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Biochemical Comparison of the Cu,Zn Superoxide Dismutases of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii*

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Cu,Zn superoxide dismutases (SODs) have been purified to homogeneity from the two varieties of *Cryptococcus neoformans*, *C. neoformans* var. *neoformans* and var. *gattii*. The N-terminal amino acid sequences of the two enzymes were similar, though not identical, and demonstrated homology with Cu,Zn SODs from other organisms. SOD activity was present in supernatants from stationary-phase cultures of isolates of *C. neoformans* var. *neoformans* and was also present from the mid-log phase onwards in cultures of an acapsular mutant of *C. neoformans* var. *neoformans*. SOD activity was practically undetectable in culture supernatants from isolates of *C. neoformans* var. *gattii*. The *C. neoformans* var. *neoformans* SOD had a reduced relative molecular mass of 19 kDa, and in its nonreduced form the enzyme was present as a 125-kDa species. Isoelectric focusing indicated that four species with pIs of 5.9, 6.15, 6.35, and 6.6 were present. The equivalent reduced molecular mass of the *C. neoformans* var. *gattii* enzyme was 19 kDa, with a single species present under nonreducing conditions (relative molecular mass of 145 kDa) with a pI of 7.5. The activities of the enzymes from both varieties were inhibited by KCN; however, the copper chelator diethyldithiocarbamate was inhibitory only against the *C. neoformans* var. *gattii* enzyme, as was sodium azide. The *C. neoformans* var. *neoformans* SOD was not affected by preincubation for 1 h at 70°C, and it also retained most of its activity when incubated at 37°C relative to its activity when incubated at 20°C, in contrast to the *C. neoformans* var. *gattii* enzyme. The pronounced differences in the physical and biochemical characteristics of the Cu,Zn SODs from the two *Cryptococcus* varieties complement recent reports illustrating the biochemical and genetic differences between *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*, and the successful purification of the two enzymes comprises the first step in determining what role, if any, the cryptococcal Cu,Zn SODs might have in protection against externally generated superoxide.

Cryptococcus neoformans is an encapsulated yeast causing human disease with clinical manifestations that may vary from asymptomatic pulmonary infiltration to fatal disseminated infection, which is characterized by meningitis. The capsular polysaccharide of this yeast contains antigenic determinants providing the basis for four serotypes, A and D (*C. neoformans* var. *neoformans*) and B and C (*C. neoformans* var. *gattii*) (2, 5, 8, 29). There are a number of clear dissimilarities between *C. neoformans* var. *neoformans* and var. *gattii*, including differences in biochemistry (18, 20), environmental source (6, 7), DNA composition (3, 21), and number of chromosomes (27). Multilocus enzyme electrophoresis also suggests that there are differences between the two varieties (3), although there is only very limited data on the differences between individual enzyme systems (14). There is also evidence that *C. neoformans* var. *neoformans* has a relative predilection for immunocompromised hosts, while *C. neoformans* var. *gattii* has a predilection for immunocompetent hosts (22).

Several virulence factors have been identified in *C. neoformans*, including the production of melanin (19) via a phenol oxidase (laccase) enzyme (28). Melanin appears to play a role in protection against oxidant damage (16, 26), and evidence that it complements the activity of superoxide dismutase (SOD) has been put forward (15). The precise role of the latter in providing protection against damage induced by externally derived superoxide has not been investigated, although it has been shown that 90% of total SOD activity results from the presence of a Cu,Zn form of the enzyme (15). An Mn-containing isoenzyme, which is almost certainly mitochondrion associated, has been purified from *C. neoformans* and partially characterized (25). However, the Cu,Zn isoenzyme has not been isolated, and in this communication we report on the purification and comparative characterization of this enzyme from the two varieties of *Cryptococcus*. The description of the purification of the *Cryptococcus* SODs provides the framework for ascertaining the potential role of this enzyme in protection against externally generated superoxide. In addition, the comparison of the SODs from the two varieties of *Cryptococcus* provides further insight into the biochemical relationship between *C. neoformans* var. *neoformans* and var. *gattii.*

