THE UNIVERSITY OF MANITOBA

THE DEVELOPMENT OF SOLID-PHASE IMMUNOASSAYS FOR THE STORAGE FUNGI, *ASPERGILLUS OCHRACEUS* AND *PENICILLIUM AURANTIOGRISEUM*

BY

PING LU

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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THE DEVELOPMENT OF SOLID-PHASE IMMUNOASSAYS FOR THE STORAGE FUNGI, *ASPERGILLUS OCHRACEUS* AND *PENICILLIUM AURANTIOGRISEUM*

BY

PING LU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Lu, Ping. Ph.D, The University of Manitoba, May, 1994. The Development of solid-phase immunoassays for the storage fungi, *Aspergillus ochraceus* and *Penicillium aurantiogriseum*. Major Professor; Marquardt, R.R. Mould contamination in cereals needs to be easily and accurately monitored as their mycotoxins may have serious detrimental effects on human and animal health. Conventional mould detection methods cannot fulfil this as they have inherent drawbacks. Two competitive enzyme-linked immunosorbent assay (ELISA) were developed for detecting and identifying *Penicillium aurantiogriseum* and *Aspergillus ochraceus* using rabbit antisera against exoantigens (ExAgs) of the two species of fungi. These ExAgs and those from other species and genera of fungi together with cereal extracts were characterized and compared using the ELISAs, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting analyses. The ELISA for *P. aurantiogriseum* was highly cross-reactive to the ExAgs of two closely related species, *P. verrucosum* and *P. citrinum,* but did not significantly cross-react with the ExAgs obtained from other fungi. Nor did it react with water extracts of cereal grains. This ELISA was also highly sensitive to the ExAgs of *P. aurantiogriseum* (sensitivity was 95 ng/ml). Silver staining and immunoblotting profiles following SDS-PAGE indicated that the ExAgs from the three related species had different protein and immunoblotting patterns and that they were different from those of other ExAgs and the grain extracts. These ExAgs had molecular weight (M_r) of between 10,000 to 120,000 with the immunodominant ExAgs having M_r of between 70,000 to 90,000. Fractionation studies suggest that the protein components were responsible for most of the inhibitory effects of the ExAgs. The second immunoassay involved the development of an immunoassay for *A.*

ochraceus. The anti-*A. ochraceus* serum did not cross-react significantly with ExAgs from any other moulds or with grain extracts, and was highly sensitive for the ExAgs of *A. ochraceus* (the limit of detection was 50 ng/ml). Immunoblotting results indicate that the immunodominant antigen had a M_r of about 30,000 with two other prominent antigens having M_r of approximately 20,000 and 30,000 and that some of the ExAgs from *A. candidus* cross-reacted with the rabbit anti-*A. ochraceus*. A series of experiments was conducted to evaluate the usefulness of the ELISA for monitoring *A. ochraceus* in wheat samples and in comparison with other methods. The results demonstrated that the assay could be used to specifically detect *A. ochraceus* in samples spiked with *P. aurantiogriseum* ExAgs, in sterile wheat samples inoculated with *A. ochraceus* and in wheat samples containing natural mycoflora and different amounts of *A. ochraceus*. The amount of *A. ochraceus* ExAgs in the sample was positively correlated with the amount of ochratoxin A ($r=0.93$, $P<0.05$), the percentage of *A. ochraceus* infection ($r=0.89$, $P<0.05$), the number of fungal propagules ($r=0.68$, $P<0.05$) and the amount of glucosamine as an indicator of fungal mass ($r=0.64$, $P<0.05$), and appeared to be inversely correlated with the percentage of *Penicillium* infection ($r=-0.32$, $P>0.05$). Immunoblotting analysis generally agreed with the ELISA results and indicated that the *A. ochraceus* ExAgs detected in mouldy-wheat extracts were similar to those obtained from liquid culture. These results suggest that an ELISA using fungal ExAgs can be used for the detection and identification of *P. aurantiogriseum* and *A. ochraceus* in wheat grains, and that the assays are simple, efficient, sensitive, and generally accurate and specific.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my supervisor Dr. R. R. Marquardt for his guidance, encouragement, understanding and humour throughout the course of this research. I would also like to thank Drs. A. A. Frohlich, K.A. Seifert, D. Kierek-Jaszczuk, R. D. Hill and J. Zawistowski for their valuable advice. I am also indebted to Dr. M.S. Madhyastha for reviewing of thesis. Special thanks are also extended to Mr. J. R. Clarke, Ms. H. Stelsovsky, Ms. N. Wang, Ms. S. Li, Mr. D. Kirsch and all the other colleagues working in rooms 106 and 124 in Animal Science building for their support and help in various aspects during my Ph.D study. I am grateful to Dr. J. T. Mills for his interest in my work and critical review of thesis and to Mr. R. M. Clear for providing of fungal spores and some advice on mycology. The technical help offered by Mr. P.A. Mills and Mr. M. Barren is greatly appreciated. The financial support from the University of Manitoba Graduate Fellowship is also gratefully acknowledged.

I appreciate the unwavering support of my husband Fayu Zhang and daughter Connie Zhang for their love, support, understanding and encouragement during the pursuit of this degree. I dedicate this dissertation to my mother Meiyu Wang, she did not have a chance to attend school but encouraged all her five daughters to go for post-secondary education.

FOREWORD

This dissertation is written in manuscript style. Three manuscripts are presented, including abstract, introduction, results and discussion. A general abstract, introduction and a literature review precede the manuscripts. They are followed by a general discussion, summary and conclusions and the cited references. The first manuscript will be submitted to "Journal of General Microbiology", the second manuscript to "Letters in Applied Microbiology" and the last manuscript to "Food and Agricultural Immunology".

The authors of the manuscripts are:

I. P. Lu[1] and R.R. Marquardt[1]

II. P. Lu and R.R. Marquardt

III. P. Lu, R.R. Marquardt, A.A. Frohlich[1] and J.T. Mills[2]

1. Department of Animal Science, University of Manitoba, Winnipeg, Manitoba.

2. Research Station, Agriculture Canada, 195 Dafoe Road, Winnipeg, Manitoba, Canada. R3T 2M9

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LIST OF ABBREVIATIONS

a_w- water activity

CFU- colony forming units

ELISA- enzyme-linked immunosorbent assay

EPS- extracellular polysaccharide

ExAg- exoantigen

HMC- Howard mould count

HPLC- high performance liquid chromatography

ID- immunodiffusion

IFA- immunofluorescent assay

Ig- immunoglobulin

IgG-immunoglobulin G

MAb- monoclonal antibody

MEA- malt extract agar

MPNT- most probable number technique

M_r- molecular weight

NC- nitrocellulose

NMR- nuclear magnetic resonance

OA- ochratoxin A

PAGE- polyacrylamide gel electrophoresis

PBS- phosphate buffered saline

PBST- phosphate buffered saline containing 0.05% Tween-20

PDA- potato dextrose agar

PPExAg- precipitated protein of exoantigen

ppm- parts per million (mg/kg)

RFC- rot fragment count

RFLP- restriction fragment length polymorphism

SDS- sodium dodecyl sulphate

TBS- Tris buffered saline

TLC- thin layer chromatography

TTBS- Tris buffered saline containing 0.05% Tween-20

INTRODUCTION

A method that can be used to reliably and efficiently monitor the contamination of grain for a specific species or genus of mould is necessary as cereal grain is an important source of foods and feeds and is often prone to contamination with moulds and their mycotoxins, particularly those from the *Aspergillus* and *Penicillium* genera (Jarvis and Williams, 1987; Pitt and Samson, 1990; Samson *et al.*, 1991). The consequence of fungal contamination can be serious as it not only causes deterioration of foods and consequently economic losses but also poses serious health hazards for humans and animals as they may produce toxic secondary-fungal metabolites (CAST, 1989). Fungal contamination, however, cannot be efficiently and accurately monitored using conventional mould detection methods because of limitations associated with these methods. Commonly used methods such as direct plating and enumeration of fungal propagules can only be used to selectively detect viable moulds with the results often being confounded by the presence of mycelia fragment and clumps of spores. These assays also do not accurately predict the biomass of the fungi present and tend to be selective for certain species as the culture may facilitate the growth of particular populations of moulds (Jarvis and Williams, 1987; Lacey *et al.*, 1991; Swanson *et al.*, 1992). Chemical analysis for chitin (glucosamine) and ergosterol have also been used as indices of mould contamination (Donald and Mirocha, 1977; Jarvis, 1977; Cousin *et al.*, 1984; Rotter *et al.*, 1989). Although these methods can detect both viable and nonviable moulds and are more sensitive and accurate than fungal propagule counts, they also have limitations. The chitin content of a mould has been shown to vary greatly among different fungi, it is present in insect exoskeletons and bacterial cell walls, and sugar amines found in food tend to interfere with its analysis (Jarvis *et al.*, 1983; Jarvis and Williams, 1987).

Ergosterol analysis which is not likely to be affected by nonfungal materials may be affected by substrate composition, type of fungi and growth conditions (Seitz *et al.*, 1977; Cahagnier *et al.*, 1983; Nout *et al.*, 1987). All these methods suffer from being tedious and time consuming, and are not capable of identifying specific species or genera of fungi. Traditional methods commonly used for the identification of fungi rely on morphological characters that are time consuming and empirical in nature, which can resulted in misidentification of species. Many newer approaches have been studied but none of them are able to overcome the difficulties associated with fungal identification (Pitt and Samson, 1990). Immunological methods have been recently used for the detection of fungi in foods and for understanding their interrelatedness (Polonelli *et al.*, 1984; Notermans and Heuvelman, 1985; Lin *et al.*, 1986; Tsai and Cousin, 1990; Fuhrmann *et al.*, 1990). Many of these assays have been of limited value as the immunogen that has been used is common to many fungal species and as a result the assays tend to be specific for fungi in general and not for certain species of fungi. The monoclonal antibody ELISA test developed by Dewey *et al.* (1990) for *Penicillium islandicum* in rice, however, is considered relatively specific and reliable but not quantitative. Kaufman and Standard (1987) have reported that the exoantigens (ExAgs) produced by fungi during growth seem to be unique and can be used for the development of tests for specific fungal species. They successfully developed specific assays for medically important fungi using the simple double-diffusion agar plate assay. It may be concluded on the basis of the literature review that there are few assays that are capable of specifically detecting fungi in food and feed and none appear to be quantitative (*P. islandicum*). It also may be concluded that the ExAgs of fungal species appear to be unique and can be readily extracted, and may therefore be useful antigens for immunological based assays.

The overall objective of this study was to demonstrate that antibodies against the exoantigens of two important storage fungi could be used to selectively identify these fungi in a grain matrix contaminated with other species of fungi. *P. aurantiogriseum* and *Aspergillus ochraceus* were the focus of this research as the former is the most common toxigenic contaminant in foods and feeds and the latter is a producer of ochratoxin A, which is of the most common mycotoxins in Canada and Europe. Currently, an immunological method for the detection of these moulds has not been developed.

The specific objectives of the research presented in this thesis were to:

1. Develop solid-phase immunoassays using rabbit antisera against the ExAgs of *P. aurantiogriseum* and *A. ochraceus* that were capable of detecting these fungi.

2. Determine if the immunoassays were species specific.

3. Further characterize the ExAgs of *A. ochraceus* and *P. aurantiogriseum* using SDS-PAGE followed by silver staining for protein or immunoblotting.

4. Test the suitability of the ELISA for detecting *A. ochraceus* in wheat contaminated with other fungi. These values were to be compared with several other commonly used methods for detecting fungal contamination.

LITERATURE REVIEW

1. Moulds and their impact on human beings and animals

1.1 Factors affecting mould contamination in foods and feeds

The moulds are ubiquitous in the environment as they commonly occur in soil, water and air, and are essential participants in ecological systems (Jarvis *et al.*, 1983). While the major factor determining the invasion of plants by fungi is the ability of microorganisms to overcome plant defence mechanisms, the occurrence of moulds in foods and feeds is affected to a considerable degree by environmental factors such as water activity (a_w), temperature, hydrogen ion concentration (pH), gas tension, consistency of substrate, nutrient status, specific solute effects, preservatives and microbial competition (Pitt and Hocking, 1985; Frisvad and Samson, 1991a; Mislivec *et al.*, 1992). Moulds are able to grow at low water activities (0.65 to 0.90) and a wide range of pH's (3 to 8) and temperatures (-7°to 45°C). Most moulds require O_2 for their growth. Carbon dioxide usually inhibits *Aspergillus* and *Penicillium* species at concentrations over 15% while it may be stimulatory at lower concentrations. Some fungi, however, can grow at high concentrations of CO_2. Most moulds favour a substrate with a firm rather than a liquid consistency as this provides better access to O_2. Fungal metabolism is best suited to a substrate having a high concentration of carbohydrate in contrast to bacteria which preferably grow on proteinaceous foods. Generally, different types of foods will contain a typical population of associated mycoflora. For example, *P. aurantiogriseum* and its varieties are common on stored cereals, while *P. commune* is common only on nuts and other lipid- and protein-rich foods (Frisvad and Samson, 1991a). Other factors including microbial competition, degree of spoilage and type of processing also affect fungal growth. Physical damage of grains, nuts and other

foods will favour the growth of *Penicillium* rather than *Aspergillus* species and will enhance the rate of the deterioration process considerably. The complex interaction of factors that influence the germination and growth of fungi has been reviewed by Lacey and Magan (1991).

1.2 The health risks and economic losses associated with fungal contamination in cereals

Cereal grains are an important source of food and feed and have several unique characters which make them an ideal ecological niche for moulds. The growth of fungi in grain is favoured by its firm textural-consistency which provides easy access to O_2, and its high content of carbohydrates and other nutrients. Storage under reduced water-activity favours the growth of fungi over bacteria. The consequences of fungal contamination in cereal grains are the loss of dry matter, an unpleasant appearance, the production of disagreeable odours, a reduction in the germination of the seed and a lowered baking quality. In addition, the digestibility and nutritional value of the grains is often reduced due to the utilisation or alteration of carbohydrates, proteins and lipids by the fungi. Spore inhalation and mycotoxin intake by humans and animals may also cause allergies, mycotoxicoses and other life-threatening health hazards (Frisvad and Samson, 1991a; Lacey and Magan, 1991).

Bennett (1987) has defined mycotoxins as being "natural products that are produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and animals by a natural route". Mycotoxins are a chemically diverse group of toxic secondary-fungal metabolites (Busby and Wogan, 1981). The toxigenic moulds which contaminate cereal grains only produce mycotoxins under certain conditions. The adverse effects of mycotoxins also vary from one toxin to another, ranging from acute death to chronic effects on vital organs and tissues (CAST, 1989). Ochratoxin A (OA), for example, is produced by *A. ochraceus* and can

cause suppression of the immune system, nephropathy, hepatopathy and a decreased rate of growth and performance in animals. It is also considered to be highly carcinogenic, mutagenic and teratogenic (Prior and Sisodia, 1982; Roschenthaler *et al.*, 1984; Bendele *et al.*, 1985; Stein *et al.*, 1985; Marquardt and Frohlich, 1992). The best known family of mycotoxins, the aflatoxins, have also been shown to be hepatotoxic and carcinogenic (CAST, 1989). Although the occurrence of acute mycotoxicoses in humans is low in the developed countries, it can be high in animals as animal feeds are not screened for the presence of these mycotoxins. The risk of the outbreaks of mycotoxicoses is high in the developing countries where food supplies are limited and harvesting and storage conditions are less than ideal. In addition, the long-term effects of a low-level of exposure still need to be established for most of the mycotoxins. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins (Mannon and Johnson, 1985). Except for a few well studied mycotoxins, the toxic effects of many mycotoxins as well as the frequency and level of contamination in foods and feeds are still not well documented. This may in part be attributable to the lack of adequate analytical methods to quantify the level of toxins and their fungi in agricultural commodities especially cereal grains (CAST, 1989). Overall, it is difficult to estimate the economic losses caused by mycotoxicoses in animals and the effect that mycotoxins have on human health. It is well known that human beings are directly exposed to mycotoxins through contaminated foods and indirectly through mycotoxin residues present in animal tissue and organs. An example of indirect contamination is the discovery of OA in pork kidney and blood (Golinski *et al.*, 1985; Marquardt *et al.*, 1988). The development of some important human diseases in certain populations has been attributed to the exposure of humans to low levels of

mycotoxins. These diseases include Balkan endemic nephropathy which has been hypothesized to be caused by OA, alimentary toxic aleukia caused by the trichothecene mycotoxins, and ergotism caused by some of the fungal alkaloids (CAST, 1989).

It is difficult to give an exact estimation of economic losses caused by food spoilage as a result of mould contamination but up to 10% of all food production may be affected (Pitt and Hocking, 1985). Experts estimated that 10 to 30% of harvested cereals, depending on storage technology and climate, is lost through mould deterioration (Chelkowski, 1991). Despite the fact that food preservation techniques have been considerably improved, mould and mycotoxin contamination remains an enormous problem throughout the world. Most of the moulds have a dual impact on human beings. One of these is the economic losses caused by damage to crops and livestock, associated regulatory costs, etc. The other, which is of increasing concern, is their possible adverse effects on human health especially the carcinogenic effects of their toxins. Therefore, an accurate, rapid and inexpensive monitoring of their presence is necessary.

1.3 *Aspergillus*, *Penicillium* and *Fusarium*; three genera of major mycotoxin producing fungi in cereal grains

Cereal grain is colonized by numerous fungi with some of them influencing grain quality before it is harvested while others damage grain after harvesting. The three major mycotoxin producing genera of filamentous moulds that are known contaminants of cereal grains are *Aspergillus, Penicillium* and *Fusarium*. *Fusaria* invade and damage grain seeds almost exclusively in the field prior to harvest and have been classified as being field fungi. The other ecological group include mainly species from *Penicillium* and *Aspergillus* genera and are often referred to as storage fungi as they invade and damage grains during storage (Christensen, 1987). There are, however, fungi

that fall between these two groups. There is a profound change in the grain ecosystem at harvest, from an environment influenced by diurnal fluctuations in weather conditions to the more stable environment of stored grain where conditions are governed chiefly by the availability of water in the grain. Harvest also redistributes fungal inoculum in the grain and introduces further inoculum (Lacey and Magan, 1991).

Penicillium and *Aspergillus*, as indicated above, dominate the spoilage mycoflora of mouldy food samples, especially cereals (Samson and Frisvad, 1991). *Aspergillus* is among the best known and most frequently recognised moulds, as well as one of the most important with regard to mycotoxin production and food spoilage. Current taxonomy recognises about 150 *Aspergillus* species, perhaps 30 of these are well defined and readily separated (Pitt and Hocking, 1985). Forty-five *Aspergillus* species have been shown to be mycotoxin producers on cereals with 17 of them being important mycotoxin producing species (Frisvad and Samson, 1991b). Among these, *A. flavus* and *A. parasiticus* are well known aflatoxin producers, *A. fumigatus* as an important pathogen for aspergilliosis and is also capable of producing several mycotoxins on cereals, *A. versicolour* as a producer of sterigmatocystin and *A. ochraceus* is capable of producing OA.

Williams and Bialkowska (1985) reported that of 294 mouldy samples examined, penicillia were isolated from 53% of the samples and among its subgenera, species of *Penicillium* was found in 90% of the samples. *Aspergillus* species was predominate in 15% of the total samples. A large number of *Penicillium* and *Aspergillus* species, as summarized by Frisvad and Samson (1991b), have been reported to produce mycotoxins. The inability to accurately and rapidly identify fungi to the species level has made it difficult to establish the

nature of the occurrence of specific toxigenic moulds in the environment.

Penicillium species are the most common moulds causing cereal spoilage, but they are seldom identified to the species level. A total of 75 species have been reported to contaminate cereals and produce known mycotoxins (Tsunoda, 1970; Welling, 1974; Hill and Lacey, 1984), with *P. aurantiogriseum* being the most important of all of the cereal borne penicillia (Frisvad and Samson, 1991b). *P. verrucosum* is considered another major species that occurs on cereals in the Northern temperate regions (Frisvad and Filtenborg, 1988). Recently, Pitt has proposed that *P. verrucosum* is the only *Penicillium* species of the subgenus *Penicillium* which produces OA (Pitt, 1987). Other *Penicillium* species of interest are *P. chrysogenum, P. citrinum* and *P. roqueforti* as they are also common cereal contaminants and important mycotoxin producers. Most of the species studied in this thesis belong to terverticillate penicillia, a classification based on the terminal structure of penicilli. They belong to the *Penicillium* subgenus which has members such as *P. aurantiogriseum* (including var. *aurantiogriseum* and *var. viridicatum*), *P. chrysogenum, P. roqueforti, P.verrucosum* and *P. commune*. They are commonly involved in food spoilage as they are able to grow at low temperatures and relatively low water-activities, and are of universal occurrence. Of the penicillia found on cereals, *P. aurantiogriseum* and *P. verrucosum* are listed as being the most common and the most dominant contaminants, followed closely by *P. citrinum* and *P. roqueforti* (Samson and Frisvad, 1991). Almost all the species in this subgenus produce mycotoxins (Frisvad and Samson, 1991b).

1.4 *Penicillium aurantiogriseum* and *Aspergillus ochraceus*

Pitt and Hocking (1985) assigned several species of fungi considered to be distinct by Raper and Thom (1949) into *P. aurantiogriseum*, including *P. cyclopium* (Westling), *P. aurantiovirens*

(Biourge) and *P. verrucosum var. cyclopium*, which is distinguished by its blue-grey conidia. *P. aurantiogriseum* is one of the most commonly encountered fungi and is ubiquitous in cereals, meat, fruits and vegetables. This complex species produces over 10 different secondary fungal metabolites and has been classified into several varieties (Frisvad and Filtenborg, 1989).

Aspergillus ochraceus, which is known to produce the ochratoxins and penicillic acid, is commonly isolated from foods. Dried foods are the most common substrate for this fungus (Pitt and Hocking, 1985; Frisvad and Samson, 1991b).

2. *Penicillium* and *Aspergillus* systematics

2.1 The importance of identifying contaminating mycoflora

Moulds and their mycotoxins in foods and feeds are highly undesirable and, therefore, their presence in these products should be accurately and quantitatively monitored. The analysis of specific mycotoxins is often inefficient as there are potentially over 100 mycotoxins that may be a contaminant and in many cases proper analytical methods and appropriate standards are still unavailable making routine measurements difficult (CAST, 1989). Many moulds are capable of producing more than one toxin with many toxins being produced by different species or genera of moulds. Under these circumstances, analysis for a few known mycotoxins would be of limited value in terms of potential toxic effects of a particular food. Frisvad (1988; 1989) concluded that an assay for mould contamination would provide the best overall index of the degree of food spoilage and mycotoxin contamination as there is a clear relationship between taxa at the species level and mycotoxins produced. The identification of the responsible mycoflora would therefore, assist in reducing the number of mycotoxin analyses that would be required. The development

of procedures for the identification of the contaminating mycoflora to the species level would also provide a better understanding about the biology and biochemistry of the organism, including the possible production of mycotoxins (Samson *et al.*, 1991).

The problem of identifying fungal species has been confused by the existence of different taxonomic schemes and compounded by inaccurate identification (Pitt and Hocking, 1985). Previous studies on the occurrence of *Penicillium* species and their production of mycotoxins have encountered several difficulties. The most serious one is that the taxonomy of *Penicillium* genus has not yet been fully settled. Frisvad and Samson (1991a) have suggested that the identification of fungi using classical procedures should be confirmed by physiological methods and secondary metabolite profiles. Confirmed data on the composition of the mycoflora of cereals, however, is limited due to the difficulties associated with their accurate identification. Also, it has not been established if moulds produce the same mycotoxins on cereals as in pure culture or if they always produce the same mycotoxins on the same substrate. In the past, the identification of food-borne fungi has often been neglected, possibly due to the lack of techniques for carrying out rapid and reliable identifications or the unavailability of skilled mycologists in regulatory laboratories.

2.2 Identification of fungal species and associated problems

The importance of understanding cereal grain mycology and for establishing the correct taxonomy of fungi causing grain deterioration has increased since the discovery of mycotoxins and their toxic effects. The modern basis of conventional *Aspergillus* and *Penicillium* taxonomy was developed by Raper and Thom (1949) and Raper and Fennell (1965) with modifications by Samson *et al.* (1976), Pitt (1979) and Ramírez (1982), and later by Samson and Gams (1985)

and Kozakiewicz (1989). The conventional methods, which continue to be popular, rely on morphology as a primary criterion for the classification of filamentous fungi. Fungi are usually grown on different media and under different incubation conditions, with biochemical and physiological characteristics providing supplementary evidence (Samson *et al.*, 1991). Colony colour, texture, diameter, exudate, conidia structure and morphology are the parameters used for determining the identity of an isolate. Pitt and Samson (1990) and Samson and Frisvad (1991) summarized the changes and developments in past decades and have concluded that the base of traditional taxonomy was too narrow and difficult to distinguish many species, particularly those in the *Penicillium* genus. In addition, the use of different taxonomic systems has resulted in different names for the same fungi and the same name for different fungi. Traditional taxonomy is also restricted by the requirement for a high level of expertise and a long period of time for a typical identification (usually 14 or more days). The former factor together with the empirical nature of the method may result in erroneous identifications. It may be concluded that the taxonomic schemes currently in use for the most important food-borne genera such as *Penicillium, Aspergillus* and *Fusarium* have been varied and in some cases inaccurate in spite of improvements in research methodology and, as a result, the systematics of these genera are still in a state of flux (Samson *et al.*, 1991). A simple, accurate and consistent method is required to assist in establishing the classification of *Penicillium* and *Aspergillus* species and for the identification of species of fungi.

2.3 New approaches to *Penicillium* and *Aspergillus* systematics

Many new physiological, chemical, molecular biological and immunological approaches have been studied in the last decade in an attempt to assist in the identification of *Aspergillus* and

Penicillium species (Pitt and Samson, 1990; Samson *et al.*, 1991; Samson and Frisvad, 1991). Physiological approaches include the use of standardized incubation media, temperatures, a_w and assay periods. The use of selective media has especially helped the differentiation of some species which would be normally difficult to distinguish using conventional media (Abe, 1956; Pitt, 1973; Pitt *et al.*, 1983; Frisvad, 1983; 1985). Although this approach is useful more attention is being given to the chemical, molecular biological and immunological approaches. This review will therefore focus on these newer approaches.

