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PURIFICATION, N-TERMINAL AMINO ACID SEQUENCE AND PARTIAL CHARACTERIZATION OF A CU,ZN SUPEROXIDE DISMUTASE FROM THE PATHOGENIC FUNGUS *ASPERGILLUS FUMIGATUS*

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A superoxide dismutase (SOD) has been purified to homogeneity from the fungal pathogen *Aspergillus fumigatus* using a combination of cell homogenization, isoelectric focusing and gel filtration FPLC. The N-terminal amino acid sequence of the purified enzyme demonstrated substantial homology to known Cu,Zn superoxide dismutases for a range of organisms, including *Neurospora crassa* and *Saccharomyces cerevisiae*. The enzyme subunit has a p*I* of 5.9, a relative molecular mass of 19 kDa and a spectral absorbance maximum of 550nm. The non reduced enzyme has a relative molecular mass of 95 kDa. The enzyme remained active after prolonged incubation at 70°C and was pH insensitive in the range 7–11. Potassium cyanide and diethyldithiocarbamate, known Cu,Zn SOD inhibitors, caused inhibition of the purified enzyme at working concentrations of 0.25 mM, whilst sodium azide and o-phenanthroline demonstrated inhibition at higher concentrations (10–30 mM). SOD activity was also detectable in culture filtrate of *A. fumigatus*. This enzyme may have a potential role as a virulence factor in the avoidance of neutrophil and phagocyte oxidative burst killing mechanisms.

KEY WORDS: *Aspergillus*, superoxide dismutase, purification.

INTRODUCTION

Aspergillus fumigatus is an opportunistic airborne pathogen affecting the respiratory system. It can cause allergy, aspergilloma by colonizing existing cavities and invasive and disseminated mycosis in immunocompromised patients. Recently invasive aspergillosis in hospitalized immunocompromised patients has increased and up to 38% of bone marrow transplant patients may have the disease in some centres[1]. Several proteins have already been assessed as potential virulence factors in *Aspergillus* sp., including a range of proteinases[2] and in particular elastases[3,4]. Other compounds have also been studied as possible virulence factors such as phospholipids which can inhibit complement activity[5,6], gliotoxins, which interfere with phagocyte function *in vitro*[7,8], spore derived anti-phagocytic substances[9], and various cell wall derived carbohydrates which interfere with phagocytic recognition and binding of non-opsonized fungal particles[10]. Whilst these substances may play roles in preventing uptake by phagocytic cells there has not yet been any attempt to study mechanisms which may protect the fungal cell from the direct killing mechanisms employed by cells such as neutrophils and macrophages.

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It has been shown that the myeloperoxidase system and its products, first described by Klebanoff[11] are used by macrophages and granulocytes as an effective killing mechanism against a range of bacteria and parasites in addition to fungi such as *Candida albicans*[12] and *A. fumigatus*[13]. To counteract this potent killing mechanism it has been suggested that in a number of parasite species anti-oxidant enzymes such as superoxide dismutases (SODs) may be used to protect the organism from toxic oxidative burst metabolites produced by the myeloperoxidase system of the immune effector cell[14]. SOD's are metalloenzymes capable of catalyzing the oxidation of superoxide with the concomitant reduction of hydrogen ions to hydrogen peroxide and molecular oxygen. Two unrelated classes of these enzymes exist. The first contain either iron (Fe SOD) or manganese (Mn SOD) at the active site[15]. The former has been found in prokaryotes and in some plant families whilst the latter is found in prokaryotes and the mitochondrial matrix[16,17]. The second unrelated class contain copper and zinc (Cu,Zn SOD). Cu,Zn SOD is usually found in the cytosol of eukaryotic cells but has been noted in plant chloroplasts and some prokaryotes[18]. All three enzyme types catalyze the same reaction comparably well.

