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ORIGINAL PAPER

Unusual location and characterization of Cu/Zn-containing superoxide dismutase from filamentous fungus *Humicola lutea*

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Abstract The present study aims to provide new information about the unusual location of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) in lower eukaryotes such as filamentous fungi. *Humicola lutea*, a high producer of SOD was used as a model system. Subcellular fractions [cytosol, mitochondrial matrix, and intermembrane space (IMS)] were isolated and tested for purity using activity measurements of typical marker enzymes. Evidence, based on electrophoretic mobility, sensitivity to KCN and H_2O_2 and immunoblot analysis supports the existence of Cu/Zn-SOD in mitochondrial IMS, and the Mn-SOD in the matrix. Enzyme activity is almost equally partitioned between both the compartments, thus suggesting that the intermembrane space could be one of the major sites of exposure to superoxide anion radicals. The mitochondrial Cu/Zn-SOD was purified and compared with the previously published cytosolic enzyme. They have identical molecular mass, cyanide- and H_2O_2-sensitivity, N-terminal amino acid sequence, glycosylation sites and carbohydrate composition. The *H. lutea* mitochondrial Cu/Zn-SOD is the first identified naturally glycosylated enzyme, isolated from IMS. These findings suggest that the same Cu/Zn-SOD exists in both the mitochondrial IMS and cytosol.

Keywords Cu/Zn-superoxide dismutase · Intracellular location · Mitochondrial intermembrane space · Enzyme glycosylation · Protein sequencing

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Introduction

Oxygen, as a substrate for energy generation, is essential for aerobic life style. But, the irony of aerobic life is that oxygen is also inherently destructive to all organisms needed by them. Its metabolism, through sequential one- to three-electron reduction of oxygen, may lead to the production of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide, and hydroxyl radical (OH·) (Fridovich 1995). When left uncontrolled, these can cause damage to DNA, lipids and proteins (Fridovich 1983; Halliwell 1994; Stadtman and Levine 2000). Mitochondria are a major endogenous source of ROS in eukaryotic cells and extremely susceptible sites towards oxidative damages. Even under normal physiological conditions, 1–2% of the oxygen consumed by mitochondria is converted into $O_2^{\cdot -}$ by the electron transport chain (Boveris 1977). Since superoxide anion radical is a charged molecule and does not diffuse early through the mitochondrial membrane, it has to be scavenged within its matrix.

The aerobic cells are able to cope with ROS toxicity by virtue of a unique set of antioxidant enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2, and prevent the formation of OH·. A first line of defense against ROS includes the superoxide dismutase (SOD) enzymes that catalyze dismutation of $O_2^{\cdot -}$ to H_2O_2 and oxygen (McCord and Fridovich 1969; Fridovich 1995). Eukaryotes possess two intracellular SODs. A copper- and zinc-containing enzyme represents 90% of the total SOD activity and is located primarily in the cytosol (Fridovich 1983). The other SOD is a manganese-containing enzyme located in the mitochondrial matrix. Mn-SOD is believed to represent the major means of protection against mitochondrial superoxide.

Although considered mainly a "cytosolic" enzyme, Cu/Zn-SOD has also been identified in peroxisomes, lysosomes, and nuclei (Chang et al. 1988; Keller et al. 1991). In plants, three subcellular locations for Cu/Zn-SOD isoforms, the cytosol, the plastid and the peroxisome, were determined (Sandalio and Del Rio 1987; Scandalios 1990; Baek and Skinner 2003; Chu et al. 2005). The Cu/Zn-SOD was reported to exist in mitochondrial intermembrane space (IMS) of insects (Hughes and Reynolds 2005) and mammalian cells (Weisinger and Fridovich 1973; Geller and Winge 1982; Inarrea et al. 2005). Furthermore, Sturtz et al. (2001) demonstrated that the mitochondrial Cu/Zn-SOD also protects the bakers' yeast mitochondrial IMS from oxidative damage. Recently, Kujumdzieva and colleagues confirmed mitochondrial location of this isoenzyme in yeasts of different genera: *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Candida*, and *Hansenula*, belonging to fermentative and respiratory subgroups (Nedeva et al. 2004). It is noteworthy that the subcellular localization of Cu/Zn-SOD is of critical importance in evaluating many aspects of intracellular metabolism and in understanding how cells are protected against intracellularly produced oxygen-based radicals (Crapo et al. 1992).

On the other hand, it has been reported that few organisms possess cytoplasmic Mn-containing SOD (Lamarre et al. 2001; Brouwer et al. 2003; Jin et al. 2005). Moreover, a novel extracellular Mn-superoxide dismutase (SOD) was isolated from a moss, *Barbula unguiculata* (Yamahara et al. 1999).

Compared to yeasts, little is known about the unusual location of SOD isoenzymes in cells of filamentous fungi. Mitochondrial Cu/Zn-SOD has been found in mitochondrial IMS of *Neurospora crassa* (Henry et al. 1980; Chary et al. 1990) and Mn-SOD as cytosolic enzyme in *Penicillium chrysogenum* (Diez et al. 1998). Cytosolic localization of Mn-SOD is suspected in many filamentous fungi among *Euascomycetes* (Frealle et al. 2006).

