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PEROXISOMAL LOCALIZATION OF MN SOD ENZYME IN *SACCHAROMYCES CEREVISIAE* YEASTS: *IN SILICO* ANALYSIS

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ABSTRACT

Superoxide dismutase (SOD) enzymes were explored in three Saccharomyces cerevisiae strains during batch-wise growth on standard YPD medium. Yeasts were grown till late exponential, early stationary phase when cells were highly enriched in peroxisomes and mitochondria. Furthermore, activity of SOD enzyme was measured in isolated mitochondrial and peroxisomal fractions after Nycodenz fractionation. The type of SOD enzyme localized in peroxisomes was investigated through in silico analysis. Sod1p and Sod2p amino acid sequences revealed that Mn SOD of S. cerevisiae possesses putative PTS1 signal (GKI), which could successively targeted the protein into yeasts peroxisomes. Results indicated that in peroxisomes of Saccharomyces cerevisiae exist in situ mechanism for scavenging of ROS, which probably comprises a cooperative action of Mn SOD and catalase.

Keywords: *Saccharomyces cerevisiae*, peroxisomes, Mn SOD

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Introduction

Peroxisomes are subcellular respiratory organelles, which contain catalase and H_2O_2-producing flavin oxidases as basic enzymatic constituents (6, 9). The oxygen consumption by peroxisomes in various metabolic states was estimated to vary from 5 to 20% of the total cellular oxygen consumed (10). Although the generation of superoxide radicals ($O_2^{\cdot-}$) in yeast peroxisomes has not been demonstrated yet, the identification of cytochrome P-450 reductase (1), cytochrome b_5 reductase (20), as well as the detected elevated levels of SOD activity during growth of yeast strains in peroxisome inducing conditions (5, 21) suggested that peroxisomal metabolism was also associated with the production of $O_2^{\cdot-}$ toxic intermediates. As the generation of superoxide anions is crucial for the normal operation of a wide spectrum of biological processes (8, 16, 32), each cell possesses defense mechanism against such toxicity. In yeast cells, like in most eukaryotes, major role in scavenging the $O_2^{\cdot-}$ radicals, formed during metabolic processes, play the superoxide dismutases. These are a family of metalloenzymes that catalyze the disproportion of superoxide anions to hydrogen peroxide and oxygen. In *Saccharomyces cerevisiae* yeasts two types of SOD enzymes have been designated: Cu/Zn SOD (product of *SOD1* gene) located in cytosol and mitochondrial intemembrane space (12, 27, 34) and Mn SOD (product of *SOD2* gene) found in mitochondrial matrix (11, 17).

Although, available data exist about the potential generation of superoxide radicals within yeast peroxisomes, no mechanism is presently known for the detoxification of these reactive oxygen species (ROS). Two contrasting hypothesis on the role of SOD enzymes in degradation of ROS generated in yeast peroxisomes have been suggested: one is focused on the function of SOD in direct scavenging of superoxide radicals in these organelles, and the other one suggests that the enzyme could be involved in the later steps of detoxification of oxygen intermediates, which take place in cytosol (32). In this respect we have examined purified yeast peroxisomes for the presence of superoxide dismutase, and reported here biochemical and bioinformatic evidence for the possible key role of Mn SOD in maintaining the antioxidant/prooxidant balance in these organelles.

Materials and Methods

Microorganisms, growth conditions and plasmids

In this investigation three strains *Saccharomyces cerevisiae* were used from National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC): *S. cerevisiae* NBIMCC 582, *S. cerevisiae* NBIMCC 583 and *S. cerevisiae* NBIMCC 584. The strains were cultivated in standard liquid YPD medium (1% Yeast Extract, 1% Bacto-Peptone, 2% glucose) at 30^0C on a reciprocal shaker (204 rpm). As positive control for peroxisomal proliferation, the coding sequences for GFP was amplified from pET-24a(+)GFP (3), by using PCR primers that added a classical PTS (peroxisomal targeting sequence; Ser-Lys-Leu) to the C-terminus of the protein. The fluorescent protein GFP^{SKL} was cloned into the 2μ plasmid pDT-PGK and was constitutively expressed from the yeast *PGK1* promoter as described previously (29).

Cell-free extract preparation

Cells from 6, 12, 20, 30, 48 and 72 h of cultivation were harvested by centrifugation at 800 x g for 10 minutes and washed twice with distilled H_2O. Cell wall disruption was carried out by spheroplasting according to the procedure of Defontaine et al. (7). The cell debris was removed by centrifugation at 1000 g, 4^0C for 10 min and the cell free extracts were triply frozen

and thawed to break open organelles, and centrifuged at 15 000 g, 4^{0}C for 10 min. The supernatants obtained were used for enzymatic analyses.