To date SODs from only six fungal genera have been isolated including; *Neurospora*[19,20], *Saccharomyces*[21] and *Schizosaccharomyces* (O'Dee and Snider, sequence registered 1992, unpublished). Although members of these genera may on extremely rare occasions cause opportunistic infections there has, as yet, been no reports of the presence of SODs in those fungi which are significant disease agents such as *A. fumigatus*. As a result it has been difficult to establish any link between the presence of SOD and pathogenicity in fungi. In this paper we describe the purification and partial characterization of a Cu,Zn SOD from *A. fumigatus* as a first step in assessing the potential role of this protein as a factor in virulence.

MATERIALS AND METHODS

Culture And Harvesting Conditions

A. fumigatus isolate NCPF 2010 (National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Colindale, London) was inoculated from water cultures onto Sabourauds' agar plates and grown at 37°C or room temperature for two to three days. Spore suspensions were made from these plates and used to inoculate 1 L Sabourauds' broth in 2 L flasks which were incubated on an orbital incubator (120 rpm) at 37°C for 65 70 h. Mycelia were harvested by filtration through fine nylon mesh bags (Boots Ltd., Nottingham, U.K.) and washed with phosphate buffered saline (PBS, 0.01 M, pH 7.4). The mycelia were either used immediately or frozen at −20°C. Culture filtrate was collected and frozen at −20°C or −70°C until use.

Production of A Growth Curve And Analysis Of Culture Filtrate For SOD Activity

A growth curve for *Aspergillus fumigatus* was established by inoculating 2.31×10^8 spores into 500 ml of Sabourauds' broth in 1 L flasks. These were grown with agitation (120 rpm) at 37°C for varying time periods and harvested as described above. The time intervals for harvesting were 19 h, 24 h, 48 h, 72 h, and 96 h after inoculation. All studies were performed in duplicate. The mycelia were then freeze dried for 5 days and the dry weight determined.

The culture filtrate was refiltered through Whatman No. 1 paper (Whatman

International Ltd., Maidstone, U.K.) and then concentrated by a factor of 5 times on Macrosep (Flowgen Instruments Ltd., Sittingbourne, U.K.) 3 kDa concentrators. The filtrate was assayed for the presence of SOD activity using the method of Beauchamp and Fridovich[22], with an Ultrospec II spectrophotometer (LKB-Pharmacia Biotech, St. Albans, U.K.), with a bovine erythrocyte SOD as a positive control. All reagents for these assays were purchased from Sigma Chemical Company Ltd. (Poole, U.K.).

Purification Of Cu,Zn SOD

Mycelia were homogenized using 0.5 mm glass ball ballotini (Jencons Scientific Ltd., Leighton Buzzard, U.K.) in 50 ml of PBS (as above) in an ice cooled bead beater (Biospec Products, Barltesville, Ok., U.S.A.). The resulting homogenate was collected and the beads were washed three times in a total volume of 150 ml PBS. The latter was pooled with the homogenate to give a total volume of 200ml which was centrifuged for 40 minutes at 4°C at 10 000g. The pellet was discarded and the supernatant was centrifuged for 2 h at 4°C at 16 000g. The supernatant was concentrated from a volume of 200 ml to 20 ml on 3 kDa Macrosep centrifugal concentrators (Flowgen) at 7000g and 4°C. The concentrate was collected and dialyzed against water overnight at 4°C.

The dialysed cytoplasmic concentrate (approx. 25 ml) was mixed with 25 ml of water and 1 ml of ampholytes (Biolyte, pH range 3–10, BioRad. Laboratories Ltd., Hemel Hempstead, U.K.), loaded onto a Rotofor isoelectric focusing system (BioRad) and electrophoretically separated (at a constant power of 12 W, in accordance with the makers guidelines) until the voltage stabilized after 4–5 h. Final voltages ranged between 800 V and 1100 V depending upon the batch of cytoplasmic antigen used. The 20 fractions removed from the Rotofor were then assayed for pH, protein content (by the Coomassie Blue method[23]) and SOD activity. Fractions were also analysed by SDS–PAGE (see below).