MATERIALS AND METHODS

Culture conditions and growth curves. Isolates of *C. neoformans* var. *neoformans* (NCPF 3168 and 3171; National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Bristol, United Kingdom), the acapsular mutant B4131 (gift of K. J. Kwon-Chung), and *C. neoformans* var. *gattii* (NCPF 3169 and 3170) were inoculated from water cultures onto Sabouraud agar plates and grown at 37°C. Yeast cells were suspended in sterile water and counted, and a volume equivalent to approximately 10^5 cells was then used to inoculate 1-liter volumes of Sabouraud broth (1% [wt/vol] mycological peptone [Unipath Laboratories, Basingstoke, United Kingdom], 2% [wt/vol] D-glucose [Sigma Chemical Co. Ltd., Poole, United Kingdom], and 0.25% [wt/vol] sodium glycerophosphate [Sigma]) in 2-liter flasks which were then incubated on an orbital incubator (100 rpm) at 37°C for periods up to 104 h. At intervals cell counts were taken and yeasts were harvested by centrifugation (10,000 × *g* for 20 min); culture supernatants were also retained. All growth curves were determined in duplicate.

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SOD activity in the supernatants was detected via a modification of the method of Beauchamp and Fridovich (1), in which the pH of the assay buffer was adjusted to 10.2 with HCl, with a bovine erythrocyte SOD as a positive control. In all assays SOD activity was calculated on the basis that 1 U of purified enzyme is capable of causing a 50% inhibition of the standard xanthine-xanthine oxidase system at 20°C (all reagents from Sigma).

Purification of the Cu,Zn SODs from *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Yeast cells harvested from mid-log- to late-log-phase cultures (from NCPF 3168, NCPF 3171, and B4131 in the case of *C. neoformans* var. *neoformans* and from NCPF 3169 and 3170 in the case of *C. neoformans* var. *gattii*) were homogenized with 0.5-mm-diameter Ballotini glass beads (Jencons Scientific Ltd., Leighton Buzzard, United Kingdom) in 50 ml of distilled water in an ice-cooled bead beater (Biospec Products, Bartlesville, Okla.). The resulting homogenate was collected, and the beads were washed twice, each time in a total volume of 150 ml of distilled water. The latter was pooled with the original homogenate, and the whole was centrifuged for 40 min at 4°C and 10,000 × *g*. The resulting supernatant was dialyzed overnight against distilled water, and 50 ml of the supernatant was then mixed with 1 ml of ampholytes (Biolyte; pH range, 3 to 10; Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom), loaded onto a Rotofor liquid isoelectric focusing system (Bio-Rad), and electrophoretically separated (at a constant power of 12 W) until the voltage stabilized after 4 to 5 h. Each of the 20 fractions harvested from the Rotofor were then assayed for pH, protein content (by the Coomassie blue method [24]), and SOD activity; fractions were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Those Rotofor fractions containing the highest ratio of SOD to protein (U/mg) were pooled and concentrated on 1-kDa Microsep centrifugal concentrators (Flowgen, Sittingbourne, United Kingdom) to a volume of less than 2 ml. Subsamples (500 μl) of this concentrate were loaded onto a Superose 12 gel filtration fast protein liquid chromatography (FPLC) column (Pharmacia Biotech, St. Albans, United Kingdom) and eluted with 50 mM Tris buffer, pH 8.4, at a flow rate of 0.5 ml/min in 1-ml fractions. SOD activity and protein content were determined as described above, and fractions with the highest ratio of SOD to protein were concentrated on centrifugal concentrators and passed down the gel filtration column a total of three times. In total, Cu,Zn SOD was purified from three separate cultures of each *C. neoformans* isolate.

SDS-PAGE and isoelectric focusing. The purification of the SODs was monitored via SDS-PAGE (on 10 and 15% polyacrylamide gels and on 4 to 20% Ready gels [Bio-Rad]), as previously described (9, 12), with either Coomassie brilliant blue R-250 (10) or silver stain (Bio-Rad). Isoelectric focusing was performed as previously described (12).

Characterization of the *Cryptococcus* Cu,Zn SODs. All characterization studies were performed using SOD from each of the three cultures of each isolate from which the enzyme had been purified and in triplicate. To measure the effect of pH on SOD activity the following buffer systems were used: pH 7.0 to 9.0 Tris HCl (50 mM) and pH 9.0 to 11.0 carbonate buffer (sodium carbonate-sodium bicarbonate) (50 mM). Two to five micrograms of SOD was used in each assay. The effects of various potential inhibitors on the activity of the *Cryptococcus* Cu,Zn SODs were determined as previously described (13). Standard deviations were calculated with StatView 512.