The aim of the present study was to obtain information about the unusual location of Cu/Zn-SOD in mitochondrial intermembrane space of filamentous fungi using *Humicola lutea* as a model system. In addition, the main properties of purified mitochondrial Cu/Zn-SOD were characterized and compared with those of Cu/Zn-SOD located in cytosol.

Materials and methods

Microorganism, cultivation, equipment and conditions

The fungal strain, *H. lutea* 103, from the Mycological Collection of the Institute of Microbiology, Sofia, was used throughout and maintained at 4°C on beer agar, pH 6.3. Composition of the seed and production media, bioreactors and culture conditions were as described previously (Dolashka-Angelova et al. 2004).

Cell-free extract preparation and isolation of cytosolic and mitochondrial fractionations

All steps during the isolation of mitochondria were performed at 0–4°C. Mycelial disruption and mitochondria isolation were performed according to Lambowitz (1979) with some modifications. Cells from 24 h cultivation were harvested using a filter paper-covered funnel (Whatman No 4 filter, Clifton, USA) with a sieve connected to a vacuum pump and washed repeatedly on the filter with distilled water and then once with isolation buffer (0.25 M sucrose, 5 mM EDTA, 0.15% bovine serum albumin, pH 7.5). For every 10 g of wet-weight hyphae, 15 g of quartz sand and 20 ml of the same buffer that contained 0.3 mM phenylmethylsulfonyl fluoride (PMSF) are added to the mortar and ground together for 1–2 min at 4°C. After addition of another 10 ml of the same buffer, the slurry was centrifuged three times at 1,500×*g* for 15 min to remove the quartz sand and undisrupted cells. The resulting supernatant was further centrifuged at 15,000×*g* for 30 min and the liquid fraction was used as the cell-free extract (CFE). Cytosol (Cyt) was obtained by centrifugation of the 15,000×*g* supernatant at 144,000×*g* for 60 min. The 15,000×*g* precipitate was resuspended in the same volume of isolation buffer and centrifuged for 40 min at 30,590×*g*. Then, the supernatant liquid was discarded and the crude mitochondrial pellet (CMP) was carefully washed with SEM buffer (0.25 M sucrose; 5 mM EDTA; 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS)/KOH, pH 7.5). This procedure was repeated three times and collected mitochondria were resuspended in deionised water and lysed by freezing and thawing. After treatment of intact mitochondria, centrifugation at 12,000×*g* for 20 min yielded the outer membranes and IMS (supernatant) and a pellet (mitoplasts) that was subjected to a second lysis by freezing and thawing. Both, mitoplasts and the 12,000×*g* supernatant were centrifuged at 144,000×*g* for 1 h, generating two sets of pellets

(the inner-membrane pellet from the mitoplasts and the outer-membrane pellet from the 12,000×*g* supernatant) and supernatants (the matrix fraction from the mitoplasts and the IMS fraction from the 12,000×*g* supernatant). All fractions were stored at −70°C until analyzed.

Analytical methods

The SOD (EC 1.15.1.1.) activity was measured by the nitroblue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units per mg protein [U/(mg protein)]. Cyanide (5 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/Zn-SOD and the cyanide-resistant Mn-SOD. The Cu/Zn-SOD activity was obtained as total activity minus the activity in presence of 5 mM cyanide.

The identity of isolated cell fractions was confirmed by the presence of corresponding marker enzymes. The cytosolic hexokinase (HK, EC 2.7.1.1.) and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49.) activities were determined according to Bergmeyer et al. (1983) and Bergmeyer and Moellering (1983), respectively. Succinate dehydrogenase (SDH, EC 1.3.99.1.), malate dehydrogenase (MDH, EC 1.1.1.37.) and isocitrate dehydrogenase (IDH, EC 1.1.1.41.) as mitochondrial matrix enzymes were measured using the methods of Veeger et al. (1969), Smith (1983), and Kornberg (1955), respectively. Sulfite oxidase (SO, EC 1.8.3.1.) as a marker enzyme from IMS was assayed as described by Cohen and Fridovich (1971). One unit equals 1 μmol of substrate reduced per min. Specific activity is given as U/mg protein.

Protein was estimated by the Lowry procedure (Lowry et al. 1951) using crystalline bovine albumin as standard. The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105°C.

Polyacrylamide gel electrophoresis (PAGE) and activity staining

Purity control of the enzyme and the molecular masses were performed on 10% polyacrylamide gels as described by Laemmli (1970), and the gels stained for protein detection were compared with duplicate gels stained for superoxide dismutase activity, as described by Misra and Fridovich (1977). The following molecular mass standards were used: (a) trypsinogen (24 kDa), (b) egg albumin (45 kDa), (c) bovine albumin (66 kDa) and (d) *Limulus polyphemus* hemocyanin (monomer, 70 kDa). To test inhibition, each subcellular fraction was preincubated with 5 mM cyanide and 10 mM H_2O_2 for 30 min and electrophoresed on 10% polyacrylamide gel prior to enzyme activity staining.

