Superoxide dismutase in *Cryptococcus neoformans* varieties *gattii, grubii,* and *neoformans*

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Some clear dissimilarities occur among the varieties of Cryptococcus neoformans *but there are few studies about the differences among individual yeast antioxidant enzymes. The total superoxide dismutase (SOD) activities and the copper, zinc-depend SOD (Cu,ZnSOD) and manganese-dependent SOD (MnSOD) isoenzymes of five reference* C. neoformans *strains belonged to A, B, C, AD and D serotypes (Table I) and other nine* C. neoformans *isolates (Table II) were determined. There were significant differences (*$p < 0.01$ *and* $p < 0.05$*) in total SOD activity among the varietie* gattii *(serotype C) and the other varieties. Cu,ZnSOD showed difference (*$p < 0.05$*) between A and D serotypes. These results point out a variety and serotype-independent SOD activity in* C. neoformans *reference strains and the other isolates that were evaluated.*

Key words: *Cryptococcus neoformans* - superoxide dismutase - antioxidant

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) are constantly generated in all aerobic biological systems, mainly by phagocytes through the respiratory burst (Babior 2002). This event is a crucial host defense against microorganisms which, paired with the non-oxidative phagocyte microbicidal mechanisms, determines death and elimination of many invader agents (Roos & Winterboum 2002). Superoxide dismutase (SOD) is a group of metalloenzymes that detoxify ROS through the conversion of $O_2^{\bullet -}$ to hydrogen peroxide and molecular oxygen (Fridovich 1995). These enzymes are virtually present in all aerobic cells and their very high degrees of conservation is testament to their importance in cellular homeostasis. Three types of SOD isoenzymes occur in living cells, whose differences are due to their prosthetic fractions. In general eukaryotic cells contain MnSOD in the mitochondrial matrix and another isoenzyme, Cu,ZnSOD, which is located principally in the cytoplasm and in a lower extent in peroxissomes (Chaturvedi et al. 2001). The third type, FeSOD, occurs in photosynthetic organisms (Okamoto et al. 1996).

Discrimination of SOD isoenzymes is based on differential inhibition or inactivation by selective chemicals. Cyanide inactivates Cu,ZnSOD while hydrogen peroxide inhibits irreversibly both FeSOD and Cu,ZnSOD (Mayer & Falkinham. 1986).

Cryptococcus neoformans is an encapsulated yeast causing human disease with clinical manifestation 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 five serotypes, A (*C. neoformans* var. *grubii*), D and AD (*C. neoformans* var. *neoformans*), and B and C (*C. neoformans* var. *gattii*).

In the last two decades, a variety of fungal antioxidants have attracted considerable interest, largely arising from their hypothetical role as virulence associated factors. The biological roles of bacterial Cu,ZnSOD contribute to the ability of invasive pathogens to survive to $O_2^{\bullet -}$ produced by macrophages and neutrophils during the respiratory burst (Hamilton & Holdom 1997, Cox et al. 2003). This work evaluated the MnSOD and Cu,ZnSOD activities from the three *C. neoformans* varieties. These strains were *C. neoformans* serotype A (ATCC 90112, USA), B (NIH –ICB 107, USA), C (NIH-ICB162, USA), AD (CBS-ICB 134, USA), D (NIH-ICB163, USA). They were subcultured in Sabouraud dextrose agar at 25°C during 24 h, ressuspended in 2 ml phosphate buffer saline solution (PBS) pH 7.8 and submitted to nitrogen cavitation (1,250 psi, 20 min, 4°C) with the protease inhibitor phenylmethionylsulphonyl fluoride (2.5 µg/ml) and the peptidase inhibitor benzamidine (1 µg/ml). The efficiences of lysis were controlled by viability analysis with cell membrane integrity measured by methylene blue coloration (Mochaba et al. 1998). Total SOD activity was measured by the inhibition of the cytochrome C (7.5 µM) reduction, mediated via $O_2^{\bullet -}$ that were generated by xanthine – xanthine oxidase system – and was monitored in a Hitachi model U-2000 dual beam spectrophotometer at 550 nm. One unit of SOD was defined as the enzyme amount required to inhibit the reduction of cytochrome C by 50% at 25°C. The activities of the isoforms MnSOD and Cu,ZnSOD were determined by a modification of the method proposed by McCord and Fridovich (1968). The MnSOD activity was determined by the inhibition of the isoform Cu,ZnSOD with the addition of KCN (5 mM) (Mayer & Falkinham 1986). The Cu,ZnSOD activity was