To determine the resistance of the Cu,Zn SODs to heat inactivation, 2 to 5 μg of *C. neoformans* var. *neoformans* enzyme and 2 to 5 μg of *C. neoformans* var. *gattii* enzyme were incubated at 50°C for up to 1 h and at 70°C for up to 1 h, assayed for residual SOD activity at 20°C, and compared to controls incubated at 20°C for the same time periods. In addition, the relative activities of the SODs from the two *Cryptococcus* varieties were compared at temperatures of 20, 30, and 37°C by setting up parallel incubations of identical standard assay tubes, in addition to SOD-negative controls, and assaying at 0 and 5 min.

N-terminal amino acid sequencing of purified Cu,Zn SODs. Ten micrograms of purified SOD from the isolates of each *Cryptococcus* variety in disruption buffer (9) was loaded into each lane of an aged multiwell SDS–15% polyacrylamide gel (13) and run at 150 V for 45 min. The gel was then electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Watford, United Kingdom) (9); blots were then stained for 1 min with Coomassie brilliant blue and destained, and the appropriate protein bands were subjected to N-terminal amino acid sequencing.

RESULTS

Growth curves and SOD production. The growth curves of three of the five *Cryptococcus* variants relative to the detection of Cu,Zn SOD activity in culture supernatants are shown in Fig. 1. SOD activity was present in culture filtrates of the encapsulated *C. neoformans* var. *neoformans* isolate (NCPF 3168) in the stationary phase (Fig. 1a). The growth curve and SOD profile for the second *C. neoformans* var. *neoformans* isolate (NCPF 3171) were similar (data not shown). The greatest Cu,Zn SOD activity in culture supernatants was produced (from the mid-log phase onwards) by the acapsular mutant of

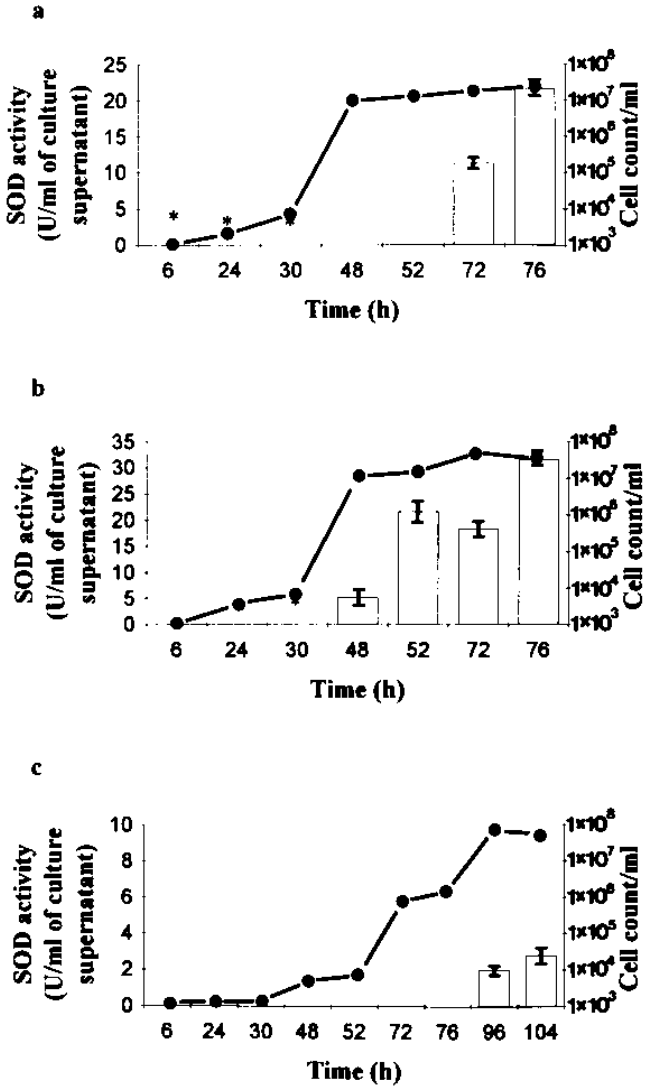

FIG. 1. Growth curves for and SOD production at 37°C in *C. neoformans* var. *neoformans* (encapsulated isolate and acapsular mutant) and *C. neoformans* var. *gattii*. (a) *C. neoformans* var. *neoformans* NCPF 3168; (b) *C. neoformans* var. *neoformans* B4131 (acapsular mutant); (c) *C. neoformans* var. *gattii* NCPF 3170. The histogram shows SOD content (error bars represent standard deviations). Solid circles represent yeast cell count. Asterisks indicate that SOD activity was not assayed.

