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Purification and ultrastructural localization of a copper–zinc superoxide dismutase (CuZnSOD) from the entomopathogenic and acaricide fungus Metarhizium anisopliae

Sílvia Elena Tolfo Bittencourt
Luiza Amaral de Castro
Sandra Estrazulas Farias
Sônia Nair Bao
Augusto Schrank
Marilene Henning Vainstein

Abstract

The entomopathogenic fungus Metarhizium anisopliae contains three superoxide dismutases. One of these enzymes was purified and partially characterized as a CuZnSOD. The enzyme has an estimated molecular mass of 30 690 Da and a specific activity of 3838.89 U mg−1. SDS-PAGE and 2D gels show a single band of protein in the fractions eluted from the gel filtration column with a molecular mass of 20 000 and ∼15 000 Da, respectively, and a pI of 6.0. These results suggest that the native enzyme is a dimer consisting of two subunits. Polyclonal antiserum were raised against purified CuZnSOD and used to determine its subcellular localization by immunoelectron microscopy. M. anisopliae CuZnSOD is present in the cell wall.

Keywords: Metarhizium anisopliae; Superoxide dismutase (SOD); Antioxidant enzyme; Active oxygen species (AOS); Oxygen free radicals (OFR); Biological control

1. Introduction

Metarhizium anisopliae is a broad host range entomopathogenic fungus first recognized as a potential candidate for biological control of agriculture pests in the 1880s. The fungus actively invades the hosts through the cuticle by mechanical pressure and synergistic action of hydrolytic enzymes [18,47,53]. The whole process of host infection occurs in successive phases of adhesion of spores, hyphal germination, differentiation (apressorium formation), penetration, colonization, reproduction and dissemination for the start of a new cycle [9]. During host colonization, fungi usually encounter a host resistance response, the generation of an environment around the pathogen rich in active oxygen species (AOS), and death of host cells surrounding the infection site. The dead host cells become a defensive layer that deprives the biotroph of a food supply. This layer also acts as a mechanical barrier, which the pathogen finds diffi- cult to breach [22,34].

Superoxide dismutases (SODs) are metalloenzymes with a redox metal at their active sites that rapidly convert two molecules of superoxide radical to hydrogen peroxide and molecular oxygen [19–21]. SODs are classified into four groups depending on their metal

cofactors: MnSOD [19], CuZnSOD [40], FeSOD [58] and NiSOD [31,59]. The CuZnSODs are typically found in the cytosol of eukaryotes, while FeSODs are mainly found in prokaryotes and chloroplasts, and MnSODs are found in prokaryotes and in mitochondria. There are, however, some exceptions to this simplified evolutionary scheme [23]. MnSOD from filamentous fungi [13–15,45] does not contain a typical mitochondrial signal peptide, characteristically rich in basic and hydrophilic amino acids [46]. FeSOD and MnSOD share a high degree of amino acid sequence homology [46], while CuZnSOD represents a distinct class. A subclass of SOD exists which is capable of utilizing either Mn or Fe as the catalytic metal ion, and it is referred to as combialistic SOD [56].

In 1993, Schrank et al. [51] described a FeSOD in M. anisopliae. The discovery of a eukaryotic FeSOD directly contradicted the prevailing scheme of SOD distribution, in which the iron type of SOD was found only in prokaryotes. Loss of infectivity has been observed in highly pathogenic strains of M. anisopliae; however, the implicated causes are still not clear. Fungal virulence is a function of many attributes and it is difficult to assess their individual contribution to host mortality [9]. One of the factors responsible for virulence variation may be the presence of superoxide dismutases (SODs) [51]. In addition to their essential role in protecting living organisms against the toxicity of free oxygen radicals generated by metabolism, SODs play a significant role in the pathogenicity of several organisms, including Salmonella typhimurium [16], Mycobacterium tuberculosis [57] and Cryptococcus neoformans [8,12,42]. The deletion of the SOD1 gene in C. neoformans resulted in defects in the expression of a number of virulence factors, i.e., laccase, urease and phospholipase. Recovery of virulence factor expression and restoration of virulence was achieved by sod1 mutant complementation [42]. Candida albicans cells lacking CuZnSOD showed increased susceptibility to macrophage attack and had attenuated virulence in mice [29].