Fractions containing high SOD activity and low protein content were pooled and concentrated on 1kDa Microsep centrifugal concentrators (Flowgen), to a volume of approx. 2 ml. Subsamples (500µl) of this concentrate were loaded on to a Superose 12 (HR 10/30, 10 × 300 mm) gel filtration FPLC column (Pharmacia) and eluted with 50 mM Tris buffer, pH 8.4, at a flow rate of 0.5 ml/min in 1 ml fractions. Protein content and SOD activity were monitored as described and fractions were pooled and/or concentrated as required. The fractions with the highest SOD activity were pooled and passed down the gel filtration column a second time and assayed as before, in order to fully purify the protein.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Isoelectric Focusing

The purification was monitored by SDS–PAGE using 15% polyacrylamide gels and sample preparation as previously described[24,25]. Protein bands were visualized using either Coomassie Brilliant Blue R-250[26] or silver stain (BioRad). Isoelectric focusing was carried out as previously described[25].

N-terminal Amino Acid Sequencing

10 µg of purified SOD in disruption buffer[24] was loaded into each track of a multi well 15% SDS–PAGE gel (as above except that the gel was aged overnight at 4°C in the presence of 2 mM thioglycolic acid in the upper buffer chamber), which was then run

at 150 V for 45 min. The gel was electrophoretically transferred (Western Blot) onto polyvinylidene difluoride membrane (Immobilon P, Millipore Corporation, Watford, U.K.), as previously described[24]. The blot was stained for 1 min with Coomassie Brilliant Blue and destained. The purified band was excised and subjected to N-terminal amino acid sequencing (Protein Sequencing Unit of the Institute of Biomolecular Sciences, SERC Centre for Molecular Recognition, University of Southampton).

Characterization Of The Cu,Zn SOD

The studies described below were undertaken using purified SOD. All assays were done in duplicate except the inhibitor assays which were done in triplicate.

Analysis Of Thermal Stability Of A.fumigatus SOD

The thermal stability of the Cu,Zn SOD enzyme was assessed. Samples containing between 2–5μg of SOD were incubated at 70°C for various time periods (1 min to 3 h), assayed for residual SOD activity at room temperature and compared to controls incubated at 25°C for the same time periods.

pH Profile

SOD activity was assayed using 2 5 ug of enzyme per assay. The following buffer systems were used; pH 8.5- 11.0 carbonate buffer (sodium carbonate/sodium bicarbonate 50 mM) and pH 7.0 9.5 Tris HCl (50 mM).

Inhibitors

The effect of different inhibitors on the activity of the Cu,Zn SOD was measured by pre-incubating 5μg of the purified enzyme with varying concentrations of inhibitors and the standard SOD assay mixture for one hour at the analysis temperature of 25°C before assaying in the normal way. The inhibitors sodium dodecyl sulphate (SDS), diethyldithiocarbamate (DCC), guanidinium chloride (GdmCl) and potassium cyanide (KCN) were all used at a final working concentration of 0.25 mM, 0.5 mM, 0.75 mM and 1 mM. All were dissolved in deionized water. The other inhibitors used were sodium azide, ethylenediaminetetraacetic acid (EDTA) and o-phenanthroline at final working concentrations of 2.5 mM, 5 mM, 10 mM, and 30 mM. The latter were dissolved in deionized water except o-phenanthroline which was dissolved in methanol. The direct effect of these inhibitors on the xanthine–xanthine oxidase assay system itself was also determined.

Absorption Spectrum

This was recorded using a Lambda 5 spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, U.K.) in the wavelength range 250 800 nm with purified SOD at a concentration of 1 mg/ml.

