

# Production of polyclonal antibodies against polypeptides from an aflatoxin strain of the fungus *Aspergillus flavus*, a pathogen of stored grain<sup>1</sup>

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## Abstract

Immunological probes can serve as a sensitive tool for an accurate and prompt detection of fungi in stored grain. Polyclonal antibodies were raised against an aflatoxigenic strain of *Aspergillus flavus* using specific isolated polypeptides (17kDa and 26 kDa) from the mycelium following separation by SDS polyacrylamide gel electrophoresis (8% to 20% gradient PAGE-SDS). Another approach was to collect the polypeptides on a nitrocellulose membrane after staining with Ponceau red dye, dissolved in DMSO followed by emulsification, and injection into the rabbits. Using Western blotting, it was found that the antibody elicited by the polypeptide of 26kDa reacted against the mycelium extract (ME). However, a low reaction was observed also against the corn extract (CE). The reaction was not against the same polypeptide as in the ME and therefore the CE was utilised to absorb nonspecific antibodies. Consequently, a positive reaction between the antibody and the ME was recorded when using antigen-coated plate (ACP) — ELISA procedure. With the CE, only low background values were recorded, using the absorbed serum. The specificity of the antibody raised is now being tested using different strains of the fungus as well as other species of storage fungi.

## Introduction

Moulds attacking foodstuffs in storage can cause serious damage which may result in reduction in quality, and in heating, mustiness and various biochemical changes. In addition, numerous moulds which commonly proliferate on foodstuffs are capable of producing mycotoxins, secondary metabolites highly toxic to humans and animals. Enumeration and identification of the predominant species in food are necessary to (a) verify if the food was prepared from high quality raw materials, (b) provide information on the way the food has been stored and (c) indicate future possible health hazards related to the food's use when there is a high incidence of mycotoxigenic species. Enumeration and classification of moulds are necessary for providing advice on storage strategy and are essential in quality assurance and quality control of both raw materials and finished products.

The methods for enumeration of moulds can be divided into two main categories: indirect and direct. The former include

the determination of indirect indices related to fungal growth, such as CO<sub>2</sub> measurements, ATP assay, and evaluation of the presence of fungal cell components (ergosterol, chitin) or of fungal volatiles. However, these methods are not specific, cannot be used for quantitative analyses, and above all are not applicable to differentiation of species. Indirect methods are therefore not recommended for use in food mycology. They provide only a general estimation of infestation, not an accurate measure.

The direct methods include direct plating and dilution plating and are the ones most commonly used in food mycology. However, numerous disadvantages are associated with their use. These are due primarily to the many factors which affect the results obtained (Jarvis et al. 1983; Lacey et al. 1980). The main factors considered to account for inaccuracy and/or disparity in the results are related to the assay technique, the choice of growth conditions, and competitive inhibition. In addition, the conventional direct methods are both time-consuming and expensive. During recent years efforts have been devoted to overcoming some of these disadvantages and particularly to develop selective media. However, there is still no universal method which will satisfy every requirement, and there remains an urgent need to develop a technique for incorporation in food legislation. Immunoassays are being viewed as one such promising alternative, since they are highly sensitive, easy to employ, inexpensive and rapid. Immunological assays have proved useful for detection of plant pathogens with antibodies (either polyclonal or monoclonal) being raised against various fungal species (Clark 1981; Clark et al. 1986). However, only limited work has been directed towards the immunological detection of fungi attacking stored grain (Banks et al. 1991; Cox 1991; Dewey et al. 1989, 1990; Notermans et al. 1986). The immunogens used to raise fungi-specific antibodies were mycelial fragments, extracts of freeze-dried mycelium, or surface washings from cultures. No data are available on the use of specific fungal polypeptide(s) to raise antibodies. The aims of the present study were to isolate a specific polypeptide from an aflatoxigenic strain of *Aspergillus flavus*. This will be used to elicit antibodies and to develop an ELISA method for the detection of the fungus based on the antibodies formed.

## Materials and Methods

The fungus *Aspergillus flavus* CBS 121 was used throughout the studies. The fungus was maintained on synthetic medium (Paster and Chet 1980). Agar discs (3 mm) covered with mycelium and taken from the edge of 7-day-old colonies were washed thoroughly with water (to remove the spores) and then served as a source of inoculum. The discs were placed (over a cellophane membrane) on irradiation-sterilised corn flour mixed with 20% agar solution (1:4 w/v). Following 3 days of incubation at 26°C, mycelia were washed several times and then collected following removal of the disc used for inoculation. The mycelium was frozen in liquid nitrogen, ground, and the powder was suspended in 0.05 M carbonate buffer, pH 9.6 (used as coating buffer in the ELISA procedure), homoge-

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nised (using an Ultra-turrax) and centrifuged (10,000 rpm for 10 minutes) at 4°C. The supernants were collected and stored at -80°C. Sclerotia collected were treated in the same manner.