Western-blot analysis

Cu/Zn- and Mn-SOD antibody preparation and immunoblot analysis were performed according to Towbin et al. (1979) as described previously (Dolashka-Angelova et al. 2004). SOD antibodies were raised in white rabbits (2.5 kg and 6 weeks old) against the Cu/Zn-SOD and Mn-SOD from *H. lutea* 103, respectively. The primary injection in complete Freund's adjuvant (day 0) and the second and third in incomplete Freund's adjuvant (days 7 and 42) were given intramuscularly (1 mg of antigen for each injection per rabbit). Bleeding occurred at days 87 and 164 after immunization.

Proteins separated by 10% SDS-PAGE gels were transferred (18 h at 12 V at room temperature) to nitrocellulose membranes (0.45 mm, Millipore) with a semidry electroblotter (Jancos, Denmark). A volume of 30 mM Tris, 240 mM glycine, and 20% methanol (v/v) was used as transfer buffer. Immobilized proteins were stained with 0.2% Ponceau dye in 3% glacial acetic acid and destained with water. The non-specific remaining binding sites were blocked with phosphate-buffered saline (PBS; 80 g/l NaCl, 2 g/l KCl, 14.4 g/l $Na_2HPO_4{\cdot}7H_2O$, 2.4 g/l KH_2PO_4, pH 7.4) containing 3% bovine serum albumin and 0.05% Tween for 1 h at 20°C. The primary antibodies were used for the first test at a 1:400 dilution. Reacting proteins were visualized by staining with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:2,000) as secondary antibody.

Purification of Cu/Zn-SOD

The mitochondrial Cu/Zn-SOD was purified by the protocol used for cytosolic enzyme (Krumova et al. 2007). Further purification was achieved by a FPLC system, equipped with a 10/10 Mono Q anion exchange column. The column was previously equilibrated with 50 mM phosphate buffer, pH 7.8, and the elution effected employing stepwise increases in sodium chloride (0–0.1 M NaCl). Active fractions were further purified by a reverse phase HPLC Nucleosil 100 RP-18 column (250 × 10 mm; 7 lm; Macherey-Nagel, Germany) eluted with eluent A, (0.1% TFA in water), and eluent B, (80% acetonitrile in A), using as gradient program 0% B for 5 min and then 0–100% B for 60 min at a flow rate of 1.0 ml/min. The UV absorbance of the eluates was monitored at 280 nm. HPLC-separated protein fragments isolated from IMS fraction were used for analysis.

Mass spectrometric analysis

Mass spectra of the native SOD, obtained after HPL chromatography, was obtained by MALDI mass spectrometry (Voyager, PerSeptive Biosystems, Wiesbaden, Germany). The sample (10–50 pmol) was dissolved in 0.1% (v/v) TFA, dialyzed against water, and applied to the target. Analysis was carried using α-cyano-4-hydroxycinnamic acid as a matrix. Chicken egg ovalbumin (44,400 Da) and bovine serum albumin (66,430 Da) were used for mass scale calibration.

Amino acid sequence determination

Purified HPLC fractions were subjected to automated Edman degradation after dissolving in 40% methanol, 1% formic acid (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany).

Glycopeptide-staining on silica gel plates

The protein was analyzed for carbohydrates with the orcinol/H_2SO_4 test (Francois et al. 1962). Fractions obtained after HPL chromatography were collected manually, lyophilized, and subsequently dissolved in water. Then, 2–4 µl of these solutions were transferred to the plate, taking care to restrict the size of the spot to 2–3 mm in diameter, and air-dried. The plate was sprayed with orcinol/H_2SO_4 and heated for 20 min at 100°C.

Statistical evaluation of the results

The results obtained in this investigation were evaluated from at least three repeated experiments. The statistical comparison between different evaluations was determined by the Student's *t* test for mean interval estimation and one-away analysis of variance (ANOVA) followed by *F*-test, with a significance level of 95% ($\alpha = 0.05$).

Results

SOD activity and isoenzyme pattern as a function of the stage of culture development

Growth phase-dependent changes of SOD biosynthesis in the fungal strain *H. lutea* 103 were quantitatively monitored by both, spectrophotometric assay and gel electrophoresis. Figure 1a shows the results of analysis of SOD activity in CFE and CMP. The time profile of total SOD production describes a similar trend in both the cell homogenates. Two maxima of enzyme activity were determined. The SOD biosynthesis started, while the cells were still growing, but the first maximum occurred in the earlier stationary phase of growth. Maximum specific activities of 89 and 99 U/mg protein were obtained in CFE (60th hour) and CMP (48th hour), respectively. The second maximum was determined at 108th hour, when the nutrients had been depleted from the medium. The SOD activity was higher (approximately 120 and 132%) in comparison with the SOD activity at the first maximum for CFE and CMP, respectively.