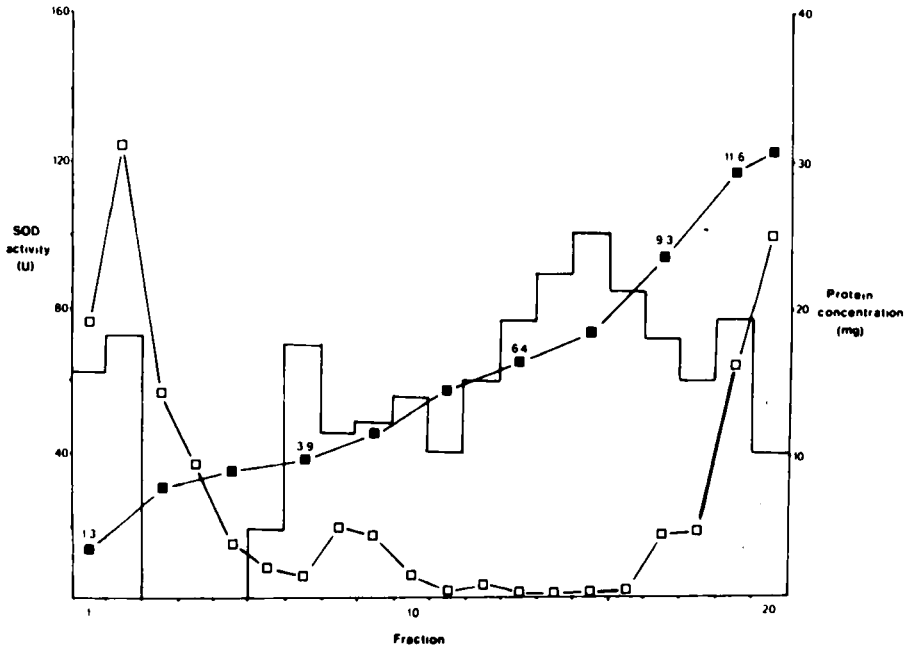

FIGURE 1 Purification of the *A. fumigatus* Cu,Zn SOD by liquid isoelectric focusing. Solid squares represent pH gradient, open squares represent protein concentration and the histogram represents SOD activity of each fraction.

RESULTS

Purification Of The SOD

The purification of the Cu,Zn SOD from *A. fumigatus* involved three main steps; homogenization of mycelia with removal of cell debris and concentration, followed by separation by liquid isoelectric focusing (Rotofor) and gel filtration FPLC. SOD activity in all assays was calculated on the basis that 1U of purified enzyme is capable of causing 50% inhibition to the standard xanthine–xanthine oxidase system. Figure 1 demonstrates the pH, protein content and SOD activity of the 20 Rotofor fractions. SOD activity was present in significant amounts in a number of fractions but was concentrated in fractions 10–17 in which protein content was relatively low in comparison to the fractions at the anodic and cathodic ends of the Rotofor. On the basis of high SOD activity and low protein content fractions 11–16 were pooled, concentrated and passed down a gel filtration column. Fraction 19 of the eluate had maximal SOD activity (Figure 2a), a pattern that was repeated after the second gel filtration run (Figure 2b).

Table 1 illustrates the purification of the protein with a final purification of 180 fold and a yield of 0.35%. The centrifugation concentration step was not a particularly efficient purification step but was invaluable in concentrating large volumes of cytoplasmic antigen to load onto the Rotofor. It is possible that the 3 kDa macrosep filter used in this step allowed loss of the SOD. The manufacturers recommend that a