The extracts were separated by SDS polyacrylamide gel electrophoresis (8–20% gradient SDS-PAGE), and specific polypeptides from the sclerotia (33 kDa) or from the mycelium (17 and 26 kDa) were identified (Fig. 1), cut out from the gel, washed several times (4 hours in sterile water), and injected intramuscularly into rabbits as emulsified gel suspension (using Fraeund's incomplete adjuvant). In another set of experiments the polypeptides were transferred to a nitrocellulose membrane by electro blotting. The membrane was stained with Ponceau red dye, cut, and the line containing the desired polypeptide dissolved in DMSO and then emulsified and injected into the rabbits (Fig. 2).

After five weekly injections, the rabbits were bled and the sera were separated and analysed using Western blotting against the extracts. Immunoassays were performed using the antigen-coated plate (ACP)-ELISA method described by Joisson et al. (1992), with small modifications. Microtitre plates were coated overnight at 5°C with antigen (100 µL/well), rinsed with distilled water, followed by two rinses with PBS containing 0.05% Tween 20, and then incubated (overnight at 5°C) with the serum [diluted 1% non-fat dry milk powder (NFDM) solution in PBS]. Plates were washed again with water and PBS containing 0.05% Tween 20 and treated for 3 hours at 37°C with goat (IgG) anti-rabbit alkaline phosphatase conjugate (diluted 1:2000 in NFDM). The conjugated enzyme binding is detected by reading (using an ELISA reader) the colour intensity developed in the plate well (A 405 nm) following the addition of the substrate p-nitrophenyl phosphate in 0.1 M diethanolamine buffer (pH 9.8).

### Results and Discussion

The use of immunological probes for diagnostic assays in grain and food mycology holds great promise. Immunological approaches in which fungi will be used as antigens to induce

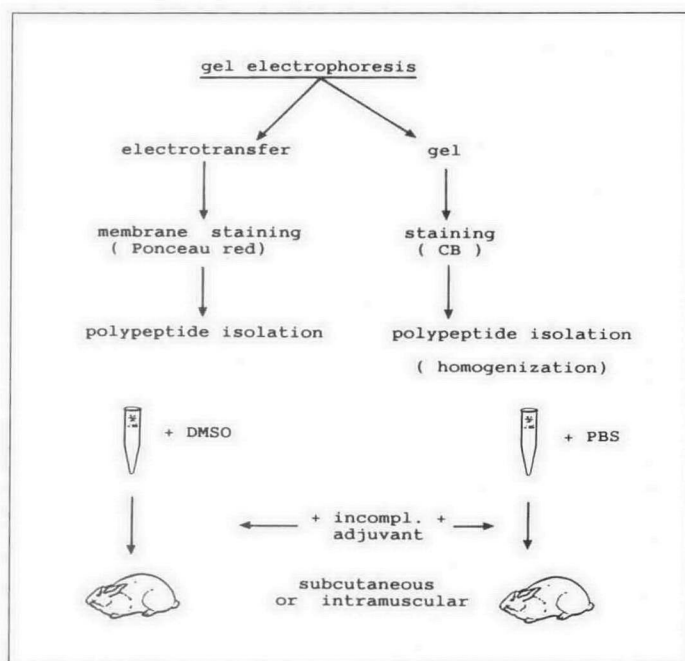


Fig. 2. Experimental procedure

antibodies can be utilised for the identification of the original antigenic fungus as well as for quantitative tests. Although several potential problematic areas are associated with production of antibodies against fungi (Clark et al. 1986; Cox 1991), both monoclonal and polyclonal antibodies have been raised to a number of plant pathogenic or medically important fungi. However, only in a very few cases have specific antibodies been raised against fungi known to invade stored grains. Usually, the immunogens used to raise antifungal specific antibodies include mycelial material, spore suspension, extracts of freeze-dried mycelia, surface washing from a solid agar culture, or extracellular polysaccharide (EPS). In