A wide variety of fungi have been shown to contain at least two forms of SOD, CuZnSOD and MnSOD [10,28, 32,35,36,44]. Thirty-two complete deduced amino acid sequences have been reported in the SwissProt, EMBL and GenBank data libraries. These include sod1, encoding a major CuZnSOD in Neurospora crassa [7]; SOD2 genes encoding MnSOD from Penicillium chrysogenum [14], Aspergillus fumigatus[13], Ganoderma microsporum [45], and Colletotrichum graminicola [15]; and sod1 and sod2, encoding a CuZnSOD and an MnSOD, respectively, in Saccharomyces cerevisiae [5,6,37], and a gene encoding FeSOD from Rhodotorula glutinis [49]. Fungal parasites have the ability to secrete enzymes in order to protect them from a hostile chemical in the environment. SODs may also play a role in fungal adhesion to the host surface or in molecular signaling, since their

substrate (superoxide anions, O− 2) and product (hydrogen peroxide, H2O2) have been implicated in these processes in animal and plant cell systems [35,48,50].

The description of the purification of the Metarhizium SODs provides the framework for ascertaining the potential role of these enzymes in protection against externally generated superoxide.

2. Materials and methods

2.1. Organisms and culture conditions

M. anisopliae strain E6 was obtained from infected insects (Escola Superior de Agronomia Luiz de Queiroz, USP, Brazil, Microbial Genetics Group Collection). Mycelia were grown in liquid Cove's medium (MCc) [11] at an initial cell density of 106 spores ml−1 on a rotator-shaking platform (180 rpm) at 28 ◦C [47]. After 48 h of incubation, mycelia were harvested by filtration onto a Whatman 1 filter paper and washed twice with sterile distilled water.

2.2. Analytical procedures

SOD activity was determined by a riboflavin-nitroblue tetrazolium (NBT) assay adapted from Beauchamp and Fridovich [1]. The photosensitive assay reagent contained 2.6 μM riboflavin, 35 μM NBT, 14 mM N ,N ,N ,N -tetramethylethylethylenediamine (TEMED) in 0.05 M phosphate buffer, pH 7.8. The reaction mixture containing the photosensitive assay reagent (0.5 ml) and the appropriately diluted enzyme preparation (up to 0.5 ml) was made up to a total volume of 1 ml. The inhibition of the NBT reduction was measured at 650 nm and a liner calibration plot was adopted. One unit of SOD represents the amount of enzyme that inhibits 50% of the rate of auto-oxidation of NBT under the defined assay conditions.

2.3. Purification of CuZnSOD

All purification procedures were carried out at 4 ◦C. The mycelia biomass (700 g) obtained from a 48-h shaking culture was collected on a sieve (160 μM), washed thoroughly with deionized water, frozen by addition of liquid nitrogen, grounded to a fine powder and suspended in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.5) containing protease inhibitors (5 mM iodoacetamide, 50 μM Nα-p-tosylL-lysine chloromethyl ketone hydrochloride TLCK, 50 μM L-1-4 -tosylamino-2-phenylethylchloromethylketone TPCK, 1 mM phenylmethanesulfonyl chloride PMSF, 2 μg ml−1 leupeptin) [52]. The cell debris were removed by centrifugation (10