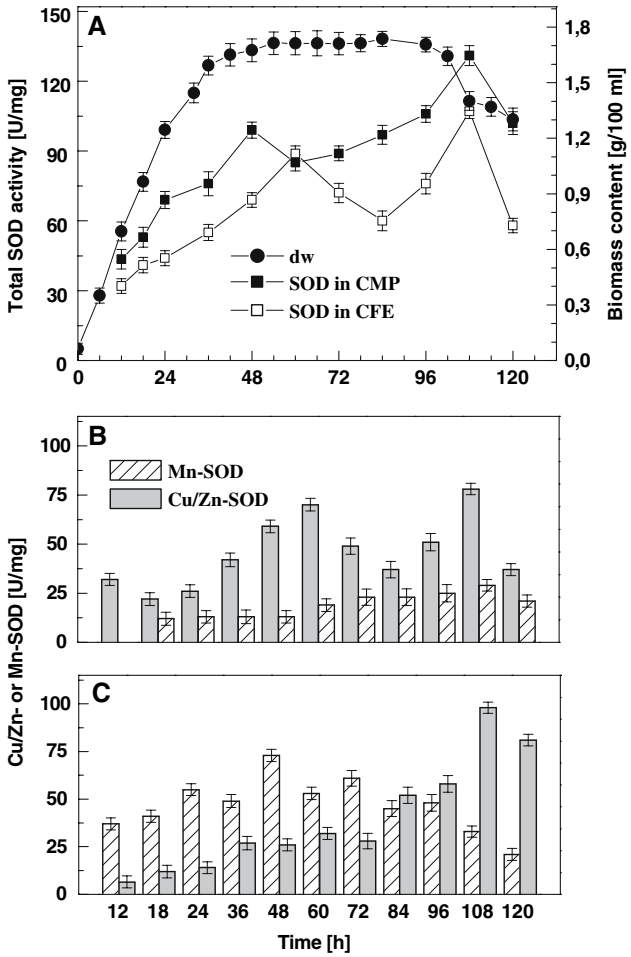

Fig. 1 **a** Time courses of mycelia growth (*filled circle*) and total SOD production in CFE (*open square*) and CMP (*filled square*); Isoenzyme profiles of SOD isolated from CFE (**b**) and CMP (**c**)

The fungal strain *H. lutea* 103 produces two SOD isoenzymes, Mn- and Cu/Zn-SOD (Fig. 1b, c). The Cu/Zn-SOD activity was obtained as a total activity minus the activity in presence of 5 mM cyanide. The isoenzyme profiles of SOD in CFE revealed the presence of both the isoenzymes, mainly Cu/Zn-SOD. Mn-SOD activity was in the range of 10–30% from the total content (Fig. 1b). In contrast, the most abundant SOD in CMP was Mn-SOD, while Cu/Zn-SOD formed a smaller portion of total SOD activity (Fig. 1c). But, in the period around the second peak of

activity (from 60 to 120 h), the ratio of Mn-SOD to Cu/Zn-SOD was changed progressively in favor of Cu/Zn-SOD (from 5.5:1 to 1:4).

The isoenzyme composition of SOD in CFE and CMP from *H. lutea* 103 was also determined using 10% polyacrylamide electrophoresis and SOD standards from bovine erythrocytes (Cu/Zn-SOD) and *E. coli* (Mn-SOD) (Fig. 2). The CFE and CMP fractions were isolated from the mycelium of 48 and 108 h-old growing cultures (1st and 2nd maxima). The isoenzymes were characterized using KCN to inhibit Cu/Zn-SOD whereas Mn-SOD is known to be resistant to this treatment (Fridovich 1982). The SOD isoenzyme profiles in the non-treated fractions (Fig. 2, panel B, lanes 3 and 5; panel C, lanes 7 and 9) revealed patterns similar to those of SOD standards (Fig. 2, panel A, lanes 1 and 2). We thus propose that CFE and CMP contain two isoforms, Cu/Zn-SOD and Mn-SOD. In all cyanide-treated fractions one sensitive and one non-sensitive isoenzyme were detected, confirming the presence of both isoenzymes (Fig. 2, panel B, lanes 4 and 6; panel C, lanes 8 and 10).

Purity of isolated cell fractions

To address the localization of fungal Cu/Zn-SOD, CFE and CMP were fractionated into cytosol, matrix and IMS (see "Materials and methods"). For fractionation experiments, fungal mycelia taken from 48 h-old cultures were used. Our previous results showed that the highest efficiency of SOD biosynthesis was achieved at the first maximum of activity for the *H. lutea* cultures (Angelova et al. 2001). Though the specific SOD activity was higher at the second maximum, the total enzyme activity (U/g biomass) was approximately 70% to that of the first maximum. This phenomenon could be explained by the higher levels of biomass and intracellular protein content at the first maximum compared to the second maximum.

The cytosol, matrix and IMS fractions were tested for purity using measurements of the activities of several enzymes, typically located in different cellular compartments: HK and G6PD in cytosol, SDH, MDH and IDH in mitochondrial matrix and SO in mitochondrial IMS. Table 1 illustrates the results of the marker evaluation. The