Subcellular fractionation and Nycodenz gradients

For cell fractionation, yeast cells were grown 20 h to reach end of logarithmic phase. Spheroplasts were generated by the procedure of Defontaine et al. (7) and unlysed cells, nuclei and cell debris were removed by centrifugation at 1000 g, 4^{0}C for 10 min. The supernatant containing the crude yeast cell organelles was again centrifuged at 25 000 g, 4^{0}C for 20 min, and the crude organelle fraction was resuspended in a total volume of 5 mM Mes/KOH buffer (pH 6.0) containing 0.24 M sucrose (to provide osmotic protection for the peroxisomes) and 1 mM EDTA. Purity of such an organelle fraction was routinely determined by Western blot analysis and activity assays of marker enzymes, such as glucose-6-phosphate dehydrogenase, hexokinase, succinate dehydrogenase and isocitrate lyase, indicating that the organelle fraction was highly enriched for yeast mitochondria and peroxisomes (results not shown). For the separation of cell organelles, in particular for separating peroxisomes from mitochondria, the crude organelle fraction was layered on the top of a Nycodenz (ICN) step gradient consisting of 17%, 25% and 35% Nycodenz in the same buffer, essentially as described by Lee et al. (22). The Nycodenz gradient was centrifuged at 133 000 g, 4^{0}C for 90 min in a SW50Ti rotor (Beckman) and the gradient was subsequently fractionated from the bottom. Each fraction (1 ml) was diluted 5-fold with Mes/KOH buffer (5 mM, pH 6.0, containing 0.24 M sucrose and 1 mM EDTA), and the Nycodenz was removed by a final centrifugation step at 15 000 g, 4^{0}C for 20 min. For enzyme assays, gradient purified organelles were ruptured by the addition of Triton X-100 to a final concentration of 1%.

Biochemical analyses

Superoxide dismutase [EC: 1.15.1.1] activity was measured as described by Beauchamp and Fridovich (2). One unit was defined as the amount of enzyme causing 50% decrease in the reduction of Nitro Blue Tetrazolium (NBT).

D-amino acid oxidase [EC: 1.4.3.3] activity was measured, as described by Lichtenberg and Wellner (23).

Fumarase [EC: 4.2.1.2] activity was assayed according to the procedure of Kanarek and Hill (18).

Analysis of carbon source utilization

Glucose was determined by the method of Somogy (33) and Nelson (28).

Cell dry weight estimation

Cell dry weight was determined gravimetrically after drying washed cells to constant weight at 105^{0}C.

Protein determination

Protein content was determined by the method of Lowry et al. (24). Bovine serum albumin (Sigma St. Louis, MO, USA) was used as a standard.

Fluorescent microscopy

Yeast cells expressing GFP^{SKL} were harvested at different cultivation hours, washed twice with PBS buffer (pH 7.4) and subsequently examined for presence of peroxisomes using BX51 fluorescence microscope (Olympus) under standard GFP settings.

Visualization of yeast mitochondria was achieved through mitochondrial DNA staining with fluorescent dye 4',6-diamidino-2-phenylindole (DAPI). To 1 ml yeast cells with OD_{600}= 0.5 was added DAPI solution with final concentration of 1 µg/ml. Cells were incubated at room temperature for 10 min and were washed twice with distilled water. Fluorescence was detected on BX51 fluorescence microscope (Olympus) through a DAPI filter.

***In silico* analysis**

The protein sequences of *S. cerevisiae* Sod1p and Sod2p in FASTA format were retrieved from *Saccharomyces* Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces/) and the intracellular localization of both enzymes were analyzed through PSORT II Prediction software (http://psort.ims.u-tokyo.ac.jp/form2.html).

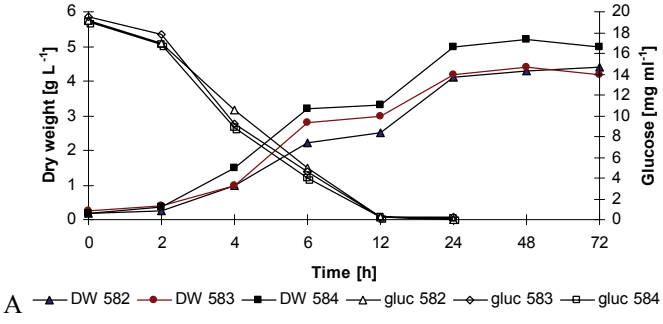
Results and Discussion

Cellular growth, proliferation of mitochondria and peroxisomes, and superoxide dismutase activity in *Saccharomyces cerevisiae*

The superoxide dismutase enzyme activity was investigated during batch-wise cultivation of three *Saccharomyces cerevisiae* wild type strains NBIMCC 582, NBIMCC 583, and NBIMCC 584. Cultivation of the cells was performed on standard YPD medium for 72 h and samples were withdrawn during different growth phases of the culture in order to determine cell dry weight and glucose consumption (**Fig. 1A**). From the growth curve it was evident that when yeast cells were inoculated into rich medium, containing high glucose concentration, after short lag period they were proliferating rapidly (38). After complete exhaustion of glucose (12th h of cultivation) a diauxic shift was observed due to the utilization of other carbon sources present in the medium (such as organic compounds from bactopeptone and yeast extract, and accumulated ethanol during fermentation) (14).