000 g, 15 min, 4 ◦C). The resulting supernatant was brought to 50% saturation by gradually adding solid (NH4)2SO4 and it was stirred for 2 h at 4 ◦C. The precipitate was removed by centrifugation (10 000 g, 15 min, 4 ◦C) and the supernatant was brought to 95% saturation with (NH4)2SO4. The precipitate was collected by centrifugation (10 000 g, 30 min, 4 ◦C), dissolved in 10 mM Tris–HCl/EDTA 1 mM, pH 7.5, and dialyzed against the same buffer for 24 h at 4 ◦C. These three fractions were denominated TE (total extract), P50% and P95%, respectively. The dialyzed P95% fraction was directly applied to a DEAE-Sephacel column (25 × 2.5 cm) equilibrated with 10 mM Tris–HCl pH 7.5. The column was washed thoroughly with 200 ml of equilibration buffer until all un- bound proteins were removed. The remaining bound proteins were eluted by a continuous linear gradient of 500 ml of the same buffer containing 0 to 1.2 M NaCl at a flow rate of 30 ml h−1. Fractions of 6 ml were collected and those containing SOD activity were pooled, concentrated by lyophilization and loaded onto a Sephadex G-150 column (100 × 2.5 cm) equilibrated with 50 mM Tris–HCl containing 0.1 M NaCl. Elution was continued with 200 ml of the same buffer at a flow rate of 12 ml h−1. Fractions of 6 ml were collected and analyzed. BSA (67 000 Da), ovalbumin (43 000 Da), chymotrypsinogen (25 000 Da) and ribonuclease A (13 700 Da) were used as molecular mass markers, and blue dextran was used for determining the void volume. During the purification steps, protein was determined spectrophotometrically at 280 nm or according to the Bradford method, by using bovine serum albumin (BSA) as standard [4].

2.4. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 15%) was according to Laemmli [33]. The purified enzyme was also analyzed by 2D gel electrophoresis [43] stained with silver nitrate [3].

For the detection of SOD activity in gels, proteins were separated on an 8.5% non-denaturing PAGE and stained using NBT, riboflavin and TEMED as previously described [17]. Potassium cyanide (KCN), an inhibitor of the CuZnSOD and not of the manganese and iron forms, and hydrogen peroxide (H2O2), an inhibitor of the CuZnSOD and FeSOD, were used at 10 mM to ascertain the SOD type [41]. Total extracts of E. coli XL1-blue containing MnSOD and FeSOD activity and purified CuZnSOD bovine (Sigma), and MnSOD and FeSOD from E. coli (Sigma), were used as controls in the activity gels.

2.5. Preparation of antisera and immunoblotting

Polyclonal antisera against purified SOD were raised in mice. Six-week-old BALB/c mice were injected intraperitoneally with 12.5 µg of purified SOD emulsified with Freund's complete adjuvant followed by booster injection of 12.5 µg in Freund's incomplete adjuvant after 25 days. Sera were obtained 8 days after the second injection and titrated by indirect ELISA. Proteins resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes. The blotted membranes were blocked with 5% (w/v) milk powder in PBS (Na2HPO4 10 mM, KH2PO4 1.7 mM, NaCl 137 mM, KCl 2.7 mM). After blocking, the membranes were incubated with anti-SOD antiserum in blocked solution (1:2000). The membranes were washed three times in PBS for 10 min, incubated with a secondary antibody (anti-mouse IgG peroxidase conjugate), washed and revealed according to the manufacturers.

2.6. Immunoelectron microscopy

Spores and mycelia of M. anisopliae harvested from Cove's complete solid medium (MCc) [11] were collected for immunogold localization, fixed following the protocol previously described and embedded in LR-gold [2]. The cured blocks were cut into ultrathin sections and mounted on copper grids, followed by immunogold labeling with 15 nm gold protein A for 1 h. Finally, specimens were stained with uranyl acetate for 20 min and Reynold's lead citrate for 5 min prior to examination in a transmission electron microscope JEOL 100 C.

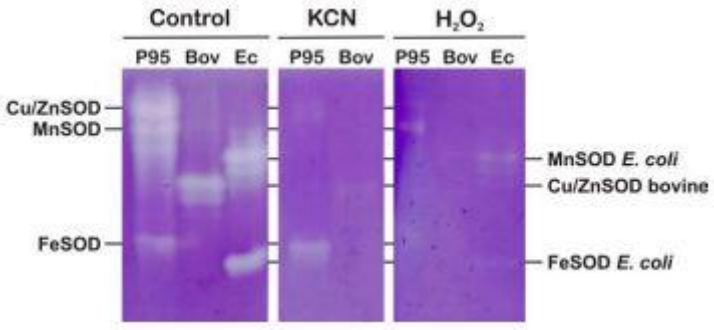

Fig. 1. Electrophoretic analysis of SODs from M. anisopliae. The non-denaturing gels were incubated with 10 mM KCN or 20 mM H2O2. (P95) P95 fraction (10 µl) containing 4.4 U of SOD; (Bov) Three units of purified bovine CuZnSOD (Sigma); (Ec) E. coli extract (10 µl) containing FeSOD and MnSOD activity (6 U).