C. neoformans var. *neoformans* (B4131) (Fig. 1b). SOD activity was very low in culture supernatants from both of the *C. neoformans* var. *gattii* isolates (data for NCPF 3170 are shown in Fig. 1c; data for the second *C. neoformans* var. *gattii* isolate were similar and are not shown). The *C. neoformans* var. *gattii* cultures grew more slowly than those of the *C. neoformans* var. *neoformans* isolates, although the former did eventually reach a cell count equivalent to the latter. The use of the specific Cu,Zn inhibitor KCN revealed no contribution to total SOD activity in the culture supernatants by Mn or Fe SOD.

Purification of the *Cryptococcus* Cu,Zn SODs. Purification of the Cu,Zn SODs from the *Cryptococcus* variants involved three main steps: first, the homogenization of harvested yeast cells, followed by the removal of cell debris and dialysis; second, separation by liquid isoelectric focusing (Rotofor); and finally sequential gel filtration FPLC. Figure 2a and c demonstrate the pH, protein content, and SOD activity of each of the 20

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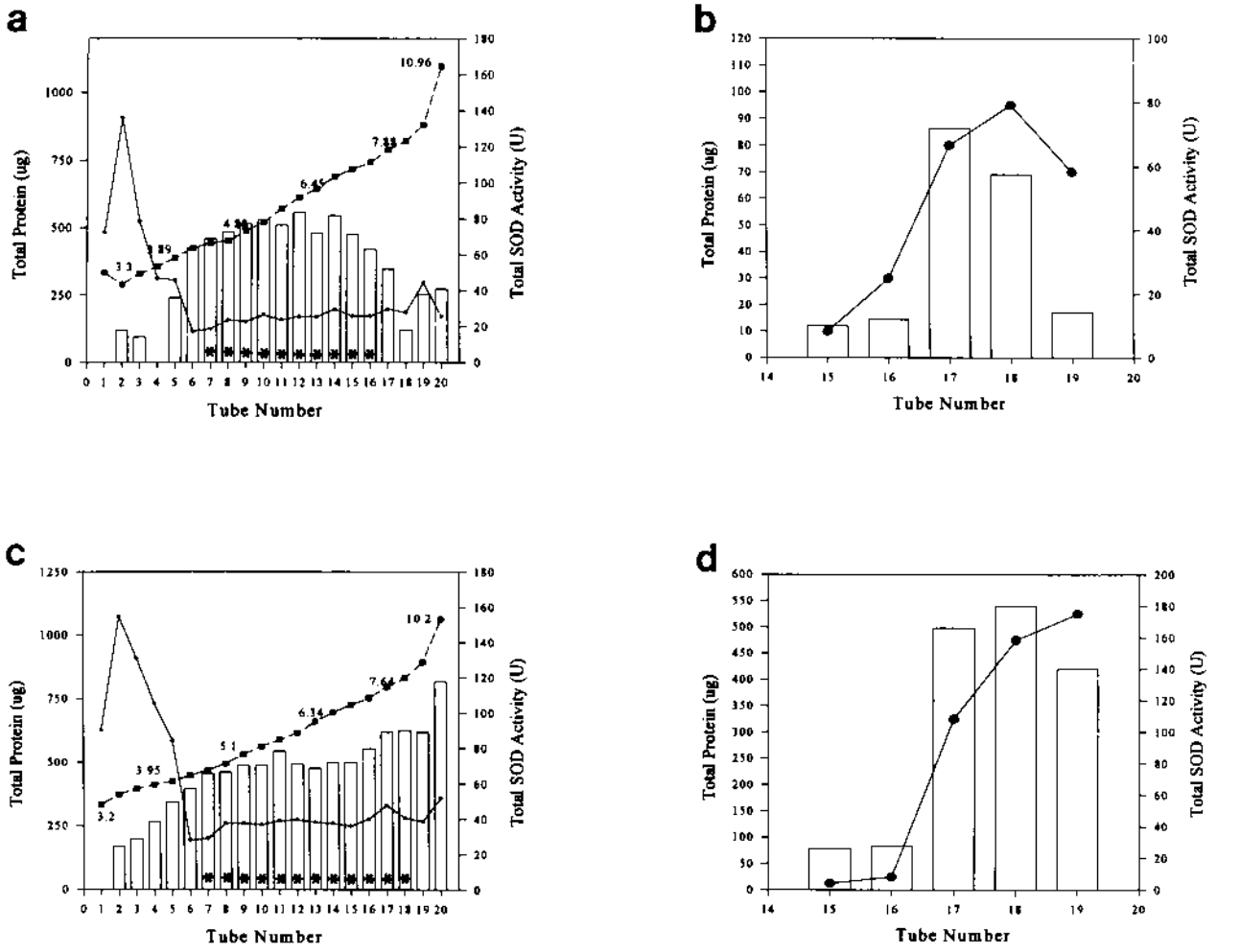

