-rich supernatant was compared with that of the unfractionated extract, there was a fourfold decrease in sensitivity (0.004 vs 0.001 µg carbohydrate µl⁻¹), which also corresponded to a fourfold decrease of protein in the sample. The latter effect may therefore be primarily attributed to the residual protein that remained in the supernatant fraction, and not to the carbohydrate component. These results suggest

Table 3. Percentage of samples infected with the indicated fungi before and after incubation on non-sterilized and moisturized wheat that was inoculated with different concentrations of *A. ochraceus* spores

Wheat samples were moisturized with distilled water to a final water content of 22% (w/w) and conditioned overnight at 4 °C. Results were obtained from Whatman no. 1 filter paper culture as outlined in Methods. Values represent mean $\pm$ SD for 3 replicates.

Species or genus of fungi identified	Incubation (d)	Percentage of samples infected			
		Control wheat*	Incubated wheat		
			No. of <i>A. ochraceus</i> spores g ⁻¹ :		
			0	10 ²	10 ⁵
<i>Penicillium</i>	0	53 $\pm$ 1	21 $\pm$ 15	41 $\pm$ 29	42 $\pm$ 24
	7	—	100 $\pm$ 0	85 $\pm$ 28	27 $\pm$ 23
	30	—	100 $\pm$ 0	76 $\pm$ 23	0 $\pm$ 0
<i>A. ochraceus</i>	0	43 $\pm$ 13	7 $\pm$ 6.6	7 $\pm$ 5.7	56 $\pm$ 10
	7	—	0 $\pm$ 0	16 $\pm$ 13	100 $\pm$ 0
	30	—	0 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
<i>A. flavus</i>	0	17 $\pm$ 5	29 $\pm$ 11	41 $\pm$ 42	21 $\pm$ 12
	7	—	1 $\pm$ 2	0 $\pm$ 0	0 $\pm$ 0
	30	—	42 $\pm$ 41	52 $\pm$ 35	25 $\pm$ 25

* Control, uninoculated and unincubated.

Table 4. Changes in level of *P. aurantiogriseum* ExAgs, OA, glucosamine and c.f.u. with time, in non-sterilized and moisturized wheat samples inoculated with different concentrations of *A. ochraceus* spores

ExAgs were quantified using the ELISA. C.f.u., glucosamine and OA were analysed using the procedures of ISO (1983a, b), Rotter *et al.* (1989), and Clarke *et al.* (1993), respectively. The correlation coefficients between the concentration of *P. aurantiogriseum* ExAgs and other parameters were: 0.63 ($P < 0.05$) for the number of c.f.u., 0.47 ($P > 0.05$) for the percentage of *Penicillium* infection, 0.43 ($P > 0.05$) for glucosamine concentration, -0.22 ($P > 0.05$) for the percentage of *A. ochraceus* infection and -0.05 ($P > 0.05$) for OA concentration, respectively. Values represent means $\pm$ SD for five replicates.

Parameter	Incubation (d)	Level of analysed constituents			
		Control wheat*	Incubated wheat		
			No. of <i>A. ochraceus</i> spores g ⁻¹ :		
			0	10 ²	10 ⁵
<i>P. aurantiogriseum</i> ExAgs (μ g ml ⁻¹)	0	0.2 $\pm$ 0.2	0.3 $\pm$ 0.05	0.09 $\pm$ 0.1	0.2 $\pm$ 0.2
	7	—	2.1 $\pm$ 1.1	1.3 $\pm$ 0.6	1.2 $\pm$ 0.5
	30	—	67 $\pm$ 32	13 $\pm$ 6.5	7.3 $\pm$ 12
C.f.u. (log ₁₀ g ⁻¹)	0	4.4 $\pm$ 4.2	4.2 $\pm$ 3.7	3.9 $\pm$ 3.9	4.2 $\pm$ 3.9
	7	—	7 $\pm$ 7.3	6.3 $\pm$ 6	8.1 $\pm$ 8.2
	30	—	12 $\pm$ 12	11 $\pm$ 11	12 $\pm$ 12
Glucosamine [mg (g grain) ⁻¹]	0	0.4 $\pm$ 0.06	0.5 $\pm$ 0.05	0.4 $\pm$ 0.06	0.5 $\pm$ 0.1
	7	—	0.6 $\pm$ 0.05	0.7 $\pm$ 0.1	1.0 $\pm$ 0.1
	30	—	3.7 $\pm$ 1	3.5 $\pm$ 1	7.0 $\pm$ 3
OA (mg kg ⁻¹)	0	0.03 $\pm$ 0.03	0.01 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0
	7	—	0.02 $\pm$ 0.02	12 $\pm$ 1	53 $\pm$ 9
	30	—	0.1 $\pm$ 0.1	100 $\pm$ 15	130 $\pm$ 7

* Control, uninoculated and unincubated.