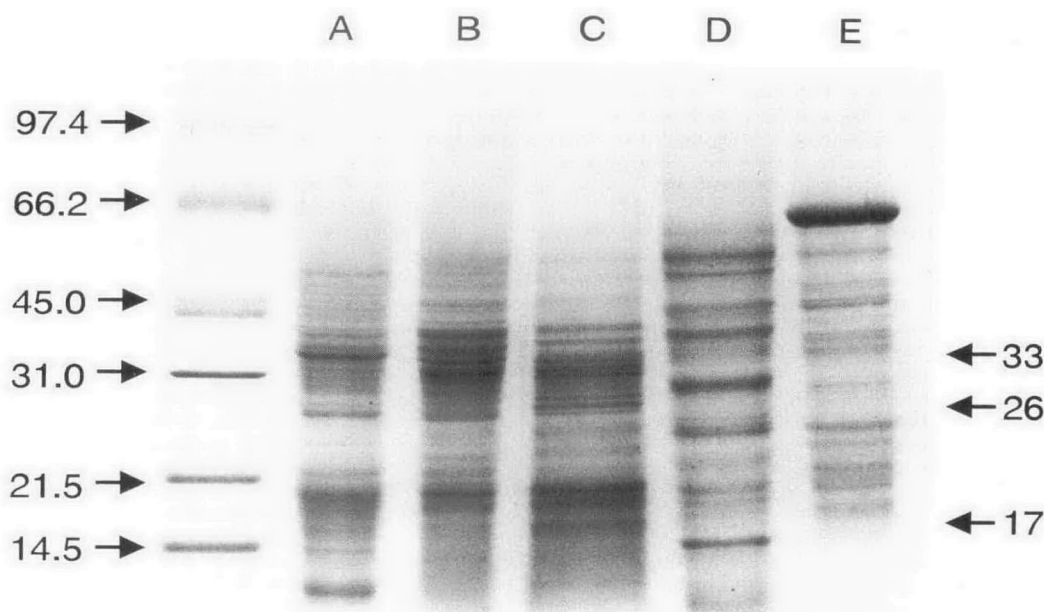


Fig. 1. Polypeptides pattern of extracts from: (A) sclerotia; (B) 3-day-old-mycelium of *Aspergillus flavus* grown on synthetic medium; (C) 3-day-old-mycelium of *Aspergillus flavus* grown on corn agar; (D) corn; (E) groundnuts.

our studies another approach was used in which antibodies were raised against polypeptides that have been identified on SDS-PAGE as specific to the fungus used (*A. flavus*), either from mycelium or from sclerotia, and did not appear in the grain material (maize or groundnuts).

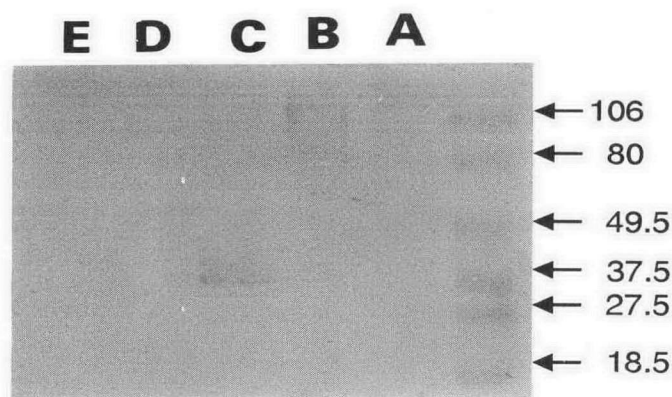
Following this concept, three bands (33 kDa from sclerotia, 26 and 17 kDa from mycelium) (Fig. 1) were identified as the target polypeptides and they were consequently used as the immunogenic materials. Results represent data obtained while using the nitrocellulose membrane technique for injection.

Immunoblot analysis of the antibody raised against the polypeptide from the sclerotia has revealed that this antibody reacted with the sclerotia extract, but not with that of the mycelium (Fig. 3). The antibodies elicited by polypeptides isolated from the mycelium (26 and 17 kDa) reacted against the mycelium extract (Figs 4 and 5) but slight reaction was observed also against the maize extract (Fig. 4I). Therefore, the maize extract was utilised to absorb nonspecific antibodies. The ACP-ELISA values recorded for the non-absorbed and absorbed antisera are presented in Figure 6. The data show that only lower background values were recorded using the absorbed antiserum, thus indicating that this antiserum was highly specific against the tested fungus.

