Analysis of *Aspergillus fumigatus* catalases possessing antigenic activity

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Summary. Analysis of *Aspergillus fumigatus* water soluble fractions by electrophoresis on non-denaturing polyacrylamide gels (PAGE) showed the presence of at least three catalase bands. They were designated F, S1 and S2 in order of descending electrophoretic mobility with respect to the anode. The multiple enzyme forms appear to be distinct in their physico-chemical properties. Enzyme bands S1 and S2 were simple catalases; the F band had an additional peroxidase function. All of the components were antigenic and differed in their binding to specific antibodies raised in rabbits with separate fractions of *A. fumigatus* mycelium. When serum from patients with aspergilloma, allergic bronchopulmonary aspergillosis, cystic fibrosis and chronic asthma were pre-incubated with *A. fumigatus* antigens and analysed by PAGE, 17 of 26 samples either abolished or reduced catalase activity. Enzyme F was a non-Concanavalin A (ConA)-binding antigen; the S1 and S2 enzymes were ConA-binding glycoprotein antigens. The major catalase band present in *A. niger* preparations represented only a minor component in *A. fumigatus*.

Introduction

A standardised, diagnostic antigen is not yet available for *Aspergillus fumigatus*, the most frequent cause of aspergillosis in man. The antigenic preparations used in different laboratories to detect antibodies to *Aspergillus* spp. are very varied; some are crude extracts, others are partially or highly purified fractions.^{1–4} Despite differences in fractionation procedures, many of these preparations have been shown to contain highly reactive antigens. However, the extent to which these reactive antigens are related, or contain identical molecules, is very difficult to determine.

In several in-vitro serological systems, fractions with enzyme activity, e.g., catalase, have been used successfully as detector antigens. In one procedure, *A. fumigatus* extracts were electrophoresed in agar and the separated components were allowed to diffuse with serum samples deposited in troughs cut in the gel. The so-called 'J' precipitin arc, possessing catalase activity, was identified in 97 of 100 serum samples from patients with aspergillosis.⁵ In another immuno-electrophoretic assay, catalase antibodies were detected by electrophoresis of serum samples in agarose gels pre-cast to contain antigen. A catalase-containing fraction had a diagnostic sensitivity for pulmonary aspergillosis of 88%.⁶ It has also proved a sensitive antigen in an indirect haemagglutination method for detection of antibodies to *Aspergillus* spp.⁷

The *A. fumigatus* catalase used as detector antigen has not been chemically defined and characterised.

The application of polyacrylamide gel electrophoresis, as a means of resolving complex mixtures, has proved an important tool in structural analyses. It has been used successfully to separate and identify mycobacterial catalases.⁸ We have used this method to examine partially purified mycelial preparations of *A. fumigatus* used in this laboratory as serodiagnostic antigens in in-vitro test systems and determined the physico-chemical and antigenic properties of the catalase enzymes obtained. The aim of the study was to detect a readily identifiable antigen that might be used as a common serodiagnostic agent to measure antibodies to *Aspergillus* spp.

Materials and methods

Preparation of A. fumigatus antigens

Preparations of *A. fumigatus* mycelium (Strains nos NCPF 2109 and 2140), were grown for 3 days in neutral glucose and peptone medium at 30°C, harvested by filtration and disrupted in a Dynomill cell disintegrator (Glen Creston, UK). Cell debris was removed by centrifugation and the supernate was concentrated before further centrifugation at 100 000 *g* to remove insoluble material. This supernate constituted the total, water-soluble material (WS) of the mycelium.⁹

Two fractions were separated from the WS material of strain 2109 on the basis of their attachment or non-attachment to ConA-Sepharose (Pharmacia Fine Chemicals, Sweden). Material that did not bind to ConA was designated the UBF (unbound) fraction; it

was protein rich. Material eluted from the column by 200 mM methyl- α -D-mannopyranoside was designated the BF (bound) fraction; it was carbohydrate rich. These fractions constituted 24% and 13%, respectively, of WS material.¹⁰ Elution with higher concentrations of methyl- α -D-mannopyranoside (600 mM), increased the yield from ConA-Sepharose by only a further 3% of the applied material. Borate buffer eluted additional carbohydrate-containing material (V. M. Hearn, unpublished results), but these fractions have not been studied further.