3. Results and discussion

Three different SOD isozymes were resolved from total protein extracts of M. anisopliae in native PAGE and NBT staining and identified by their cyanide and H2O2 sensitivities (Fig. 1). The most active enzyme, the slowest moving band, was sensitive to both

inhibitors and was classi- fied as a CuZnSOD; the fastest moving band was resistant to cyanide and sensitive to H2O2 as characteristic of FeSOD; the intermediary moving band was the least prominent isozyme in a total protein extract when compared to CuZnSOD and FeSOD. This enzyme was resistant to both H2O2 and low concentrations of cyanide (10 mM) resembling the characteristics of MnSOD; however, the sensitivity to higher concentrations of cyanide suggests that a different structure or different metals may be present in the active site (Fig. 1). The presence of a cyanide-insensitive and H2O2-sensitive FeSOD was reported as being specifically and concomitantly expressed during differentiation of hypha/bud cell into chlamidospore in Fusarium oxysporum f. sp. Raphani [32]. These results suggest that active intermediates of oxygen and/or those detoxifying enzymes participate in the differentiation of the fungus upon response to starvation [32]. However, direct evidence confirming the presence of a FeSOD in F. oxysporum, was not provided [39]. Functional analysis of the SOD isozyme profile from C. graminicola identified two major SOD activities, representing FeSOD and MnSOD differentiated by their sensitivity to H2O2 [15]. More recently Raychaudhuri et al. (2003) reported for the first time the presence of cytosolic FeSOD in a yeast system, Rhodotorula glutinis, confirmed by atomic absorption spectroscopy and assay with different inhibitors. In silico analysis of the sequence data suggested that it was not a classical cytosolic SOD, as it showed similarity towards mitochondrial FeSOD/MnSOD [49].

Table 1
Purification steps of Metarhizium anisopliae SOD

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold) partial/total
TE	478	135 084.74	282.60	100/–	–
P50%	116	5021.23	43.29	3.72/–	
P95%	173	59 104.42	341.65	43.75/100	1.05/1.05
DEAE-Sephacel	5.32	9382.72	1763.67	6.95/15.87	5.18/5.44
Sephadex G-150	1.06	4069.23	3838.89	3.01/6.88	2.18/11.86

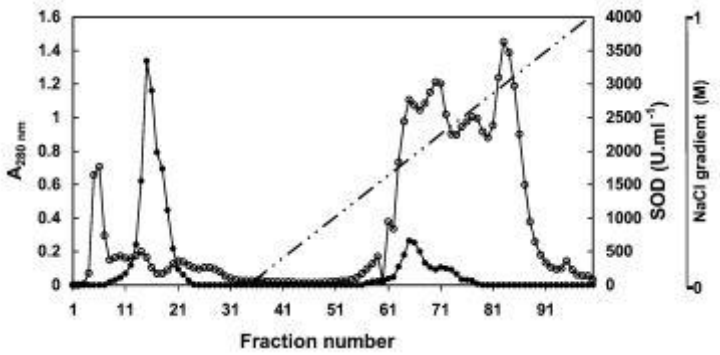

Fig. 2. Anion-exchange chromatography profile of M. anisopliae SOD on DEAE-Sephacel. Concentrated sample (173 mg of protein) was pumped into a column (25 × 2.5 cm) at a flow rate of 30 ml h−1. The column was washed with 200 ml 10 mM Tris–HCl, pH 7.5, and the proteins were eluted in a continuous

linear 0–1.0 M NaCl gradient (500 ml). Fractions of 6 ml were collected. (O) Protein absorption at 280 nm, (•) SOD activity and (- -) NaCl gradient