Chemical methods have included the analysis of long-chain fatty acids, secondary metabolite profiles, isozyme electrophoretic patterns and the distribution of the ubiquinone system. Dart *et al.* (1976), for instance, analyzed the relationship between some *Penicillium* species based on their long-chain fatty acids, but this method did not agree well with morphological taxonomy (Pitt, 1984). Cruickshank and Pitt (1987) studied the electrophoretic patterns of extracellular isozymes (including polygalacturonase, pectinesterase, amylase and ribonuclease) of species belonging to the *Penicillium* subgenus by incorporating the respective enzymatic substrate into the gels. The enzyme patterns correlated well with most of the currently accepted species except *P. aurantiogriseum* and *P. viridicatum*. This and other studies indicate that enzyme and protein electrophoretic patterns could be used in fungal taxonomy (Bent, 1967; Paterson *et al.*, 1989; Yamatoya *et al.*, 1990). Kuraishi *et al.* (1991) compared the ubiquinone systems of 118 species from *Penicillium* and related genera using High-Performance Liquid Chromatography (HPLC). Partial homogeneity in the ubiquinone system was observed among related species which suggest that it may also be useful as a taxonomic criterion. Frisvad and coworkers developed a taxonomic system based on the pattern of secondary metabolites produced

by different fungi, using several different chromatographic methods. The agar plug Thin-Layer Chromatography (TLC) system was easy to carry out and relatively fast, and allowed thousands of strains of *Penicillium* and *Aspergillus* to be screened for secondary metabolite profiles (Filtenborg and Frisvad, 1980; Frisvad and Filtenborg, 1983; 1989). A HPLC system for comparing secondary metabolite profiles has been shown to be more accurate and reliable but had the disadvantages of requiring costly equipment and well trained workers (Frisvad and Thrane, 1987). The revised scheme for the classification of the subgenus *Penicillium* of Frisvad and Filtenborg (1989) which is based on secondary metabolite profiles agrees with that of other leading mycologists who have used the more classical methods (Pitt, 1979; Stolk and Samson, 1972). A possible pitfall of this procedure is that the same fungal species does not always produce the same secondary metabolites (Chelack *et al.*, 1991).

Molecular biological analyses of genetic materials for DNA homology, DNA restriction fragment length polymorphism (RFLP), various RNA and DNA restriction mapping and rRNA sequence comparisons have also been studied. Kurtzman *et al.* (1986) proposed, on the basis of a high degree of nuclear DNA complementarity and similar genome size, that *A. flavus* and three closely related species (*A. parasiticus, A. oryzae* and *A. sojae*) be considered to be one species. Other studies, however, have reported differences between the DNA of *A. flavus* and the three other related species (Klich and Mullaney,1987; Gomi *et al.*, 1989). Logrieco *et al.* (1990) confirmed on the basis of their ribosomal RNA sequences that some *Penicillium* species were distinct. The results of most of the genetic analyses have in general correlated well with traditional taxonomy, suggesting, as would be expected, that these methods are useful as taxonomic tools.

Scanning electron microscopic examination of conidia (Stolk and Samson, 1972; Kozakiewicz, 1989) and immunological methods including the detection and identification of fungal cell wall antigens and exoantigens (ExAgs) (Polonelli *et al.*, 1984, 1987; Hearn *et al.*, 1990; Fuhrmann *et al.*, 1992) have also been used as tools for solving taxonomic problems. Mycologists in general have not widely utilized immunological methods as an aid in solving taxonomic problems which in part may be attributed to their inability to identify specific antigens. Previous studies, however, have shown that these methods could be useful for such purposes. Nemergut *et al.* (1977) studied the immunological relationship between five species of *Penicillium* including *P. citrinum, P. chrysogenum, P. italicum, P. notatum* (should be *P. chrysogenum* according to Samson *et al.*, 1991) and *P. roqueforti* using an immunodiffusion (ID) technique and rabbit antisera raised against extracts from both mycelia and culture fluid in an assay. Most of the antisera were species specific except for the cross-reactivity between *P. chrysogenum* and *P. notatum* (should be *P. chrysogenum* according to Samson *et al.*, 1991). Polonelli *et al.* (1984) observed a high degree of serological relatedness among the *Aspergillus* species belonging to subgenus *Circumdati*, section *Flavi*, notably *A. flavus, A. parasiticus, A. oryzae* and *A. sojae*. Polonelli *et al.* (1987) subdivided 24 *Penicillium* isolates related to *P. camemberti,* on the basis of their ExAgs, into nine groups and further concluded that *P. commune* Thom was the wild-type ancestor of *P. camemberti*. Fuhrmann *et al.* (1989) studied the taxonomic relationships among 44 strains of *Aspergillus* and *Penicillium* species using antisera against *P. verrucosum var verrucosum* in an immunofluorescent assay (IFA) and an indirect ELISA. Antigenically this species appeared to be similar to strains belonging to subgenus *Furcatum* but was different from *P. frequentans* (subgenus *Aspergilloides*). This

immunological study suggested that there were common epitopes on *P. frequentans*, *A. versicolour* and *A. fumigatus*. Fuhrmann *et al.* (1990) used an indirect-monoclonal antibody (MAb)-ELISA and IFA to analyze the taxonomic relationship among five species of *Aspergillus* and 13 species of *Penicillium* belonging to subgenera *Aspergilloides, Furcatum, Penicillium*. Common antigenic determinants were found for all moulds that were tested, for *Penicillium* and *Aspergillus* genera, for subgenus *Aspergilloides* and genus *Aspergillus*, and for subgenus *Aspergilloides*. No distinction between subgenera *Furcatum* and *Penicillium* was observed. Fuhrmann *et al.* (1992) produced a MAb using mycelia of *P. frequentans* which recognized an antigen on the inner spore wall layer and cross-reacted with various strains of the *Penicillium* and *Aspergillus* genera. Neucere *et al.* (1992) used rabbit antibodies against mycelial surface washings of *A. flavus* to compare the immunochemical profile of *A. flavus* and *A. parasiticus* by immunoabsorption, and by crossed- and line-immunoelectrophoresis. The results successfully indicated the phylogenetic closeness and differences among these species. The results of these studies suggest that immunological methods may contribute significantly to the refinement of the taxonomic classification of moulds particularly if they are based on the detection of unique fungal antigens. They may, therefore, provide a useful means for the characterization of moulds and for revising the taxonomic classification of *Penicillium* genus. Immunoassays that are currently being used for the detection and characterization of bacteria, yeasts and moulds are fast and easy to perform.

In addition to the individual methods discussed above more integrated approaches have been used in *Penicillium* taxonomy (Klich and Pitt, 1985; 1988; Paterson *et al.*, 1989; Bridge *et al.*, 1990; Stolk *et al.*, 1990). The classification of terverticillate penicillia have received the

greatest attention because of their importance as major toxigenic food contaminants and the difficulties in their identification. For instance, Stolk *et al.* (1990) reexamined and delimited the terverticillate penicillia on the basis of morphology, taking growth characters and profiles of secondary metabolites into account. The production of OA was also used as one of the key factors in the identification of *P. verrucosum* (Pitt, 1987). Bridge *et al.* (1990) examined 348 strains of terverticillate penicillia for their physiological, biochemical and morphological characteristics. These included the assessment of growth on specific carbon and nitrogen sources, screening for enzyme production, TLC analysis of secondary metabolites, and analysis of conidia by scanning electron microscopy. They also carried out additional studies on strain variation and analysed fungal extracts for the isozyme electrophoretic patterns. They concluded that the terverticillate penicillia were a group of very similar fungi. The integrated multidisciplinary approach has generated very interesting and useful results but will not be adopted for routine practical applications because of the many analyses required.

3. Exoantigen test and solid-phase immunoassays for fungal detection and identification

3.1 The principles of solid-phase immunoassays used and their advantages.

Solid-phase immunoassay requires the immobilization of a reactant involved in the antigen-antibody reaction onto a solid phase. Plates, beads and membranes can be used to separate bound from free reactants through extensive washing. The formation of the antigen-antibody complex on the solid-phase is detected by different systems such as a colour reaction produced by an enzyme label. The enzyme-linked immunosorbent assay(ELISA) and the immunoblotting technique (immunoblotting) are the two most commonly used solid-phase immunoassays. Many

new forms of immunoassay exist including both indirect and direct, and competitive and non-competitive formats. In the indirect (or antibody capture) non-competitive ELISA, the antigen is immobilized on the solid phase and the binding of antibody is normally detected by an enzyme-labelled second antibody (Tijssen, 1985). In the indirect competitive ELISA, the antigen is immobilized and the free test or standard antigen compete with immobilized antigen for the restricted amount of antibody. This prevents a certain fraction of the antibodies from being indirectly immobilized onto the solid phase. The indirectly immobilized antibodies are detected by enzyme labelled anti-immunoglobulin (Tijssen, 1985; Harlow and Lane, 1988).

The ELISA as developed by Engvall and Perlmann (1971) has high sensitivity, detectability and specificity and uses equipment and reagents that are stable, commercialized and relatively inexpensive. The assays are rapid, simple, highly reproducible, feasible under field conditions and involve no radiation hazards as compared to radio-immunoassay (RIA). It has many advantages over more conventional methods in detection, identification, diagnosis and quantitation (Tijssen, 1985; Kemeny and Challacombe, 1988; Butler, 1991).

Immunoblotting involves the transfer of electrophoretically separated proteins to activated cellulose or nitrocellulose (NC) membranes and their subsequent immunodetection (Towbin *et al.*, 1979). Immunoblotting combine the high resolution of polyacrylamide gel electrophoresis (PAGE) with the sensitivity and flexibility of ELISA for probing selected components of protein mixtures with specific antibodies. Compared with the ELISA, the solid phase used in immunoblotting has a much higher binding capacity (approximately 100-fold) for protein antigens. In immunoblotting, both electrostatic and hydrophobic interactions are involved in the binding of antigens to the membranes while in polystyrene ELISA hydrophobic interactions are

believed to be nearly the exclusive force. Overall, immunoblotting is a more powerful technique than the ELISA for comparing degree of diversity among antigens (Brown *et al.*, 1991) as immunoblotting can be used for determining the presence and quantity of antigens, their relative molecular weights, the efficiency with which they are extracted and some of the properties of antibodies. It can also be used to purify antigens or antibodies (Harlow and Lane, 1988).

3.2 Search for specific fungal antigens: -the exoantigens test and its application

The required specificity of the immunoassays for fungal antigens vary depending on the objective of the assay. Broad screening assays that are specific for moulds in general would be required under certain circumstances while species specific assays would be required for the detection and identification of specific toxigenic moulds. The proper choice of the antigen is therefore essential for the success of the assay.

The nature of species-specific fungal antigens and their site of production are still unknown (Dewey, 1988). Conidia, mycelia, culture fluid and surface washings have all been used with varying degree of success, possibly because most of the research on food mycology has concentrated on the polysaccharide antigens (Lin and Cousin, 1987; Kamphuis *et al.*, 1989; Tsai and Cousin, 1993). Studies with exocellular polysaccharides (EPS) by Preston *et al.* (1970) on six *Penicillium* species including *P. chrysogenum, P. raistrickii*, *P. patulum* (should be *P. griseofulvum* according to Samson *et al.*, 1991), *P. claviforme, P. varians* and *P. charlesii* demonstrated that galacto-furanosyl residues were contained in the EPS of all of these species except *P. varians* and, as a result, these species cross-reacted with the *P. charlesii* EPS rabbit antiserum. The EPS of *P. varians* did not contain these residues and did not cross-react with this antibody. Notermans *et al.* (1988b) demonstrated that (1→5)-linked ß-D-galactofuranosides were

immunodominant in the EPS of *Penicillium* and *Aspergillus* species. Leal *et al.* (1992) isolated a water-soluble fraction of cell wall material from some *Penicillium*, *Eupenicillium* and *Aspergillus* species. Nuclear magnetic resonance (NMR) spectra analysis demonstrated that all the fungal species had a characteristic ß-(1-5) linked galactofuranose. This antigen may therefore be suitable for a general mould assay but not for the detection of specific moulds.

Structurally proteins possess more diverse antigenic determinants and thus theoretically should offer the possibility of developing more specific tests. Protein antigens should therefore, be used for the development of species or genus specific assay. Dewey *et al.* (1990) were able to develop a species specific assay for detecting *P. islandicum* using a glycoprotein. Huppert and Bailey (1963) first introduced an ID test for mycotic diseases while Manych and Sourek (1966) first identified fungi by use of their soluble antigens. Kaufman and Standard (1987) carried out extensive studies on the specific and rapid identification of medically important fungi using ExAgs. They defined ExAgs as antigens or soluble immunogenic macromolecules produced by fungi early in their development, some of which may be unique to certain species or genera of fungi and can be readily detected in culture broths or aqueous extracts of slant cultures. Using this method, specific identification of fungi could be accomplished within 2 to 5 days of the receipt of mature cultures, which by conventional methods would take as long as weeks or months. This is of significant importance for clinical applications which demand both speed and accurate identification of pathogenic fungi. The techniques for performing ExAg tests using the mere ID assay have been standardized and commercialized (Standard *et al.*, 1985). Johnson *et al.* (1984) evaluated five commercial ExAg test kits for the serodiagnosis of coccidioidomycosis and histoplasmosis and observed a 52 to 100% correlation between the ID kit and results from

clinical and mycology research laboratories. All commercial kits tested were 100% specific for both diseases but the sensitivity varied. Exoantigen tests also appear to be extremely useful for determining the taxonomic inter-relatedness among morphologically similar and dissimilar fungi. For instance, Notermans and Heuvelman (1985), Notermans *et al.* (1986a) and Polonelli *et al.* (1988) observed cross-reactivities between species of *Mucor* and *Rhizopus* but not with *Aspergillus* and *Penicillium* species. Exoantigen tests can also be used to overcome the difficulties encountered with identifying dimorphic or multimorphic fungi and even contaminated or nonviable fungi as they appear to be a constant manifestation of fungal growth.

The specificity and sensitivity of the ID assay can be further improved by the application of antigen purification techniques combined with the production of MAbs as well as by the use of newer techniques such as solid-phase immunoassays including immunoblotting and ELISA (Polonelli *et al.*, 1986). The specificity offered by the ExAg has lead to the development of several ExAg assays, mostly for medically important fungi including some species of *Aspergillus* and *Penicillium* (Sekhon *et al.*, 1982; 1986; Polonelli *et al.*, 1985). *Aspergillus* species, particularly *A. fumigatus* as the cause of aspergillosis and allergy, have been extensively studied using several purified antigens, and poly- and monoclonal antibodies in ELISA, immunofluorescent and immunoblotting assays (De Magaldi and Mackenzie, 1984; Burnie *et al.*, 1989; Ste-Marie *et al.*, 1990; Hearn *et al.*, 1990; Latgé *et al.*, 1991). In addition to their application in medical mycology, ExAg tests have also been successfully used to detect plant fungal pathogens (Wycoff *et al.*, 1987; Clausen *et al.*, 1991; Xia *et al.*, 1992).

4. Mould detection as related to food and feed quality

4.1 Conventional methods and associated problems

The official methods for detecting mould contamination in tomatoes, fruits and related commodities are the Howard Mould Count (HMC) and Rot Fragment Count (RFC) (Howard, 1911; AOAC Official Methods of Analysis, 1984). These methods are based on the direct microscopic examination of a number of standardized fields to give an estimation of the extent of mould contamination. Other methods include the enumeration of fungal propagules on solid media [colony forming units (CFU), King *et al.*, 1986], Most Probable Number Technique (MPNT) on liquid culture and other culture methods (Koburger and Norden, 1975). New methods under development include fluorescent and other examination (Preece, 1971a), direct and indirect immunofluorescent techniques (Preece, 1971a, 1971b; Warnock, 1973; Denizel, 1974), solid-phase immunoassays including latex agglutination assays and the ELISA (Notermans and Heuvelman, 1985; Lin *et al.*, 1986; Kamphuis *et al.*, 1989; Tsai and Cousin, 1990). Also, biochemical tests such as chitin and ergosterol analysis as indirect measurements of fungal biomass, and the measurement of metabolic activity such as CO_2 production and ATP formation (Ingram, 1960; Sharpe, 1973; Cousin *et al.*, 1984), as well as the measurement of electrical impedance (Fleischer *et al.*, 1984). The advantages and disadvantages of these methods have been reviewed by Jarvis *et al.* (1983) and Jarvis and Williams (1987).

The methods available to measure fungal growth in stored grains have been reviewed by Lacey *et al.* (1991). Direct plating and enumeration of fungal propagules (CFU) are commonly used for cereal grains due to their solid and non-sterile nature (Mislivec *et al.*, 1992). The estimation of the level of contamination by viable mould conidia is frequently confounded by the presence of mycelia fragment produced during homogenization and by clumps of spores,

particularly when contaminating moulds are actively growing. Results under such conditions are often difficult to interpret (Jarvis and Williams, 1987). These methods cannot detect nonviable mould contaminants and are not related to the biomass of the fungi present in the grains. The culture media and incubation conditions may also selectively facilitate the growth of only some populations of moulds as different fungi have different requirements and rate of growth (Swanson *et al.*, 1992). These methods therefore have some serious inherent limitations. A collaborative study was conducted to compare methods and media for enumeration of fungi in foods in 15 laboratories. The results indicated that the major differences among the methods were the media used. Counts varied widely depending upon the food being examined with the most frequently identified moulds being *Penicillium* and *Aspergillus* species (King, 1992).

Compared to the counting of CFU, chitin and ergosterol analyses seem to be more reliable for cereals, as chitin (poly-ß-1: 4-N-acetyl-glucosamine) is a major component of the fungal cell wall while ergosterol is the major sterol in the fungal membrane. Fungal mycelia usually contain approximately 20-80 μg chitin per mg of dry weight (measured as glucosamine, Donald and Mirocha, 1977; Jarvis, 1977; Cousin *et al.*, 1984; Rotter *et al.*, 1989). Chitin analysis is sensitive but tedious. Other drawbacks of this method include the possible interferences by bacteria and insect contamination as insect exoskeletons also contain chitin and bacterial cell walls contain glucosamine (Jarvis and Williams, 1987; Lacey *et al.*, 1991). The chitin content and composition also varies among species or genus of fungi and with the age of the fungus. In comparison to chitin analysis, the ergosterol assay is not likely to be affected by non-fungal materials but the effect of substrate composition, type of fungi and growing conditions on ergosterol level is not known (Seitz *et al.*, 1977; Cahagnier *et al.*, 1983; Nout *et*

al., 1987). Overall, conventional mould detection methods for cereal grains have many limitations, making them imprecise, nonspecific and often time consuming to conduct.

4.2 The use of immunoassays in the detection of moulds in food

The extraordinary discriminatory power of immunological methods have also proved to be useful in food mycology. Direct and indirect immunofluorescent techniques have been applied on a wide range of moulds (Preece, 1971a, Warnock, 1973, Denizel, 1974) and several sensitive and specific ELISA tests have been developed for the purpose of monitoring minute mould contaminants in food including assays for *Mucor, Fusarium, Alternaria, Geotrichum, Rhizopus, Cladosporium* fungal genera.

Notermans and Heuvelmen (1985) isolated heat-stable mould antigens from *M. racemosus, F. oxysporum* and *P. verrucosum var. cyclopium* (correct name is *P. aurantiogriseum* according to Samson *et al.*, 1991) by a simple water-extraction of mycelia and culture fluid followed by column chromatography. A sandwich ELISA was used to detect these moulds in samples while an indirect competitive ELISA was used to evaluate the cross-reactivity of the antibodies to 27 species of moulds from nine genera. Although they claimed the assay as genus specific, cross-reactions were observed between some genera of moulds. The antibody raised against *P. verrucosum var. cyclopium* was cross-reactive with both *Aspergillus* and *Penicillium* species as well as wheat. In further studies, Notermans and Soentoro (1986) purified the EPS antigens of *P. verrucosum var. cyclopium, P. digitatum, M. racemosus, C. cladosporioides, F. oxysporum* and *G. candidum* from the freeze-dried culture fluid using $(NH_4)_2SO_4$ precipitation followed by column chromatography of the supernatant. A sandwich ELISA was developed using rabbit antibodies against the EPS antigens and 92 species of moulds from 10 genera were

screened for the production of EPS antigens. The antibodies against *Cladosporium* and *Geotrichum* EPS were genus specific and all other antibodies cross-reacted with EPS from species of other genera. It is evident that almost all moulds studied produced detectable amount of the same EPS antigens. Notermans *et al.* (1986b) used the same ELISA methods to test the antigen production under different growth conditions. The production of antigens appeared to be correlated with mycelium weight. There was no influence, however, of the type of medium, type of culture (surface or submerged), incubation temperature or water activity on the production of these antigens. The monosaccharide residues of the purified EPS antigens, as indicated previously, were partially characterized and identified by gas chromatography (Notermans *et al.*, 1987). Galactose residues were immunodominant in the EPS antigens of *Penicillium* species and *A. repens* and were later determined to be (1→5)-linked ß-D-galactofuranosides (Notermans *et al.*, 1988b).

Several immunoassays including a latex agglutination assay for detecting the EPS produced by *Aspergillus* and *Penicillium* species at a concentration of 5 to 10 ng per ml of purified EPS, a sandwich ELISA using acid hydrolysed EPS which was more specific and sensitive, and a *Mucorales* order specific ELISA were developed and used to test various food samples (Notermans *et al.*, 1988a; Kamphuis *et al.*, 1989; 1992; De Ruiter *et al.*, 1993). These assays in general suffer the drawbacks of undesirable cross-reactions. For instance, agglutination was reported in the negative control, some commodities especially walnuts gave clearly false positive results (Van der Horst *et al.*, 1992; Notermans and Kamphuis, 1992). In some cases the interference of different food matrices to the assay were not studied.

Lin *et al.* (1986) developed a sandwich ELISA for detecting *Alternaria alternata, G.*

candidum and *R. stolonifer* in tomato puree using rabbit antisera against homogenates of the lyophilized boiled moulds. Cross-reactivity among the three species was less than 10% with detection limits being approximately 1 μg dried mould per g of sample with the ELISA values being correlated with the amount of mould added to the puree. The specificity of this ELISA was further characterized using 21 species and isolates of moulds and seven species of yeasts. Antibodies against *Alternaria alternata* cross-reacted with other species of fungi. The antibodies, nevertheless, could be used in an ELISA to detect both viable and nonviable moulds for the three species in different processed foods spiked and inoculated with these fungi with results being comparable with those of the HMC. The partially purified and characterized antigenic fractions were found to be heat-stable and therefore, may have been polysaccharides (Lin and Cousin, 1987). Tsai and Cousin (1990) also developed quantitative immunoassays for detecting *Aspergillus, Cladosporium, Geotrichum*, *Mucor* and *Penicillium* genera in dairy products using rabbit antisera against EPS antigens. All of the antibodies cross-reacted to varying degrees with other moulds when tested for specificities using a sandwich ELISA. In a competitive ELISA, the *Mucor*, Geotrichum and *Cladosporium* antibodies were genus specific. The sensitivity of the sandwich ELISA for detecting moulds was 1 ng to 1 μg/ml. In further studies, Tsai and Cousin (1993) purified and partially characterized the extracellular and mycelial antigens and demonstrated that the molecular weight (M_r) of the antigens ranged from 450,000 to 670,000, contained 10 to 50% protein and 13 to 75% neutral sugars.

A fundamental rule for all immunological based methods is that the specificity of an antibody should be demonstrated in the same system in which it will be used. In most of the ELISAs developed by Notermans, Kamphuis and De Ruiter, the test samples were mostly

random samples and the effect of food matrix on the assay sensitivity was not fully studied. Results obtained from testing random food samples using ELISA and other methods are difficult to interpret as there are no reliable methods to reflect the true status of mould contaminations.

Several commercialized latex agglutination assays for detecting moulds in foods have been evaluated in collaborative studies (Stynen *et al.*, 1992; Notermans and Kamphuis, 1992; Van der Horst *et al.*, 1992; Karman and Samson, 1992; Braendlin and Cox, 1992). Most of these tests involve assays against galactomannan and therefore they tend to be general assays for *Aspergillus* and *Penicillium* but are not specific for species or genera of fungi. Also, foodstuffs that contain galactomannan, which is a common occurrence, would cross-react with the antibody and therefore invalidate test results.

4.3 The use of immunological methods for the detection and identification of moulds in cereals

In cereals, Warnock (1973) used antibodies against soluble mycelial extracts of *Alternaria alternata, A. flavus* and *P. cyclopium* (correct name is *P. aurantiogriseum* according to Samson *et al.*, 1991) to detect these moulds in stored barley grains by an IFA. The antibodies had a low degree of cross-reaction with fungi of other species and genera. A correlation was obtained between IFA values and percentage of infection as determined by plate culture isolation for *Aspergillus* and *Penicillium*. Using fresh cell-free surface washings from slant cultures, Dewey *et al.* (1989) produced MAbs that were relatively specific for *Humicola lanuginosa*, and developed an indirect ELISA, dot-blot and dip-stick immunoassays to detect the thermophilic fungus on inoculated and naturally infected rice grains. In a further study, Dewey *et al.* (1992) used extracts from freeze-dried mycelium as a reference in the ELISA to determine mycelial

growth. The ELISA results were in the same order of magnitude as those determined by the ergosterol analysis of the fungus and a theoretical calculation for estimating the total mycelial length in freeze-dried material. The ELISA method also compared favourably with direct linear measurement of live mycelium and gave a better estimation of fungal biomass than the dilution plate count method. The ELISA method was also more sensitive and specific than ergosterol or the direct plating method for the assay of inoculated rice grains. Using a similar method for preparing antigen, Dewey *et al.* (1990) produced species-specific MAbs for detecting *P. islandicum* in rice grains with the specific antigen being characterized as a glycoprotein having a molecular weight of greater than 90, 000 dalton. An indirect ELISA and dip-stick assay were developed to detect *P. islandicum* in inoculated and naturally infected rice grains.

Banks *et al.* (1992) produced rabbit polyclonal antibodies against *P. aurantiogriseum var. melanoconidium* that cross-reacted with 33 of the 37 fungi tested, including 27 storage and 10 field fungi. The antigen was prepared from the washings of the disrupted mycelium with the fungal hyphae being successfully attached onto microtiter plates (Banks and Cox, 1992). Rabbit IgG was purified and labelled with horseradish peroxidase for a sandwich ELISA. Preliminary optimization indicated that intra-test variability was acceptable but inter-test variability was greater with the antibody being more suitable for the detection of the three major groups of storage fungi (*Aspergillus, Eurotium* and *Penicillium*) than field fungi. The assay was unable to detect spores in the medium, mycelium and spores present in spiked barley and was highly cross-reactive with unspiked barley extracts. These undesirable features prevented the successful development of this assay (Cox, 1991).