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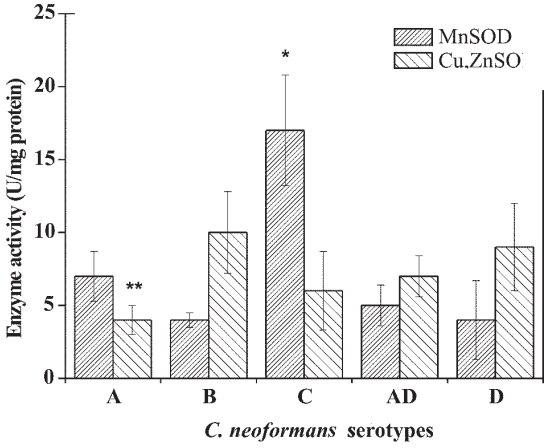
determined by the difference between the total SOD and MnSOD activities. All samples were submitted to total protein determination using bovine serum albumin as standard and the final enzymes units (U) were plotted per mg of total protein (Bradford 1976). The data shown are means ± sd from experiments performed in triplicate that were statistically analyzed using one way ANOVA and Bonferroni Multiple Comparisons Tests. $p < 0.05$ was considered significant. The specific activities of total SOD and of the both isoenzymes MnSOD and Cu,ZnSOD are shown the Tables I, II and Figure. *C. neoformans* var. *gattii* serotype C showed the higher total SOD activity. Var. *grubii* displayed the lower total SOD activity among the analyzed reference yeast strains with (4 ± 1) U of Cu,ZnSOD/mg protein and 7 U of MnSOD/mg protein. Var. *neoformans* serotypes D and AD showed no significant differences in both isoenzymes. There were significant differences ($p < 0.01$) in MnSOD activity from the serotype C strain in comparison with all the other reference varieties tested. Cu,ZnSOD showed differences ($p < 0.05$) between A and D reference serotypes.



MnSOD and Cu,ZnSOD activities in five reference serotypes of *Cryptococcus neoformans*. Cultured *C. neoformans* cells were lysed by nitrogen cavitation and both superoxide dismutase isoenzymes were determined. Results expressed as means ± sd, n = 3; *$p < 0.01$ serotype C against all other serotypes and **$p < 0.05$ serotype A against serotype D in respect to the same isoenzyme.

In recent studies, the variety *gattii* has been considered, as a separated species, *C. gattii* (C. *bacillisporus*) (Sorrel 2001, Barreto de Oliveira et al. 2004). There are a number of clear dissimilarities among *C. neoformans* var. *grubii*, var. *neoformans* and var. *gattii* (*C. gattii = C. bacillisporus*), including differences in biochemistry, environmental source, DNA composition, number of chromossomes, clinical manifestation of the disease, and response to antifungal therapy. There are differences with regard to the enzyme production in the three varieties, although there is only very limited data on the differences among individual enzyme system (Lacaz et al. 2002). It is shown here that SOD activity is one biochemical parameter to be consider among these discrepances. *C. neoformans* var. *gattii* predominantly infects immunocompetent individuals, whereas var. *grubii* and *neoformans* are common in immunocompromised individuals. The mechanism of diferences in host prediction remain largely unknown, except for two experimental studies that reported that *C. neoformans* var. *gattii* inhibits phagocyte response whereas the other two varieties are readily killed by ROS that are released by phagocytic cells. It was hypothesized that this difference could result from innate diverse responses among the antioxidants of these varieties (Chaturvedi et al. 2001). The comparison of the total SOD from the three varieties of *C. neoformans* provides further insight into the biochemical relationship among them. We refined these studies showing here that the isoenzymes Mn- and Cu,ZnSOD vary significantly among

TABLE I

Total superoxide dismutase and isoenzymes MnSOD and Cu,ZnSOD activities in *Cryptococcus neoformans* reference strains

Strain	ATCC90112	NIH-ICB107	NHI-ICB162	CBS-ICB134	NIH-ICB163
Serotype	A	B	C	AD	D
Varieties	*grubii*	*gattii*	*gattii*	*neoformans*	*neoformans*
Total SOD [a]	11 ± 2.8	14 ± 0.5	23 ± 10.6*	12 ± 0.5	13 ± 8.8
MnSOD [a]	7 ± 1.7	4 ± 0.5	17 ± 3.8	5 ± 1.4	4 ± 2.7
Cu,ZnSOD [a]	4	10	6	7	9

a: activity (U/mg protein); results (in triplicate) are expressed as means ± sd, *$p < 0.01$ (serotype C against all other serotypes).