The isolation and purification of CuZnSOD was performed from mycelia of M. anisopliae. Although the enzyme has been purified from numerous sources, only a few reports exist concerning SOD purification from fungal sources [26–28,41], especially from entomopathogenic fungi. The purification to homogeneity of CuZnSOD from M. anisopliae involved four steps: homogenization of mycelia with removal of cell debris and dialysis; precipitation with (NH4)2SO4; ion exchange and gel filtration chromatography (Table 1). Fractional precipitation of the proteins was performed first at 50% saturation of (NH4)2SO4. The extensive protein removal during this precipitation step was an advantage since all three types of SOD remained present in the upper phase, increasing the specific activity 1.05-fold when they were further precipitated with a 95% saturation of (NH4)2SO4. The P95% fraction was submitted to DEAESephacel and three peaks with SOD activity were eluted (Fig. 2). The pooled fractions containing CuZnSOD were concentrated and further purified by gel filtration chromatography on Sephadex G-150. The enzyme was eluted with an estimated molecular mass of 30 690 Da and a specific ac tivity of 3838.89 U mg−1 (Table 1). SDS-PAGE (Fig. 3A), non-denaturing PAGE (Fig. 3B) and 2D-gels (Fig. 3C) show a single band of protein in the fractions eluted from the gel filtration column with an apparent molecular mass of 20 000 and ∼15 000 Da, respectively, and a pI of 6.0 (Figs. 3A and 3C). This value is approximately half of the molecular mass of the whole protein determined by gel filtration (30 690 Da) and indicates that the enzyme is composed of dimers of equal size, whose association depends upon noncovalent interactions. On the basis of inhibitory action of the known CuZnSOD inhibitor potassium cyanide [20] the newly described SOD is clearly a member of the CuZnSOD group. To date, most of the eukaryotic CuZnSOD have all been shown to be homodimers with each subunit being approximately 15 000 Da in size [21,25,30]. One exception is the CuZnSOD of Phanerochaete crysosporium, a homodimer with identical molecular mass of 22 000 Da [44]. For SOD from Aspergillus spp., a different pattern has been described in which the corresponding enzyme has revealed tetrameric or pentameric structures [26,28]. The pI 6.0 encountered for CuZnSOD purified from M. anisopliae was almost identical to that of the SODs from Aspergillus flavus and Aspergillus niger [26,28].