It may be concluded that immunoassays are faster, more specific and simple than the

fungal propagule counting method for detecting cereal grain mould contaminants with the added advantages of being able to detect quantitatively or semi-quantitatively both viable and nonviable moulds. Compared to chitin and ergosterol analysis, immunoassays are simple, specific and not as tedious. Currently very few ELISAs have been developed that can detect the presence of a specific fungal species while some of the more general assays tend to cross-react with the matrix. Methods therefore need to be developed that can accurately and simply detect moulds in grains with no or little interference.

Manuscript I

The detection of exoantigens from *Penicillium aurantiogriseum* and related fungal species by ELISA and immunoblotting

ABSTRACT

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed using rabbit antiserum against the exoantigens (ExAgs) of *Penicillium aurantiogriseum* and cross-reactivities were determined with 16 ExAgs from other species and genera of fungi and with the water-soluble extracts from four grains. Antiserum had a high sensitivity to three species of *Penicillium* (95 ng/ml for *P. aurantiogriseum*, 260 ng/ml for *P. verrucosum* and 210 ng/ml for *P. citrinum*) with little or no cross-reactivity with the ExAgs from the other fungi that were tested including three other species of *Penicillium*, four species of *Aspergillus*, three species of *Fusarium*, two species of Mucor and one species of *Alternaria*. The ELISA was used to detect naturally contaminating *Penicillium* species in wheat samples in comparison with conventional mould detection methods including an analysis for the number of colony forming units (CFU), amount of chitin (glucosamine), concentration of ochratoxin A (OA) as an indicator of competing mycoflora including *Aspergillus ochraceus*. The results indicated that the ELISA could accurately detect *P. aurantiogriseum* in the presence of *Aspergillus* and other moulds. Immunoblotting qualitatively confirmed the ELISA results in both liquid culture and wheat samples. The data also indicates that *P. aurantiogriseum, P. citrinum* and *P. verrucosum* are more closely related to each other than to other *Penicillium* species that were tested. The data suggest that the immunoassay developed for *P. aurantiogriseum* ExAgs is useful for the detection and identification of *P. aurantiogriseum* and related species with the advantages of being more efficient, simple and reliable than conventional techniques.

INTRODUCTION

Mould contamination not only can cause deterioration of the entire spectrum of foods and feeds but also can adversely affect human and animal health as toxic metabolites (mycotoxins) may be produced. The latter is of increasing concern as many different fungi have been shown to produce one or more mycotoxins (CAST, 1989). The three major genera of mycotoxin-producing fungi are *Aspergillus*, *Penicillium* and *Fusarium*. Fusaria invade food and feed supplies mainly during growth and harvesting, 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 some of the most economically important fungi, as they are universally present, can cause food spoilage and bio-deterioration, and are capable of producing many different mycotoxins (Pitt and Hocking, 1985; Pitt and Samson, 1990). Species in the *Penicillium* genus are particularly abundant with *P. aurantiogriseum* being the most commonly occurring species of *Penicillium*. It has been shown to produce over 10 different secondary metabolites (Frisvad and Filtenborg, 1989). It is therefore necessary not only to quantitatively monitor for mould contamination in foods and feeds but also to accurately and reliably identify the species or, if possible, strains of contaminating fungi as specific moulds are associated with the production of certain specific families of mycotoxins (Frisvad, 1988; 1989). Traditional fungal taxonomy based primarily on morphology and growth characteristics are generally considered to be tedious and time-consuming, and have a requirement for a high level of expertise which can lead to erroneous identification, even for experienced workers. This problem is serious, particularly with penicillia as the large number of species and their close morphological similarities make their identification especially difficult (Pitt and Samson, 1990;

Samson and Frisvad, 1991). Conventional mould detection methods include the use of culture methods, electrical measurement, detection of enzymes, microscopic detection, and chitin and other chemical analysis. These have been used with success but suffer from low sensitivities and lack of specificity (Jarvis *et al.*, 1983; Notermans and Heuvelman, 1985; Jarvis and Williams, 1987).

Pitt and Samson (1990) have reviewed most of the new approaches for *Aspergillus* and *Penicillium* systematics and consider none of them to be sufficiently accurate when used alone to reliably identify and detect particular species of storage fungi. Kaufman and Standard (1987) reported that the exoantigens (ExAgs) which are soluble and in some cases unique, could be used as indicators of the presence of fungal species or genera. They produced antibodies against these antigens and developed a simple immunodiffusion method for diagnosing medically important fungi. This method, however, is relatively insensitive and requires large amount of antibody. Immunoassays have been developed for detecting and identifying several species of moulds but with variable success (Notermans and Heuvelman, 1985; Notermans *et al.*, 1986b; Lin *et al.*, 1986; Dewey *et al.*, 1990; Fuhrmann *et al.*, 1992; Tsai and Cousin, 1990; Cox, 1991). Currently no ELISA method has been developed that can specifically and quantitatively detect the presence of the common storage fungi. There is, therefore, a need for an immunoassay that is capable of not only identifying the presence of *Penicillium* species, particularly the most common species, but also for estimating their concentration in a grain sample. The objective of this study was to develop a rapid, specific and sensitive assay for detecting and identifying *P. aurantiogriseum* in grain samples. Exoantigens of *P. aurantiogriseum* were utilized to develop an indirect competitive ELISA and for immunoblotting analysis. These assays were capable of

detecting *P. aurantiogriseum* in grain samples.

MATERIALS AND METHODS

Reagents, grains and supplies

Potato Dextrose Agar (PDA) and Yeast Extract from Becton Dickinson and Co., Cockeysville, MD; microtiter plates (Falcon 3911 Microtest III Flexible Assay Plates) from Becton Dickinson and Co., Oxnard, CA; ammonia sulphate from Fisher Scientific Chemical Manufacturing Division, Fair Lawn, NJ and reagents for preparing phosphate buffered saline (PBS) from Mallinckrodt Canada Inc., Pointe-Claire, PQ were obtained. The reagents used for electrophoresis and immunoblotting were of ultra-pure grade from ICN, Schwarz/Mann Biotech, Division of ICN Biomedical Inc., Cleveland, OH and Bio-Rad Laboratories, Hercules, CA. Other reagents were obtained from Sigma Chemical Company, St. Louis, MO. All solvents and reagents were of analytical grade. The microplate reader (Model 450), electrophoresis tank (Mini-PROTEAN II) and electrotransfer device (Transblot SD Semi-Dry Transfer Cell) were from Bio-Rad, Richmond, CA. Grains and soybean were from local sources and did not contain any fungal biomass as detected visually.

Fungal sources

Isolates of *P. verrucosum* Dierckx (798), *P. aurantiogriseum var. aurantiogriseum* Dierckx Chemotype I (3298) (Frisvad and Filtenborg, 1989) and *P. citrinum* Thom (832) were provided by Dr. G. Platford, Plant Pathology Laboratory, Manitoba Agricultural Services Complex, Winnipeg, MB. The identity of these species were checked by Dr. J.T. Mills using procedures described by Filtenborg and Frisvad (1980). Cultures of *Aspergillus ochraceus* Wilhelm (NRRL,

3174) and *A. versicolour* (Vuill.) Tiraboschi (NRRL 573) were 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. Isolates of *A. candidus* Link (ATCC 44054), *A. flavus* var. *columnaris* Link (ATCC 44310), *P. 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 Station.

Antigen preparation

Exoantigens from all the fungi 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 PDA were used to inoculate a sucrose-yeast-mineral (SYM) liquid media. After two weeks of growth at 30°C the mycelia were separated from the liquid media by filtration through two layers of Whatman No 1 filter paper (Whatman, Clifton, NJ). The liquid fractions which contained the ExAgs were freeze-dried and stored at -20°C. The freeze-dried ExAgs that were used for the immunization or ELISA were redissolved in 0.1 M pH 7.2 PBS, dialysed 24 h against 5 changes of PBS and concentrated in a Minicon CS-15 spinal fluid concentrator (AMICON-Division, W.R.Grace & Co.-Conn., Beverly, MA). Particulate matter was removed by centrifuge at 20,000 x *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 (fungicide, sodium ethylmercurithiosalicylate). Thimerosal-free antigens used for immunization were sterilized by passing through a 0.22 μm filter (Nalgene, Nalge Company, Subsidiary of Sybron Corporation, Rochester, NY). These preparations were referred to as ExAgs. Protein concentrations in these

and subsequent samples were determined using the Bradford procedure (Bradford, 1976).

Extracellular protein (PPExAg) and extracellular polysaccharide (EPSAg) antigens were prepared from ExAg by precipitation (Tsai and Cousin, 1990). Briefly, the freeze-dried ExAgs of *P. aurantiogriseum* was redissolved in 0.1 M pH 7.2 PBS and precipitated by adding ammonia sulphate to a final concentration of 80% (w/v). The precipitate was redissolved in PBS and the two fractions (supernatant as EPSAg and precipitate as PPExAg) were dialysed against saline and concentrated. Protein quantification was as described above and sugar quantification according to the method of Dubois *et al.* (1956).

Preparation of water extracts of grain

Clean wheat, barley, corn and soybean samples were ground using a Cyclotec sample mill equipped with a 1-mm screen (Tecator AB, Höganäs, Sweden), 50 g of the ground sample was mixed with 100 ml of SYM liquid media and the mixture was shaken vigorously for 1 h on a Wrist Action Shaker (Model 75, Burrell Corporation, Pittsburgh, PA). The mixtures were centrifuged at 20,000 x *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 while the working solutions were stored at 4°C.

Immunization and preparation of antibody

Two rabbits (Dutch Belted, female, 1-1.5 kg in body weight) were injected subcutaneously (3 sites) with sterile ExAg of *P. aurantiogriseum*. The antigen preparation was emulsified with Complete Freund's Adjuvant (CFA, first injection) or Incomplete Freund's Adjuvant (IFA, booster injections) and administered at a dosage of 1 mg protein at 3-week intervals. One rabbit died prior to obtaining antiserum. The remaining rabbit was bled one week after the third

injection and serum antibody response was assayed by immunodiffusion (Standard *et al.*, 1985). The rabbit was exsanguinated when the desired antibody response was achieved, blood was collected, and serum was harvested, aliquoted and stored at -80°C. The antibody is subsequently referred to as the antiserum or the rabbit anti-*P. aurantiogriseum*.

ELISA titer assay

The protocols followed were according to Harlow and Lane (1988). In brief, microtiter plates were coated with 0.1 μg per well of *P. aurantiogriseum* ExAgs in 100 μl 0.1 M PBS, pH 7.2 and incubated at 4°C overnight. After 3 washings with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 5% skim milk in PBS for 2 h at 37°C. Serial dilutions of the antiserum (100 μl/well) together with the negative control serum (preimmune serum) were added to the plates, and after 2 h incubation at 37°C the plates were washed 3 times with PBST. The plates were incubated for an additional 1.5 h with a goat anti-rabbit-IgG alkaline phosphatase conjugate. p-nitrophenyl phosphate was added after 6 washings with PBST, and the plates were read at 405 nm. The optimal antigen and antibody concentrations were determined using a checker board titration assay.

Competitive ELISA

The procedures were essentially the same as those of the titer assay except 50 μl of a single fixed dilution of the antiserum (1:3000) was used after the addition of 50 μl of known quantities of ExAgs from different fungi. The concentrations of proteins which inhibit 50% of the antibody binding were calculated from the inhibition curve and referred to as the sensitivity (Tsai and Cousin, 1990). The degree of reactivity (expressed as percent cross-reactivities) of different fungal ExAgs to anti-*P. aurantiogriseum* was calculated by comparing the sensitivity of the

fungal ExAgs with that of *P. aurantiogriseum* (scale set at 100). The assays were replicated twice in duplicate and the results were pooled.

Gel electrophoresis and immunoblotting analysis

Exoantigens were solubilized in 1.5% sodium dodecyl sulphate (SDS) at 100°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli *et al.*, 1970) using a 4-20% T gradient gel (Mini-PROTEAN II Ready Gel, Bio-Rad, Hercules, CA). The prestained molecular weight (M_r) standards (Bio-Rad, low range) were: phosphorylase B, bovine serum albumin, ovine albumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. The respective apparent M_r were 106,000; 80,000; 49,500; 32,500; 27,500 to 18,500. Gels were either visualized by silver staining (Bio-Rad, Silver Plus Kit) or transferred onto nitrocellulose membrane (0.22 μm, Bio-Rad) in a semi-dry transblot cell (Bio-Rad) using 25 mM Tris, 192 mM glycine buffer (pH 8.3) containing 20% methanol (v/v) and 1.3 mM SDS (0.00375%) (50 V for 30 min). The membrane was washed with Tris-buffered-saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5) containing 10% methanol (v/v) for 1 h and blocked with 3% bovine-serum-albumin (BSA, w/v) for 2 h. After washing with TBS containing 0.05% tween-20 (TTBS, v/v), the membrane was incubated for 1.5 h with the antiserum (1:5000) diluted with TTBS containing 1% 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 (NBT) 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, Mini-

Protean II Ready Gel Instruction Manual and Transblot SD Semi-Dry Electrophoretic Transfer cell Instruction Manual).

Assay of different antigen fractions

The procedures were the same as for the competitive assay except that PPExAgs and EPSAgs of *P. aurantiogriseum* were used as inhibiting antigens at known concentrations. *P. aurantiogriseum* ExAgs were used as the control. The sensitivity and reactivities were calculated on the basis of the protein and sugar concentrations of the different fractions.

Detection of *P. aurantiogriseum* ExAgs in wheat

Wheat samples were moisturized with distilled water to 22% (w/w), inoculated with 0, 10^2 or 10^5 *A. ochraceus* spores per g and incubated for 30 days. 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 analyzed for *P. aurantiogriseum* ExAgs using the ELISA and immunoblotting procedures, and for glucosamine (chitin, Rotter *et al.*, 1989), ochratoxin A (OA) (Clarke *et al.*, 1993), the number of colony-forming unit (CFU, ISO, 1983a; 1983b) and mycoflora (Mills and Wallace, 1979). The procedure for detecting *P. aurantiogriseum* ExAgs in wheat samples using the ELISA was the same as for the 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 of 0.1M pH 7.2 PBS followed by vigorous shaking for 1 h and centrifuging at 20, 000 x *g* for 45 min. The supernatant was filtered through Whatman No 1 filter paper and used for the inhibition assay along with properly diluted rabbit anti-*P. aurantiogriseum*. The amount 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 analyzed using the immunoblotting SDS-PAGE procedure described for pure fungal ExAgs and again, pure *P. aurantiogriseum* ExAgs were included.

RESULTS

ELISA titer and optimization

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

Competitive ELISA

Typical competitive inhibition curves of four different *Penicillium* ExAgs with rabbit anti-*P. aurantiogriseum* are illustrated in Figure 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*, *P. verrucosum* and *P. citrinum*, that it reacted to a negligible degree with the ExAgs of the other fungal species ($< 5\%$), and that it did not react with extracts from grain or soybeans. The sensitivity of the assay against *P. aurantiogriseum* ExAgs, as measured by the

Figure 1. Typical competitive inhibition curves of different fungal antigens for rabbit antibodies raised against *P. aurantiogriseum* ExAgs: P. aur, *P. aurantiogriseum*; P. ver, *P. verrucosum*; P. cit, *P. citrinum*; P. roq, *P.roqueforti*. Arrows indicate the protein concentration which caused a 50% reduction in antibody binding and is the value used to denote sensitivity. The data was the average of three duplicate assays with the intra-assay coefficient of variation (CV) being 10% and that of inter-assay being 30%. See Materials and Methods for further detail.

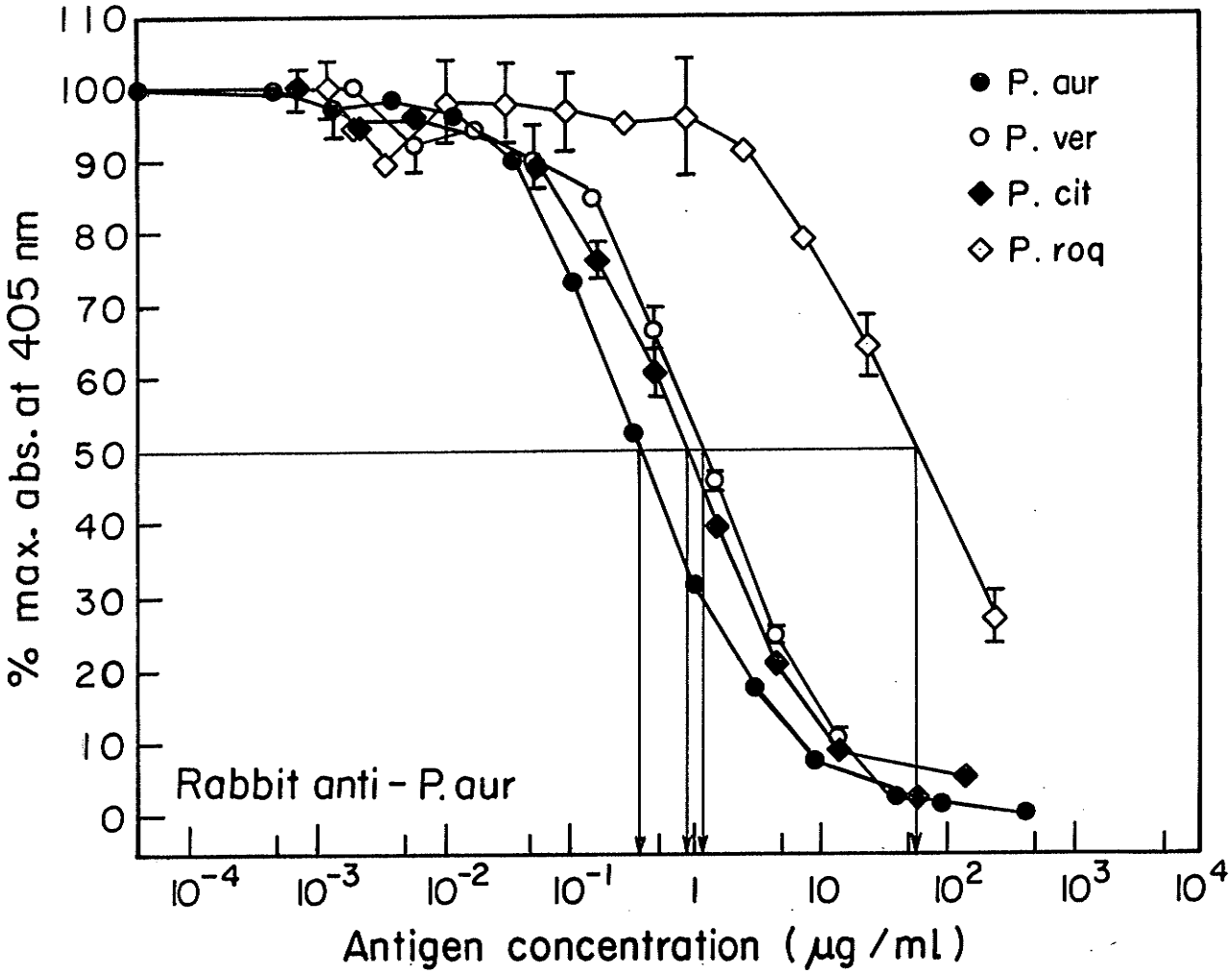

110
100
90
80
70
60
50
40
30
20
10
0
% max. abs. at 405 nm
P. aur
P. ver
P. cit
P. roq
Rabbit anti - P. aur
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
10
10^{2}
10^{3}
10^{4}
Antigen concentration (μg / ml)

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

EXAgs tested[1]	Percent cross-reactivity[2] (%)	EXAgs tested	Percent cross-reactivity (%)
P .aurantiogriseum	100	*F. avenaceum*	1±0
P. citrinum	45.4±10.8[3]	*F. equiseti*	1±0
P. verrucosum	34.6±0	*F. poae*	0.2±0
P. commune	0.2±0.01	*M. spp1*	1±0
P. roqueforti	0.9±0	*M. spp2*	1±0
P. chrysogenum	0.1±0.03	*Al. alternata*	4.8±1.9
A. candidus	0.7±0.3	Barley extract	0
A. flavus	1±0	Corn extract	0
A. ochraceus	4.5±0.1	Soy extract	0
A. versicolour	0.7±0.3	Wheat extract	0

1. Abbreviations of the fungal antigens: *P.*, *Penicillium*; *A.*, *Aspergillus*; *F.*, *Fusarium*; *M.*, *Mucor* (only identified to genus level); and *Al.*, *Alternaria*.
2. Percent 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* (as 100%).
3. Standard deviation of mean of replicate assays.

concentration of protein which inhibited antibody binding by 50%, was 95 ng per ml.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting pattern of selected ExAgs are shown in Figures 2 and 3, respectively. The protein silver staining patterns for *P. aurantiogriseum* (lane 1), *P. verrucosum* (lane 2) and *P. citrinum* (lane 3) and to a lesser degree *P. roqueforti* (lane 4) appeared to be different to each other (Figure 2). The relative amounts of individual proteins as indicated by the intensity of the band, however, appeared to vary among the four penicillia. The other two species of *Penicillium* (*P. chrysogenum*, lane 5 and *P. commune*, lane 6), the two species of *Aspergillus* (*A. ochraceus*, lane 7 and *A. flavus*, lane 8) and the one species of *Fusarium* (*F. poae*, lane 9) appeared to have unique protein staining patterns that were different from those in lanes 1 to 4. There were, however, several proteins from all species that had M_r that were similar to each other.

Immunoblotting analysis indicates that *P. aurantiogriseum*, *P. verrucosum* and *P. citrinum* not only yielded many distinct bands but that they also had different immunoblotting profiles (Figure 3). The M_r for the ExAgs varied from a low of less than 18,500 to more than 106,000 with approximately 3 or 4 yielding dark bands, 10 to 12 light bands and 5 to 8 faint bands. Most of the immunodominant bands had M_r of between approximately 70,000 and 90,000. Also, individual band intensity varied somewhat among species. The reaction of the antibody with the ExAgs of *P. roqueforti* (lane 4), *A. ochraceus* (lane 5), *A. flavus* (lane 6) and *F. poae* (lane 7) was much less pronounced than that obtained with the other ExAgs. Only one antigen had a pronounced reaction with *P. roqueforti* (M_r of approximately 80,000, lane 4) with traces of the same band

Figure 2. SDS-PAGE profiles of ExAgs from: *P. aurantiogriseum* (lane 1); *P. verrucosum* (lane 2); *P. citrinum* (lane 3); *P. roqueforti* (lane 4); *P. chrysogenum* (lane 5); *P. commune* (lane 6); *A. ochraceus* (lane 7). *A. flavus* (lane 8) and *F. poae* (lane 9). Samples were solubilized by boiling in 1.5% SDS and separated on 4-20% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). Gels were visualized by silver staining. The molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, low range). See Materials and Methods for further detail. The amount of proteins applied on each lane was 1.7 μg. A indicates the band unique for a pattern present in lane 1, while b indicates unique polypeptide present in *P. citrinum*.

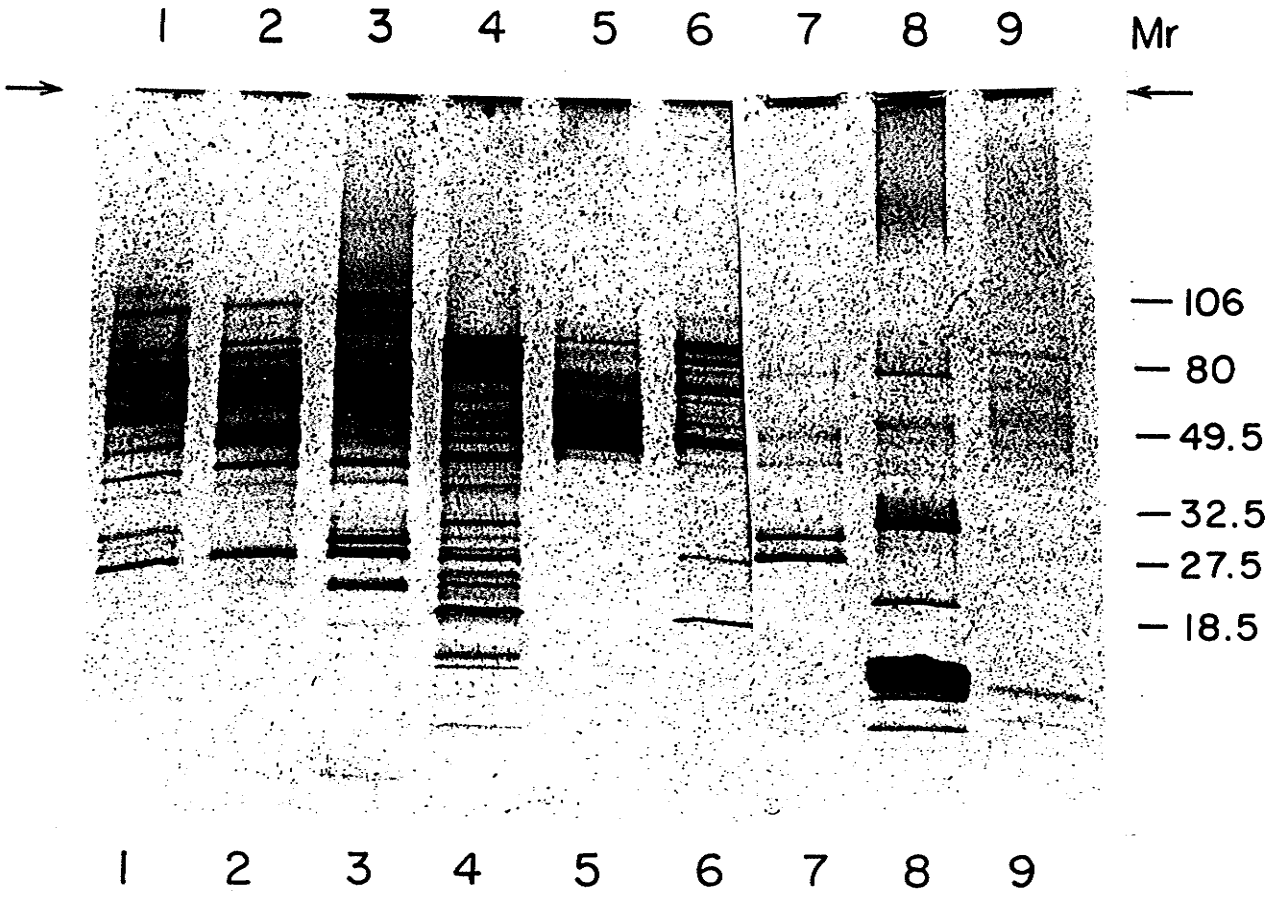
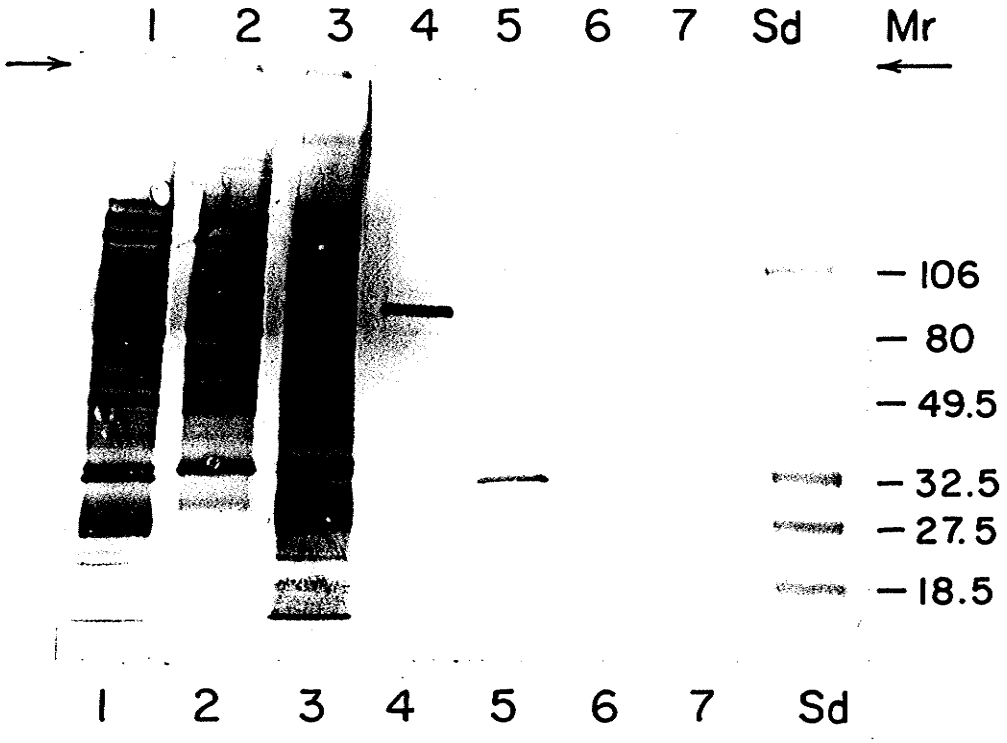

1 2 3 4 5 6 7 8 9
Mr
106
80
49.5
32.5
27.5
18.5
1 2 3 4 5 6 7 8 9

Figure 3. Immunoblotting fingerprints of different fungal ExAgs using rabbit anti-*P. aurantiogriseum*. The specific ExAgs are *P. aurantiogriseum* (lane 1); *P. verrucosum* (lane 2); *P. citrinum* (lane 3); *P. roqueforti* (lane 4); *A. ochraceus* (lane 5); *A. flavus* (lane 6); *F. poae* (lane 7). The molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, low range). See Materials and Methods for further detail. The amount of proteins applied on each lane was 1.7 μg. Replicate analysis gave identical patterns.