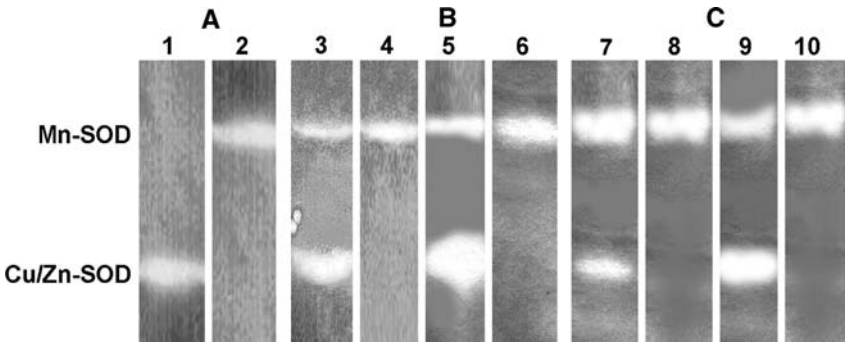

Fig. 2 Polyacrylamide gel electrophoresis of SOD from CFE and CMP fractions of *H. lutea* . **a** SOD standards: Cu/Zn-SOD from bovine erythrocytes (*lane 1*); Mn-SOD from *Escherichia coli* (*lane 2*); **b** SOD from CFE; **c** SOD from CMP; Protein samples (60 µg) from mycelia taken from first (*lanes 3*, *4*, *7*, and *8*) and second (*lanes 5*, *6*, *9* and *10*) maximum, respectively, were loaded on 10% non-denaturing gel and than stained for superoxide dismutase activity. Isoenzymes were differentiated based on their sensitivity to KCN. *Lanes 3*, *5*, *7*, and *9* no inhibitor; lanes *4*, *6*, *8*, and *10* with 5 mM KCN

Table 1 SOD and marker enzyme activities in cytosol and submitochondrial fractions of the fungal strains *H. lutea* 103

Specific enzyme activities [U/(mg protein)]	Cytosol fraction	Mitochondrial fractions	
		Matrix	IMS
Hexokinase	0.89 ± 0.032	ND	ND
Glucose-6 phosphate dehydrogenase	22.82 ± 1.54	ND	ND
Succinate dehydrogenase	ND	0.55 ± 0.016	ND
Malate dehydrogenase	ND	0.71 ± 0.004	ND
Citrate synthase	ND	0.52 ± 0.002	ND
Sulfite oxidase	ND	ND	1.46 ± 0.09
Cu/Zn-SOD	39.64 ± 1.32	ND	40.21 ± 2.56
Mn-SOD	ND	31.24 ± 2.03	ND

ND not detected

purity of submitochondrial fractions was demonstrated as enzymatic activities, characteristic for the cytosol, were not detected in it. The matrix fraction contained primarily the matrix markers SDH, MDH and IDH, while the intermembrane space contained abundant SO and was practically free of other markers.

The localization of SODs in cytosolic and submitochondrial fractions

The purified fractions (cytosol, matrix and IMS) were studied for their SOD-isoenzyme profile (Table 1). After inhibition with KCN, the soluble cytosolic fraction (144,000×*g* supernatant) contained abundant Cu/Zn-SOD and no detectable Mn-SOD. At the same time, when the outer membrane of the mitochondria was ruptured, Cu/Zn-SOD was released into the supernatant. Therefore, fungal Cu/Zn-SOD is located inside the mitochondria in the IMS fraction. By contrast, Mn-SOD was accumulated in the mitochondrial matrix only.

In the subsequent experiments, total proteins in purified fractions taken from first and second maxima were separated by non-denaturing PAGE and subsequently stained to detect SOD activity (Fig. 3a). The analysis revealed the existence of one slow migrating isoenzyme band (molecular mass of about 90 kDa) in mitochondrial matrix and one fast migrating isoform (molecular mass of about 36 kDa) in the cytosol and IMS. The intensities of these bands were very similar to each other, but they indicated clearly different SOD isoenzymes. The activity of the bands from the mitochondrial matrix was not affected by treatment with 5 mM KCN and 10 mM H_2O_2, indicating the absence of a Cu/Zn-SOD (Fig. 3b, lanes 4, 5, 6). However, the gel loaded with the protein from cytosol and IMS fractions showed complete inactivation of SOD activity by both the inhibitors (5 mM KCN and 10 mM H_2O_2) (Fig. 3b, lane 1 vs. lanes 2 and 3; and lane 7 vs. lanes 8 and 9), confirming the presence of Cu/Zn-SOD. Immunoblotting confirmed that the intermembrane space contained Cu/Zn-SOD, but not Mn-SOD, while the matrix contained Mn-SOD, but not Cu/Zn-SOD (Fig. 3c).