Fig. 3. Immunoblotting detection of ExAgs in mouldy wheat extracts (lanes 1–8) as compared to *P. aurantiogriseum* isolate 3298 ExAgs from liquid culture (lane 9) using rabbit anti-*P. aurantiogriseum* serum. The specific extracts were control (lane 1); from non-sterilized wheat (replicates in lanes 2, 3 and 4); and non-sterilized wheat inoculated with a low (replicates in lane 5 and 6) or a high (replicates in lanes 7 and 8) number of *A. ochraceus* spores following the incubation of non-sterilized grain for 30 d. Samples were solubilized by boiling in 1.5% SDS and separated on 4–15% gradient gels. Lane 10 contains pre-stained molecular mass standards. See Table 3 and Methods for further detail. The quantity of reference standard *P. aurantiogriseum* ExAgs applied on lane 9 was 1.7 µg. Replicate analysis gave identical patterns.

that the protein and/or the glycoprotein, and not the carbohydrate components, were primarily responsible for the inhibitory effects of the ExAgs.

Detection of *P. aurantiogriseum* contamination in wheat samples

Grain naturally infected with fungal spores was inoculated with different concentrations of *A. ochraceus* spores, and the profile of fungal infection was followed after moisturization of the grain and incubation for 0, 7 or 30 d at 30 °C. Analysis of the grain for particular genera and species of fungi prior to incubation demonstrated that it contained spores of *Penicillium* spp., *A. ochraceus* and *A. flavus* (Table 3). A visual inspection of the grain indicated that there was considerable mould growth in all samples after 7 d incubation and very pronounced growth after 30 d. A dramatic increase in contamination of grain with *Penicillium* spp. (21 to 100%) occurred when moisturized, uninoculated grain was incubated for 30 d. There was a corresponding decrease in *A. ochraceus* (7 to 0%) and an increase in *A. flavus* (29 to 42%) contaminations. Samples in the different treatments were also analysed for total number of c.f.u. and glucosamine as indicators of total fungal biomass in the grain, and for OA and *P. aurantiogriseum* ExAgs as indicators of the presence of *A.*

ochraceus and *P. aurantiogriseum*, respectively (Table 4). The data showed that the concentration of *P. aurantiogriseum* ExAgs in naturally infected (uninoculated) grain, as detected by the ELISA, increased dramatically during the time course of the study, from 0.3 µg ml⁻¹ in the uninoculated wheat to 67 µg ml⁻¹ after 30 d. This increase was accompanied by increases in the concentration of glucosamine, an indicator of fungal mass, and c.f.u. but not in the concentration of OA. In contrast, samples of grain which were inoculated with *A. ochraceus* spores and incubated for 7 or 30 d showed a much lower increase in the concentration of *P. aurantiogriseum* ExAgs (a maximum difference of 10-fold), but a great increase in OA concentration (maximum difference of 300-fold). The glucosamine content and the number of c.f.u. in inoculated groups increased dramatically (e.g. 0.5 to 7.0 mg for glucosamine, and 10⁴ to 10¹² c.f.u. per g grain) and were similar to each other and to those in the uninoculated samples after 30 d of incubation. The concentration of *P. aurantiogriseum* ExAgs in the grain samples was positively correlated with the c.f.u. counts (footnotes of Table 4; $r = 0.63$, $P < 0.05$). The correlation coefficients between the concentration of *P. aurantiogriseum* ExAgs and the other parameters were much lower ($P > 0.05$).

The results of immunoblotting on selected wheat samples

showed that the rabbit anti-*P. aurantiogriseum* serum detected more antigens in samples where *Penicillium* predominated than in samples where *A. ochraceus* predominated (Fig. 3), and did not react with control (uninoculated and unincubated) wheat. The immunoblotting pattern of *P. aurantiogriseum* ExAgs obtained from liquid culture had a similar pattern to those obtained from the wheat extracts prepared from inoculated grain. The same antigenic bands were present in all samples, except some which were present at very low levels as indicated by the faint bands. These results are consistent with the concentration of *P. aurantiogriseum* ExAgs (Table 4) that was detected using the ELISA. They demonstrate that the growth of *P. aurantiogriseum* in wheat naturally infected with this fungus was suppressed when the wheat was inoculated with *A. ochraceus*.

DISCUSSION

The ELISA employed in this study demonstrated that the rabbit anti-*P. aurantiogriseum* serum was specific for *P. aurantiogriseum*, and that it had little or no cross-reactivities with five other *Penicillium* species, four species of *Aspergillus*, three *Fusarium* species, two *Mucor* species and one *Alternaria* species. The ExAg profiles obtained by immunoblotting following SDS-PAGE also confirmed these observations, as there were at least 15 common bands among *P. aurantiogriseum* isolates, and at most only a few of these bands (one or two) were common to other species. Immunoblotting analysis was a particularly useful tool, as it not only detected the presence of very small quantities of antigens, but also revealed some physico-chemical properties of the antigenic components. The potential usefulness of the ELISA was further confirmed in naturally infected wheat that was inoculated with *A. ochraceus* spores. There was a dramatic increase in total fungal biomass for all treatments after a 30 d incubation period as indicated by the glucosamine level and the number of c.f.u., with the final composition of fungi being influenced by the degree of infection with *A. ochraceus*. Grain samples that had a high percentage of *Penicillium* had a much higher concentration of the *P. aurantiogriseum* ExAgs than those that had a high percentage of *A. ochraceus*.