1 2 3 4 5 6 7 Sd Mr
106
80
49.5
32.5
27.5
18.5
1 2 3 4 5 6 7 Sd

being observed with *A. ochraceus*, *A. flavus* and *F. poae* ExAgs (lanes 5 to 7). Two antigens from *A. ochraceus* (lane 5) having M_r of approximately 32,500 and 34,000 also reacted either moderately well or weakly with the antibody. One antigen from each of *A. flavus* (lane 6) and *F. poae* (lane 7) ExAgs having respective M_r of approximately 14,000 and 16,000 slightly reacted with the antibody.

Analysis of ammonium sulphate fractionated antigens

Ammonium sulphate fractionated ExAgs from *P. aurantiogriseum* compared to the unfractionated sample yielded a precipitate that had the same concentration of protein (120 and 120 μg/ml) but a 35-fold lower concentration of carbohydrates (500 vs 17,000 μg/ml) and a supernatant fraction that had similar content of carbohydrates (11,000 vs 17,000 μg/ml) but a greatly reduced content of protein (30 vs 120 μg/ml). Removal of the carbohydrate antigens from the extract only slightly increased the amount of extract required to achieve the same ELISA absorbance value (i.e. sensitivity, 0.3 vs 0.2 μg protein/ml, Table 2). In contrast, when the sensitivities of the carbohydrate rich supernatant was compared with the unfractionated extract there was a four fold decrease in sensitivity (4 vs 1 μg of carbohydrate/ml) which also corresponded to a four fold decrease of protein in the sample. The latter effect may therefore be primarily attributed to an effect of the residual protein that remained in the supernatant fraction and not to the carbohydrate component. These results suggest that the protein compared to the carbohydrate components were responsible for most of the inhibitory effects of the ExAgs.

The detection of *P. aurantiogriseum* contamination in wheat samples

Grain naturally contaminated with fungal spores was inoculated with different amounts

TABLE 2. Inhibition of different *P. aurantiogriseum* ExAg fractions by anti-*P. aurantiogriseum* serum.

Fraction[1]	Sensitivity (ng/ml)[2]		Percent cross-reactivity (%)[2]	
	Protein	Sugar	Protein	Sugar
ExAg	200	1000	100	100
EPSAg	NR[3]	4000	NR	25
PPExAg	300	NR	67	NR

1. ExAg, exoantigens from crude extract; EPSAg, extracellular polysaccharides; and PPExAg, extracellular proteins. The amount of protein and carbohydrate in ExAg, EPSAg and PPExAg were 120 and 17,000 μg/ml; 30 and 11,000 μg/ml; 120 and 500 μg/ml, respectively.
2. See Table 1.
3. NR, not reported.

of *A. ochraceus* spores and the nature of fungal infection was followed after moisturization of the grain and incubation for 0, 7 and 30 days. Analysis of the grain for specific genera and species of fungi prior to incubation demonstrated that it contained spores of *Penicillium, 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 day incubation and a very pronounced growth after 30 days. Moisturized, uninoculated grain when incubated for 30 days as compared to zero days resulted in a dramatic increase in the percent of the grain that was contaminated with *Penicillium* species (21 to 100%), a decrease in its contamination with *A. ochraceus* to zero and little change in its contamination with *A. flavus* (29 to 42%). Corresponding changes over time in the percent infection of the grain when it was inoculated with a high concentration of *A. ochraceus* spores (10^5/g) were 42 to 0% for *Penicillium*, 56 to 100% for *A. ochraceus* and 21 to 25% for *A. flavus*. Intermediate results were obtained with the shorter incubation period or with grain that had been inoculated with a lower number of *A. ochraceus* spores. The results demonstrated that the percentage of *Penicillium* infection of wheat after an appropriate incubation period can be greatly decreased by the introduction of spores from other genera of fungi. Samples in the different treatments were also analyzed for total number of CFU, glucosamine as an indicator of total fungal biomass, and OA as an indicator of the presence of *A. ochraceus* and *P. aurantiogriseum* ExAgs as indicators of the amount of *P. aurantiogriseum* in the grain (Table 4). The data showed that the amount of *P. aurantiogriseum* ExAgs as detected by the ELISA in the uninoculated wheat samples increased dramatically towards the end of the incubation period. This increase was accompanied by increases in the concentration of glucosamine (chitin) and number of CFUs but not in the concentration of OA. In contrast,

TABLE 3. The time course production of mycoflora on non-sterilized and moisturized wheat samples that were inoculated with different concentrations of *A. ochraceus* spores[1].

Species or genera of fungi identified	Incubation time (days)	Contamination of grain by fungi after inoculation with *A. ochraceus* spores (%)[2]		
		0	10^2/g	10^5/g
Penicillium	0	21±15	41±29	42±24
	7	100±0	85±28	27±23
	30	100±0	76±23	0±0
A. ochraceus	0	7±6.6	7±5.7	56±10
	7	0±0	16±13	100±0
	30	0±0	100±0	100±0
A. flavus	0	29±11	41±42	21±12
	7	1±2	0±0	0±0
	30	42±41	52±35	25±25

1. Moisture adjusted to 22% (w/w) with distilled water and conditioned overnight at 4°C.
2. Uninoculated and nonincubated with different number of *A. ochraceus* spores. Results were obtained from filter paper culture as outlined in Materials and Methods. Percent infection refers to percent of grain samples that were contaminated with the indicated fungi. The technical help from Mr. M. Barren is greatly appreciated.

TABLE 4. Time course production of *P. aurantiogriseum* ExAgs, ochratoxin A (OA), glucosamine and colony forming units (CFU) in non-sterilized and moisturized wheat samples inoculated with different concentrations of *A. ochraceus* spores.

Parameters analyzed	Incubation time (day)	Inoculated wheat, number of *A. ochraceus* spores		
		0	10^2/g	10^5/g
P. aurantiogriseum ExAgs (μg/ml)	0	0.3±0.05	0.09±0.1	0.2±0.2
	7	2.1±1.1	1.3±0.6	1.2±0.5
	30	67±32	13±6.5	7.3±12
CFU ($\log_{10}$/g)	0	4.2±3.7	3.9±3.9	4.2±3.9
	7	7±7.3	6.3±6	8.1±8.2
	30	12±12	11±11	12±12
Glucosamine (mg/g grain)	0	0.5±0.05	0.4±0.06	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 (ppm)	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

1. Control, uninoculated and nonincubated.
2. The correlation between the amount of *P. aurantiogriseum* ExAgs and other parameters were: 0.63 ($P < 0.05$) for the number of CFU, 0.47 ($P > 0.05$) for the percentage of *Penicillium* infection, 0.43 ($P > 0.05$) for glucosamine, -0.22 ($P > 0.05$) for the percentage of *A. ochraceus* infection and -0.05 ($P > 0.05$) for OA concentration, respectively.

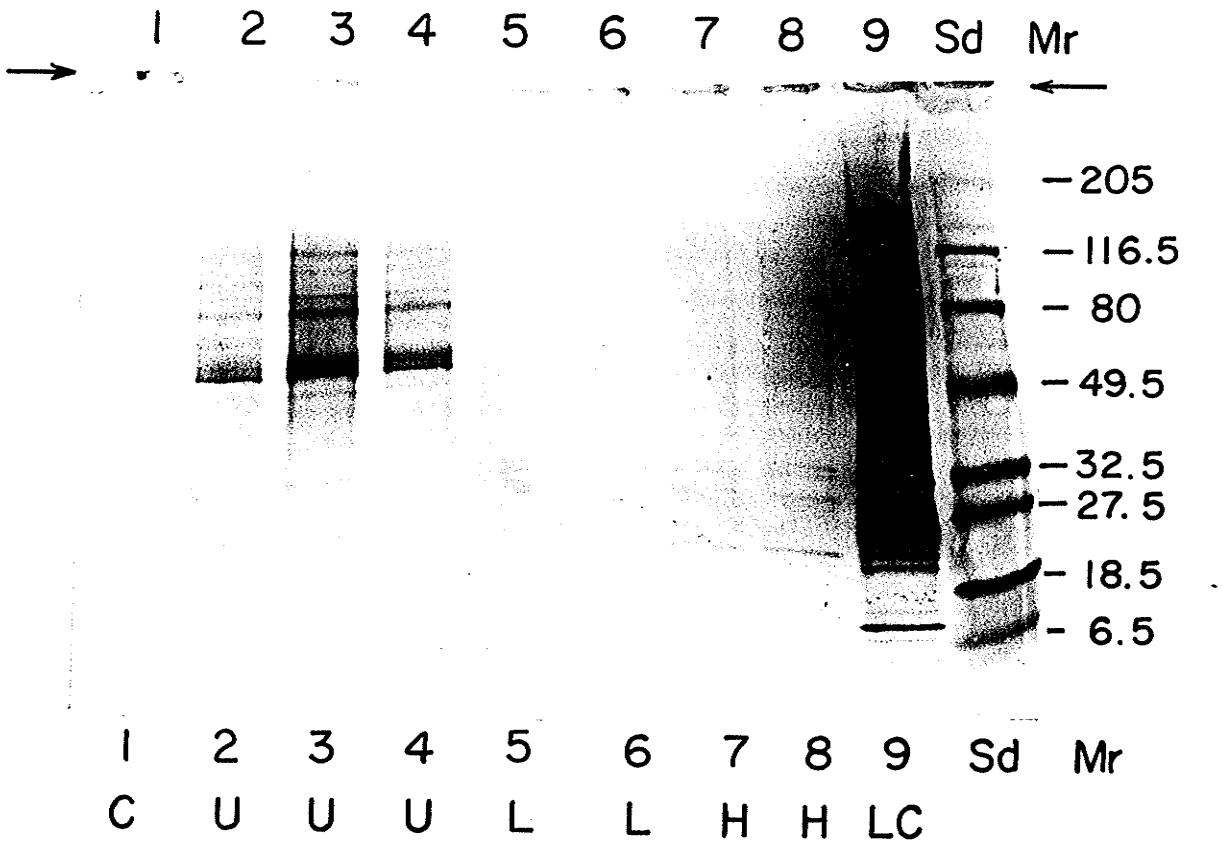
samples from those groups which were inoculated with *A. ochraceus* spores and incubated for 7 or 30 days (groups with 10^2/g, low, L and 10^5/g, high, H) had much lower concentrations of *P. aurantiogriseum* ExAgs (a maximum difference of 10-fold) and a corresponding or greatly increased concentration of OA (maximum difference of 130-fold). The chitin (as glucosamine) content and the number of CFU in these groups increased dramatically (eg, 0.5 to 7.0 mg for glucosamine and 10^4 to 10^{12} CFU/g grain) and were similar to each other and to those in the uninoculated samples after 30 days of incubation. The concentration of *P. aurantiogriseum* ExAgs in the grain samples were positively correlated with the number of CFU counts (footnotes of Table 4, $r=0.63$, $P<0.05$) and negatively with the concentration of OA ($r=-0.05$, $P>0.05$). The coefficient of correlation 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 indicate that the rabbit anti-*P. aurantiogriseum* detected more antigens in samples where *Penicillium* predominated than in samples where *A. ochraceus* predominated (Figure 4). Furthermore, no ExAgs were detected in the control wheat samples. The immunoblotting pattern of *P. aurantiogriseum* ExAgs obtained from liquid culture also tended to exhibit a similar pattern to those obtained from the wheat extracts. Most of the same antigens were present in all samples except some were present at very low concentration as indicated by the faint bands. Presumably the inoculation of the wheat with *A. ochraceus* suppressed the growth of penicillia particularly *P. aurantiogriseum*.

DISCUSSION

The semi-quantitative ELISA employed in this study demonstrated that the rabbit anti-*P.*

Figure 4. Immunoblotting of ExAgs in mouldy wheat extracts (lanes 1-8) as compared to P. aurantiogriseum ExAgs from liquid culture (LC, lane 9) using rabbit anti-*P. aurantiogriseum*. The specific extracts were control (C, lane 1); and extracts from non-sterilized wheat (U lane 2, 3, 4); from non-sterilized wheat inoculated with a low (L, lane 5, 6) or a high (H, lane 7, 8) number of A. ochraceus spores following the incubation of nonsterilized grain for 30 days. Lanes with the same letter and different number represent patterns for replicate samples. Samples were solubilized by boiling in 1.5% SDS and separated on 4-15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, low range). See Table 3 and Materials and Methods for further detail. The amount of reference standard *P. aurantiogriseum* ExAgs applied on lane 9 was 1.7 μg. Replicate analysis gave identical patterns.


1 2 3 4 5 6 7 8 9 Sd Mr
205
116.5
80
49.5
32.5
27.5
18.5
6.5
1 2 3 4 5 6 7 8 9 Sd Mr
C U U U L L H H LC

aurantiogriseum was relatively specific for three fungal species (*P. aurantiogriseum, P. citrinum* and *P. verrucosum*) and that it had little or no cross-reactivities with three other *Penicillium* species, four species of *Aspergillus*, three *Fusarium* species, two *Mucor* species and one *Alternaria* species. The immuno-fingerprinting profiles following SDS-PAGE also confirmed these observations as there were many common antigens among the cross-reacting species and at most only few common antigens (one or two) among the other species. Similar ELISA and immunoblotting analyses have not been reported before, as far as the authors are aware. 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 antigens such as the relative molecular mass and their relative abundance. The potential usefulness of the assay was further confirmed in the wheat samples that were inoculated with *A. ochraceus* spores. In this study there was a dramatic increase in total fungal biomass for all treatments after a 30 day incubation period as indicated by the glucosamine assay and the number of CFU with the final composition of fungi being influenced by the degree that the competing mycoflora, *A. ochraceus* was present. Both the ELISA and the immunoblotting pattern for *P. aurantiogriseum* ExAgs indicated that there was a much higher concentration of the ExAgs in the grains samples that cross-reacted with the antibodies that had a high percentage of *Penicillium* compared to those that had a high percentage of *A. ochraceus*.

The results from this study therefore suggest that antiserum against the ExAgs from *P. aurantiogriseum* can be used to specifically detect three fungal species (*P. aurantiogriseum, P. verrucosum* and *P. citrinum*) in the presence of other species of *Penicillium* or other genera of fungi, that the method is reproducible and sensitive with a detection limit being as low as 95

ng/ml of ExAg proteins. In addition, anti-*P. aurantiogriseum* did not cross-react with water-soluble plant proteins (wheat, barley, corn and soybean, see Table 1) indicating that *P. aurantiogriseum* ExAgs ELISA can be used to specifically detect fungi in a plant matrix. Further studies, however, must be carried out to more conclusively demonstrate that ExAgs from *P. aurantiogriseum* can be not only used to specifically identify the three species of *Penicillium* in the presence of other fungi but that it can also be used to quantify the amount of fungi present in the sample. Several other researchers have also developed ELISA for common moulds in foods including the detection of *Mucor* and *Fusarium* as well as *Aspergillus* and *Penicillium* genera in foods (Notermans and Heuvelman, 1985), the detection of *Alternaria, Geotrichum* and *Rhizopus* genera in tomato puree (Lin *et al.*, 1986) and the detection of *Cladosporium, Geotrichum* and *Mucor* genera in dairy products (Tsai and Cousin, 1990). All of these assays appear to have limitations as they are not specific for species or even genera of fungi and in some cases tended to cross-react with food components (Cox, 1991; Van der Horst *et al.*, 1992; Notermans and Kamphuis, 1992). One possible reason for this lack of specificity is that in these studies the antigens were mostly heat-stable and may be a polysaccharide which is not only present in several species and genera of fungi but also potentially present in food products such as walnuts (Preston *et al.*, 1970; Van der Horst *et al.*, 1992; Notermans and Kamphuis, 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 may be considered to be the first and only species-specific assay that has ever been developed for the detection of *Penicillium* species in grain. The assay, however, was not quantitative and its cross-reactivity was not tested for many other fungal species especially those

belonging to the *Penicillium* genus.

Traditionally, the *Penicillium* species that were used in this study belong to several subgenera. Some of them belong to the subgenus *Penicillium* including *P. aurantiogriseum, P. chrysogenum, P. roqueforti*, *P. commune* and *P. verrucosum*. Some have been assigned to other subgenera, including *P. citrinum* which belong to subgenus *Furcatum* (Pitt and Hocking, 1985). The assignment of different species of *Penicillium* to different subgenera and their degree of phylogenetic relationship have been mostly determined on the basis of traditional taxonomic methods. Immunological identification methods have a great potential to be used as an aid to traditional fungal identification, owing to the high specificity of antibody-antigen reactions. Fuhrmann *et al.* (1990), for instance, used an indirect-monoclonal antibody-ELISA to analyze the taxonomic relationships between fungi. They observed that all tested moulds shared one common antigenic determinant and at least one epitope was shared by *Penicillium* and *Aspergillus*, one was likely common to all *Penicillium* species belonging to subgenera *Aspergilloides* and *Aspergillus* species, and additionally an antigenic epitope was common to all tested species of *Penicillium* subgenera *Aspergilloides*. Fuhrmann *et al.* (1992) developed a monoclonal antibody that would only cross-react with *Aspergillus* and *Penicillium* species. Results in the current study, using ELISA and electrophoresis followed by immunoblotting have demonstrated that polyclonal antibodies against the ExAgs of a specific fungal species can also be used to establish the relationships among fungal species. The results of this study, however, differed slightly in the assignment of fungal species as reported by other authors using more traditional taxonomic methods. It is clear that several immuno-dominant ExAgs are shared by *P. aurantiogriseum*, *P. verrucosum* and *P. citrinum*, suggesting that these three species are

probably the closest taxonomically related fungi tested. The closeness between *P. aurantiogriseum* and *P. verrucosum* agrees with the conclusion of Samson *et al.* (1976), suggesting that *P. aurantiogriseum* should be regarded as a variety of *P. verrucosum*, but not with those of Pitt and Hocking (1985). The relative similarity among ExAgs of these two species and *P. citrinum* is surprising as *P. citrinum* has been considered to be more distantly related taxonomically, but Fuhrmann *et al.* (1990) also did not find any distinction between subgenera *Penicillium* and *Furcatum*. In contrast to *P. aurantiogriseum, P. verrucosum* and *P. citrinum* cross-reactivities, the species of fungi belonging to the subgenus *Penicillium* (*P. roqueforti* and *P. chrysogenum*) showed much less similarity with *P. aurantiogriseum*. Further studies will be required to determine the degree of cross-reactivity with other commonly occurring and closely related species of *Penicillium*. It should be pointed out that most of the taxonomic interrelatedness established in this study agreed well with traditional taxonomy, and some of the minor disagreement with traditional taxonomy may be due to the inherent difficulty in *Penicillium* systematics for accurately distinguishing among different species. The phylogenetic relationship between other species and subgenera within *Penicillium* genus needs to be further established.

The ExAgs used in this study were a mixture of the excreted macromolecules (M_r 10,000 to 120,000) with their nature being generally unknown. Ammonia sulphate fractionation, however, demonstrated that they may have been composed of both proteins and glycoproteins or carbohydrates with most of the reactivity of the anti-*P. aurantiogriseum* being attributable to the protein rather than the carbohydrate component of the ExAgs. Among the many protein bands that were separated by electrophoresis, only three or four with M_r from 70,000 to 90,000

yielded immuno-dominant bands. One of the immuno-dominant bands in *P. aurantiogriseum* was also present in *P. verrucosum* and *P. citrinum* but was only weakly developed in the presence of the ExAgs from *P. roqueforti* and was either not present or did not react with the same antigen from *Aspergillus* and *Fusarium* species. Purification of ExAgs and possibly the use of monoclonal antibody may therefore be necessary for developing more specific assays for some species in the *Penicillium* genus. 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.

It is concluded that the ExAg-ELISA and immunoblotting tests are sensitive, semi-quantitative and relatively specific which can be useful as a tool not only for the identification and detection of *P. aurantiogriseum* and closely related species in cereal grains but also to assist in the refinement of fungal taxonomy. The ELISA developed in this study can specifically be used to detect quantitatively ng or μg levels of the ExAgs of *P. aurantiogriseum, P. citrinum* and *P. verrucosum*, which are the most common grain contaminants and major mycotoxin producers.

Manuscript II

Aspergillus ochraceus exoantigens and their characterization by ELISA and immunoblotting

ABSTRACT

Aspergillus ochraceus Wilhelm is a common grain storage fungus known for its production of highly toxic, ochratoxin A (OA). Rabbit antiserum was raised against the exoantigens (ExAgs) of *A. ochraceus* and an indirect competitive enzyme-linked immunosorbent assay (ELISA) and an immunoblotting procedure were used to characterize the ExAgs of *A. ochraceus* and other storage and field fungi. ExAgs of 17 closely (four *Aspergillus*) and distantly (seven *Penicillium*, three *Fusarium*, two *Mucor* and one *Alternaria* species) related fungal species and water-soluble extracts of three grains were tested. The rabbit antiserum was highly sensitive to ExAgs from *A. ochraceus* and essentially did not cross-react with ExAgs of any of the storage fungi or field fungi and with the water-soluble grain components in the ELISA. The sensitivity of the ELISA for the detection of *A. ochraceus* ExAgs was between 120 and 220 ng of protein/ml with a detection limit of 50 ng/ml. Immunoblotting confirmed the results obtained from the ELISA as this antiserum reacted strongly with the ExAgs of *A. ochraceus*, and to a much smaller degree with the ExAgs of *A. candidus* Link with the limit of detection being 3.8 ng/lane. The taxonomic relationship between fungal species revealed by the results of this study agreed with conventional fungal systematics. Immunoassay for ExAgs, therefore, could be used for both fungal detection and as an aid for fungal taxonomy with the distinct advantages of being highly sensitive, efficient and reliable. The specificity and sensitivity of this antiserum should allow the development of a specific and reliable ELISA that can be used for the monitoring of *A. ochraceus* contamination in grain samples.

INTRODUCTION

Aspergillus ochraceus Wilhelm is a well known producer of ochratoxin A (OA, Van der Merwe *et al.*, 1965). This fungus belongs to the family of grain storage fungi which frequently contaminate cereal grains and can cause considerable health hazards and economic losses due to deterioration of grain and mycotoxin contamination. OA has been shown to be present in animal products, is a prevalent nephrotoxin and hepatotoxin, and is able to produce cumulative toxic effects including suppression of the immune function and induction of cancer (Prior and Sisodia, 1982; Roschenthaler *et al.*, 1984; Bendele *et al.*, 1985; Stein *et al.*, 1985; Golinski *et al.*, 1985; Marquardt *et al.*, 1988). As *A. ochraceus* has been associated with OA contamination, it is of considerable importance to utilize assays that rapidly and accurately detect its presence to avoid or at least to reduce the hazardous effects of its toxins. Conventional methods for the detection of moulds in food, including mould counting and chemical analysis for compounds such as ergosterol and chitin, suffer from being tedious and nonspecific (Jarvis *et al.*, 1983; Jarvis and Williams, 1987). Traditional methods for the identification of fungi are based primarily on morphological characteristics which are time consuming, require a high level of expertise and are empirical. This has resulted in many misidentifications. New approaches have investigated their physiological characteristics, secondary metabolite profiles, enzyme electrophoretic patterns, genetic composition, ultrastructure and immunological properties (Pitt, 1979; Polonelli *et al.*, 1984; Frisvad and Filtenborg, 1989; Fuhrmann *et al.*, 1990; Pitt and Samson, 1990). Chemotaxonomy based on the analysis of secondary metabolite or enzyme profiles have proved to be useful due to their specificity (Cruickshank and Pitt, 1987; Frisvad and Filtenborg, 1989) but may not always be accurate (Chelack *et al.*, 1991). The development

of immunological assays for the identification and quantification of specific storage fungi would be of considerable benefit because of their simplicity and high degree of specificity. This would be greatly facilitated by the use of an antigen that is unique, specific, highly immunogenic and representative of the fungus under different environmental conditions (Polonelli *et al.*, 1984; Fuhrmann *et al.*, 1992). Kaufman and Standard (1987) have demonstrated that fungi produce soluble macromolecules (exoantigens) which appear to be unique. They have developed, using these antigens, a simple immunodiffusion test to identify medically important fungi. This method is specific but is inefficient, insensitive and time consuming. Among various immunological techniques, the enzyme-linked immunosorbent assay (ELISA) has been increasingly adopted for the detection and identification of fungi in foods, as it has been shown to be sensitive, efficient and reliable (Notermans and Heuvelman, 1985; Lin *et al.*, 1986; Dewey *et al.*, 1990; Tsai and Cousin, 1990). A sensitive ELISA, however, that is capable of specifically detecting and identifying *A. ochraceus* in cereal grains has not been developed. The objective of this study was to demonstrate that the exoantigens (ExAgs) of *A. ochraceus* can be used to produce antibodies that can be utilized for the specific detection of this species of fungi. Rabbit polyclonal antibodies were raised against ExAgs of *A. ochraceus* and an indirect competitive ELISA was developed and used to characterize this antiserum. Immunoblotting was used to confirm the ELISA results and to partially characterize ExAgs of *A. ochraceus*. Both methods demonstrated that the antiserum against ExAgs was specific for *A. ochraceus* and allied species, and suitable for monitoring *A. ochraceus* contamination in grain samples.