FIG. 2. Purification of the *C. neoformans* var. *neoformans* (NCPF 3168) and *C. neoformans* var. *gattii* (NCPF 3170) SODs by Rotofor isoelectric focusing (a and c, respectively) (fractions chosen for subsequent purification are indicated by asterisks) and gel filtration FPLC (b and d, respectively). The histogram shows the SOD content of each fraction. Circles represent the protein content of each fraction, and squares represent the pH profile in Rotofor fractions.

Rotofor fractions for *C. neoformans* var. *neoformans* (NCPF 3168) and var. *gattii* (NCPF 3170) homogenates, respectively. The profiles (data not shown) for the other three isolates (NCPF 3171 and B4131 in the case of *C. neoformans* var. *neoformans* and NCPF 3169 in the case of *C. neoformans* var. *gattii*) were similar to the respective profiles given in Fig. 2. There were very large quantities of precipitated protein in fractions 1 to 5, 19, and 20. In each case those fractions with the highest SOD/protein ratio were pooled, concentrated, and passed down a gel filtration FPLC column. The purification profile of the first of the three sequential gel filtration runs is shown in Fig. 2b and d (for *C. neoformans* var. *neoformans* NCPF 3168 and *C. neoformans* var. *gattii* NCPF 3170, respectively; profiles for NCPF 3171, B4131, and NCPF 3169 are not shown but are similar to profiles shown for the respective varieties). The results of the purification of the *Cryptococcus* SODs are illustrated in Table 1. The specific activities of the two enzymes at 20°C were similar: 5,714 U/mg for the *C. neoformans* var. *neoformans* (NCPF 3168) enzyme and 5,000 U/mg for the *C. neoformans* var. *gattii* (NCPF 3170) enzyme. All subsequent results presented relating to the characterization of the Cu,Zn SODs were obtained with purified enzyme from NCPF 3168 (*C. neoformans* var. *neoformans*) and NCPF 3170 (*C. neoformans* var. *gattii*). Data for NCPF 3171, B4131 (*C. neoformans* var. *neoformans*), and NCPF 3169 are not shown since in each case results were virtually identical to those for the respective *C. neoformans* var. *neoformans* and var. *gattii* isolates provided.

The purification of the *C. neoformans* var. *neoformans* and var. *gattii* SODs was also monitored by SDS-PAGE and silver staining (Fig. 3). Table 2 summarizes the reduced and nonreduced relative molecular masses of the two enzymes and their pIs. As regards the latter, the occurrence of the Cu,Zn SODs within their respective Rotofor pH profiles was suggestive of similar enzyme pIs. However, isoelectric focusing of the purified Cu,Zn SODs on gels revealed the presence of four isoforms with different pIs in the case of the *C. neoformans* var. *neoformans* enzyme. In contrast, in the case of the *C. neoformans* var. *gattii* enzyme, only one isoform was present. On repeated freeze-thaw cycling the *C. neoformans* var. *neoformans* SOD generated diffuse bands on nonreduced gels (data not shown).

Effect of pH on SOD activity. The effect of pH on the activity of the two *Cryptococcus* enzymes is shown in Fig. 4. Both *Cryptococcus* enzymes demonstrated some degree of pH dependence over the range 7 to 11. The effect of a pH below 7

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TABLE 1. Purification of *C. neoformans* Cu,Zn SODs

Variety and method	Total protein (mg)	Total activity (U)	Sp act at 20°C (U/mg of protein)	Yield (%)	Purification (fold)
C. neoformans var. *neoformans*					
Homogenization	155	3,200	20.6	100	1
Liquid isoelectric focusing	3.5	941	268.8	29.4	13
Gel filtration (1st run)	0.46	401	871.7	12.5	42.3
Gel filtration (3rd run)	0.014	80	5,714	2.5	277
C. neoformans var. *gattii*					
Homogenization	180	2,915	16.2	100	1
Liquid isoelectric focusing	4	851	212.5	29	13
Gel filtration (1st run)	0.8	346	432.5	12	26.7
Gel filtration (3rd run)	0.021	105	5,000	3.6	309

was not assessed since xanthine oxidase is inactive below pH 6.5. The pH dependence of xanthine oxidase above pH 7 was controlled by varying the amount of xanthine oxidase added to each assay to provide similar quantities of superoxide.