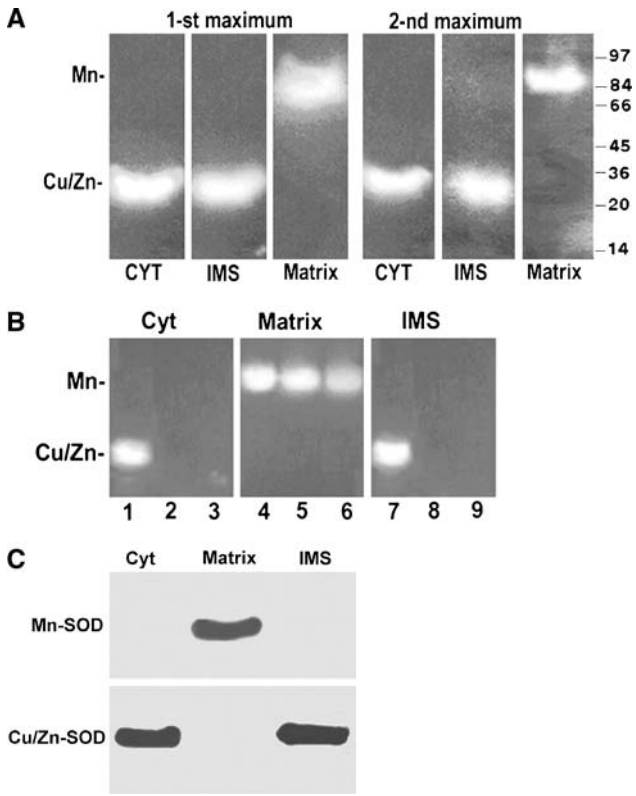

Fig. 3 Localization of Mn-SOD and Cu/Zn-SOD in purified cell fractions (cytosol, mitochondrial matrix and IMS). **a** Polyacrylamide gel electrophoresis (10% gel) of proteins from fungal culture grown up to *1-st* or *2-nd maximum*, stained for enzyme activity. Mn-SOD from *Escherichia coli* and Cu/Zn-SOD from bovine erythrocyte (data not shown) were used as standards . **b** Inhibitor studies of Mn- and Cu/Zn-SOD using 5 mM cyanide and 10 mM H_2O_2 (see "Materials and methods"). *Lanes 1*, *4* and *7*: untreated proteins; *lanes 2*, *5* and *8*: proteins, treated with 5 mM KCN; *lanes 3*, *6* and *9*: proteins treated with 10 mM H_2O_2. **c** Western blot analysis of extracts from identical cell fractions. The separated proteins were transferred to nitrocellulose membranes and probed with anti-*H. lutea* polyclonal antibodies. The mobilities and molecular masses (in kDa) of marker proteins are indicated on the *right*

Characterisation of mitochondrial Cu/Zn-SOD

Figure 4 presents the reverse phase HPLC isolation profile of the fractions, which were collected and subsequently analyzed by MALDI-MS and amino acid sequencing.

Two methods were used for molecular mass determination of SOD: MALDI-MS and PAGE. After purification of the native proteins on HPLC, the samples were subjected to MALDI-MS analysis. Molecular masses of fractions 1, 2 and 3 were determined to be about 8 and 12 kDa (data not shown). The mass spectrum obtained for fraction 4 is presented in Fig. 5a. The observed highest mass peak at 15,912 Da corresponds to monomeric SOD. Under the acidic pH of 0.1% (v/v) TFA, the homodimer SOD dissociated into monomers. The molecular mass deduced from 10% PAG electrophoresis, 32 kDa (data not shown), correlates to the native molecule and confirms that the enzyme is a dimer.

To investigate, if the mitochondrial Cu/Zn-SOD is glycosylated, the carbohydrate content of fractions collected after HPLC was estimated applying the orcinol/H_2SO_4 assay (Francois et al. 1962) (Fig. 5b). For comparison, the cytosolic Cu/Zn-SOD from *H. lutea*, which is known to contain oligosaccharides (Dolashka-Angelova et al. 2004), and non-glycosylated Cu/Zn-SOD from bovine erythrocytes were used. After adding orcinol/H_2SO_4 to the spotted cytosolic enzyme and fraction 4 (Fig. 5b, spots 4 and 5) the color was

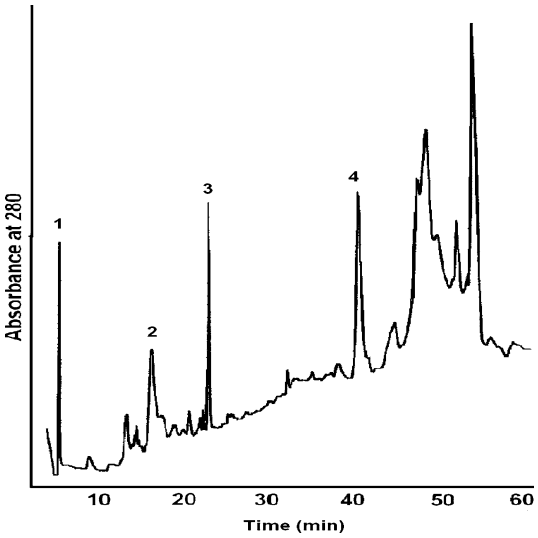

Fig. 4 HPLC profile of IMS fraction isolated from *H. lutea*. Chromatographic conditions: column (100 × 2.1 mm, Nucleosil RP C18); linear gradient elution [solvent A (0.1% TFA in water) and solvent B (0.085% TFA in acetonitrile)] for 60 min at a flow rate of 1 ml/min; detection UV at a wavelength of 280 nm

changed, providing initial information that the mitochondrial Cu/Zn-containing isoenzyme is also glycosylated, while the test was negative for bovine SOD (Fig. 5b, spot 6) and HPLC fractions 1, 2, and 3 (Fig. 5b, spots 1, 2, and 3).

The N-terminal sequence of the intact protein was determined. Two programs, LALIGN and Fasta, were used to analyze the alignment of N-terminal amino acid sequence of SOD, isolated from the mitochondrial IMS fraction, and the cytosolic Cu/Zn-SOD (Dolasha-Angelova et al. 2004), isolated from the same strain (Fig. 6). While the N-terminal sequences of fractions 1, 2, and 3 did not correlate to those of SODs (data not shown), fraction 4 demonstrated 100% identity with Cu/Zn-SOD, localized in the cytosol.