MATERIALS AND METHODS

Reagents and equipment

Potato Dextrose Agar (PDA) and Yeast Extract were from BBL, Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD; microtiter plates (Falcon 3911, Microtest III Flexible Assay Plates) were from Becton Dickinson and Co., Oxnard, CA; and reagents for preparing phosphate buffered saline (PBS) were from Mallinckrodt Canada Inc., Pointe-Claire, PQ. Ultra-pure reagents for electrophoresis and immunoblotting were from ICN, Schwarz/Mann Biotech, Division of ICN Biomedical Inc., Cleveland, OH and Bio-Rad Laboratories, Hercules, CA. Other reagents were obtained from Sigma Chemical Company, St. Louis, MO. All solvents and reagents were of analytical grade or better. The microplate reader (Model 450), electrophoresis tanks (Mini-PROTEAN II) and electrotransfer device (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell) were from Bio-Rad Laboratories, Richmond, CA.

Fungal sources

Isolates of *Penicillium verrucosum* Dierckx (798), *P. aurantiogriseum var aurantiogriseum* Dierckx Chemotype I (Frisvad and Filtenborg, 1989) (3298), *P. citrinum* Thom (832) and *P. cyclopium* Westling were provided by Dr. G. Platford, Agricultural Services Complex, Winnipeg, MB. Cultures of *A. ochraceus* Wilhelm (NRRL 3174) and *A. versicolour* (Vuill.) Tiraboschi (NRRL 573) were maintained in this lab. 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. Isolates of *A. candidus* Link (ATCC 44054), *A. flavus* Link var. *columnaris* (ATCC 44310), *P. chrysogenum* Thom (IBT 3359), *P. commune* Thom (IBT 10501)

and *P. roqueforti* Thom var. *roqueforti* (IBT 5229) were provided by Dr. J.T. Mills, Agriculture Canada, Winnipeg Research Station, Winnipeg, MB.

Antigen preparation

Exoantigens from all the fungal species listed above were prepared according to Standard *et al.* (1985) with minor modifications. Briefly, fungal spores from different genera were propagated on PDA and then used to inoculate a sucrose-yeast-mineral (SYM) liquid media. After two weeks of growth at 30°C, the mycelia were separated from the liquid media by filtration through two layers of Whatman No 1 filter paper (Whatman, Clifton, NJ), and the liquid fraction freeze-dried and stored at -20°C. The freeze-dried liquid fractions for immunization or the ELISA were redissolved in 0.1 M PBS (0.075 M NaCl, pH 7.2), dialysed 24 h against 4 changes of PBS and concentrated using a Minicon CS-15 spinal fluid concentrator (AMICON-Division, W.R.Grace & Co.-Conn., Beverly, MA). Particulate matter was removed by centrifugation. The antigen preparation was aliquoted and stored at -80°C. Antigen used for immunization was sterilized by passing through a 0.22 μm filter (Nalgene, Nalge Company, Subsidiary of Sybron Corporation, Rochester, NY). The protein concentration of this and other fractions were determined using the Bradford procedure (Bradford, 1976). These preparations are referred to as exoantigens (ExAgs).

Extraction of water-soluble grain proteins

Clean wheat, barley and corn samples were ground using a Cyclotec sample mill equipped with a 1-mm screen (Tecator AB, Höganäs, Sweden), 50 g of the grain sample was mixed with 100 ml of SYM liquid media and the mixture was shaken vigorously for 1 h on a Wrist Action Shaker (Model 75, Burrell Corporation, Pittsburgh, PA). The sample mixtures were centrifuged at 20,000 x g at 4°C for 30 min, and the supernatants were carefully removed, filtered,

concentrated and aliquoted. The stock antigen preparations were stored at -20°C and the working solutions at 4°C.

Immunization and antibody isolation

One rabbit (Dutch Belted, female, 1 to 1.5 kg in body weight) was subcutaneously injected (3 sites) with sterile ExAg of *A. ochraceus* at the dosage of 1 mg protein at 3-week intervals. The antigen preparation was emulsified with Complete Freund's Adjuvant (CFA, first injection) or Incomplete Freund's Adjuvant (IFA, booster injections). The rabbit was bled and serum antibody response was assayed by double-immunodiffusion one week after the second and subsequent injections. When the desired antibody titer (1:32) was achieved the rabbit was exsanguinated, blood was collected, and serum was harvested, aliquoted and stored at -80°C.

ELISA titre assay

The assay was carried out essentially as described by Harlow and Lane (1988). Briefly, microtiter plates were coated with 0.1 μg per 100 μl per well of *A. ochraceus* ExAg in 0.1 M pH 7.2 PBS and incubated at 4°C overnight. After 3 washings with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 5% skim milk for 2 h at 37°C. Serial dilutions of antisera (100 μl/well) together with negative control serum (preimmune serum) were added to the plates and after 2 h incubation at 37°C, the plates were washed 3 times with PBST. The plates were incubated an additional 1.5 h with goat anti-rabbit-IgG conjugated to alkaline phosphatase. p-nitrophenyl phosphate was added after 6 washings with PBST and the plates were read at 405 nm when the optical density was between 1.5 and 2.0 absorbance units. Checker board titrations were used to determine the optimal coating antigen concentration and antibody dilution.

Competitive ELISA

The protocols for the competitive assays were similar to those of the titre assay except 50 μl of known quantities of serially diluted ExAgs from different fungi were added before the addition of 50 μl of a single fixed-dilution of the rabbit anti-fungal ExAgs serum (1:3000). Goat anti-rabbit IgG conjugated with alkaline phosphatase and p-nitrophenyl phosphate (pNPP) were successively applied onto the plates after incubation. The sensitivity of the assay was the concentration of ExAg protein which inhibited 50% of the antibody binding. This value was calculated from the competitive inhibition curve. The reactivities to other antigens were expressed as percent cross-reactivities. These values were calculated by comparing the sensitivity of different fungal ExAgs with that of *A. ochraceus*. Each assay was replicated twice in duplicate and the results were averaged. The general procedures for the ELISA were similar to those given in Harlow and Lane (1988).

Electrophoresis and immunoblotting analyses

Exoantigens from different fungal species were solubilized in 1.5% SDS at 100°C for 5 min and separated by non-reducing SDS-PAGE according to the method of Laemmli *et al.* (1970) using 4 to 20% gradient gels (Mini-PROTEAN II Ready Gel, Bio-Rad, Hercules, CA). Pre-stained standards that were used and their corresponding molecular weight (M_r) were myosin, 205,000; ß-galactosidase, 116,500; phosphorylase B, 106,000; bovine serum albumin (BSA), 80,000; ovine albumin, 49,500; carbonic anhydrase, 32,500; soybean trypsin inhibitor, 27,500; and lysozyme, 18,500 (Bio-Rad, Low Range). The ExAgs were either visualized by silver stain (Bio-Rad, Silver Plus) or transferred onto a 0.22 μm nitrocellulose membrane (Bio-Rad, Richmond, CA) by semi-dry transfer in 25 mM Tris, 192 mM glycine, pH 8.3 buffer containing 20%

methanol and 1.3 mM SDS according to the manufacturer's instructions (Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell Instruction Manual). The membrane was washed with Tris-buffered-saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5) containing 10% methanol and blocked with 3% BSA for 2 h. The membrane was then washed three times with TBS containing 0.05% Tween-20 (TTBS) for 5 min and incubated for 1.5 h with a 1:5000 dilution of the rabbit antiserum raised against *A. ochraceus* ExAgs. After washing, the membrane was incubated with goat-anti-rabbit IgG conjugated with alkaline phosphatase for 1 h. Nitro-blue tetrazolium (NBT) in combination with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as the substrate (Blake *et al.*, 1984).

RESULTS

Characteristics of the ELISA

Typical competitive inhibition curves for some of the antigens are shown in Figure 5 while the sensitivities and cross-reactivities of all the antigens are shown in Table 5. The ELISA results obtained with the ExAgs of 17 fungal species from five different genera and the water-soluble extracts from three grains demonstrated that the ELISA was highly specific for *A. ochraceus* ExAgs as only three species cross-reacted with the degree of cross-reactivity being low (less than 3%, Table 5). It also did not cross-react with any of the grain extracts. The competitive ELISA was quantitative and highly sensitive for *A. ochraceus* ExAgs as the average sensitivity of the assay was 190 ng/ml with the lowest detectable concentration of *A. ochraceus* ExAgs being 50 ng/ml. The limits of detection of fungal biomass would be much lower as the ExAgs only constitute a small portion of total fungal biomass.

Figure 5. Typical inhibition curves of different fungal antigens for rabbit antibody raised against *A. ochraceus* ExAgs: Ao, *A. ochraceus*; Ac, *A. candidus*; Af, *A. flavus*; Av, *A. versicolour*. Arrows indicate the protein concentration which caused 50% reduction in antibody binding and is the value used to denote sensitivity.

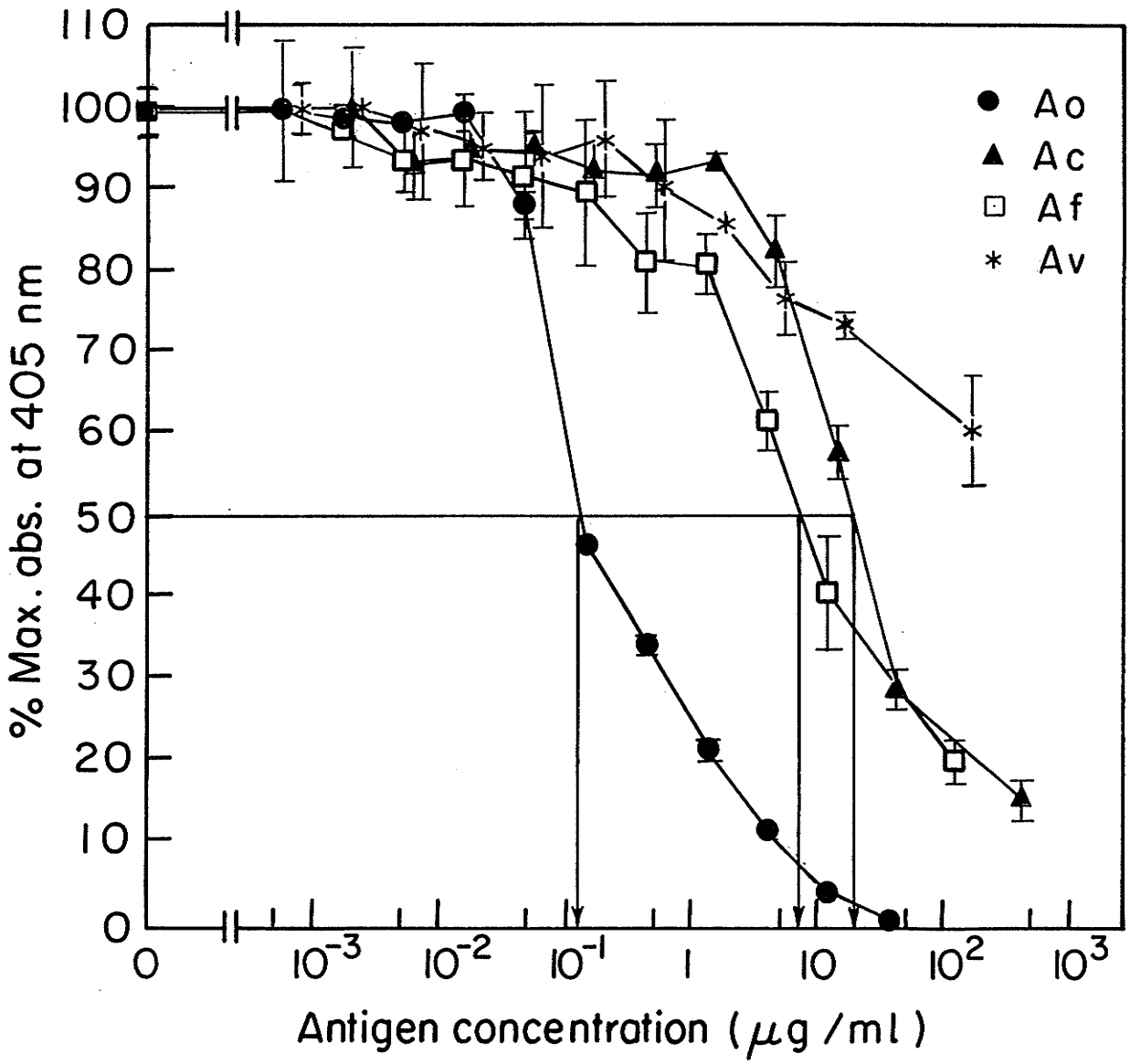

110
100
90
80
70
60
50
40
30
20
10
0
% Max. abs. at 405 nm
0
10^{-3}
10^{-2}
10^{-1}
1
10
10^{2}
10^{3}
Antigen concentration (μg /ml)
Ao
Ac
Af
Av

TABLE 5. Reactivities in the ELISA of different ExAgs to antibodies against ExAgs of *A. ochraceus*.

Antigens tested[1]	Antigen concentration for 50% inhibition (μg/ml)[2]	Percent cross-reactivity[2]
A. ochraceus	0.19±0.02	100
A. candidus	18±2	1±0.2
A. flavus	12±8	2.5±1.6
A. versicolour	Nil[3]	0
P. cyclopium	Nil	0
P. roqueforti	Nil	0
P. verrucosum	Nil	0
P. aurantiogriseum	29±22	1.3±1
P. chrysogenum	Nil	0
P. citrinum	Nil	0
P. commune	Nil	0
F. avenaceum	Nil	0
F. equiseti	Nil	0
F. poae	Nil	0
M. species	Nil	0
M. species	Nil	0
Al. alternata	Nil	0
Barley extract	63±18[4]	0.4±0.1
Corn extract	50±20[4]	0.5±0.2
Wheat extract	170±50[4]	0.1±0.05

1. *P*, *Penicillium*; *A*, *Aspergillus*; *F*, *Fusarium*; *M*, *Mucor* (species which were only identified to genus); *Al*, *Alternaria*.

2. Percent cross-reactivity was the concentration of *A. ochraceus* ExAgs required to give 50% inhibition divided by the concentration of test ExAgs required to give the same degree of inhibition X 100. Values are mean ± SD.

3. Nil, not able to inhibit antibody binding by 50% at a concentration of 100 to 500 μg/ml.

4. Higher amounts were required in other studies which would suggest that these grains may have contained traces of contaminating fungi.

SDS-PAGE analyses

Silver staining of the proteins following electrophoresis demonstrated that the ExAgs from the different species tended to exhibit a unique pattern (Figures 6 and 7). *A. ochraceus* (lane 1) exhibited three prominent bands; two positively stained bands (4 and 5) and one negatively stained band (6) having M_r of approximately 30,000; 29,000 and 20,000, respectively (Figures 6 and 7). In addition, a faint band (1) having a M_r of approximately 100,000 was observed. Bands 2 and 3 having M_r of approximately 90,000 and 50,000 were not readily visible but were detected (Figure 8), as discussed subsequently, using immunoblotting. The different species of *Aspergillus* (lanes 2-4) appeared to have protein bands with similar M_r to those of *A. ochraceus* but none was as prominent as bands 4, 5 or 6. The faintly stained protein band in *A. ochraceus* (band 1) appeared to be more abundantly present in some of the other species of fungi. The very prominent negatively stained band (6) in *A. ochraceus* (lane 1) appeared to be present in smaller amount in extracts prepared from *A. candidus* (lane 3), *A. flavus* (lane 2), *P. chrysogenum* (Figure 7, lane 5) and *P. citrinum* (Figure 7, lane 6). The electrophoretic patterns of cereal extracts also exhibited a unique spectrum of protein bands with the intensely stained bands tending to be different from those of the fungal ExAgs (Figure 6 and 7).

Characteristics of immunoblotting analysis

Immunoblotting results demonstrated that this antibody did not cross-react with ExAgs of five *Penicillium* species (*P. aurantiogriseum, P. verrucosum, P. roqueforti, P. citrinum* and *P. chrysogenum*), *A. versicolour*, one *Fusarium* species (*F. poae*), one *Mucor* species and, most important, it did not show any cross-reactivity to water-soluble wheat and barley proteins (Figure

Figure 6. Silver stained SDS-PAGE profiles of different fungal ExAgs: *A. ochraceus* (lane 1); *A. flavus* (lane 2); *A. candidus* (lane 3); *A. versicolour* (lane 4); *P. aurantiogriseum* (lane 5); *P. verrucosum* (lane 6); wheat extract (lane 7). Samples were solubilized by boiling in 1.5% SDS and separated on 4-15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, low range). The amount of proteins applied on each lane was 125 ng. Bands 2 and 3 were not readily visible but were detected using immunoblotting.

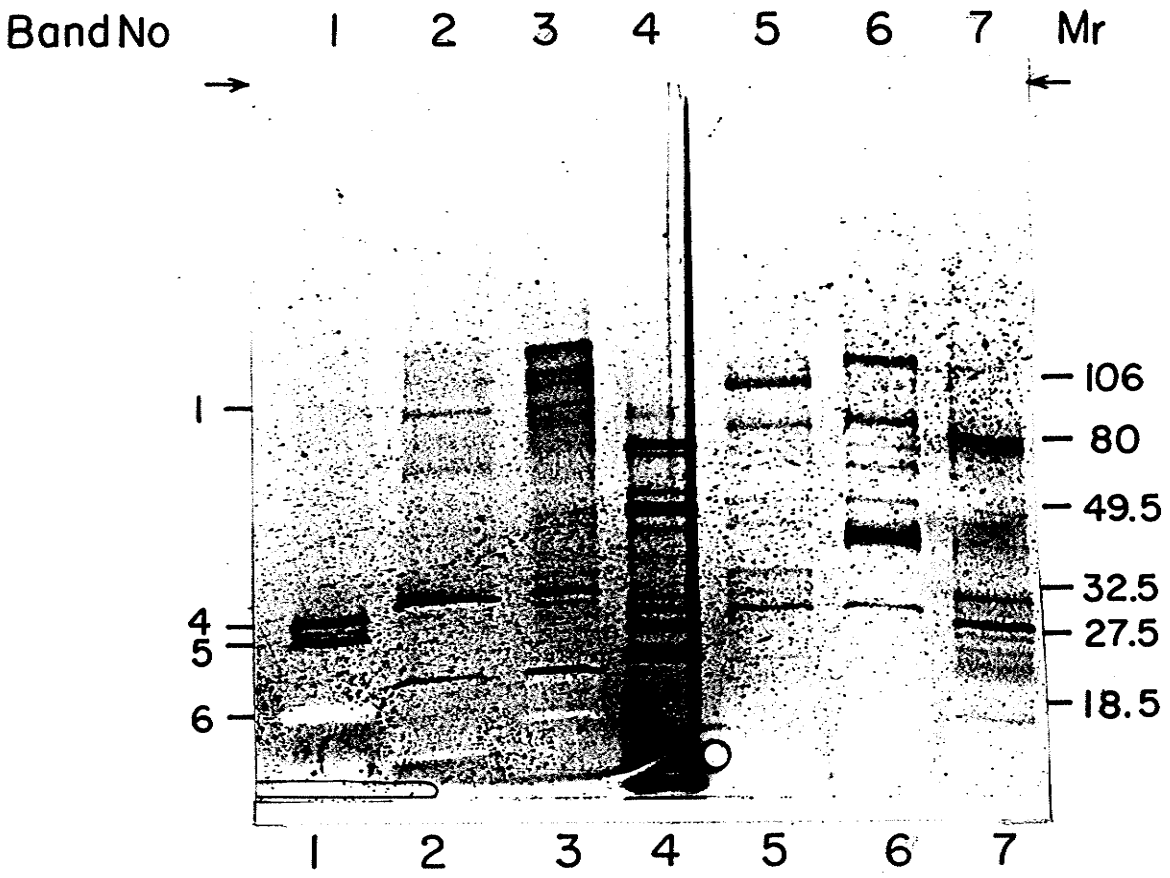

Band No
1
2
3
4
5
6
7
Mr
106
80
49.5
32.5
27.5
18.5
1
4
5
6
1
2
3
4
5
6
7

Figure 7. Silver stained SDS-PAGE profiles of different fungal ExAgs: *A. ochraceus* (lane 1); *F. poae* (lane 2); barley extract (lane 3); *P. cyclopium* (lane 4); *P. chrysogenum* (lane 5); *P. citrinum* (lane 6); *Mucor spp1.* (lane 7). Samples were solubilized by boiling in 1.5% SDS and separated on 4-15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, low range).

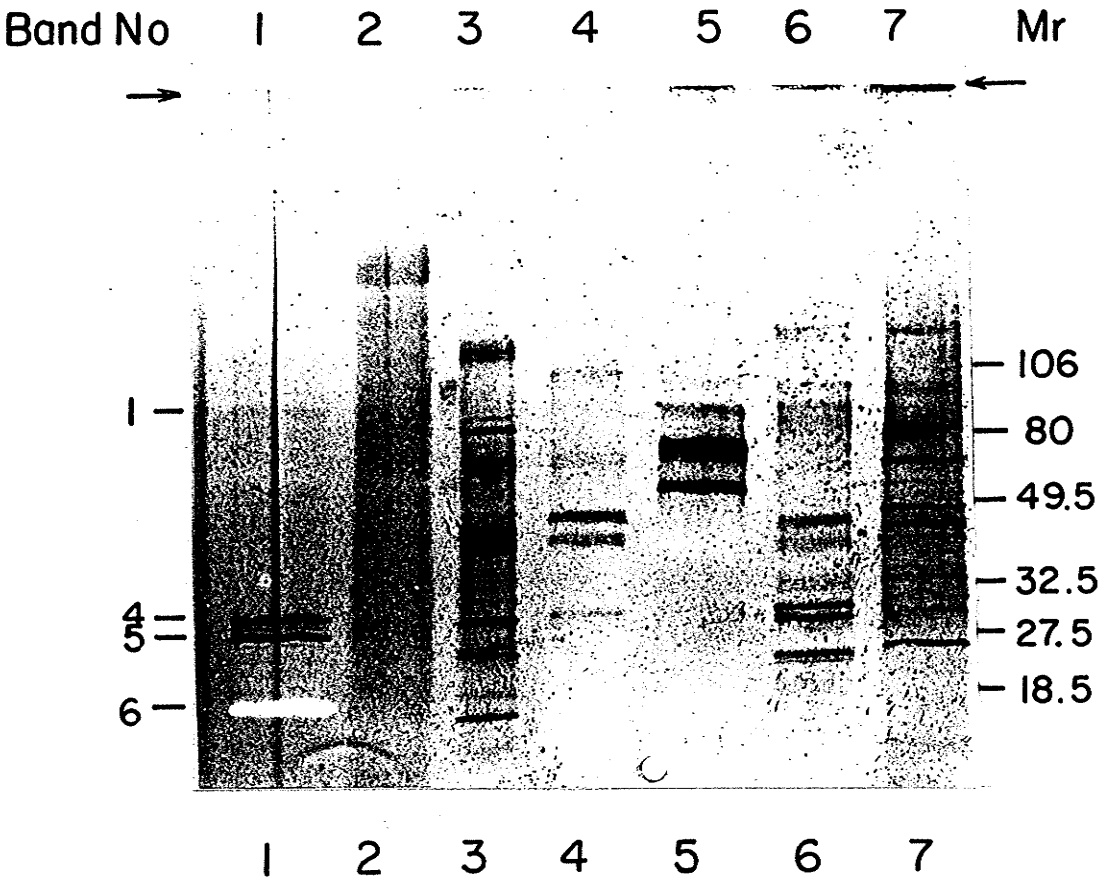
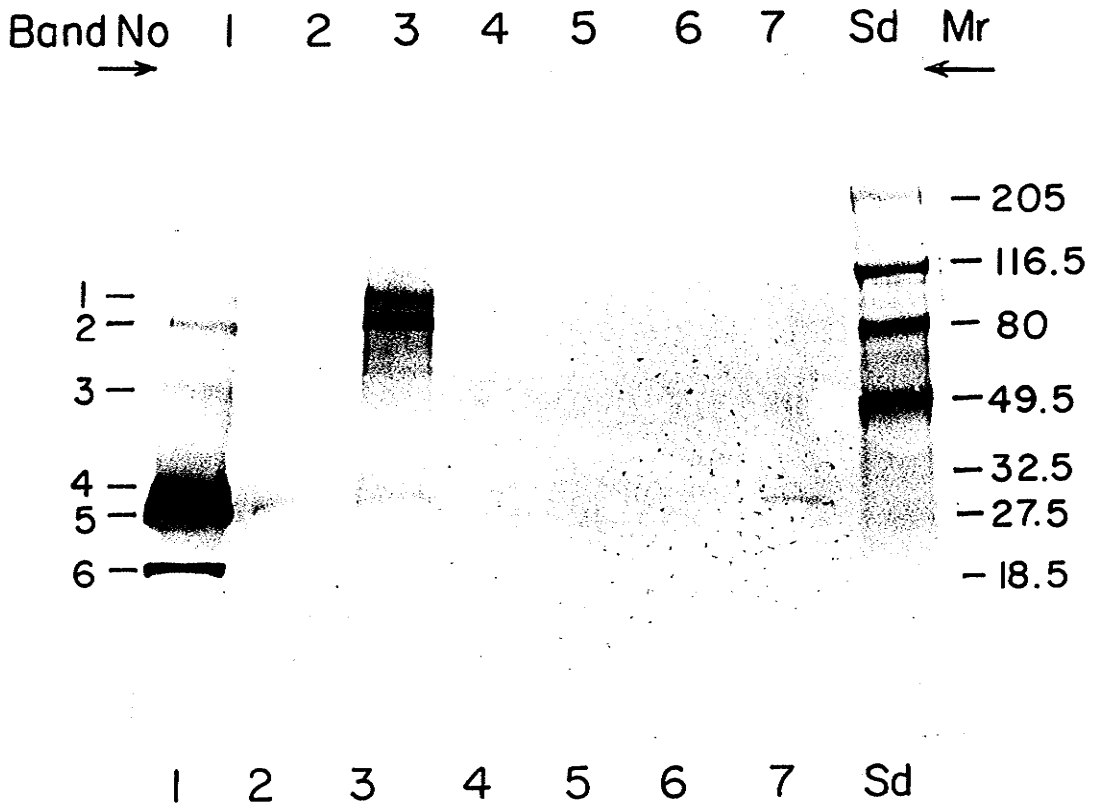

Band No
1
2
3
4
5
6
7
Mr
106
80
49.5
32.5
27.5
18.5

Figure 8. Immunoblotting fingerprints of different fungal ExAgs using rabbit anti-*A. ochraceus* (1:5000 diluted): *A. ochraceus* (lane 1), *A. flavus* (lane 2), *A. candidus* (lane 3), *A. versicolour* (lane 4), *P. verrucosum* (lane 5), *P. aurantiogriseum* (lane 6), wheat extract (lane 7). The apparent molecular weight of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, high and low range). The amount of proteins applied on each lane was 125 ng while the antiserum was diluted 1 to 5000.