Effects of known SOD inhibitors on the activities of the purified SODs. The effects of a number of potential enzyme inhibitors are demonstrated in Table 2. In the case of the *C. neoformans* var. *neoformans* enzyme, diethyldithiocarbamate (DDC), sodium azide, and EDTA had virtually no inhibitory activity, while KCN demonstrated full inhibition at all working concentrations. The other inhibitors had no effect whatsoever. The *C. neoformans* var. *gattii* enzyme was also inhibited by KCN, and in contrast to the *C. neoformans* var. *neoformans* enzyme, it was also inhibited to various extents by both DDC and sodium azide.

Resistance of the *Cryptococcus* SODs to heat inactivation and relative activities at 20, 30, and 37°C. Both of the *Cryptococcus* enzymes retained activity after pretreatment at 50°C for 1 h (Table 2). However, while the *C. neoformans* var. *neoformans* enzyme survived pretreatment for 1 h at 70°C, the *C. neoformans* var. *gattii* enzyme lost all activity under these conditions.

The relative activities of the two *Cryptococcus* enzymes at incubation temperatures of 20, 30, and 37°C are shown in Table 2. The *C. neoformans* var. *neoformans* SOD retained significantly more of its activity at the higher incubation temperatures than did the *C. neoformans* var. *gattii* enzyme.

N-terminal amino acid sequences of the purified SODs. There was clear sequence homology between the *Cryptococcus* SODs and Cu,Zn SODs from a range of organisms, although the N-terminal amino acid sequences of the SODs from the two *Cryptococcus* varieties were not identical, with differences appearing after the initial 10 amino acids (Table 3). The *C. neoformans* var. *neoformans* SOD demonstrated the greatest homology with Cu,Zn SODs from *Neurospora crassa* (Table 3) and *Aspergillus fumigatus*. The *C. neoformans* var. *gattii* SOD demonstrated the greatest homology with Cu,Zn SODs from *Schizosaccharomyces pombe* and *A. fumigatus* (Table 3).

DISCUSSION

The cryptococcal SODs described here demonstrate obvious N-terminal amino acid sequence homology with Cu,Zn SODs from other organisms, including fungi (13), and there was no homology with the previously described *Cryptococcus* Mn SOD (25) or with other representatives of this form of the enzyme. The homology between the two *Cryptococcus* enzymes was 100% over the first 10 amino acids, although clear differences in composition occur over the next 9. It is of note that while KCN inhibits both cryptococcal enzymes, another previously defined inhibitor of Cu,Zn SODs, the copper chelator DDC, has a significant inhibitory effect only on the *C. neoformans* var. *gattii* SOD. This might suggest that in the *C. neoformans* var. *neoformans* enzyme, in contrast to the *C. neoformans* var. *gattii* enzyme, the Cu ion is inaccessible to DDC and is therefore indicative of a structural difference between the two enzymes.

The clearest evidence for differences between the two enzymes in structure and composition comes from the observations of nonreduced relative molecular masses and pIs. The two enzymes appear to have similar reduced molecular masses. However, while the *C. neoformans* var. *gattii* enzyme appears to be comprised of a single species with a nonreduced molecular mass of 145 kDa and a pI of 7.5, isoelectric focusing revealed that the *C. neoformans* var. *neoformans* enzyme is composed of four species with pIs of 5.9, 6.15, 6.35, and 6.6 and has a nonreduced relative molecular mass of 125 kDa. Cu,Zn SOD isoforms from other organisms have been described (23), and it is unclear at this point whether the *C. neoformans* var. *neoformans* isoforms arise from differences in amino acid content (possibly resulting from the existence of multiple genes with different sequences) or from differences in glycosylation, although the latter appears less likely. Cu,Zn SODs are often multimeric, and indeed, tetrameric forms have been identified (4). It is unclear what the intact structures of the two *Crypto-*