The WS material of the same strain was treated with ammonium sulphate. Components were separated to yield two fractions, one insoluble in ammonium sulphate in the range 0–50% w/v (AS50 fraction), the other insoluble in ammonium sulphate 50–75% (AS75 fraction), constituting approximately 42% and 6.2%, respectively, of WS. Also retained were those components which were not precipitated in a saturated solution of the salt (ASS fraction), and which constituted approximately 13.8% of WS.¹¹

Production of rabbit antisera

Antiserum was obtained by hyperimmunisation of New Zealand White rabbits with the WS fraction of strain NCPF 2109 of *A. fumigatus*. This fraction (at a concentration of 60 mg/ml) was mixed with an equal volume of Freund's incomplete adjuvant and 1 ml was injected subcutaneously at weekly intervals for 6–8 weeks.

For predominantly carbohydrate antigens (cell wall and the BF fraction) the following protocol was used. Antigen (10 mg dry wt) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a buttock; 10 days later the same amount of antigen was mixed with an equal volume of Freund's incomplete adjuvant and injected into the other buttock; 10 days later the same amount of antigen with Freund's incomplete adjuvant was injected into several subcutaneous sites; 10 days later, 5 mg of antigen was given intravenously without adjuvant; 1 week later the animal was test-bled. Sometimes another intravenous injection was required to obtain maximal immunisation, as judged by ELISA titres.¹⁰

Antisera were prepared to two protein-enriched antigens (cytoplasm and the UBF fraction). Cytoplasmic components were obtained as protoplasts, free of wall material.⁹ Antigen (0.5 mg dry wt) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a buttock; 10 days later a similar injection was made into the other buttock. The rabbits were left for 5 months and then given 0.5 mg doses of antigen mixed with Freund's incomplete adjuvant by subcutaneous injection at many sites; after 10 days the animals were given 0.1 mg of antigen intravenously without adjuvant; a test-bleed was taken 10 days later. Sometimes an additional intravenous injection was required.^{12, 13}

An antiserum was prepared to a water-soluble wall fraction, WS/TE. A strong precipitin line revealed by immuno-electrophoresis with this antigen and polyvalent antiserum raised in rabbits to crude wall material was cut from a total of eight separate gels, pooled and homogenised in 2 ml of saline in a mortar and pestle.¹⁴ It was mixed with an equal volume of Freund's complete adjuvant and the suspension was injected intradermally in multiple sites (>40) in a New Zealand White rabbit.¹⁵ A single injection of 1 ml of *Bordetella pertussis* vaccine (Wellcome) was administered intramuscularly in the animal's buttock to boost antibody production. The animal was test bled after 14 days and required booster doses of soluble antigen by intravenous injection at monthly intervals for a total of 6 months. Serum was collected from rabbits before injection of antigens for use as a negative control.

Human sera

Serum specimens were selected from those submitted to the Mycological Reference Laboratory from patients with aspergilloma (3), allergic bronchopulmonary aspergillosis (ABPA; 5), cystic fibrosis (6), chronic asthma (5) and atopy (2). Samples used were those that gave high ELISA values when tested for anti-*Aspergillus* IgG class antibodies. A total of 21 serum samples was examined. Some ELISA-positive serum samples were used as pools (four or five individual samples constituting a single pool) in an attempt to obviate any differences in the individual immune response. A total of five pools was prepared and tested. Control serum samples (9) were from laboratory personnel and from those samples that gave negative readings for *Aspergillus* antibodies in ELISA.

Preparation of an IgG fraction

Serum samples were treated with caprylic acid and the resultant precipitate was discarded after centrifugation.¹⁶ The IgG component (and a portion of the IgA) remained in the supernate which was dialysed and reduced in volume to that of the original serum.

Specific immunoglobulin-binding experiments

Partially-purified IgG fractions were prepared from rabbit-antiserum to *A. fumigatus* antigens. Fractions from human serum were either from individual samples or, in some cases, from specimens combined as a pool. IgG fractions were prepared from three of these pools. A total of nine control serum specimens was used—two pools of serum, one pool of IgG, four individual serum samples, negative on ELISA for *Aspergillus* antibodies, and two individual samples from laboratory personnel. In this assay, the WS fraction (at protein concentrations of 10–25 μ g), was allowed to react with 25 μ l of antiserum or immuno-

globulin preparation for 1.5 h at room temperature. Initial investigations showed that removal of any immune complexes formed, by incubation with staphylococcal protein A before electrophoresis, did not affect the final result; therefore, this step was omitted in subsequent experiments.