Band No
1
2
3
4
5
6
7
Sd
Mr
205
116.5
80
49.5
32.5
27.5
18.5
1
2
3
4
5
6
7
Sd

8, some of the negative data are not shown). The antiserum, however, was highly reactive to *A. ochraceus* ExAgs (lane 1) and exhibited lower cross-reactivities to the ExAgs of *A. candidus* (LANE 3) and had only slight reactivity with the ExAgs of *A. flavus* (lane 2). A total of six bands were visually detected from the electrophoretogram with the *A. ochraceus* ExAgs with band 5 being the most prominent and tending to merge with band 4. Band 6 was readily visible, band 2 was faint while the other bands were hardly visible. The application of a higher amount of protein to the gel (2000 vs 125 ng/lane) yielded an additional 4 to 6 bands, all of which were faint while the application of a 160-fold lower amount of protein demonstrated that bands 4 and 5 were distinct with band 4 having a colour intensity similar to that of band 6. Under these conditions only bands 4, 5 and 6 were evident. The colour intensity of band 5 was also the same as that of bands 4 and 6 when the ExAg preparation was additionally diluted (16-fold) suggesting that antigen number 5 contributed substantially more to the total antigen-antibody reaction than any of the other antigens (data not shown). These results demonstrate that there was one immunodominant antigen (band 5), two antigens that yielded readily visible bands (bands 4 and 6) while the others were only slightly visible. Bands 1, 4, 5 and 6 of *A. ochraceus* (lane 1) as detected by immunoblotting appeared to be the same as the corresponding silver stained bands. The apparent M_r of the different antigens are indicated in Figure 8.

Results presented in Figure 8 also demonstrate that the rabbit anti-*A. ochraceus* serum in the presence of *A. candidus* ExAgs (lane 3) yielded one prominent band (band 2), one clearly visible band (band 1) and up to 6 faint bands, some of which overlapped with those produced by the *A. ochraceus* ExAgs (bands 5 and 6). In the presence of a higher concentration of *A. candidus* (2000 vs 125 ng ExAg protein/lane) a total of 10 to 13 bands became visible whereas

upon the application of a much lower concentration of the ExAgs, many of the bands were not visible (data not shown). A visual comparison of immunoblotting patterns of serially diluted antigens from *A. ochraceus* and *A. candidus* demonstrated that the two respective immunodominant bands had similar colour intensity at protein concentrations of approximately 4 and 32 ng per lane, an 8-fold higher concentration for the latter compared to the former antigen. These results would suggest that *A. candidus* has many antigens that cross-reacted with the *A. ochraceus* antiserum with most of the reactions being weak although a few showed significant cross-reactivities (band 1 and 2). Approximately an eight fold higher concentration of *A. candidus* ExAgs was required to produce the same band intensity as obtained with the ExAgs from *A. ochraceus*. These observations would suggest that the antigens represented by bands 4, 5 and 6 of *A. ochraceus* tend to produce antibodies that have low or no cross-reactivities with similar proteins in other fungal species. The high sensitivity of the immunoblotting assay is indicated by its ability to detect band 5 of *A. ochraceus* ExAgs when only 4 ng of ExAgs was applied to the gel.

DISCUSSION

A competitive ELISA was developed using rabbit antibodies against the ExAgs of *A. ochraceus*. Various fungal ExAgs and water extracts from grains were tested for their cross-reactivities with the antibody using the ELISA and immunoblotting techniques. The results of both methods indicate that this assay, for the fungi that were tested, was specific and highly sensitive for *A. ochraceus* ExAgs. Of the limited ELISAs developed for detecting moulds in foods, only a few were quantitative and none of them were specific for species of *Aspergillus* or *Penicillium*

genera. Notermans and Heuvelman (1985) developed an ELISA test for detecting moulds in foods using heat-stable extracellular polysaccharide antigens. The antigens tended to be common to several species and genera of fungi and therefore tended not to be species or genus-specific. Lin *et al.* (1986) using antigens extracted from boiled mycelium, developed a sandwich ELISA for detecting fungi within genera of *Alternaria*, *Geotrichum* and *Rhizopus* in tomato puree but the cross-reactivity of species among genera was not tested. The assay for Alternaria, however, was shown in further studies to cross-react with other genera of fungi (Lin and Cousin, 1987). The detection limit of these assays was 1 mg per kg (ppm) dried moulds. Dewey *et al.* (1990), however, developed an ELISA that was specific for *P. islandicum* but this assay was not quantitative. The current ELISA, as indicated above, tended to be highly specific for *A. ochraceus* when tested against many different fungal species, whereas the other assays were generally not specific for the particular fungal species from which the antibody was developed. The high sensitivity of the rabbit anti-*A. ochraceus* ExAgs for *A. ochraceus* together with its low degree of cross-reaction with grain components and other fungi would suggest that the antiserum would be suitable for the specific detection of *A. ochraceus* in grain samples in the presence of other moulds.

Immunoblotting profiles of the ExAgs of *A. ochraceus* (Figure 8) demonstrated that one of the proteins was the immunodominant antigen (band 5) and two produced good reactions (bands 4 and 6). Antigens 4 and 5, as shown by silver staining, were also the most abundant ExAgs and had an apparent M_r of about 30,000. They appeared to be unique for *A. ochraceus*, but the results are not conclusive as the resolution of the protein bands in this region was not distinct. A smaller ExAg having an apparent M_r of approximately 20,000 produced a distinct

negatively stained band (band 6) which, as indicated above, was also highly immunogenic.

Aspergillus candidus also had several antigens that cross-reacted with the antiserum. The two more prominent bands (1 and 2, Figure 8, lane 3) appeared to be different in M_r from the prominent bands (4, 5 and 6) obtained with the *A. ochraceus* ExAgs. Although both ExAgs from the two species of fungi produced prominent immunoblots, approximately 8-fold more antigen was required to produce the same band intensity with the ExAgs from *A. candidus* compared to that from *A. ochraceus*. This suggests that the ExAgs in *A. candidus* were present in either smaller amounts in the extracts or tended to have a weaker cross-reactivity with the antiserum than that of the ExAgs from *A. ochraceus*. The data, therefore, are in agreement with the results obtained for the ELISA which also indicated the same pattern of inhibition between the antiserum and the two ExAgs. In addition, it was also shown that when the concentration of the ExAgs were increased there were several other common cross-reacting proteins between *A. ochraceus* and *A. candidus*. On the basis of these observations it may be concluded that *A. candidus* and *A. ochraceus* have many ExAgs which are probably mainly proteinaceous in nature and which will cross-react with each other. The degree of cross-reactivity of *A. candidus* ExAgs with the antiserum, however, tends to be weaker and therefore does not greatly interfere in ELISA. The other species of *Aspergillus* and other fungi did not produce immunostained bands or produced only faint bands suggesting that at most they contained antigens that only weakly cross-reacted with the antiserum. Compared with the ELISA, immunoblotting has similar sensitivities (50 ng/ml vs less than 5 ng/lane). Unfortunately, this technique which is not only highly sensitive but also specific, has not been used for detection and identification, and for studying interrelatedness among storage fungi. The results of this study also suggest that even

greater specificity may be obtained for the detection of *A. ochraceus* as three proteins (4, 5 and 6) were identified that are relatively abundant, appeared to be highly antigenic and exhibited low cross-reactivities with the other fungal species. These can be isolated in pure form for either polyclonal or monoclonal antibody production.

In addition, the immunoblotting results indicate that *A. flavus*, *A. candidus* and *A. ochraceus* have proteins that tend to cross-react with each other to a greater degree than with *A. versicolour* suggesting that these species may be taxonomically more closely related to each other than with *A. versicolour*. The interrelatedness among the *Aspergillus* species revealed by this study agreed with conventional taxonomy. In traditional *Aspergillus* classification, *A. flavus, A. candidus* and *A. ochraceus* belong to the same subgenus *Circumdati* while *A. versicolour* belongs to another subgenus *Nidulantes* (Gams *et al.*, 1985).

Currently, all conventional mould detection and classification methods for cereal grain involve the culture of the moulds which is time consuming and require a high level of expertise. The conventional methods also do not monitor mould contamination effectively under the circumstances where fungal deterioration occurs and the fungus is not viable because of processing or other treatment. Under such conditions there would not be a close association between amount of fungal biomass present in the samples and the number of viable spores. Other methods such as the ergosterol or chitin assay have been used as a measure of fungal contamination (Donald and Mirocha, 1977; Nout *et al.*, 1987; Rotter *et al.*, 1989). These methods, however, lack accuracy and specificity, and are difficult to carry out. The ExAgs, which are secreted into the environment by fungi during their growth, have been shown to be useful for detecting medically important fungi using a simple double-diffusion type

immunological assay (Standard *et al.*, 1985), and have been shown in the current study to be useful for the detection of *A. ochraceus* antigens by use of ELISA or immunoblotting techniques. These data suggest that these ExAgs, which appear to be protein in nature, can be used to specifically detect fungi in foods or feeds. The advantage of the ELISA compared to the other assays are numerous and as indicated above, a distinct advantage is that sample preparation only involves extraction of the sample in an aqueous solution without any cleanup or further preparation.

In conclusion, the ELISA test developed in this study is highly sensitive for *A. ochraceus* and at most, weakly cross-reacts with other related species. These results suggest that an immunoassay can be developed for the detection and identification of specific fungi using fungal ExAgs. Compared to conventional methods, this method is simple, fast, reliable and quantitative. Additional research, however, should further improve the specificity of the assay as several antigens have been identified in *A. ochraceus* that are immunodominant and may be unique, and, most of all, the assay should be validated if it is to be used in practical applications such as the detection of fungi on cereal samples.

Manuscript III

The development of a solid-phase immunoassay for the detection and identification of *Aspergillus ochraceus* in wheat grain

ABSTRACT

Aspergillus ochraceus Wilhelm is a common contaminant of stored foods especially cereal grains and is able to produce the mycotoxin, ochratoxin A (OA). An indirect competitive ELISA using rabbit anti-*A.ochraceus* exoantigens (ExAgs) was evaluated in a number of different studies. All of the studies indicated that the solid-phase immunoassays (ELISA and immunoblotting) for *A. ochraceus* ExAgs were specific and that they could be used to provide an index of the degree that the sample was contaminated with *A. ochraceus*. The presence of *Penicillium* including the common fungus, *P. aurantiogriseum*, other fungal ExAgs, and the wheat matrix had little effect on the detection and quantitation of *A. ochraceus* ExAgs. Sterilized wheat samples, for example, that had been inoculated with spores from both *A. ochraceus* and *P. aurantiogriseum* gave a positive ELISA result when the samples were inoculated with *A. ochraceus* spores but not when they were only inoculated with *P. aurantiogriseum* spores. The detected amounts of *A. ochraceus* ExAgs in naturally moulded wheat and in natural moulded wheat enriched in *A. ochraceus* correlated favourably with the amount of OA detected ($r=0.93$, $P<0.05$), the percentage of *A. ochraceus* infection ($r=0.89$, $P<0.05$), the chitin (glucosamine) content ($r=0.64$, $P<0.05$) and the number of colony forming units ($r=0.68$, $P<0.05$), and tended to be inversely correlated with the percentage of *Penicillium* infection ($r=-0.32$, $P>0.05$). Immunoblotting patterns of extracts from a liquid and wheat culture of *A. ochraceus* demonstrated that antibodies were developed against several antigens with the immunodominant antigens having molecular weights of approximately 20,000 and 30,000. The results also indicated that there was a change in the immunoblotting pattern with other antigens tending to give a more pronounced staining pattern compared to that obtained in pure culture. Overall, the

assay was sensitive (limit of detection was as low as 50 ng/ml), appeared to be specific and was highly correlated with the amount of *A. ochraceus* in the sample.

INTRODUCTION

The mould *Aspergillus ochraceus* Whilhem is economically important as it is a common storage fungus and produces ochratoxin A (OA). This mycotoxin is nephrotoxic, hepatotoxic and immunosuppressive (Prior and Sisodia, 1982; Roschenthaler *et al.*, 1984; Bendele *et al.*, 1985; Stein *et al.*, 1985; Marquardt and Frohlich, 1992) and has been reported to be present in a wide range of foods including cereal grains and animal products (Pitt and Hocking, 1985; Golinski *et al.*, 1985; Marquardt *et al.*, 1988). The contamination of food with *A. ochraceus* is therefore of concern and, as a result, a reliable and sensitive method should be developed to not only detect its presence but also to quantify its amount in food and feed. This cannot be achieved using traditional methods such as direct plating and propagule dilution counting as these methods are time consuming to carry out and have several other weaknesses including a lack of reliability and sensitivity. Other alternative chemical analyses for compounds such as ergosterol and chitin also suffer from being nonspecific, complicated, tedious and require expensive equipment (Jarvis *et al.*, 1983; Cox, 1991). Immunochemical methods have been increasingly used for the detection of specific fungi in foods owing to their high sensitivity, specificity and simplicity (Notermans and Heuvelman, 1985; Lin *et al.*, 1986; Tsai and Cousin, 1990). Kaufman and Standard (1987) have demonstrated that fungi produce soluble macromolecules (exoantigens) which appear to be unique to particular species or genera of fungi. The development of an assay that will detect these exoantigens (ExAgs) may not only be useful for indicating the level of contamination of fungi in food and the corresponding presence of mycotoxins, but also would assist in clarifying the nature of the interrelatedness among different species of fungi, particularly those in the *Penicillium* genus. Among the various immunological techniques, only

a few have utilized the enzyme linked-immunosorbent assay (ELISA) for the detection of fungi in cereal grains. Dewey *et al.* (1990) developed a monoclonal antibody based ELISA for detecting *P. islandicum* in rice grains, which indicated that it is feasible to develop such an assay for the specific monitoring of mould contamination. Currently, an efficient and sensitive ELISA-based assay that can detect and differentiate *A. ochraceus* from other *Penicillium* and *Aspergillus* species has not been developed. A previous study, however, has reported on the successful development of an indirect competitive ELISA that would specifically detect *A. ochraceus* ExAgs (Lu and Marquardt, Manuscript 2). The purpose of the present investigation was to evaluate the suitability of this ELISA for detecting and quantifying *A. ochraceus* in various samples containing different combinations of ExAgs from *A. ochraceus* and another common fungus, *P. aurantiogriseum*, in *A. ochraceus* and *P. aurantiogriseum* inoculated wheat samples, and in naturally moulded wheat samples inoculated with *A. ochraceus* spores.

MATERIALS AND METHODS

Materials

Potato Dextrose Agar (PDA) and Yeast Extract were from BBL, Microbiology System, Becton Dickinson and Co., Cockeysville, MD; microtiter plates (Falcon 3911 Microtest III Flexible Assay Plates) were from Becton Dickinson and Co., Oxnard, CA; reagents for preparing phosphate buffered saline (PBS) were from Mallinckrodt Canada Inc., Pointe-Claire, PQ; D-glucosamine-HCL were from Calbiochem, San Diego, CA, and Ultra pure reagents for electrophoresis and immunoblotting were from ICN, Schwarz and Mann Biotech, Division of ICN Biomedical Inc., Cleveland, OH and Bio-Rad Laboratories, Hercules, CA. Other reagents

were obtained from Sigma Chemical Company, St. Louis, MO. All solvents and reagents were of analytical grade or of better quality. The microplate reader (Model 450), electrophoresis tanks (Mini-PROTEAN II) and electrotransfer device (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell) were from Bio-Rad Laboratories, Richmond, CA. Isolates of *P. aurantiogriseum var. aurantiogriseum* (3298) were provided by Dr. G. Platford, Plant Pathology Laboratory, Manitoba Agricultural Services Complex, Winnipeg, MB. This species was re-identified on the basis of both morphological characteristics and secondary metabolite profiles (Frisvad and Filtenborg, 1983). The culture of *A. ochraceus* (NRRL 3174) was maintained in our laboratory.

General Procedures

Procedures for the preparation of ExAgs, extraction of the soluble grain components, immunization and antibody characterization by immunoblotting and ELISA were the same as in a previous report (Lu and Marquardt, Manuscript 2). Protein, glucosamine and galactosamine were quantified according to procedures outlined by Bradford (1976) and Rotter *et al.* (1989), respectively. Glucosamine or galactosamine which is a measure of the chitin content of the mould was used to estimate the amount of fungal biomass.

Experiment 1, Exoantigen spiking assay

Aspergillus ochraceus ExAgs were premixed with those of *P. aurantiogriseum* at concentrations given in Table 6. They were added into microtiter plates precoated with *A. ochraceus* ExAgs followed by the addition of rabbit anti-*A. ochraceus* and a competitive assay was then carried out. Alkaline phosphatase labelled goat anti-rabbit IgG and p-nitrophenyl phosphate were used in the detection system. The amount of ExAgs in the sample was calculated from a standard inhibition curve obtained by using pure *A. ochraceus* ExAgs. The assays were carried out in

duplicate and the entire procedure was repeated.

Experiment 2, Spore load and the production of ExAgs by two fungi

Aspergillus ochraceus and *P. aurantiogriseum* were grown on PDA slants at 25°C for 7 days, and spores were harvested by adding 5 ml of distilled water containing 0.1% Tween 80 onto each slant followed by dislodgement of the spores with a sterile inoculation needle. A stock solution of the spore suspension from each fungus was prepared either alone or in combination so that the spore counts were approximately 10^5 per ml. Each of the three stock solutions were subjected to 10-fold serial dilutions (1:10 to $1:10^6$). Clean non-mouldy wheat (100 g in 500 ml Erlenmeyer flasks) was mixed with 50 ml of distilled water, conditioned overnight at 4°C and autoclaved for 20 min to sterilize the grain. The sterilized wheat was inoculated with 10 ml of the serially diluted spore suspension (10-fold, 1:10 to $1:10^6$), sealed with a sterile sponge plug, and the samples were incubated at 30°C for 6 days. Extracts were prepared by mixing 100 ml of 0.1 M pH 7.2 PBS with the wheat samples, soaking with frequent mixing for 30 min followed by filtration (50 ml) through 2 layers of Whatman No 1 filter paper (Whatman, Clifton, NJ). The filtrate was aliquoted and stored at -20°C. The washed samples were then mixed with 160 ml of PBS followed by blending with a commercial blender for 3 min and centrifuging for 30 min at 20,000 x g. Aliquots of the supernatants were filtered and stored at -20°C.

The assay for the *A. ochraceus* ExAgs involved the same protocol as for the competitive assay (Lu and Marquardt, Manuscript 2) and in general followed the procedures outlined by Harlow and Lane (1988). The serially diluted (1:10 to $1:10^3$) extracts were added to the microtiter plates which were coated with *A. ochraceus* ExAgs and an equal volume of rabbit anti-*A. ochraceus* solution was then added. The amount of ExAgs presented in the samples were

calculated from the standard inhibition curves.

Experiment 3, Inoculation of autoclaved wheat with two species of fungi

Clean wheat samples were moisturized by adding 50 ml of distilled water to 100 g of sample in 500 ml Erlenmeyer flasks, conditioned overnight and autoclaved. The samples were aseptically inoculated with 1 ml spore suspension containing approximately 10^6 spores of *A. ochraceus* or *P. aurantiogriseum* and were sealed with a sponge plug. The inoculated samples were incubated at 30°C together with the non-inoculated control samples. Fungal growth was visually scored to indicate the degree of fungal growth (none, slight and considerable). Three flasks were then taken from each inoculation group on days 0, 2, 4, 6, 8, 10, 12 and from the controls on days 0, 6, and 12, and were dried by a stream of air at ambient temperature in a fume hood. The samples were ground in a Cyclotec mill equipped with a 1-mm screen (Tecator AB, Höganäs, Sweden) and stored at -20°C.

The exoantigens were isolated by extraction of 10 g of ground dried grain with 40 ml of 0.1M pH 7.2 PBS. The mixtures were shaken vigorously using a Wrist Action Shaker (Model 75, Burrell Corporation, Pittsburgh, PA) for 1 h, centrifuged at 20, 000 x g for 45 min, and the supernatant was removed, filtered, aliquoted and stored at -20°C. The amount of ExAgs was assayed by the indirect competitive ELISA as described above. Briefly, the plates were coated with *A. ochraceus* ExAgs and, after blocking and washing, 50 μl samples that were subjected to 10-fold serial dilutions in PBST (1 to $1:10^4$) were added to the microtiter plates followed by an equal volume of the working solution of rabbit anti-*A. ochraceus*. Goat anti-rabbit IgG conjugated with alkaline phosphatase and p-nitrophenyl phosphate were added, and absorbance at 405 nm was determined. The concentrations of ExAg in the extracts were calculated from the

standard inhibition curves.

Individual glucosamine and galactosamine analyses were carried out on 150 mg of each triplicate sample. Selected ExAg extracts as indicated in Figure 4 were solubilized directly in 1.5% sodium dodecyl sulphate (SDS) at 100°C for 5 min and analyzed by immunoblotting (Lu and Marquardt, Manuscript 2). The *A. ochraceus* ExAgs from pure liquid culture were used as standard reference antigens and approximately 125 ng of protein was applied per lane while the other samples were applied directly without dilution.

Experiment 4. Studies with non-autoclaved wheat

Hard spring wheat samples that were not heat-treated were used. These grains had been previously exposed to the indigenous fungal spores that occur naturally and therefore would become mouldy under high moisture conditions. 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 moisturized grain was inoculated with 0, 10^2 or 10^5 *A. ochraceus* spores per g, sealed with a sponge plug and the samples were incubated at 30°C for 0, 7 and 30 days. There were a total of five replicates for each time period in each group. The non-moisturized grain also served as a control. The individual treatments are presented in Table 7. The samples were prepared for analysis following procedures outlined above for Experiment 3.

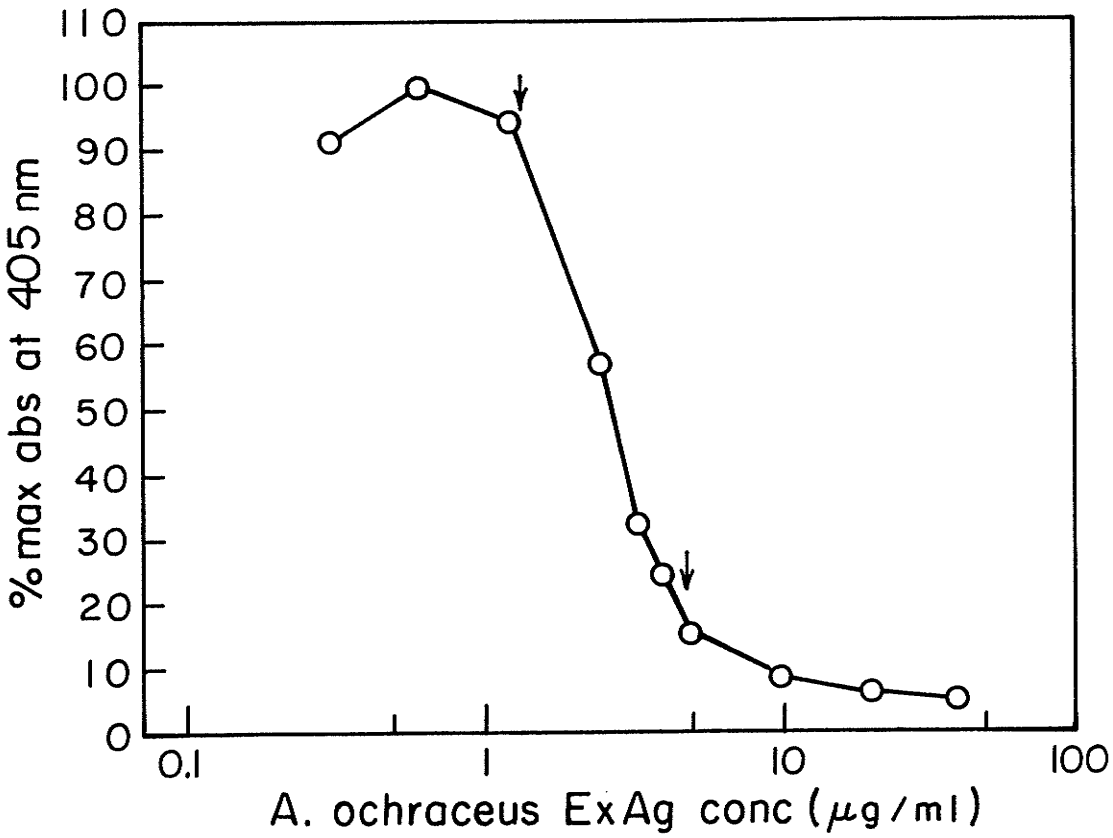
The number of fungal propagules were determined according to standard procedures (ISO, 1983a; 1983b). In brief, 90 ml of sterile diluent (0.1% peptone saline, pH 7.0) was mixed with 10 g of sample, the mixture was shaken for 30 min on a Wrist Action shaker, and 1 ml of the mixture was immediately transferred into 9 ml of sterile diluent and mixed. Each sample was then subjected to 10-fold serial dilutions. Samples (1 ml) from each dilution were poured onto

4 malt-extract-agar (MEA. 2% malt extract, pH 6 to 6.5, containing 0.0006% Tetracycline) plates and incubated upright at room temperature (20-25°C) for 5 days. Plates having between 5 to 50 colonies (occasionally up to 100) were counted. The mycoflora of each sample was also analyzed following standard procedures (Mills and Wallace, 1979). The amount of OA in each sample was determined by High-Performance-Liquid-Chromatography (HPLC) analysis using a procedure similar to that described by (Clarke *et al.*, 1993). In brief, 1 g of sample was extracted with 15 ml of 80% methanol: 20% H_2O (pH 2.1, acidified with H_3PO_4), the mixture was shaken for 30 min and centrifuged for 15 min at 10,000 x g, and the supernatant was transferred into optical clear vials and diluted if necessary. The sample (10 μl) was injected onto a C18 RP column (Beckman, San Ramon, CA) having a flow rate of 1.5 ml per min. The mobile phase was composed of 67% methanol, 10% isopropanol and 33% H_2O (pH 2.1, acidified with H_3PO_4). ExAgs were extracted using a protocol similar to that described in Experiment 3 except that the proportion of sample to PBS was changed from 10 g per 40 ml to 5 g per 25 ml. Exoantigen analysis, the immunoblotting protocols, and the total fungal biomass as estimated by glucosamine analysis were essentially the same as described in Experiment 3.