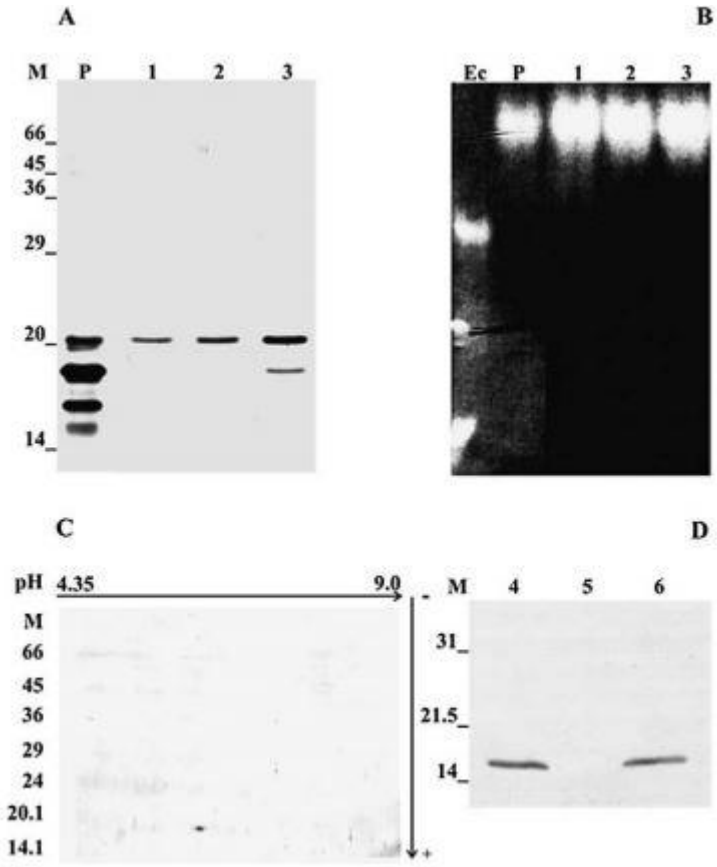

Fig. 3. Electrophoretic and immunoblot analysis of purified CuZnSOD from M. anisopliae. (A) SDS-PAGE (15%) stained with silver nitrate; (B) Non-denaturing PAGE stained with nitroblue tetrazolium; (C) 2D SDS-PAGE stained with silver nitrate; (D) Western blot. Lanes: (M) Mw markers (panels A and C); (P) Pool of fractions eluted from the first peak of the DEAE-Sephacel chromatography (panels A and B); (1–3), fractions 58, 59, and 60 (10 µl each), respectively eluted from the Sephadex G-150 chromatography, (panels A and B); (4) Proteins extracted from mycelium of M. anisopliae (30 µg); (5) Concentrated supernatant of M. anisopliae culture (30 µg), (6) CuZnSOD purified from M. anisopliae (10 µg) (panel D).

M. anisopliae SOD was inhibited by 2 mM KCN. This was not surprising, since CuZnSODs are generally known to be CN−-sensitive enzymes [30]. The activity of the purified SOD was susceptible to inactivation by hydrogen peroxide. The inactivation of CuZnSOD has been attributed to the reduction of the enzyme-bound Cu2+ to Cu1+ by H2O2, followed by a Fenton-type reaction of Cu2+ with additional H2O2 to form CU2+–OH. This could oxidatively attack an adjacent histidine residue of the enzyme [38].

The specificity of polyclonal antisera raised against puri- fied M. anisopliae CuZnSOD was verified by western blot. These antisera showed specificity for a protein with apparent molecular mass of 15 000 Da from mycelia extracts and not for proteins present in the culture supernatant, indicating that CuZnSOD in M. anisopliae might not be secreted in the investigated conditions. MnSOD and FeSOD did not share major antigenic determinants with CuZnSOD (Fig. 3D).