Electrophoresis in native gels

Vertical polyacrylamide gel electrophoresis (PAGE) with gel slabs 1.5 mm thick and a separating polyacrylamide gradient of 5–15% w/v was performed according to the method of Laemmli.¹⁷ The electrode buffer was 25 mM Tris–190 mM glycine, pH 8.3; sodium dodecylsulphate (SDS) was omitted from the system. Samples for analysis were applied in a mixture containing glycerol 10% v/v plus a trace of bromophenol blue in 64 mM Tris HCl buffer, pH 6.8. Other details of electrophoretic separation were as already described.¹⁸ High-mol.-wt protein standards (Gibco BRL) were run in parallel.

Detection of catalase activity

The ferricyanide-negative stain of Woodbury *et al.*¹⁹ was used to locate catalase bands in PAGE gels as described by Wayne and Diaz.⁸

Sensitivity of the detection system

Catalase was detected in WS and AS50 preparations when the protein concentration was 10 µg/track; WS was applied routinely at 25 µg of protein/track. UBF was applied at a concentration of 50 µg/track. The carbohydrate-enriched fractions (BF, AS75 and ASS) were applied at a protein concentration of 5–8 µg/track.

Control. A commercial sample of *A. niger* catalase (Sigma; 6900 units/mg of protein) was included in several electrophoresis experiments for comparative purposes.

Mol.-wt estimations. Samples were electrophoresed on 7.5%, 10% and gradient (5–15%) polyacrylamide gels with appropriate standards for mol.wt determinations. Markers included ovalbumin, bovine albumin (monomer and dimer) and urease from Jack Bean (trimer and hexamer) (Sigma).

Detection of peroxidase activity. A diaminobenzidine stain was used to detect peroxidase activity associated with catalase bands on PAGE gels.²⁰

Heat sensitivity. Enzyme lability was monitored by incubation of fractions at 68°C for 1 min²¹ before electrophoresis.

Inhibition studies. Attempts were made to determine the effect of several reagents on the expression of enzymic activity. Heavy metals (CuSO₄, HgCl₂ and ZnSO₄, 5 mM solutions) were each incubated with *A. fumigatus* fractions for 1 h at 37°C before electrophoresis. Fractions were incubated with the anionic detergent SDS (0.5% and 1.0% w/v) for 0.5 h at 37°C.

Mercaptoethanol treatment. *A. fumigatus* fractions in 25 mM Tris–192 mM glycine buffer, pH 8.3, were treated with 30 mM β-mercapto-ethanol, overnight at 4°C. This reagent causes reduction of disulphide linkages.²² The pH of the samples was adjusted to pH 6.8 before electrophoresis as already described.

Lectin binding experiments. *A. fumigatus* fractions were pre-incubated with ConA to assess the possible interaction of this lectin with enzyme molecules. Each fraction, containing carbohydrate (sugar) 50 µg, was mixed with ConA (Sigma) 500 µg in 1 mM Tris HCl, pH 7.0, containing 1 mM CaCl₂ and 1 mM MnCl₂; the mixture was held overnight at 4°C.

Protein and sugar analysis

The total protein content of the WS, UBF, BF, A50, AS75 and ASS fractions was estimated by the Coomassie Blue dye-binding method of Read and Northcote,²³ with bovine serum albumin as a standard. Total neutral sugar was determined by the method of Dubois *et al.*,²⁴ with glucose as a standard.

Results

Distribution of enzyme activity

All fractions contained catalase bands that were separated on polyacrylamide gels and were detectable with the ferricyanide reagent (fig. 1). The ASS fraction

Fig. 1. Electrophoresis on a 5–15% gradient polyacrylamide native gel; catalase bands were visualised with the ferricyanide-ferric chloride stain. Lane: 1, *A. niger* catalase; 2, *A. fumigatus* NCPF 2140 WS; 3, *A. fumigatus* NCPF 2109 WS; 4, UBF; 5, BF; 6, AS75.

Table I. Physico-chemical properties of *Aspergillus fumigatus* catalases

Test	F enzyme	S enzyme
Distribution	WS, UBF	WS, UBF, BF, AS50, AS75
Peroxidase activity	Present	Absent
Heat sensitivity	Sensitive	Insensitive
Metal ions	Sensitive	Insensitive
β -Mercaptoethanol	Sensitive	Sensitive
ConA-binding	Unbound	Bound

possessed only trace amounts of enzyme activity (results not shown).