RESULTS

A typical standard inhibition curve of *A. ochraceus* ExAgs from pure liquid culture is shown in Figure 9. The ability of the assay to detect the ExAgs from *A. ochraceus* in the presence of a background of *P. aurantiogriseum* ExAgs is illustrated in Table 6 (Experiment 1). The results demonstrated that at concentrations of *A. ochraceus* ExAgs greater than 1 μg per ml

Figure 9. Typical standard inhibition curve of *A. ochraceus* ExAgs prepared from pure liquid culture. The line between the arrows indicate the linear region which was used for determining the concentration of *A. ochraceus* ExAgs in all samples. Values represent the mean of two 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%.


110
100
90
80
70
60
50
40
30
20
10
0
% max abs at 405 nm
0.1
1
10
100
A. ochraceus ExAg conc (μg/ml)

and in the presence of 0.05 μg per ml ExAgs from *P. aurantiogriseum* there was no apparent reactions of *P. aurantiogriseum* with the *A. ochraceus* antiserum. Overall, the degree of cross-reaction of *P. aurantiogriseum* with the anti-*A. ochraceus* serum was relatively insignificant as its value was less than 1 % for all concentrations of *P. aurantiogriseum* ExAgs including samples that contained 100 μg per ml *P. aurantiogriseum* ExAgs. The assay, however, became limiting for the detection of *A. ochraceus* ExAgs at concentrations above 50 μg per ml. Other studies demonstrated that this can be corrected by the appropriate dilution of the samples.

The influence of inoculation size, type of inocula and source of ExAgs on the amount of *A. ochraceus* ExAgs detected in water extracts is illustrated in Figure 10 (Experiment 2). The results demonstrated that the *P. aurantiogriseum* ExAgs did not react with the *A. ochraceus* antiserum, that the antiserum can be used to detect the presence of *A. ochraceus* ExAgs in the presence of *P. aurantiogriseum*, and that the yield of ExAgs was approximately 10-fold greater when obtained from a homogenate of the grain compared to that obtained by surface washing. Future studies were, therefore, conducted with homogenates of the grain.

The objective of Experiment 3 was to further confirm that the *A. ochraceus* antiserum could be used to predict the presence and amount of *A. ochraceus* in the presence of *P. aurantiogriseum* in a grain sample as well as to establish the relationship between the content of *A. ochraceus* ExAgs and the content of fungal biomass as estimated from the glucosamine and galactosamine assay. Results shown in Figure 11 demonstrated that mycelial growth as indicated by the concentration of glucosamine was obvious on day 2 and that extensive and progressively more growth occurred afterwards on all of the samples. Visual scoring yielded the same trend. The ExAg immunoassay indicated that there was a parallel increase in the amount of

TABLE 6. ELISA of samples spiked with different amounts of *A. ochraceus (A. och)* and *P. aurantiogriseum (P. aur)* ExAgs using rabbit anti-*A. ochraceus* serum[1].

Sample number	Amount of ExAgs added (μg/ml)		Detected amount of *A. och.* ExAgs (μg/ml)[2]
	A. och.	*P.aur.*	
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

1. See Materials and Methods (Experiment 1) for further detail.
2. Values for samples 1 and 2 are low because of the limiting nature of this particular assay. Value represent mean±SD of two replicate analyses.

Figure 10. Comparison of the amount of *A. ochraceus* ExAgs detected in the washings (A) or the homogenate (B) of wheat samples inoculated with different concentrations of fungal spores including A.o., *A. ochraceus*; P.a., P. *aurantiogriseum*; A.o.+P.a., *A. ochraceus* plus *P. aurantiogriseum*. The cultures were inoculated with 10-fold serial dilutions of spores. Values are expressed as ppm. Assays were as described in Materials and Methods, Experiment 2. Values represent an average of duplicate analysis with the average coefficient of variability (CV) being 30% for A.o., 5% for A.o+P.a. and 50% for P.a. in the washings, and 61%, 31% and 25% for the corresponding homogenates.

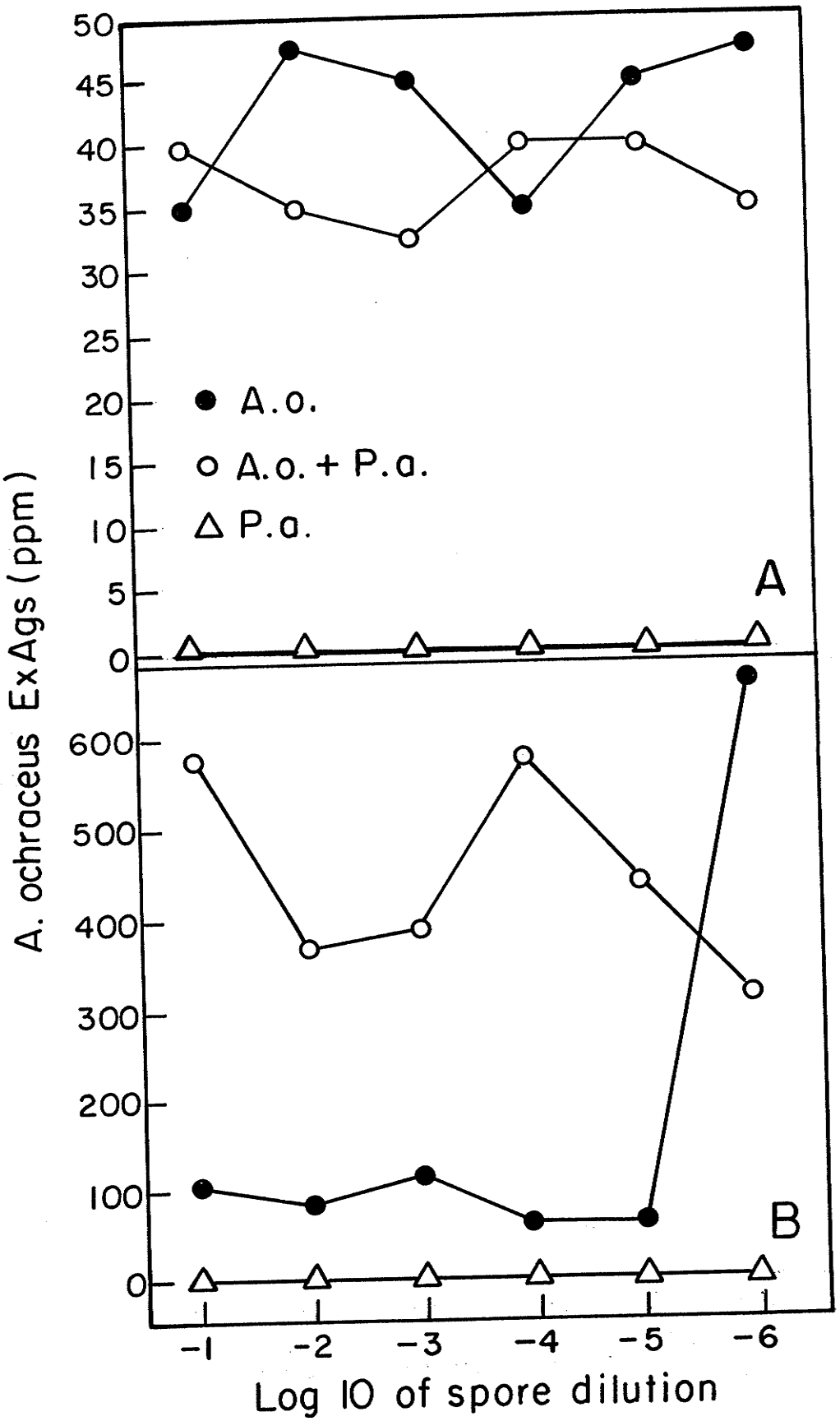
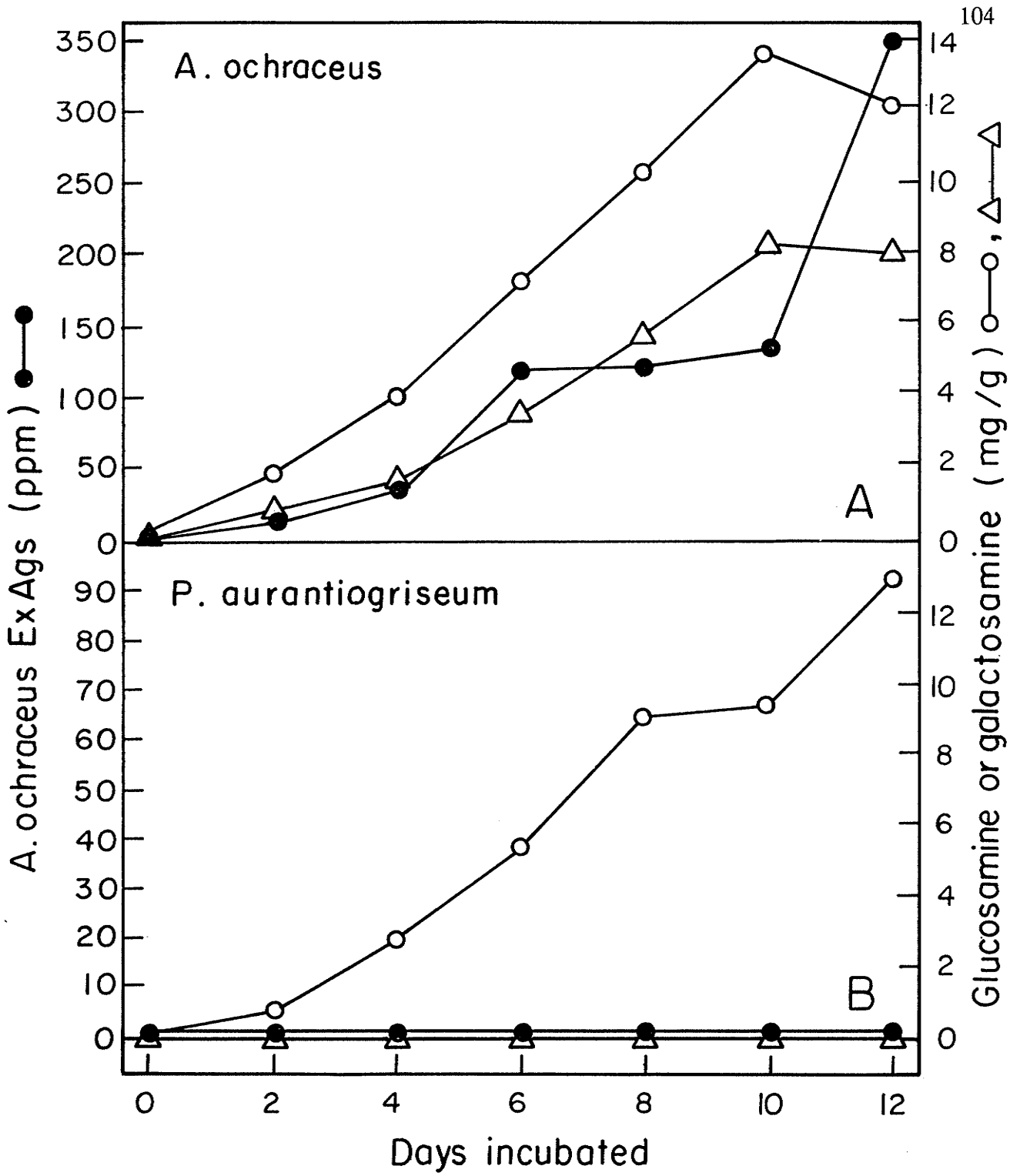

50
45
40
35
30
25
20
15
10
5
0
● A.o.
○ A.o. + P.a.
△ P.a.
A
600
500
400
300
200
100
0
B
-1
-2
-3
-4
-5
-6
A. ochraceus ExAgs (ppm)
Log 10 of spore dilution

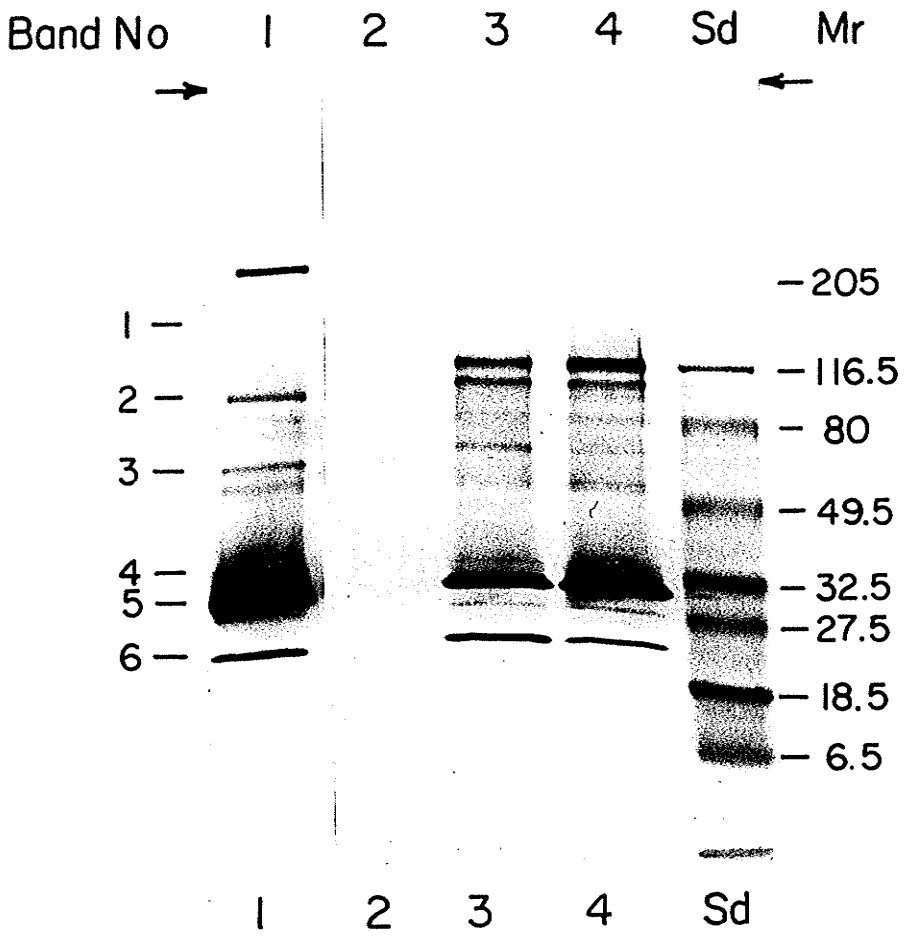
Figure 11. Comparison between the amount of *A. ochraceus* ExAgs as detected by the ELISA and the amount of chitin in moisturized and autoclaved wheat samples that were inoculated with *A. ochraceus* (A) and *P. aurantiogriseum* (B). Glucosamine and galactosamine values provided an index of the chitin content of the sample. The ExAgs were extracted from wheat samples and assayed using procedures described in Materials and Methods, Experiment 3. Visual score of fungal growth were zero, day 0; slight, day 2 and highly positive for days 4 to 12. Values represent average of three replicate samples with the average coefficient of variability (CV) being 45.2% for *A. ochraceus* ExAgs, 15.4% for glucosamine and 16.9% for galactosamine (Figure 11A) and 3.9% for *A. ochraceus* ExAgs and 27.3% for glucosamine (Figure 11B).


A. ochraceus
P. aurantiogriseum
A
B
A. ochraceus ExAgs (ppm)
Glucosamine or galactosamine (mg/g)
Days incubated
0
2
4
6
8
10
12
50
100
150
200
250
300
350
20
30
40
60
70
80
90

A. ochraceus ExAgs over time on wheat samples inoculated with *A. ochraceus* (over 100-fold) but not on wheat inoculated with *P. aurantiogriseum*. The increase in glucosamine in the former comparison was correlated with the concentration of *A. ochraceus* ExAgs as detected by the ELISA test ($r = 0.78$, $P < 0.05$). There was also a time dependent increase in the galactosamine content on *A. ochraceus* inoculated samples whereas no galactosamine was detected in wheat samples inoculated with *P. aurantiogriseum*. It is not known whether this is a general phenomena for the two genera; if so, this difference could also provide a basis to distinguish between the two genera of fungi. The immunoblotting results shown in Figure 12 indicate that the *A. ochraceus* ExAgs were not present on non-mouldy wheat samples and were produced during fungal growth. The ExAgs produced on the wheat matrix appeared to be slightly different from those produced in liquid media. A total of 3 or 4 major ExAgs and as many as 9 minor ExAgs were detected in samples prepared from the liquid media with a similar number being obtained in samples extracted from the grain. The most intensely stained bands in all preparations were those having a molecular weight (M_r) of approximately 30,000 and to a slightly lesser degree, those with a M_r of approximately 20,000.

The objective of experiment 4 was to determine if the ELISA could be used to specifically detect the presence of *A. ochraceus* in a sample of grain that was naturally contaminated with fungal spores. In this study total fungal propagule counts (number of colony forming units, CFU), type of mycoflora, the content of fungal biomass (glucosamine assay) and OA production were assessed in non-autoclaved and moisturized wheat that was inoculated with different concentrations of *A. ochraceus* spores and incubated for 0, 7 and 30 days. The

Figure 12. Detection of *A. ochraceus* ExAgs in wheat extracts by immunoblotting using rabbit anti-*A. ochraceus*: *A. ochraceus* ExAgs from liquid culture (lane 1) and extracts of wheat inoculated with *A. ochraceus* and incubated for different time periods; day 0 (lane 2); day 6 (lane 3) and day 12 (lane 4). Samples were solubilized by boiling in 1.5% SDS and separated on 4-15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The molecular weight (M_r) of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, broad range). Arrows represent points of application of samples. See Materials and Methods (Experiment 3) for further details. The amount of *A. ochraceus* ExAgs standard applied to the gel was 125 ng per lane.


Band No
1
2
3
4
Sd
Mr
1
2
3
4
5
6
205
116.5
80
49.5
32.5
27.5
18.5
6.5
1
2
3
4
Sd

naturally contaminated grain had a relatively low rate of infection with *A. ochraceus* on day zero (<10%) with the percent infection being 20% for *Penicillium* species (Figure 13). *A. ochraceus* infection in the un-inoculated samples decreased to near zero after 7 or 30 days of incubation while 100% of the same samples were infected with *Penicillium* species. The pattern for grain inoculated with a high concentration of *A. ochraceus* spores was different as initially the infection with *A. ochraceus* was much higher than that observed with the un-inoculated grain while the corresponding infection with the *Penicillium* species was not greatly changed. Incubation of the grain, however, resulted in a dramatic increase in percent infection with *A. ochraceus* (100% infection) while there was a progressive time course decrease in percent infection of the *Penicillium* species with the values at 30 days being zero. Inoculation of the grain with 10^2 *A. ochraceus* spores per g yielded a pattern of response that was intermediate to the un-inoculated grain and that inoculated with 10^5 of *A. ochraceus* spores per g. These data demonstrated that the composition of the mycoflora was influenced by several factors such as spore load and incubation period.

The data in Table 7 demonstrated that in all samples the number of fungal propagules increased in a near logarithmic manner from approximately 10^4 to 10^{12} CFU during the 30 day incubation period suggesting that the total number of fungal propagules was not greatly affected by pre-inoculation of wheat with *A. ochraceus*. The concentration of glucosamine in the samples also increased over time with the most pronounced increase occurring between days 7 and 30. The total increase in glucosamine also tended to be two fold greater for the grain inoculated with a high concentration of *A. ochraceus* spores compared to the un-inoculated grain with the maximal change been 14-fold (0.5 vs 7.0 mg per g grain).

Figure 13. The change in the composition of mycoflora of raw wheat inoculated with zero (natural), 10^2 (low Ao) or 10^5 spores of *A. ochraceus* (high Ao) per g of wheat. The moisture 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 coefficient of variability (CV) among samples was 38%. See Materials and Methods in Experiment 4 for further detail.

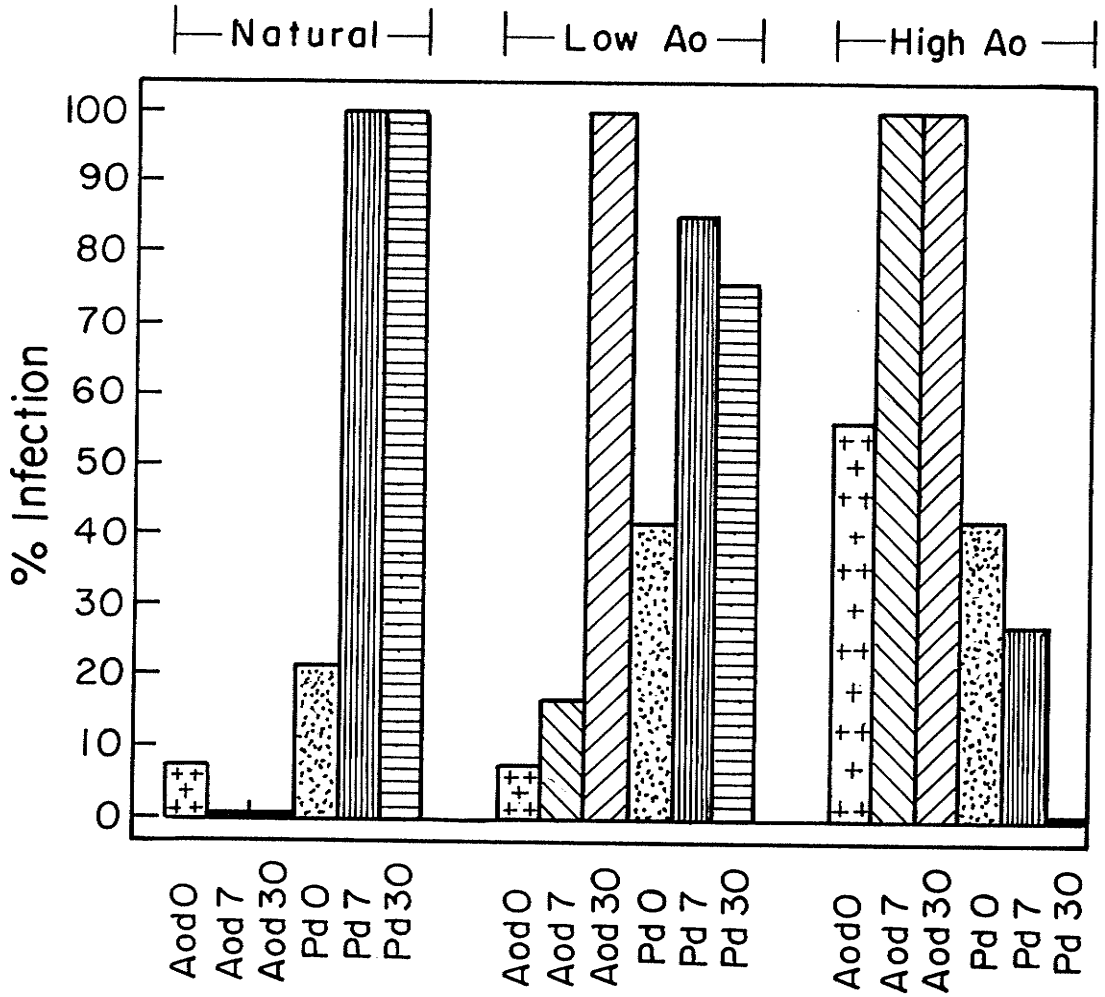

Natural
Low Ao
High Ao
% Infection
100
90
80
70
60
50
40
30
20
10
0
Aod 0
Aod 7
Aod 30
Pd 0
Pd 7
Pd 30

TABLE 7. The number of colony forming units (CFU) and the concentration of glucosamine, ochratoxin A (OA) and *A. ochraceus* ExAgs on moisturized wheat samples that were inoculated with different numbers of *A. ochraceus* spores and incubated for different periods of time (Experiment 4).