Taken together, our results show that fraction 4, isolated from IMS of *H. lutea* is a Cu/Zn-SOD. Table 2 represents a comparison between the biochemical and biophysical characteristics of our Cu/Zn-SOD (present study) and those described earlier for the Cu/Zn-SOD, isolated from the cytosolic fraction of the same strain (Dolashka-Angelova et al. 2004). Overall, the data revealed that both products were almost equal in their properties.

Discussion

The present data confirm our previous studies that *H. lutea* 103 is a high producer of SOD (Angelova et al. 2001; Dolashka-Angelova et al. 2004; Krumova et al. 2007). Time courses of total SOD biosynthesis in both, CFE and CMP show equal trends in which significant changes in SOD biosynthesis during different growth phases are observed. These changes in enzyme activity might reflect the various antioxidant needs during the development of fungal mycelium. The enzyme profiles included two production maxima. A similar phenomenon has been reported for *Candida maltosa* (Shilova et al. 1989) and *Cordyceps*

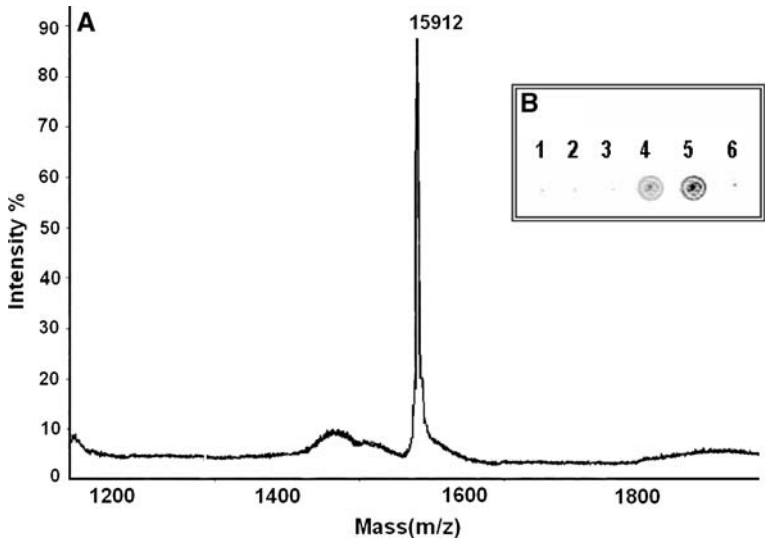

Fig. 5 **a** MALDI-MS spectrum of Cu/Zn superoxide dismutase isolated from IMS fraction. α-Cyano-4-hydroxycinnamic acid was used as a matrix. A total of 4,500 shots were acquired in the MS mode at a collision energy of 5,180. Solutions of human albumin (66,347.7 Da) and rabbit actin (43 kDa) were used to calibrate the mass scale. The mass values assigned to the amino acid residues are average masses. **b** Orcinol/sulphuric acid test of fractions eluted by HPLC (*spots 1*, *2*, *3*, and *4*), cytosolic Cu/Zn-SOD isolated from *H. lutea* (*spot 5*), and bovine Cu/Zn-SOD (*spot 6*)

	1				5				10				15				20				25				30				35			
IMS	V	K	A	V	A	V	L	R	G	D	S	K	I	T	G	T	V	T	F	E	Q	A	N	E	S	A	P	T	T	V	S	W	N	I	T	G	H	D
Cyt	V	K	A	V	A	V	L	R	G	D	S	K	I	T	G	T	V	T	F	E	Q	A	N	E	S	A	P	T	T	V	S	W	N	I	T	G	H	D

Fig. 6 Comparison of N-terminal amino acid sequences of Cu/Zn-SODs isolated from IMS fraction (*IMS*, present study) and cytosol fraction (*Cyt*, Dolashka-Angelova et al. 2004) of *H. luitea* mycelium

Table 2 Comparison of the main properties of cytCu/Zn-SOD and mitCu/Zn-SOD

Property	cytCu/Zn-SOD[a]	mitCu/Zn-SOD[b]
Structure	Homodimer	Homodimer
Molecular mass PAGE (Da)	32,000	32,000
Molecular mass MALDI (Da)	15,935	15,912
Glycosilation	Yes	Yes
Inhibitory test	KCN, H_2O_2	KCN, H_2O_2
Western immunoblot	Cu/Zn-SOD	Cu/Zn-SOD
N-terminal sequences	100%	100%

[a] Dolashka-Angelova et al. (2004)

[b] Present study

militaris (Wang et al. 2006). Secondary increase in SOD activity during the late stationary phase can be explained by an increase in the rate of ROS generation when the hyphae emerge from the newly-formed spores and they start to grow utilizing endogenous sources of carbon and nitrogen (Shilova et al. 1989). Moreover, our results demonstrate almost the same levels of total SOD activity in CMP and CFE with some superiority of the mitochondrial fraction. It is possible that SODs provide the same degree of protection for both the compartments. A similar relative proportion (1:1) was reported for fermentative and respiratory yeast strains of genera *Saccharomyces, Kluyveromyces, Pichia, Candida* and *Hansenula* (Nedeva et al. 2004).