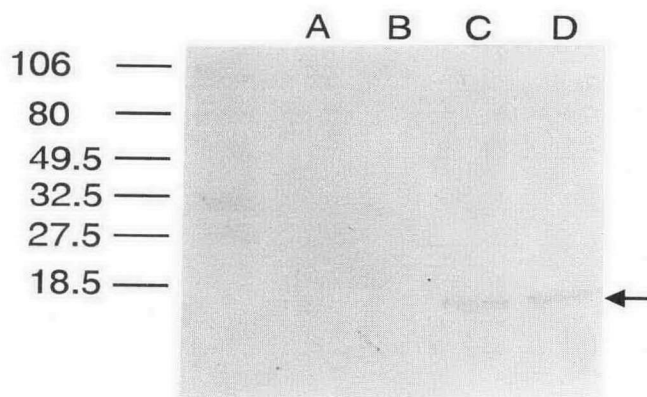
Immunoassays provide rapid, sensitive and easy-to-use tools for detecting fungal inoculum of stored grains and foods. Of the antibodies that have been raised for detecting mycelium and spores of spoilage fungi, very few are species-specific. However, the advantages of the immunoassays over the traditional methods currently used encourage further studies toward their application in grain and food mycology. The antibodies we have raised are now being examined for their specificity by analysing mycelium at different ages, spores, sclerotia, different strains of the fungus, as well as other fungal species. These studies will include also maize from different varieties and maturation stages as well as other grains, e.g. wheat groundnuts and soybeans.

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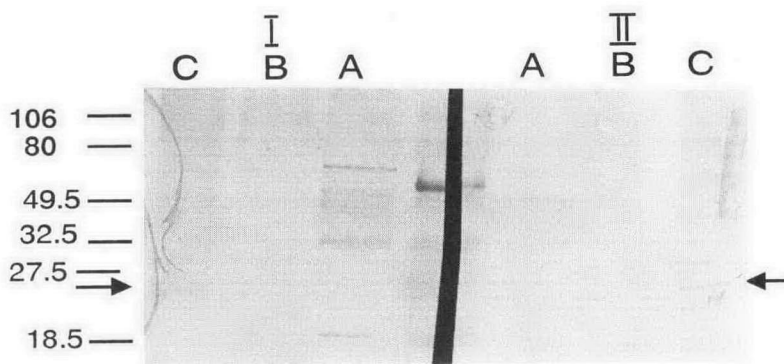
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**Fig. 3.** Immunoblot analysis of different extracts incubated with rabbit antiserum against a 33 kDa polypeptide from sclerotia. Extracts: A, B. *Aspergillus flavus* mycelium C. sclerotia D. corn E. groundnuts.



**Fig. 4.** Immunoblot analysis of different extracts incubated with rabbit antiserum against a 17 kDa polypeptide from *Aspergillus flavus* mycelium. Extracts: A. corn B. groundnuts. C, D. *Aspergillus flavus* mycelium.



**Fig. 5.** Immunoblot analysis of different extracts incubated with rabbit antiserum against a 26 kDa polypeptide from *Aspergillus flavus* mycelium. I. Antiserum. II. Antiserum absorbed with corn extract (1:3). A. corn B. groundnuts. C. mycelium

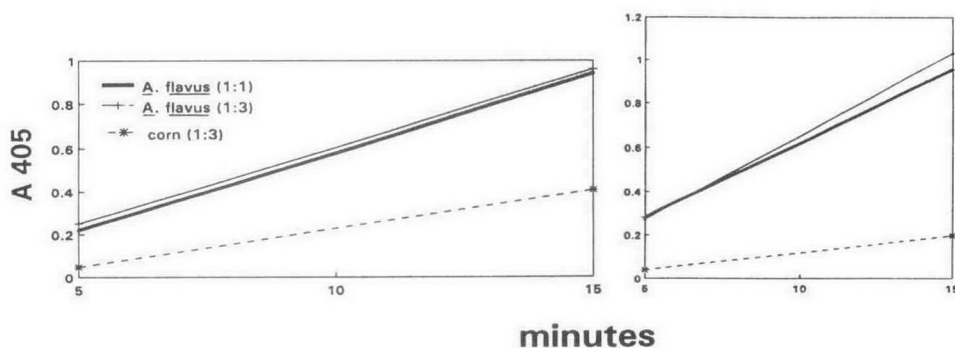


Fig. 6. Antigen-coated plate (ACP) — ELISA values using rabbit 26 kDa antiserum either non-absorbed (left) or absorbed with corn extract (right).

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