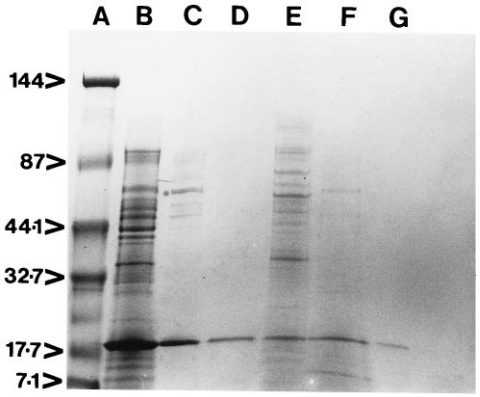

FIG. 3. Purification of the *C. neoformans* var. *neoformans* (NCPF 3168) and *C. neoformans* var. *gattii* (NCPF 3170) SODs as determined by SDS-PAGE and silver staining. Lane A, Bio-Rad molecular mass markers (numbers at left are in kilodaltons); lane B, crude *C. neoformans* var. *neoformans* homogenate; lane C, *C. neoformans* var. *neoformans* Rotofor fraction 12; lane D, *C. neoformans* var. *neoformans* purified SOD; lane E, crude *C. neoformans* var. *gattii* homogenate; lane F, *C. neoformans* var. *gattii* Rotofor fraction 12; lane G, *C. neoformans* var. *gattii* purified SOD (all samples reduced with 2-mercaptoethanol).

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TABLE 2. Characteristics of purified *C. neoformans* Cu,Zn SODs

Source	Reduced relative molecular mass (kDa)	Nonreduced relative molecular mass (kDa)	pI(s)	% Effect (SD) of inhibitor[a]:							% Activity			
				None (control)	1.0 mM KCN	1.0 mM DDC	1.0 mM GdmCl	30 mM sodium azide	30 mM EDTA	30 mM *o*-phenanthroline	After 60 min of pretreatment at:		At incubation temp of:	
											50°C	70°C	20°C	30°C	37°C
C. neoformans var. *neoformans*	19	125	5.9, 6.15, 6.35, 6.6	0	100 (0)	5 (1.5)	0 (0)	0 (0)	3.5 (1.4)	0 (0)	100	100	100	94	82
C. neoformans var. *gattii*	19	145	7.5	0	100 (0)	100 (0)	0 (0)	40 (6.4)	0 (0)	7 (1.0)	100	0	100	82	43

[a] The values for full activity (0% inhibition) are equivalent to the specific activities given for each variety (see text).

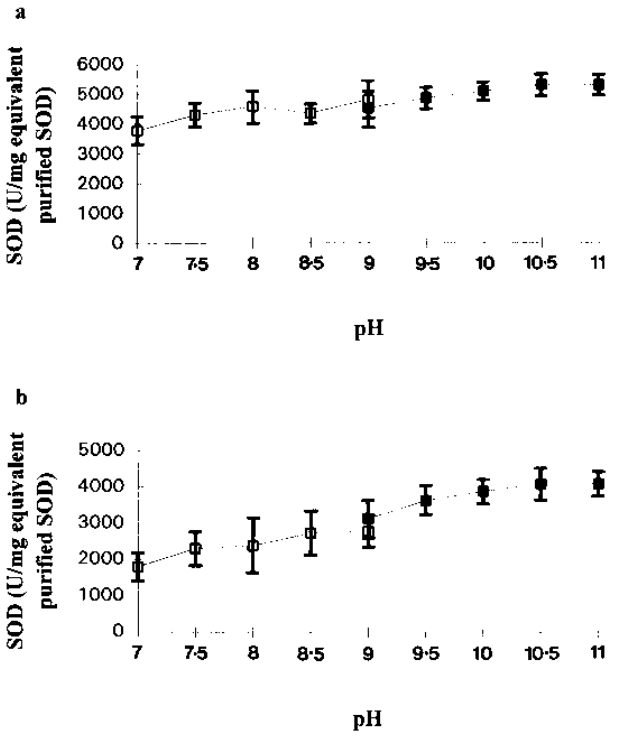

FIG. 4. Activity of purified *C. neoformans* var. *neoformans* (a) and *C. neoformans* var. *gattii* (b) Cu,Zn SODs over a pH range of 7 to 11 (error bars represent standard deviations). Open squares, Tris HCl buffer system; solid squares, carbonate buffer system.

coccus enzymes are, although they are evidently different from one another. That the Cu,Zn SODs should be apparently different in structure and composition is perhaps surprising for fungi which are classified as varieties of the same species. However, recent studies have stressed the biochemical and genetic differences between the two varieties (17, 20), and the differences among Cu,Zn SODs described here tend to coincide with this pattern of observation. The Cu,Zn SODs from both *C. neoformans* var. *neoformans* and var. *gattii* displayed some apparent pH dependence, in contrast to previously described fungal Cu,Zn SODs (13).