To assess the level of induction of peroxisomal proliferation during growth on YPD medium and to select the time-point for biomass collection, the wild type *Saccharomyces cerevisiae* strains were additionally transformed with pDT-GFP^{SKL} plasmid, where GFP was C-terminally extended with the natural preoxisomal targeting signal 1 - SKL (**Fig. 1B**). Furthermore, yeast cells collected from different hours of cultivation were stained with fluorescent dye DAPI and the cellular mitochondrial status was evaluated (**Fig. 1C**). As shown in **Fig.1 B** and **C**, it was evident that at 24th h an active growth took place and yeast cells were highly enriched in peroxisomes and mitochondria. These results are in correlation with the already published data about the role of glucose as

main regulator of peroxisomal synthesis and mitochondrial biogenesis via glucose catabolite repression (35, 36).



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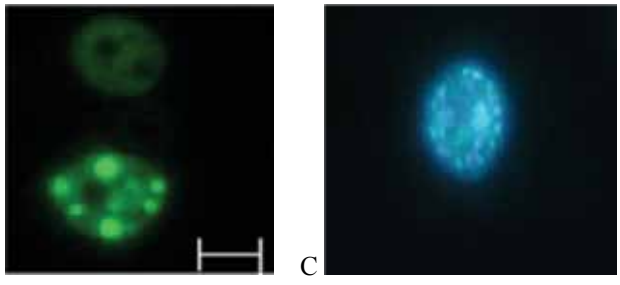

Fig. 1. Yeast cell growth and utilization of carbon source

Growth curves of the three *Saccharomyces cerevisiae* strains, expressing pDT-GFP^{SKL} plasmid, were followed up after their cultivation on standard YPD medium (A). The highest quantity of cellular peroxisomes (GFP fluorescence) (B), as well as mitochondria (DAPI fluorescence) was detected at 24th h of cultivation

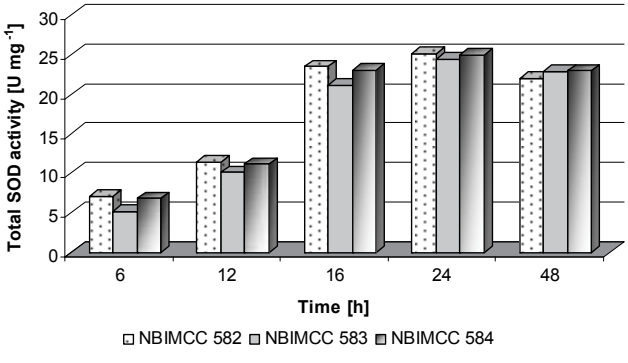

Fig. 2. Total SOD activity during growth on YPD standard medium

Cell-free extracts from different hours of cultivation were prepared and total SOD activity was measured in different phases of yeast cellular growth

Next, samples of biomass harvested at different time-points of cultivation, were processed and crude enzyme extracts were prepared. A spectrophotometrical analysis of the total SOD activity is shown in **Fig. 2**. At the 6th h of cultivation low SOD activity was detected due to the presence of glucose in the media and the weak respiration activity (26). The highest SOD activity was measured after 24th h of cultivation, which coincided, with the late exponential – early stationary growth phase of the cultures. This increase in SOD activity, after the exhaustion of glucose, is consistent with the available data for the induction of mRNA levels of SOD enzymes upon shift to a nonfermentable carbon source (13). These changes in gene expression were partly carbon source specific, because the activity of the electron transport chains in mitochondria and peroxisomes appears to be the major source of electrons that generate $O_2^{\cdot-}$. In this respect, the shift from growth on fermentable to a nonfermentable carbon source is a condition leading to increased levels of intracellular SOD activity. The comparative analysis between of SOD activities in the three *Saccharomyces cerevisiae* strains showed that this dependency is common for all studied strains.