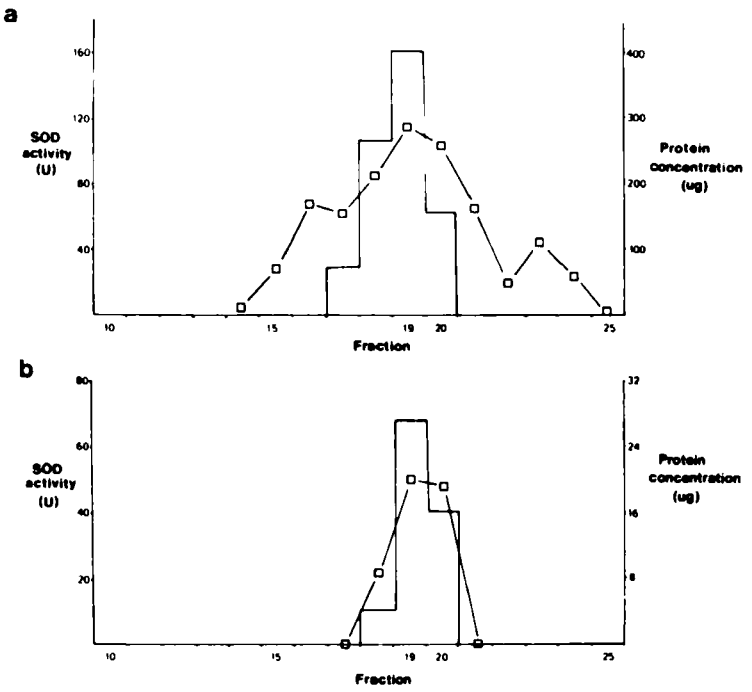

FIGURE 2 a and b Purification of the *A. fumigatus* Cu,Zn SOD by gel filtration FPLC. 2a: pooled fractions from isoelectric focusing (see text for details), 2b: fraction 19 from first gel filtration run passed down the column a second time. In both cases open squares represent protein concentration and the histogram represents SOD activity.

filter size of between 3 and 6 times smaller than that of the molecule to be retained should be used and the 3 kDa filter used only just fell into these limits (there is no smaller pore size available in this range).

SDS–PAGE analysis (Figure 3) demonstrates the purification of the SOD to homogeneity. The reduced form of the enzyme has a relative molecular weight of 19 kDa and the non reduced form of the enzyme has a relative molecular weight of 95 kDa. The p*I* of the enzyme subunit was determined as 5.9.

The purification of this protein has been repeated in triplicate without variation in its properties, demonstrating the stability of this enzyme and the reproducibility of the methods.

TABLE I
Purification of *A. fumigatus* SOD

	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Yield (%)	Purification (fold)
Homogenization	529.2	9720	18.36	100	1
3 kDa concentration	167.4	1209	7.2	12	0.39
Liquid Isoelectric Focussing	2.76	448	162.3	4.6	8.8
Gel Filtration (1st Run)	0.28	160	162.3	1.6	31
Gel Filtration (2nd Run)	0.01	34	3400	0.35	185

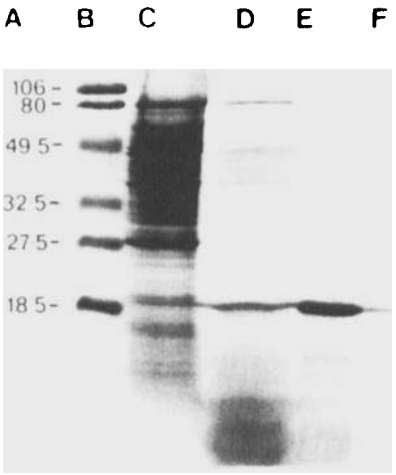

FIGURE 3 Purification of the *A. fumigatus* SOD as determined by silver stained SDS PAGE gel. Track A; BioRad molecular weight markers (in kDa), Track B; crude mycelial antigen, Track C; Rotofor fraction 14, Track D; gel filtration FPLC fraction 19, Track E; fraction 19 passed down second gel filtration column. Tracks A–E reduced. Track F; purified protein, non reduced.

N-Terminal Amino Acid Sequencing

N-terminal sequencing of the protein was repeated three times with little variation in the sequence obtained. The two possible amino acid ambiguities to the sequence shown were at position 1 where a lysine residue may be present and at position 14 where an arginine maybe exchanged. Sequencing of the *A. fumigatus* SOD band and comparison of the amino acid sequence with other known sequences showed extensive homology

TABLE 2.
N-terminal amino acid sequence comparison of *A. fumigatus* SOD with most homologous proteins from the GENEMBL bank