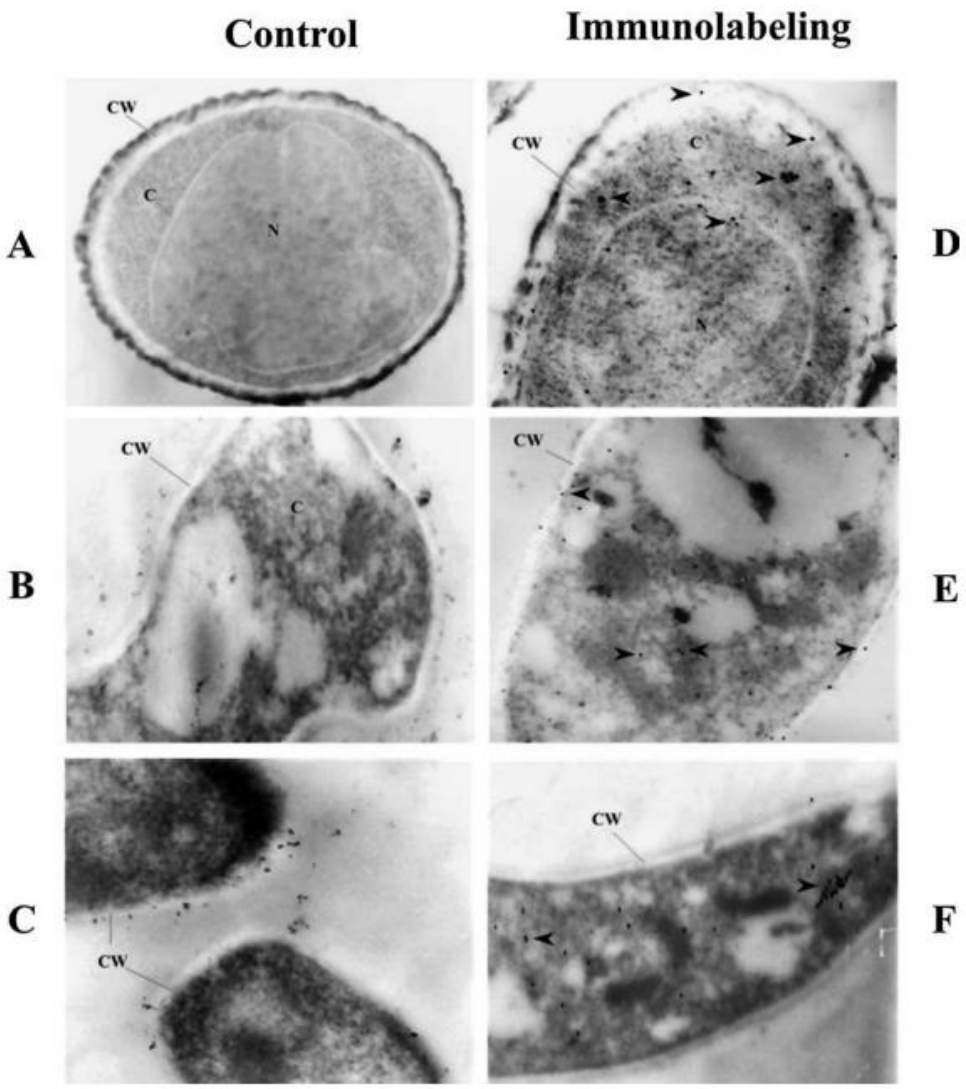

Fig. 4. Ultrastructural localization of CuZnSOD of M. anisopliae. (A) Spores; (B) mycelium from 24-h culture; (C) mycelium from 48-h culture. Lower case letters are: (c) cytoplasm; (cw) cell wall; (n) nucleus. Arrows heads show the depositions of gold particles.

Immunolocalization with CuZnSOD resulted in extensive gold labeling in the cell walls and peripheral cytoplasm of mycelia, while control sections incubated with pre-immune serum showed only a very low level of non-specific binding of gold particles (Fig. 4). Labeling of sections from 24- and 48-h-old mycelia with antiserum to CuZnSOD indicated that the enzyme accumulated within the cell walls. Different cells exhibited different labeling intensities (Figs. 4D, 4E, 4F). Intense labeling within and around the circumference of the fungal cell wall and, to a lesser extent, within the peripheral cytoplasm-cell membrane regions, was observed, suggesting a location for a rapid secretion. A. fumigatus CuZnSOD is present in substantial quantities in the cell wall and it is secreted into culture supernatants, making it available to exogenous sources of superoxide [24]. Usually, in eukaryotic cells CuZnSOD is found in the cytosol, where it fulfills a housekeeping function, by protecting the cell against endogenous

superoxide production. In prokaryotes like Caulobacter crescentus, SODs are located in different cellular compartments, CuZnSOD in the periplasm and FeSOD in the cytoplasm [54,55].

The search for thigmotropically controlled proteins identified a CuZnSOD that was posttranslationally modified upon appressorium induction in Uromyces appendiculatus, an important structure for host penetration [35].

The cell wall location of CuZnSOD in M. anisopliae may have important implications for further understanding its function. As an entomopathogenic fungus M. anisopliae lives in an ecological habitat that creates extracellular or extracytoplasmic oxidative stress against which a cytoplasmic SOD might not be effective. This CuZnSOD might be essential for tolerance to that oxidative stress, while the other cytoplasmic SODs produced by the fungus detoxify superoxide derived from cytoplasmic metabolism. Presumably, in filamentous fungi the membrane and the thick cell wall are impermeable to superoxide. If CuZnSOD functions as proposed, then the CuZnSOD form might more likely be found in fungi that are tolerant of extracellular oxidative stress, like the entomopathogenic ones

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