At least two electrophoretic variants of catalase were seen in the WS fraction. A third component was present in the UBF fraction. These variants were designated F, S1 and S2, in order of decreasing anodic mobility. The WS preparation, which contained the F and S1 bands, may also contain the S2 enzyme but because of "tailing" of the S1 band, its presence was not established unequivocally. The UBF sub-fraction differed from the other WS sub-fractions in that only it contained the F enzyme band and apparently lacked the S1 component. The S2 band constituted a minor enzyme variant in *A. fumigatus* which was detectable only at high concentrations of UBF.

It was ascertained that AS50 contained the S1 catalase band (results not shown). However, this fraction was not examined further because of its known antigenic variability.²⁵ Some strain differences were apparent; WS from strain NCPF 2140 differed from the equivalent fraction from strain NCPF 2109 in containing at least four catalase bands, one of which had very limited anodic mobility. The F band constituted a trace component of this catalase mixture in the conditions of these tests. It may be seen more clearly at higher sample concentrations but this results in overloading of the other catalase components.

Species differences were also noted. The major catalase component of *A. niger* had a similar mobility to the minor, S2 component of the UBF fraction of *A. fumigatus*.

A band with mobility similar to that of the F enzyme had a peroxidase function associated with its catalase activity. It was detected in both WS and UBF preparations.

Properties of catalase enzymes

Mol.-wt estimations. The WS and UBF fractions were used for mol.-wt determinations. The F enzyme had an apparent mol.wt (10^3) of 240 and the S1 and S2 bands mol.wts (10^3) of 420 and 501, respectively.

Batch variation. Batch variation occurred and was most obvious in the amounts of detectable F-catalase. Of 10 batches of WS from strain NCPF 2109 tested, four contained only trace amounts of F catalase and of the two batches of WS from strain NCPF 2140 tested, one contained trace amounts and the other was

highly active. In contrast, levels of S-catalases detected were much more constant in all the fractions examined. The properties of *A. fumigatus* catalases are summarised in table I.

Enzyme stability. All catalases were stable to repeated freeze-thawing. Some S1 enzyme activity appeared to be stable to freeze-drying but there was loss of the F-component.

Heat sensitivity. Heating the WS preparation inactivated the F catalase. The S components of the mixture were unaffected.

Inhibition by heavy metal ions. Pre-incubation of WS fractions with heavy metals resulted in loss of the F band on electrophoresis; the S bands were unaffected.

Effect of detergent. Under the conditions used, SDS had no effect on enzyme activity.

Effect of β -mercaptoethanol. Pre-incubation of WS and UBF fractions with β -mercaptoethanol resulted, in all cases, in a total loss of the F band. All fractions containing S bands (WS, UBF, BF and AS75) showed much reduced S1 activity and total abolition of S2 activity relative to controls. This result is in agreement with previous findings that disulphide bridges play a structural role in catalases.²⁶

Lectin binding. Pre-incubation of the WS preparations from strains NCPF 2109 and 2140 with ConA resulted in a loss of the S1 and S2 components. The F-band was unaffected.

Reactivity with rabbit antiserum

The interaction of catalase-containing fractions with antibodies raised to *A. fumigatus* mycelial fractions is shown in table II and fig. 2a. Antiserum to WS caused a streaking of enzyme activity of the homologous antigen. When an antiserum to the UBF fraction was pre-incubated with WS antigen, the bulk of F-catalase activity was lost. An IgG fraction of serum raised against the BF preparation, when pre-incubated with the same WS antigen, caused a loss of S catalases on subsequent PAGE. Antisera to wall components had no detectable effect.

Reactivity with human serum

The interaction of catalase-containing fractions with 26 serum samples from patients with high anti-

Table II. Reactivity of *Aspergillus fumigatus* catalases with rabbit and human sera

Serum (n)	Activity of	
	F enzyme	S enzymes
Rabbit WS	Reduced	Reduced
Rabbit UBF	Reduced	0
Rabbit BF	0	Abolished
Rabbit wall	0	0
Rabbit WS/TE	0	0
Human positive (26)	Reduced(4) Abolished (3)	Reduced (7) Abolished (9)
Human negative (9)	0	0

0, no apparent effect.

Aspergillus titres on ELISA was investigated. Interpretation of the results was sometimes difficult because immunoglobulin subunits often obscured the catalase bands (fig. 2b). When catalase bands were abolished, two *Aspergillus*-positive serum samples abolished both F and S bands, one abolished F only and seven S only. When bands were reduced, four serum samples reduced both F and S bands and three reduced only the S band. Of the two major catalase components,

the S enzyme, a ConA-binding glycoprotein, reacted most consistently with anti-*Aspergillus* antibodies. Only one serum sample appeared to contain antibodies exclusively to the non-ConA-binding moiety. The loss of catalase-reactive bands depended upon the concentration of material tested. At higher concentrations of WS with a constant level of antiserum, the bands were still visible.