SOD source	Sequence	% Identity*
Aspergillus fumigatus SOD	VAVAVLRGDSKITGTVTFEQARXN 1 10 20	
Schizosaccharomyces pombe SOD mRNA	RAVAVLRGDSKVSGVVTFEQVDQN 10 20	73.9%/23aa
Neurospora crassa Cu,Zn SOD (*sod1*)	RPVAVVRGDSNVKGTVIFEQESES 130** 140 150	56.5%/23aa
Saccharomyces cerevisiae Cu,Zn SOD	QAVAVLKGDAGVSGVVKFEQASES 140** 150 160	56.5%/23aa
Synthetic Human Cu,Zn SOD gene	KAVAVLKGDGPVQGIINFEQKESN 10 20	57.9%/19aa
Mouse Cu,Zn SOD mRNA	KAVCVLKGDGPVQGTIHFEQKASG 10 20	57.9%/19aa
Mouse Cu,Zn SOD (SOD-1) gene	KAVCVLKGDGPVQGTIHFEQKARP 120** 130	57.9%/19aa

* % sequence identify in amino acid overlap.
** Derived from DNA sequences – does not represent mature protein.

with SOD proteins from various sources (Table 2). All sequences shown by the database to have homology with the *A. fumigatus* sequence were SOD proteins and in the majority of cases were stated as Cu,Zn SODs. There was no homology with Mn SODs or Fe SODs. The highest homology found was with a SOD protein sequence from the yeast *S. pombe* of 74% over an amino acid overlap of 23 amino acids. The next most homologous sequences were the filamentous fungi *N. crassa* and bakers' yeast *S. cerevisiae*. These are the only fungal SOD sequences registered or published to date. It should be noted that the sequence homology with the higher eukaryotic SOD sequences also showed reasonable identity thus demonstrating profound protein conservation.

Properties Of The Enzyme

Temperature Stability of SOD Enzyme

The *A. fumigatus* SOD enzyme was unaffected by incubation at 70°C for 30 minutes, although after 60 minutes at this temperature the activity was reduced to 40% of the standard reaction.

The Effect of pH on SOD Activity

Figure 4. shows the effect of pH on the activity of the *A. fumigatus* SOD enzyme. It can be seen from the figure that the specific activity is not effected by the change in pH over the range 7–11, particularly in the carbonate buffered assay solution. A slight decrease in activity is observed in the Tris buffered solution, possibly due to the differing buffer capabilities of this system. The pHs below 7 were not assayed because the xanthine oxidase is inactive below pH 6.5. The xanthine oxidase enzyme itself has a pH optimum as do many enzymes. This pH dependence effects the activity of the xanthine oxidase

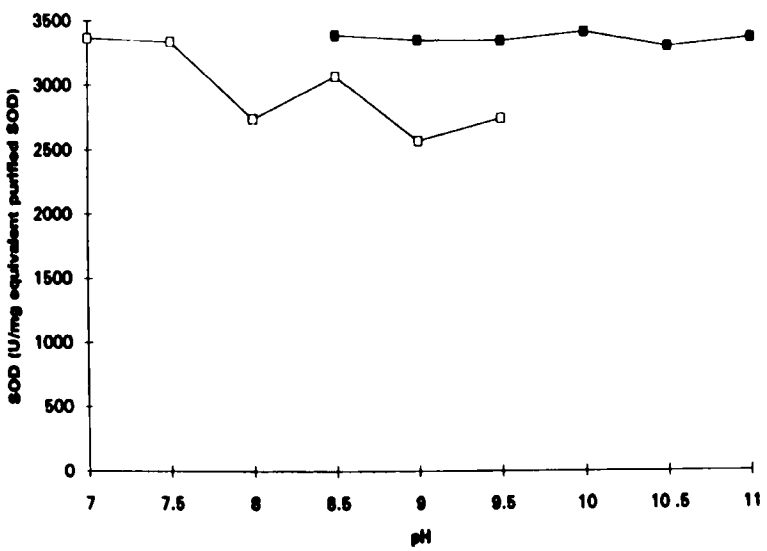

FIGURE 4 Activity of purified *A. fumigatus* SOD over pH range 7–11 (■ – carbonate buffer, □ – Tris HCl buffer).