TABLE II

Total superoxide dismutase and isoenzymes MnSOD and Cu,ZnSOD activities in nine *Cryptococcus neoformans* strains

Strain	ICB154	ICB170	ICB107A	ICB184	ICB88	ICB108	ICB134A	ICB173	ICB110
Serotype	A	A	B	B	C	C	AD	D	D
Varieties	*grubii*	*grubii*	*gattii*	*gattii*	*gattii*	*gattii*	*neoformans*	*neoformans*	*neoformans*
Total SOD [a]	15	62±1	52.5±10.5	33±0.5	160±1.5*	186*	48	51.5±10.5	16
MnSOD [a]	*10.5±0.5*	*9±1*	*11±0.5*	*23*	*75*	*32±1.5*	*36*	*14±0.5*	*11*
Cu,ZnSOD [a]	4.5	53	41.5	10	85	154	12	37.5	5

a: activity (U/mg protein); results (in triplicate) are expressed as means ± sd, *$p < 0.05$ (serotype C against all other serotypes).

all serotypes. After phagocytosis by polymorphonuclear cells or macrophages, pathogens in the phagolysosomes are exposed to a variety of ROS, including $O_2^{\bullet -}$. Microorganism SODs are important housekeeping antioxidants and have an additional hypothetical role in virulence. Despite these enzymes have been biochemically characterized from some fungus as *Aspergillus* and *Cryptococcus*, there is as yet no strong evidence that these enzymes are involved in pathogenicity. The Cu,ZnSOD was previously pointed in *C. neoformans* as the more abundant form of the enzyme, and its cytoplasmic location was thought to be more relevant for a possible protection against phagocyte-derived ROS (Chaturvedi et al. 2001). Our results showed a discrepancy from these results in respect to serotype C, although we confirmed the higher total SOD activity inside this serotype. Previous report showed similarities with regard to amino acid sequences among Cu,ZnSOD isolated from *C. neoformans* and from other organisms, including fungus, and there was no homology with the previous described *C. neoformans* MnSOD with other representatives of this isoform of the enzyme. It was noted that, while KCN inhibited both cryptococcal Cu,ZnSOD enzymes, another previously defined inhibitor of the isoenzyme, the Cu^{2+} chelator diethyldithiocarbamate (DDC) had 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*, the Cu^{2+} is inaccessible to DDC indicating a possible structural differences between the two enzymes. These apparent differences in structure are surprising for fungi that still today are classified as varieties from the same species. In addition, the SOD from all the *C. neoformans* varieties displayed some apparent pH dependence, in contrast to previously described fungal SOD (Hamilton & Holdom 1997). The characterization of a Cu,ZnSOD gene knock-out *C. neoformans* mutant has been realized (Chaturvedi et al. 2001). In the mutant for this gene, no defects were seen in growth, capsule synthesis, mating, sporulation but it was markedly attenuated in virulence in a mouse model and it was significantly susceptible to in vitro killing by human neutrophils. This report constituted the first instance in which SOD has been directly implicated in the virulence of a fungal pathogen. In some bacteria, SOD has been shown to be important for survival within macrophages and for virulence in animal models. It is known that *C. neoformans* resides in macrophages during many stages of experimental and human infections and that the resistance to macrophage killing, in first instance mediated by SOD activity may be important for virulence in this fungus (Chaturvedi et al. 1996).

The prevalence of invasive fungal infections is increasing simultaneously in occurrence to the growing of immunocompromised patient numbers. So, it is important to develop new strategies to control fungal invasions. As *C. neoformans* is a successful intracellular pathogen, it is believed that it must have efficient mechanisms for the detoxification of ROS. It seems plausible that the role of microbial SOD in pathogenicity should be closely associated with defense against phagocyte attack (Chaturvedi et al. 1996).

In a first moment we evaluated the activities of SOD isoenzymes of 14 *C. neoformans* strains but additional studies have already being performed to give support to this previous one. Then, detailing antioxidant enzymes from these fungi could provide insights that can help us in the diagnosis and treatment of this important human disease.

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