The main findings from our study are the following. Firstly, our data represent new information concerning the intracellular localization of mitochondrial cyanide-sensitive SOD in a strain belonging to filamentous fungi. As is typical for eukaryotes, the fungus *H. lutea* possesses two different evolutionarily unrelated forms of the enzyme superoxide dismutase (Cu/Zn- and Mn-containing SOD) that, as scavengers of superoxide, appear to provide a front-line defense against superoxide-mediated toxicity. Fractionation of fungal cells into CFE and CMP showed that both the compartments contained a mix of Cu/Zn- and Mn-SOD. Unusual location of the SOD isoenzymes in CFE and CMP may be attributed to contamination with enzymes from the other compartments. Further fractionation of the CFE and CMP by ultracentrifugation yielded fractions that were, on the basis of marker enzyme activities, pure cytosol, mitochondrial matrix and IMS. Cytosol contained only Cu/Zn-SOD. Pure submitochondrial fractions demonstrated that the Cu/Zn-SOD was in the intermembrane space, while the Mn-SOD was in the matrix. Several lines of evidence support the existence of Cu/Zn-SOD in the purified IMS fraction of mitochondria, isolated from *H. lutea*: electrophoretic mobility, sensitivity to KCN and H_2O_2 and immunoblot analysis.

Based on the present study, Cu/Zn-SOD seems to protect both cytosolic and mitochondrial components from oxidative damage. Related results have been observed for yeasts (Chang and Kosman 1990; Slekar et al. 1996; Sturtz et al. 2001), plants (Hernandez et al. 1994), mammalian cells (Geller and Winge 1982; Okado-Matsumoto and Fridovich 2001; Inarrea et al. 2005), and filamentous fungus *Neurospora crassa* (Henry et al. 1980). According to Nedeva et al. (2004), the existence of Cu/Zn-SOD in yeast mitochondria can be assumed as a general phenomenon. Similar distribution of SOD isoenzymes may be due to: (1) existence of multiple sites of superoxide generation or (2) superoxide does not readily cross membranes (Okado-Matsumoto and Fridovich 2001). We suggest that superoxide, produced on the outer surface of the inner membrane and hence entering the intermembrane space will be scavenged thereby Cu/Zn-SOD, superoxide produced in the matrix space will be dealt by the matrix Mn-SOD (Okado-Matsumoto and Fridovich 2001).

We noted that the activity of mitochondrial superoxide dismutase is almost equally partitioned between the matrix and the intermembrane compartments space (Mn- and Cu/Zn-containing isoenzyme, respectively) (Fig. 3), thus suggesting that the intermembrane space could be one of the major sites of exposure to $O_2^{\cdot-}$ (Inarrea et al. 2005). Sturtz et al. (2001) provided direct biochemical evidence, that yeast cells lacking Cu/Zn-SOD exhibit increased carbonylation of mitochondrial proteins. The authors suggested that Cu/Zn-SOD in the mitochondria appears to be important for reactive oxygen physiology and the partitioning of this enzyme between the cytosol and mitochondria is greatly influenced by the cellular location of copper chaperone for superoxide dismutase (CCS) that donates copper to the Cu/Zn-SOD. Notably, lowering of CCS levels resulted in a dramatic loss of mitochondrial Cu/Zn-SOD, whereas cytosolic Cu/Zn-SOD was largely unaffected.

Secondly, our study provides information on the main properties of mitCu/Zn-SOD. This study is one of the very few reports dealing with the comparison of both cytosolic and IMS Cu/Zn-SODs, isolated from one and the same organism (Table 2). There are only data from experiments with rat liver cells (Okado-Matsumoto and Fridovich, 2001) and *Neurospora crassa* (Henry et al. 1980). Purified mitCu/Zn-SOD (present study) and cytCu/Zn-SOD (Dolashka-Angelova et al. 2004), isolated from the fungal strain *H. lutea* 103, were found to be homodimers with identical molecular mass, 15,912 and 15,935 Da (measured by MALDI), respectively. They have cyanide- and H_2O_2-sensitivity, typical for Cu/Zn-containing isoenzymes. PAGE and western blot analysis confirmed that both isoenzymes belong to the Cu/Zn-SOD family. Furthermore, 100% identity was found for a 38 amino acid overlap between the N-terminus of the cytosolic enzyme and the

mitCu/Zn-SOD. It is noteworthy that the glycosylation is a general characteristic of the enzymes from both the compartments. To our knowledge, the *H. lutea* mitochondrial Cu/Zn-SOD is the first identified naturally glycosylated enzyme, isolated from IMS. Until now, there is no other report describing a Cu/Zn-SOD from the IMS as glycoprotein. Naturally glycosylated cytosolic SODs are also isolated in very few cases only. According to Tibell et al. (1987), Edlund et al. (1992), and Watanabe et al. (2003) the secretory tetrameric extracellular SOD (EC-SOD) is the only glycosylated SOD besides our *H. lutea* enzyme, described so far.

Taken together, the present results reveal that the same Cu/Zn-SOD exists in both, the mitochondrial IMS and cytosol. Culotta and associates suggested, that the apoenzyme in yeast can cross the outer membrane and is trapped in the intermembrane space, when metallated by the copper chaperone of superoxide dismutase in that space (Field et al. 2003). According to Okado-Matsumoto and Fridovich (2001), the identity in properties of both forms of Cu/Zn-SOD suggests that they might be the product of one gene.

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