TABLE 3
Effect of various inhibitors on the activity of *A. fumigatus* SOD.

Inhibitor	% Inhibition of SOD activity at final working concentration of inhibitor*			
	0.25 mM	0.5 mM	0.75 mM	1.0 mM
KCN	100	100	100	100
SDS**	0	0	0	0
DDC	100	100	100	100
GdmCl	0	0	0	0
	2.5 mM	5 mM	10 mM	30 mM
Azide	52	61	76	76
EDTA	0	0	0	0
o-Phenanthroline***	0	16	29	35

*Control with water = 0% inhibition = 3400 U/mg equivalent
**Residual effect due to the effect on the xanthine oxidase system (at higher concentrations)
***Residual effect due to the effect on the xanthine oxidase system

causing the production of the superoxide anion (the substrate for SOD) to be different at varying pH's. In this study to define the pH dependence or otherwise of the SOD the amount of substrate provided was controlled by varying the concentration of the xanthine oxidase added and therefore provide a more constant amount of substrate to the assay.

Inhibitors

The effect of various potential enzyme inhibitors at various working concentrations is shown in Table 3. Potassium cyanide and DCC completely inhibited the *A. fumigatus* Cu,Zn SOD at 0.25 mM. Both sodium azide and o-phenanthroline had some inhibitory effect at higher concentrations. Guanidinium chloride, SDS and EDTA did not have any effect upon the activity of the Cu,Zn SOD. None of these inhibitors with the exception of o-phenanthroline and SDS (at the higher concentrations) affected the ability of the xanthine–xanthine oxidase system to itself generate superoxide radicals.

Absorption spectrum

The absorption spectrum of the purified *A. fumigatus* SOD was measured between the wavelengths 260 800 nm (spectra not shown). A peak of absorbance was observed at 550 nm. There was no obvious shoulder in the spectrum at 280 nm suggesting a lack of tryptophan and tyrosine residues.

Growth curve

A. fumigatus mycelia when harvested after the different time produced the growth curve seen in Figure 5. SOD activity was detected in the culture filtrate at 48h (8.8 U/ml of X5 concentrated culture filtrate) and thereafter (Figure 5), i.e. from mid log phase onwards.

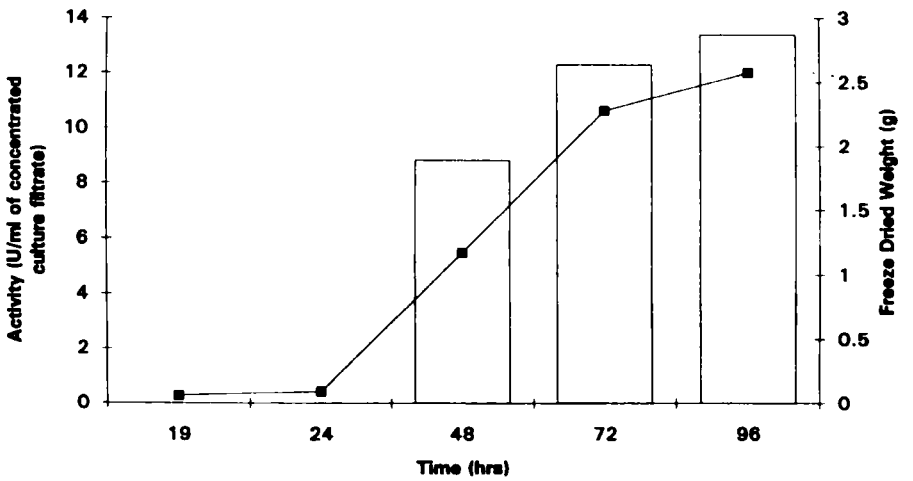

FIGURE 5 Detection of *A. fumigatus* SOD in culture filtrate during mycelial growth (line graph freeze dried weight, histogram SOD activity in culture filtrate, U/ml).