It is known that isolates of *C. neoformans* var. *gattii* are typically more sensitive to higher temperatures, with lower growth rates at 37°C than those of *C. neoformans* var. *neoformans* isolates (17), and the *C. neoformans* var. *gattii* isolates used in this study conformed to this growth pattern. We have demonstrated that the *C. neoformans* var. *neoformans* Cu,Zn SOD retains more of its activity than the equivalent *C. neoformans* var. *gattii* enzyme when incubated at 37°C. If the ability of the Cu,Zn SOD to retain activity at 37°C is representative of the abilities of other enzymes from *C. neoformans* var. *neoformans*, then this may go some way in accounting for the higher growth rate of this variety relative to isolates of *C. neoformans* var. *gattii* at 37°C.

The total Cu,Zn SOD activity in isolates of *C. neoformans* is higher when cells are cultured at 37°C than when cells are cultured at 25°C (15), leading to the suggestion that this higher level of activity in some way compensates for the converse lower level of phenol oxidase activity and hence for less production of the antioxidant melanin. This higher level of total Cu,Zn SOD activity must result from an upregulation in Cu,Zn SOD production—as demonstrated in this communication,

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TABLE 3. N-terminal amino acid sequence comparison of *C. neoformans* SODs and the most homologous proteins in the GenEMBL database

Protein	Comparison with *C. neoformans* var. *neoformans* SOD		Comparison with *C. neoformans* var. *gattii* SOD	
	Sequence[a] (amino acid positions)	% Similarity (n = 19)	Sequence[a] (amino acid positions)	% Similarity (n = 19)
C. neoformans var. *neoformans* SOD	VKAVAVLKGDSHVYGTITF (1–19)	100	VKAVAVLKGDSHVYGTITF (1–19)	84.2
C. neoformans var. *gattii* SOD	VKAVAVLKGDSPVTGVITF (1–19)	84.2	VKAVAVLKGDSPVTGVITF (1–19)	100
A. fumigatus Cu,Zn SOD	VKAVAVLRGDSKITGTVTF (1–19)	73.6	VKAVAVLRGDSKITGTVTF (1–19)	73.6
N. crassa Cu,Zn SOD	VKAVAVVRGDSNVKGTVIF (2–20)	68.4	VKAVAVVRGDSNVKGTVIF (2–20)	63.2
S. pombe mRNA SOD	VRAVAVLRGDSKVSGVVTF (2–20)	68.4	VRAVAVLRGDSKVSGVVTF (2–20)	73.6
Rat Cu,Zn SOD	MKAVCVLKGDGPVQGVIHF (2–20)	63.2	MKAVCVLKGDGPVQGVIHF (2–20)	73.6

[a] Shared amino acids are underlined.

even in the comparatively temperature-insensitive *C. neoformans* var. *neoformans* Cu,Zn SOD, there is still a decrease in the activity of the purified enzyme on the order of 20% during incubation at 37°C compared to its activity during incubation at 20°C. The *C. neoformans* var. *neoformans* enzyme resembles the previously described *A. fumigatus* Cu,Zn SOD (13) in its ability to retain activity after pretreatment at 70°C, whereas the *C. neoformans* var. *gattii* enzyme loses its activity when pretreated this way, which emphasizes its relative sensitivity to higher temperatures.

In contrast to a previous report (15) we were able to demonstrate the presence of Cu,Zn SOD in a culture supernatant of *C. neoformans*. However, SOD activity was detectable only in the stationary phase of the four encapsulated isolates (with very low levels in the two *C. neoformans* var. *gattii* isolates); it is not clear which point in the growth curve the previous study (15) focused on, but it is apparent that sampling before the stationary phase would have produced a negative result. This is in contrast to the situation seen in *A. fumigatus*, where Cu,Zn SOD activity is detectable from the mid-log phase onwards (13), and this observation makes it probable that the presence of Cu,Zn SOD activity in culture supernatants arises more from cell lysis than from an active process of export from living cells. However, we have previously demonstrated that substantial amounts of Cu,Zn SOD are present within the cell wall of *A. fumigatus* (11), and it is possible that Cu,Zn SOD may also accumulate in both the cell wall and capsule of *C. neoformans*. The possibility of capsule retention of the enzyme is suggested by the observation that the culture supernatant from the acapsular mutant of *C. neoformans* contains the greatest amount of Cu,Zn SOD activity of the five isolates assayed—indeed, Cu,Zn SOD activity is detectable from the mid-log phase onwards in the acapsular mutant. The question of the intracellular location of Cu,Zn SOD in *Cryptococcus* will be studied in our laboratory via the production of a polyclonal antibody to the enzyme.

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