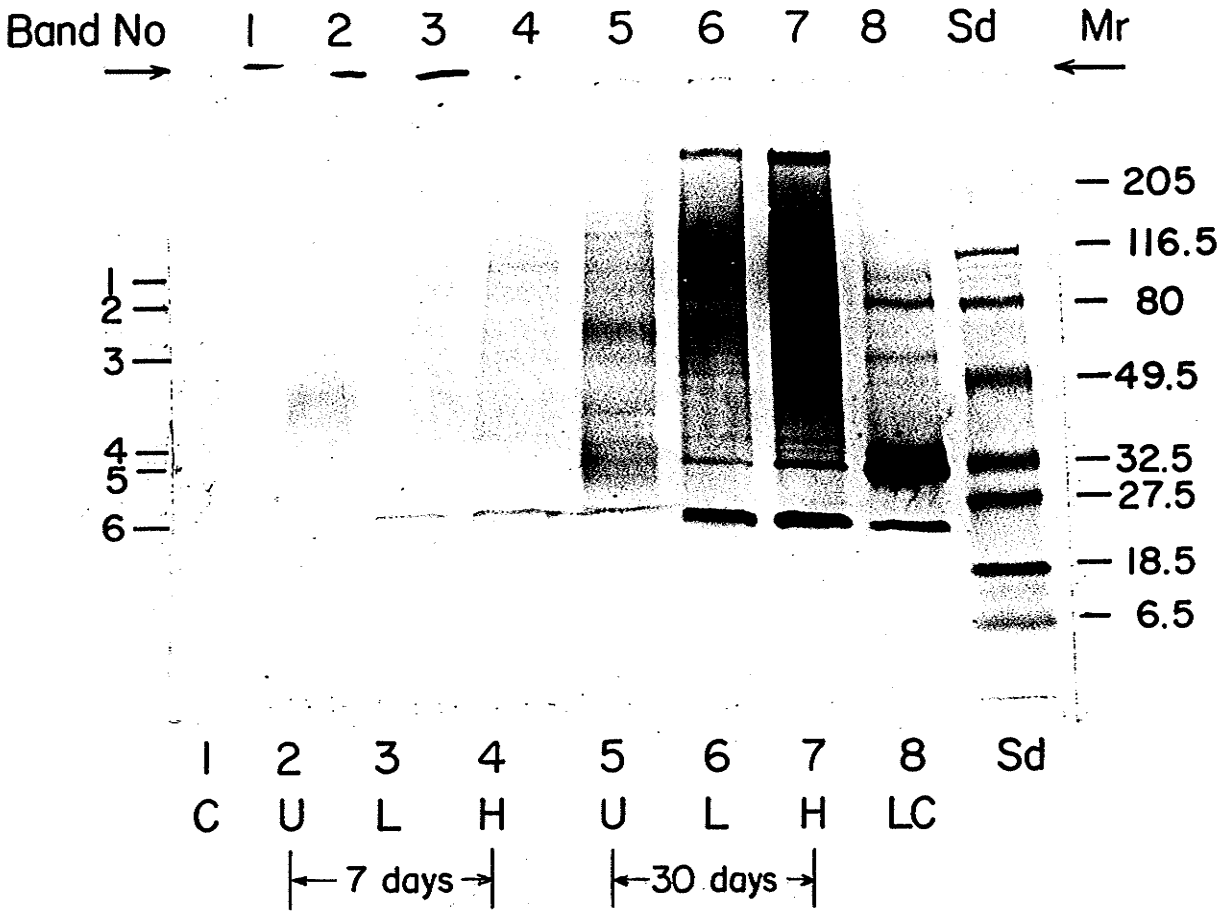
Parameters assayed	Incubation time, days	Inoculated wheat, number of *A. ochraceus* spores[1]		
		0	10^2/g	10^5/g
CFU ($\log_{10}$/g)	0	4±4	4±4	4±4
	7	7±7	6±6	8±8
	30	12±12	11±11	12±12
Glucosamine (mg/g)	0	0.5±0.05	0.4±0.1	0.5±0.1
	7	0.6±0.05	0.7±0.1	1.0±0.1
	30	3.7±1	3.5±1	7.0±3
OA (ppm)	0	0.01±0.01	0	0
	7	0.02±0.02	12±1	53±9
	30	0.1±0.1	100±15	130±17
A. ochraceus ExAgs (ppm)	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

1. The moisture of the wheat was adjusted to 22% (w/w) with water and conditioned overnight at 4°C prior to inoculation. Values represent means ±SD of five replicate samples.

The pattern of response of OA was different to that observed with the number of fungal propagules and glucosamine as there was no production of OA in the naturally contaminated grain. There was, however, a dramatic linear increase in the production of OA in those samples inoculated with *A. ochraceus*, particularly for those inoculated with the high concentration of *A. ochraceus*. The total amount of *A. ochraceus* ExAgs also followed a pattern similar to that observed for the production of OA. The concentration of ExAgs detected after 7 and 30 days of incubation were approximately 10-fold greater in the inoculated compared to the un-inoculated wheat.

Figure 14 illustrate the immunoblotting results of *A. ochraceus* ExAg of all four groups of wheat sampled at different incubation times. The control sample (C) and the un-inoculated grain (U) after 7 days incubation only had trace amount of *A. ochraceus* ExAgs while the immunoblotting pattern for the ExAgs obtained from liquid culture (LC) was the same as obtained in Experiment 3. The other two groups of samples after seven days incubation appeared to have produced additional bands, including a predominant *A. ochraceus* ExAg band (M_r approximately 20,000). The staining pattern was particularly pronounced in those samples that had been inoculated with the high concentration of *A. ochraceus* spores (H) and incubated for 30 days but was much less intense for the corresponding uninoculated samples which may have also contained *A. ochraceus* as indicated by the ELISA and mycoflora analysis. The reaction of the antiserum with the ExAgs from wheat samples were also somewhat different than that obtained with the ExAgs from pure liquid culture. One noticeable difference was that the

Figure 14. Detection of *A. ochraceus* ExAgs on unincubated wheat samples (C, lane 1), wheat samples incubated for 7 (lanes 2, 3, 4) or 30 days (lanes 5, 6, 7) and in liquid culture (LC lane 8) by immunoblotting using rabbit anti-*A. ochraceus*. The incubated wheat samples were un-inoculated (U) or were inoculated with 10^2 (L) or 10^5 (H) spores of *A. ochraceus* per g. Samples were solubilized by boiling in 1.5% SDS and separated on 4 to 15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The molecular weight (M_r) of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, broad range). The arrows indicate points where samples were applied. See Figure 12 for the amount of sample applied and Materials and Methods, Experiment 4 for further detail.


Band No
1
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3
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6
7
8
Sd
Mr
205
116.5
80
49.5
32.5
27.5
18.5
6.5
1 2 3 4 5 6 7 8 Sd
C U L H U L H LC
7 days
30 days

predominant ExAg in liquid culture (M_r approximately 30,000) did not appear to be nearly as abundant in the wheat samples. The ExAg with a lower molecular weight (with apparent M_r about 20,000) and many other higher molecular weight ExAgs also appeared to be more abundant in the inoculated wheat samples relative to those in the sample obtained from liquid culture.

A further comparison among the 30 days incubated wheat samples (Figure 15) indicated that group H (high inoculum) samples had apparently more *A. ochraceus* ExAgs than those in group L (low inoculum) samples and that the staining patterns were similar. The anti-*A. ochraceus* ExAg antibody also detected some ExAgs in group U (uninoculated) samples, with the composition of these ExAgs being different to the *A. ochraceus* ExAgs in both the wheat and liquid media that were inoculated with *A. ochraceus* (groups L, H and LC). Control samples again had only a trace amount of *A. ochraceus* ExAgs. Immunoblotting data also indicate that the composition of ExAgs appear to be similar within each inoculation group (groups U, L or H), but that the amount of ExAgs varied greatly from one sample to another.

The relationship between the different quantitative parameters are shown in Table 8. The results indicate that the amount of *A. ochraceus* ExAgs was highly correlated with the amount of OA produced ($r= 0.93$, $P<0.05$) and the percentage of *A. ochraceus* infection ($r= 0.89$, $P<0.05$), and to a much lesser degree with the amount of glucosamine ($r= 0.64$, $P<0.05$), and with the number of fungal propagules ($r=0.68$, $P<0.05$). The amount of *A. ochraceus* ExAgs was weakly and inversely correlated with the percentage of *Penicillium* infection ($r=-0.32$, $P>0.05$).

Figure 15. Immunoblotting assay for *A. ochraceus* ExAgs in wheat extracts (lanes 1-8) and in liquid culture (LC, lane 9) using rabbit anti-*A. ochraceus*. The specific extracts were: control (C, 0 time, lane 1), samples incubated for 30 days from un-inoculated wheat (U, lanes 2-4), and from wheat inoculated with 10^2 (L, lanes 5-6) or 10^5 spores of *A. ochraceus* (H, lanes 7-8) per g. Different lanes in the same group represent replicate samples from different flasks. Samples were solubilized by boiling in 1.5% SDS and separated on 4-15% gradient gels (Bio-Rad, Mini-PROTEAN II Ready Gels). The arrows indicate point of application of sample. The molecular weight (M_r) of the bands shown were estimated from prestained standards (Sd) (Bio-Rad, broad range). See Materials and Methods, Experiment 4 for further detail. See Figure 12 for the amount of protein applied.

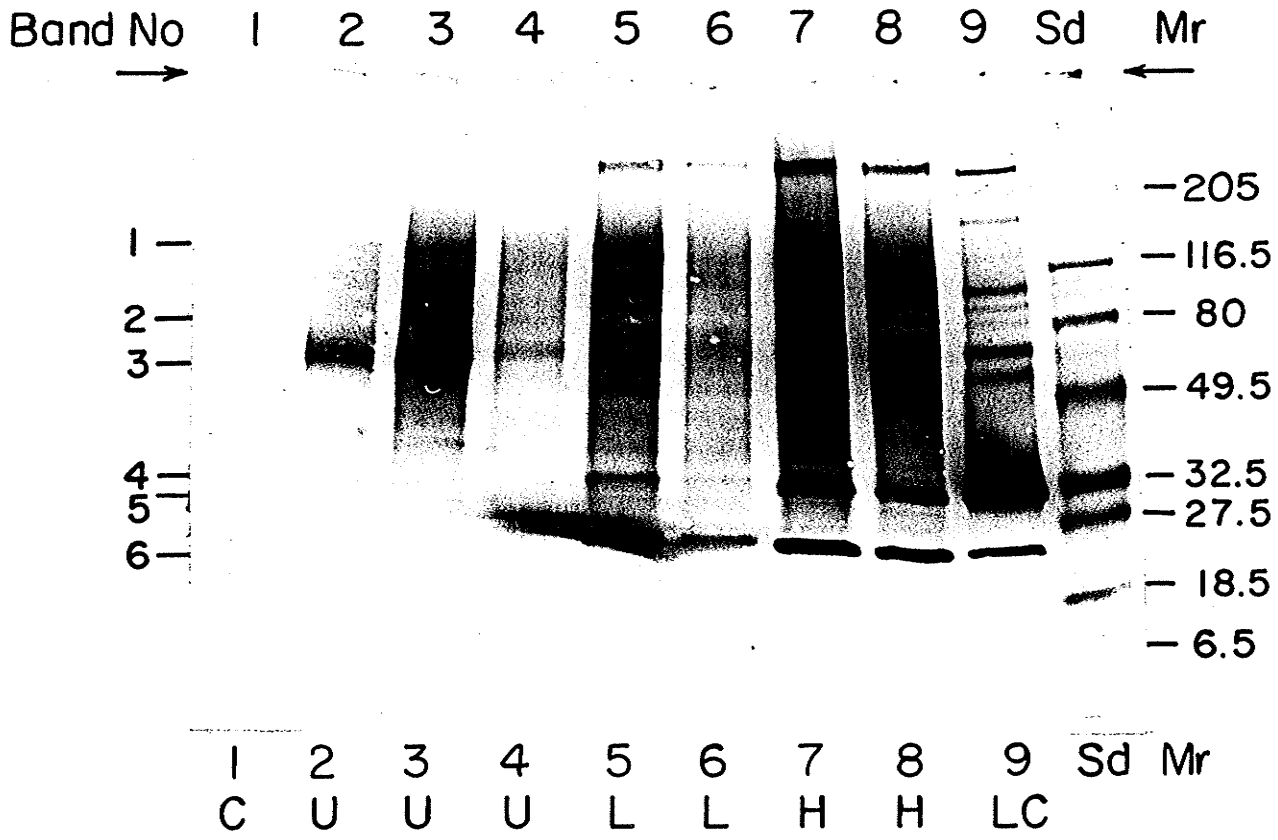
Band No
1
2
3
4
5
6
7
8
9
Sd
Mr
205
116.5
80
49.5
32.5
27.5
18.5
6.5
1 C
2 U
3 U
4 U
5 L
6 L
7 H
8 H
9 LC
Sd
Mr

TABLE 8. Correlation between the amount of *A. ochraceus* ExAgs detected using the ELISA and other methods for estimating *A. ochraceus* contamination in wheat samples[1].

X Variable	Y Variable	Correlation coefficient (r)	Confidence level (P)
A. ochraceus ExAgs	OA content	0.93	<0.05
	Percent *A. ochraceus* infection[2]	0.89	<0.05
	Glucosamine concentration	0.64	<0.05
	Number of CFU	0.68	<0.05
	Percent *Penicillium* infection[2]	-0.32	>0.05

1. See Table 7, Figure 13 and Materials and Methods of Experiment 4 for further detail.
2. Percent infection was based on the percentage of the samples that were infected with either *A. ochraceus* or *Penicillium*.

DISCUSSION

The results demonstrate that antibodies developed against the ExAgs from *A. ochraceus*, within the limits studied, can be used to specifically detect the presence of the fungus in the presence of other fungi that naturally occur on grain including the presence of the most common storage fungus, *P. aurantiogriseum* (Williams and Bialkowska, 1985). The results also demonstrate that the ELISA as outlined in this study is a reliable indication of the amount of OA produced in the sample, presumably because it was the only fungus that produced a significant amount of this toxin. In addition, the assay was a much better predictor of the amount of *A. ochraceus* in the grain than the glucosamine assay which presumably is a predictor of the total fungal biomass (Donald and Mirocha, 1977). The glucosamine assay is also much more difficult to conduct than the ELISA. One interesting observation in this study was the presence of both galactosamine and glucosamine in *A. ochraceus* inoculated grain but not in that of *P. aurantiogriseum*. This difference, if shown to be general, could also be used to distinguish between species of fungi.

In a previous study it was shown that the degree of cross-reactivity between the *A. ochraceus* ExAgs antibodies 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 and Marquardt, Manuscript 2). This further supports the data that the assay is specific. Another important feature of the assay was that the antiserum did not cross-react with the wheat matrix and, as shown in the previous study (Lu and Marquardt, Manuscript 2), did not cross-react with corn or barley extracts. Other assays that have been developed to detect the presence of fungi have not shown this specificity. Notermans and Heuvelman (1985), for example, reported that

the antibody against extracellular polysaccharide (EPS) of *P. verrucosum var. cyclopium* cross-reacted with wheat. In another study, Cox (1991) developed an ELISA for detecting fungal mycelia on cereal grains. The assay was unable to detect spiked fungal mycelia and spores in barley and was highly cross-reactive with the unspiked barley extracts.

The current assay, although having certain limitations as discussed later, also appears to be much more specific than that obtained with many other assays that have been developed, as most of the previous attempts to develop a specific assay for *Aspergillus* and *Penicillium* species appear to have failed (Notermans and Heuvelman, 1985; Tsai and Cousin, 1990; Cox, 1991). This may be related to the nature of the antigens used in their assays, as the assays developed by researchers such as Notermans and coworkers have utilized the EPS which contain galactofuranoside residue. These EPS tend to be highly conserved in nature and are not only found in fungi but probably other sources including food components (Van der Horst *et al.*, 1992; Notermans and Kamphuis, 1992). As a result, these assays are relatively nonspecific. The current assay also seemed to be highly sensitive as it could detect as little as 50 ng ExAgs per ml of sample. The detection level for fungi would be much lower as the ExAgs presumably constitute only a small portion of the total fungal biomass.

Immunoblotting studies have also demonstrated that the *A. ochraceus* ExAgs were not present in non-contaminated wheat samples. They did demonstrate, however, that there were several different ExAgs produced in liquid culture and that although the same ExAgs were produced on wheat when inoculated with *A. ochraceus*, their relative concentrations were often very different. In addition, there were a few ExAgs that were produced in liquid culture but not in the wheat matrix and vice versa, which indicate that some metabolic pathways have changed.

These studies, therefore, indicate that environmental conditions may not only affect the total amount of ExAgs produced but also the amount of each individual ExAg. As a result, it may not be possible to accurately predict mycelial dry weight on the basis of its content of ExAgs. These shortcomings, however, are probably no worse than those obtained with other mould assays that have been used as an index of fungal biomass including the assays for chitin (glucosamine), ergosterol and number of fungal propagules.

These results suggest that additional research is required to determine the degree that ExAg production under different environmental conditions is associated with the production of fungal biomass and in the case of toxigenic fungi, the production of the toxin of interest, and to identify the specific protein or epitope on the protein that is the best indicator of fungal biomass. Further research is also required to fully establish the overall specificity and sensitivity of the assay with regards to food products naturally contaminated with fungi. The assay nevertheless, seems to be superior in many respects to other types of assays that have been developed for the detection of specific fungi as it appears to offer the potential of specifically detecting and semi-quantitatively determining the amount of *A. ochraceus* in a contaminated sample of grain.

GENERAL DISCUSSION

Mould infestation and the associated contamination of cereals with mycotoxins remains a serious problem despite developments in modern drying and storage techniques (Pitt and Hocking, 1985; Chelkowski, 1991). This not only results in economic losses but can also adversely affect human and animal health (CAST, 1989). The availability of reliable and efficient assays for the identification and quantitation of the predominant fungal species is necessary as this will provide information on the fungi present and the mycotoxins that could be produced (Samson *et al.*, 1991).

Although there are many fungal species that commonly occur on cereal grain, the current research focused on one of the most common contaminants, *Penicillium aurantiogriseum*, and *Aspergillus ochraceus*, a producer of ochratoxin A. Conventional methods for detecting mould contamination in cereals including direct plating, fungal propagule counting and chemical analysis (mainly glucosamine and ergosterol) are tedious and time consuming and are unable to detect specific moulds (Donald and Mirocha, 1977; Jarvis and Williams, 1987; Rotter *et al.*, 1989; Swanson *et al.*, 1992). The classical methods for identifying moulds based on morphological characters, some of which are carried out in conjunction with other procedures, are widely used, as many of the newer techniques are not practical because of certain limitations. The lack of a simple and reliable method for the identification of fungi has resulted in many misidentified species, particularly those in the *Penicillium* genus, and has decreased the usefulness of previous studies on the occurrence of moulds and their production of mycotoxins (Pitt and Hocking, 1985; Pitt and Samson, 1990; Samson and Frisvad, 1991). Several ELISAs have been developed for detecting moulds in foods but most of the previous attempts to develop

a specific assay for species or genus of *Aspergillus* or *Penicillium* have failed because of undesirable cross-reactivities (Notermans and Heuvelman, 1985; Tsai and Cousin, 1990; Cox, 1991; Notermans and Kamphuis, 1992; Van der Horst, 1992). Dewey *et al.* (1990), however, were able to develop a monoclonal antibody-ELISA for detecting *P. islandicum* in rice grain that was relatively specific but did not appear to be quantitative. Kaufman and Standard (1987) using a simple double-diffusion immunoassay and a different type of antigen, the ExAgs, demonstrated that they could be used for the specific assay of medically important fungi.

The current studies attempted to combine the high specificity and uniqueness of the exoantigens with the ELISA for the detection of two species of fungi, *A. ochraceus* and *P. aurantiogriseum* using rabbit antisera raised against their ExAgs. The antisera and their corresponding ExAgs were characterized using ELISA and SDS-PAGE followed by immunoblotting analyses. The results of the competitive ELISA indicated that the antibodies against the ExAgs of *P. aurantiogriseum* was highly sensitive and relatively specific for three of the most common toxigenic penicillia (*P. aurantiogriseum, P. verrucosum* and *P. citrinum*). SDS-PAGE followed by silver staining or immunoblotting confirmed this observation by showing a similar protein and immunoblotting patterns among the ExAgs from the more closely related fungi but not among those that are more distantly related. The assay could also be used to detect *P. aurantiogriseum* ExAgs in wheat samples in the presence of a background of different mycoflora.

The antiserum against ExAgs of *A. ochraceus* was shown to be even more specific than that of *P. aurantiogriseum* ExAgs as it only weakly cross-reacted with the ExAgs from a closely related species, *A. candidus*. The experiments also involved the detection of *A. ochraceus* ExAgs

in spiked samples; in moisturised sterile wheat that was inoculated with *A. ochraceus* and *P. aurantiogriseum*; and in naturally contaminated wheat samples that had been inoculated with *A. ochraceus*. The results demonstrated that the ELISA test could specifically detect *A. ochraceus* contamination in wheat grain and possibly other cereals that were contaminated with other fungi. Also, that there were high correlations between the values obtained with the ELISA test and those of the other methods and that it offered many advantages compared to other commonly used methods. Immunoblotting studies provided confirmatory information on the reliability of the ELISA test and information on the antigens that could be purified and used for antibody production so as to further improve the specificity of the assay.

The specificities of the two ELISAs appeared to be superior to previously developed quantitative ELISAs (Notermans *et al.*, 1986a; Lin *et al.*, 1986; Tsai and Cousin, 1990). The apparent reason for the greater specificity is that the ExAgs that were used in the current studies were probably proteinaceous in nature and therefore would have contained more heterogenous epitopes than the extracellular polysaccharides that were used by the other researchers. These saccharides had repeating structural determinants that were common to several species of fungi (Preston *et al.*, 1970; Notermans and Soentoro, 1986; Notermans *et al.*, 1987). More physico-chemical studies, however, are necessary in order to provide a better understanding of the nature and properties of the ExAgs that were used in the current studies.

It may be concluded that both assays were relatively specific and can be used to quantify the amount of ExAgs in fungi and thereby indirectly provide an index of the amount of fungal biomass in grains and probably other foods. The assays are also much faster and are easier to perform than other assays and may also be useful in *Aspergillus* and *Penicillium* taxonomy as

they can indicate degree of interrelatedness among fungi species. The assays, however, need to be further evaluated and the specificity of the assay should be further improved, perhaps by the use of more specific antigens as a source of the immunogen.

SUMMARY AND CONCLUSIONS

Two indirect competitive ELISA tests for detecting *Penicillium aurantiogriseum* and *Aspergillus ochraceus* contamination in cereal grain were developed using rabbit antisera against their ExAgs. A total of 16 or 17 species of fungi from five genera and three or four water-soluble grain extracts were used to test the specificity of these assays. SDS-PAGE followed by protein staining of the gels or immunoblotting was used to further characterize the ExAgs and to confirm the ELISA results. The anti-*P. aurantiogriseum* serum had a high sensitivity to three closely related *Penicillium* species (95 ng/ml for *P. aurantiogriseum* and 260 ng/ml for *P. verrucosum* and 210 ng/ml for *P. citrinum*) with little or no cross-reactivity with the ExAgs from the other fungi that were tested including three other species of *Penicillium*, four species of *Aspergillus*, three species of *Fusarium*, two species of *Mucor* and one species of *Alternaria*. The ELISA was able to detect the three closely related *Penicillium* species in wheat samples naturally contaminated with other fungi and *A. ochraceus* with quantitative values being related to the number of fungal propagules, amount of chitin (glucosamine), concentration of ochratoxin A (OA) as an indicator of a competing species of fungi (*A. ochraceus*) and percent of the grain contaminated with *Penicillium* species. Immunoblotting patterns qualitatively confirmed the ELISA results in naturally contaminated wheat samples and in liquid culture.

The rabbit anti-*A. ochraceus* serum appeared to be specific as the ELISA demonstrated that it essentially did not cross-react with ExAgs of any of the storage or field fungi (four *Aspergillus*, seven *Penicillium*, three *Fusarium*, two *Mucor* and one *Alternaria* species) and with three water-soluble grain extracts. The sensitivity of the ELISA for the detection of *A. ochraceus* ExAgs was also high being between 120 to 220 ng of protein/ml with a detection limit of 50

ng/ml. The presence of *Penicillium* including the common species, *P. aurantiogriseum*, other fungal ExAgs, and the wheat matrix had little effect on the detection and quantitation of *A. ochraceus* ExAgs. Sterilized and moisturised wheat samples also gave a positive ELISA when the samples were inoculated with *A. ochraceus* but not when they were only inoculated with *P. aurantiogriseum*. The amount of *A. ochraceus* ExAgs detected in naturally moulded wheat and in natural mouldy wheat enriched with *A. ochraceus* correlated favourably with the amount of OA detected ($r=0.93$, $P<0.05$), the percentage of *A. ochraceus* infection ($r=0.89$, $P<0.05$), the chitin (glucosamine) content ($r=0.64$, $P<0.05$) and the number of fungal propagules ($r=0.68$, $P<0.05$), and tended to be inversely correlated with the percentage of *Penicillium* infection ($r=-0.32$, $P>0.05$). Immunoblotting analysis confirmed the results obtained with ELISA as this antiserum reacted strongly with ExAgs of *A. ochraceus* and to a much lesser degree with other ExAgs. The immunoblotting patterns of *A. ochraceus* ExAgs indicated that antibodies were developed against several antigens with the immunodominant antigens having molecular weight (M_r) of approximately 20,000 and 30,000. The other species of fungi, especially *A. candidus*, also had ExAgs that reacted with the antiserum but this reaction appeared to be weak. Several of these antigens, however, seemed to have similar M_r to those of the *A. ochraceus* ExAgs. Immunoblotting analysis on extracts from wheat inoculated with *A. ochraceus* demonstrated that the ExAgs produced on wheat were similar to those in liquid culture.

Based on the results obtained it is concluded:

1. The immunoassays developed for the ExAgs of two commonly occurring species of fungi (*P. aurantiogriseum* and *A. ochraceus*) can be used for the detection of these moulds and in some cases related species with the advantages of being more efficient,

simple and reliable than conventional techniques.

2. The combination of ELISA and immunoblotting techniques enhanced our knowledge on the relationships among fungi within the economically important genera (*Aspergillus* and *Penicillium*). The taxonomic relationship among fungal species as revealed in this study agreed with conventional fungal systematics.

3. The solid-phase immunoassays can be used for the specific quantitative monitoring in grains and foods of *P. aurantiogriseum* or *A. ochraceus* alone and probably both fungi by combining the antibodies.

4. The detection of toxigenic moulds in cereal grains should also provide useful information on the potential contamination of hazardous mycotoxins and therefore reduce the number of mycotoxin analyses required.

5. The immunoblotting data obtained in these studies can be used to identify the immunodominant and unique antigens which would facilitate their isolation and purification. These ExAgs could then be used for antibody production which should improve the sensitivity and specificity of the assay.

Suggestions for future research are to:

1. isolate and purify the unique ExAgs of these species to improve the specificities of the antibodies and therefore the assays, and to purify the polyclonal antibodies obtained in the current studies by use of affinity column chromatography. Production of monoclonal antibodies would be also of great benefits.

2. study the nature and comparative properties of the unique ExAgs to provide a better understanding of similarities and differences among fungi. This should also facilitate

improvements in the accuracy and reproducibility of these assays.

3. further examine the specificities of these assays by testing a broader range of fungal species in a wider variety of foods and feeds. Also determine if there is a direct relationship between amount of ExAgs present and amount of fungal biomass under different physiological conditions.

4. further optimize the assay conditions and utilize a direct ELISA format to simplify and shorten the assay time. The development of a simple "dip-stick" type assay would be of considerable benefits.

Overall, the results of this study suggest that the ExAgs can be used to develop specific immunobased assays not only for the two species of fungi investigated in this study but also for other storage and field fungi of concern. Additional research will be required to identify unique and immunodominant antigens or unique epitopes within these antigens so as to further improve the specificity of the assays, and to more closely establish the relationships between concentration of ExAgs in the fungi and total fungal biomass.

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