DISCUSSION

In this paper we have described the purification to homogeneity and partial characterization of a Cu,Zn SOD from the pathogenic filamentous fungi *A. fumigatus*. Although SODs have been purified from other fungi such as *N. crassa*[19] this is the first report of the isolation of this enzyme from an important fungal pathogen. Purification of the molecule to homogeneity was a relatively straightforward procedure, involving isoelectric focusing and gel filtration. This contrasts with, for example, the first report of the isolation of a Cu,Zn SOD from *N. crassa* which used a complex purification method, involving homogenization, treatment with chloroform/ethanol, recovery of the SOD from the organic phase, precipitation with cold acetone, dialysis against potassium phosphate buffer and separation by ion exchange chromatography[19]. Purification of the *Aspergillus* SOD was aided by the apparent stability of the enzyme, as exemplified by its thermal stability, a property which has been observed in other SODs[16].

Enzyme activity of the *A. fumigatus* Cu,Zn SOD molecule appears to be pH independent within the range 7.0 to 11.0. This pH independence is common to many Cu,Zn SODs[15,27] although there are also pH dependent members of this class of enzyme[28,29]. The enzyme activity was inhibited by potassium cyanide and DCC, which are known Cu,Zn SOD inhibitors[30], and which have been shown not to effect the activity of either Mn or Fe containing SODs[31]. DCC is a copper chelator and KCN may interfere with copper centres within the enzymes.

The functional similarities of Cu,Zn SODs are almost certainly due to the highly conserved active site. In fact three areas of Cu,Zn SODs are completely conserved; a. the subunit interface, b. the metal binding ligands, and c. the residues responsible for the electric field gradient which channels superoxide radicals and other small ions to the Cu(II) active site[27]. This homology at the active site extends to a more limited extent to the rest of the protein. Areas where variability seems to occur are typically at the surface of the molecule[18,29]. The Cu,Zn SODs thus far isolated from fungi demonstrate

approximately 68% homology over the whole molecule. Overall Cu,Zn SOD sequence homology between the higher and lower eukaryotes is 50–56%[27]. It is apparent that this amino acid identity extends to the N-terminal sequence of the protein, as the SOD isolated in this study demonstrates clear similarity both to several fungal Cu,Zn SODs and to Cu,Zn SODs from more unrelated sources. There was no significant amino acid sequence identity between the Cu,Zn SOD isolated in this study and sequence data for either Fe SODs or Mn SODs, an observation which concurs with the known lack of sequence homology between the two classes of SODs.

SDS PAGE of the mercaptoethanol reduced *Aspergillus* SOD indicates a relative molecular weight of 19 kDa, whereas when the enzyme is in its non reduced form it has a relative molecular weight of 95 kDa. All SODs described to date appear to be multimeric, and inter-monomer disulphide bridges are known to play a major role in the intact enzyme structure, together with various non covalent bonds. The majority of prokaryotic Mn SODs and Fe SODs are dimers. The Cu,Zn SODs isolated to date have also been shown to be homodimers, each subunit being approximately 16 kDa in size, although they may be as large as 18 kDa[16,32]. The protein described in this paper appears to be a little larger than those so far reported and the apparent dimer formation commonly seen in cytosolic Cu,Zn SODs cannot be identified in the *Aspergillus* enzyme. However, the 95 kDa species seen in non reducing conditions may be indicative of a tetrameric or pentameric structure for the intact *Aspergillus* enzyme. A tetrameric form of a Cu,Zn SOD has been reported from human blood plasma[33]. This protein has an estimated molecular weight of 135 kDa and was described as being extracellular. The Cu,Zn SOD described in this report has been detected in culture filtrate, although this does not preclude cell lysis as a source of apparent extracellular SOD activity. We are currently attempting to make monoclonal antibodies against the *Aspergillus* SOD in order to determine by immunohistochemistry if this enzyme can be detected *in vivo* as an extracellular product. If this enzyme were to have a role as a virulence factor in *Aspergillus* infections, particularly in regard to avoidance of reactive oxygen intermediates produced by immune effector cells, it is likely that it would be a secreted product, emphasising the importance of such a study.

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