The results from this study therefore suggest that antibodies against the ExAgs from *P. aurantiogriseum* can be used to specifically detect *P. aurantiogriseum* in the presence of other species of *Penicillium* or other genera of fungi, and that the method is reproducible and sensitive, with the detection limit being as low as 50 ng ExAg protein ml⁻¹. In addition, anti-*P. aurantiogriseum* serum did not cross-react with water-soluble plant proteins (wheat, barley, maize and soybean), indicating that *P. aurantiogriseum* ExAgs ELISA can be used to specifically detect this fungus in a plant matrix. Several other researchers have also developed ELISA for the detection of common moulds in foods, including *Mucor*, *Fusarium*, *Aspergillus* and *Penicillium* (Notermans & Heuvelman, 1985), *Alternaria*, *Geotrichum* and *Rhizopus* in tomato purée (Lin *et al.*, 1986), and *Cladosporium*, *Geotrichum* and

Mucor in dairy products (Tsai & Cousin, 1990). All of these assays appear to have limitations, as they are not species- or even genus-specific (Notermans & Kamphuis, 1992; Van der Horst *et al.*, 1992; Banks *et al.*, 1993). One possible reason for this lack of specificity is that in these studies the antigens were mostly heat-stable and may be polysaccharide in nature. These polysaccharides are not only present in several fungal genera, but may also be present in food products such as walnuts (Preston *et al.*, 1970; Notermans & Kamphuis, 1992; Van der Horst *et al.*, 1992). One exception is the monoclonal-antibody-based ELISA and 'DIP-STICK'-type assay for *P. islandicum* that was developed by Dewey *et al.* (1990). The antibody was generally species-specific, and the assay may be considered to be the first species-specific assay that has been developed for the detection of *Penicillium* species in grain.

The ExAgs used in this study were a mixture of excreted macromolecules (10–120 kDa), their nature being generally unknown. Ammonium sulfate fractionation, however, demonstrated that they may have been composed of proteins, glycoproteins and carbohydrates, with most of the reactivity of the anti-*P. aurantiogriseum* serum being attributable to the former two rather than the latter component of the ExAgs. Among the many protein bands that were separated by electrophoresis, only three or four, of molecular mass 70–90 kDa, were immunodominant. Purification of ExAgs, and possibly the use of monoclonal antibody, may therefore be necessary for developing more specific assays. The ExAgs, nevertheless, appear to be particularly useful immunogens, as extraction procedures are simple, they appear to be unique for certain fungal species and they can be readily utilized in an ELISA format.

Previous studies, however, have been inconclusive with regard to the nature of the antigens obtained from fungal extracts. Polonelli *et al.* (1986), working with the ExAgs of *Microsporium canis*, reported that the antigenic components in isolates of this species were protein in nature and could be utilized for immunoidentification and typing of dermatophytes. Yu *et al.* (1990), in studies with homogenates of *A. fumigatus*, also concluded that the antigens from this fungus were protein. Hearn *et al.* (1990) concluded that the electrophoretically separated antigenic moieties from *A. fumigatus* mycelial extracts were predominantly glycoprotein, and that both carbohydrate and protein residues were involved in the binding of the antigen to the antibody. Similarly, MacDonald *et al.* (1989) demonstrated that the low molecular mass surface glycoproteins were the major specific antigens in different species of fungi. Notermans & Kamphuis (1992), as discussed above, developed antibodies against extracellular polysaccharides.

The procedure for producing the ExAgs is simple, and antiserum against the ExAgs can be readily produced in rabbits, the yolk of the laying hen (unpublished data), and probably other animal species in large amounts. The antibodies can be used in an ELISA, for immunoblotting studies and probably in a double-diffusion assay as

originally used by Kaufman & Standard (1987). As a result, this procedure can be used under a wide variety of conditions, and could be readily adapted to conditions in third-world countries as the requirement for equipment is minimal and the cost per assay is low. It is concluded that the ExAgs-ELISA and immunoblotting tests are sensitive, quantitative and sufficiently specific to be used for the identification and detection of *P. aurantiogriseum* and closely related species in cereal grains. Further studies on a broader spectrum of fungi, under different growth conditions, are needed to show that the ELISA developed in this study can be used for routine screening of *P. aurantiogriseum* contamination in grain. The results also suggest that the ExAgs can be used to develop similar specific assays for other toxicologically important fungi. Recent studies in our laboratory have demonstrated that solid-phase immunoassays of *A. ochraceus* ExAgs were also specific and could be used to detect the presence of this fungus in stored grain (Lu *et al.*, 1994).

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