Discussion

Examination of the WS preparations of two strains of *A. fumigatus* showed that the S catalases were consistently present, whereas the presence of the F enzyme and its mobility were variable. Lack of the F catalase may reflect differential synthetic activity but the final pattern could also be modified by differential lability of the enzymes.

The catalase enzymes described in this study resemble the two classes of catalase described for mycobacteria, *Escherichia coli* and *Saccharomyces cerevisiae*, i.e., a heat-labile catalase that also has a peroxidase-like function and a more common, heat-stable, non-peroxidative type.^{8, 27, 28} However, they did not display the disulphide-sulphydryl interconver-

Fig. 2. Electrophoresis on a 5-15% gradient polyacrylamide native gel of *A. fumigatus* NCPF 2109 WS pre-incubated with rabbit or human serum (a) Lane: 1, WS + normal rabbit IgG; 2, WS + BF IgG; 3, WS + homologous IgG; 4, WS + UBF IgG; 5, WS control. (b) Lane 1, WS only; 2, WS + freeze-dried human positive IgG pool; 3, WS + human positive IgG pool; 4, WS + human positive serum pool; 5, WS + individual positive human serum; 6, WS + individual human serum from a healthy control.

sion noted with catalases from man and horse.²⁶ The inhibitory effect of mercaptoethanol showed that they appeared to require an S-S grouping to maintain their structural integrity.

A. niger appeared to lack any component corresponding to either the F or S1 catalases found in *A. fumigatus*. A major component in *A. niger* had a mobility similar to the minor (S2) component present in *A. fumigatus*. Both enzymes were bound by ConA and were not affected by heat or heavy metals. In this context, the findings of Schønheyder *et al.*⁶ are of interest; they reported that an antiserum to the 250-Kda fraction of *A. fumigatus* cross-reacted with catalase antigens of *A. flavus*, *A. terreus* and *A. nidulans*, but not of *A. niger*.

Both F and S electrophoretic variants of catalases have been detected in 10 strains of *A. fumigatus*.²⁹ In a separate study, Schønheyder *et al.*⁶ analysed 14 *A. fumigatus* isolates and found that only one possessed two immunologically distinct but electrophoretically identical catalases. This was in a strain other than NCPF 2109 but the authors stressed that the occurrence of the second catalase was not constant. It should be noted that they used agarose whereas we used a polyacrylamide matrix for electrophoresis.

After reaction with antibody, electrophoresis of the WS fraction showed a large amount of catalase activity streaking from the origin of the gel. Removal or displacement from the gel of catalase antigens by antibody may be due to the size of the immune complex. It is known that catalase can retain its activity after reaction with antibody.²¹ When fractions were allowed to react with serum from *Aspergillus*-positive patients, the concentration of antibody to the F component appeared generally lower than that of

antibody towards the S components. The latter are heat-stable, ConA-binding glycoproteins which have been shown to be immunologically reactive in several in-vitro assay systems. Schønheyder and Andersen¹ have partially characterised the catalase-containing fraction of *A. fumigatus*. SDS-PAGE of this fraction gave six protein bands, two precipitating antigens by crossed immuno-electrophoresis and two glycoprotein antigens by crossed immuno-electrophoresis-ConA intermediate gel. It is possible that the same major catalase is being described in both instances, although discrepancies in estimated mol. wts remain to be resolved. Because it is consistently present in all strains of *A. fumigatus* examined, may be readily identified as an antigen-enzyme, and is immunologically highly reactive, it may be a potential standardised diagnostic reagent.

The system used in our experiments will detect only high levels of anti-catalase antibodies. Other methods, including ELISA, should prove more sensitive.^{6, 7} The presence of an additional catalase moiety that is present in most strains examined and differs in its chemical and physical properties has not been reported previously. Resolution on native polyacrylamide gel of the 250-Kda catalase complex described by Schønheyder and Andersen¹ and the catalase purified by Senet *et al.*⁷ would reveal whether more than one catalase enzyme is also present in these preparations. It would be interesting to establish whether these antigens detect specific anti-*Aspergillus* antibodies to one or multiple forms of catalase in patients.

We are grateful to Dr M. Kelemen, The School of Pharmacy, Brunswick Square, London, WC1, for advice in the preparation of the manuscript.

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