Compartmentalization of SOD activity in *Saccharomyces cerevisiae* yeast cells

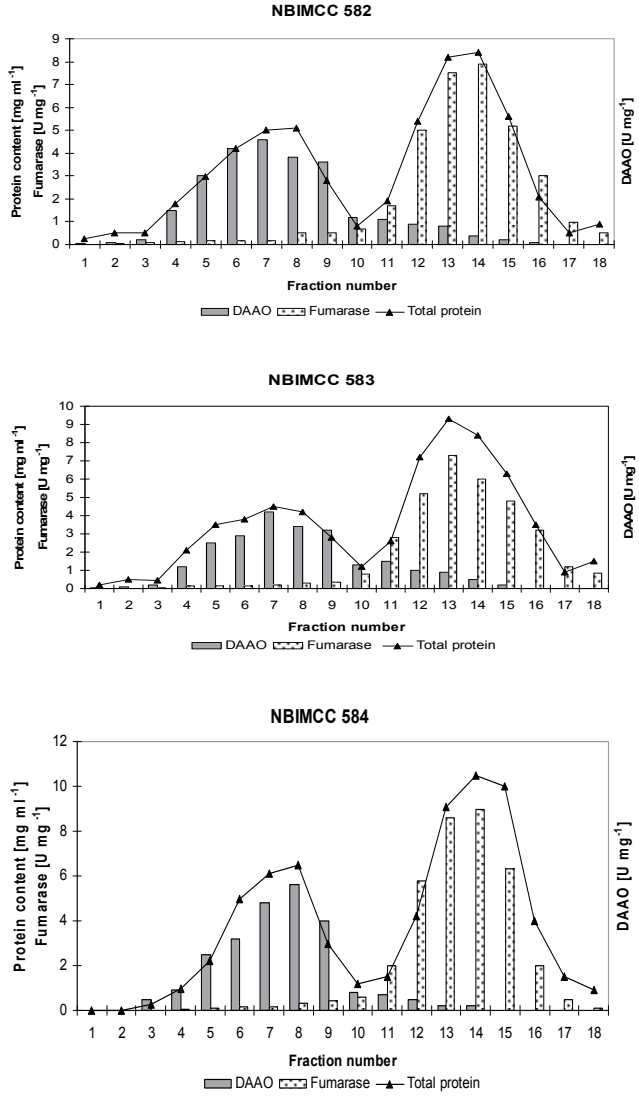

Fig. 3. Subcellular fractionation of *S. cerevisiae* peroxisomes and mitochondria

An organelle pellet was isolated from the three *S. cerevisiae* strains and was further purified and fractionated by Nycodenz equilibrium density gradient centrifugation. Fractions 1 and 18 represent the bottom and top fractions of the gradient. Activities of D-amino acid oxidase (D-AAO) and fumarase were determined as peroxisomal and mitochondrial marker enzymes, respectively. Protein concentration (▲) in each gradient was also determined

To obtain cell fractions with enhanced SOD activity an ultracentrifugation step of cell free extracts isolated at 24th h of

cultivation was carried out. The resulted subcellular fractions were purified by ultracentrifugation through a Nycodenz step gradient and subsequently analyzed for protein content, D-amino acid oxidase and fumarase activity. The results presented in **Fig. 3** clearly indicated that there were two peaks in protein distribution: in fraction 7 and fraction 13. As the maximum of D-amino acid oxidase activity was located in fraction 7 and the maximum of fumarase activity was detected in fraction 13, it could be concluded that these fractions were highly enriched in peroxisomes and mitochondria, respectively.

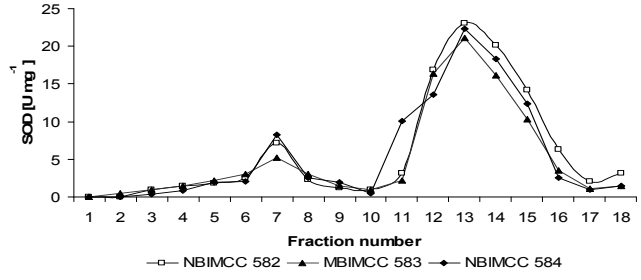

Fig. 4. Activity and distribution of peroxisomal and mitochondrial SOD activity during cell growth on YPD standard medium

Aliquots of each gradient fraction were spectrophotometrically analyzed for presence of superoxide dismutase (SOD) activity and specific enzyme activity was calculated

The measured SOD activity in isolated fractions (**Fig. 4**) indicated that significant part of the enzyme was located in mitochondrial fraction (21÷23U mg^{-1}), which is most probably due to the presence of matrix Mn SOD and intermembrane space Cu/Zn SOD one (12, 27). These data were in agreement with the already published results of Nedeva et al. (27), where the targeting of Mn and Cu/Zn superoxide dismutases in yeast mitochondria is shown to be a general phenomenon. Furthermore, reliable SOD activity (5÷8U mg^{-1}) was detected in fraction 7, which is highly enriched in cellular peroxisomes. In recent years the presence of different types of superoxide dismutases have been demonstrated in peroxisomes from several plant species (4, 8, 31), and more recently the occurrence of SOD has been extended to peroxisomes from human and transformed yeast cells (10, 19). Here, for the first time the presence of SOD activity was also demonstrated in peroxisomes of yeast *Saccharomyces cerevisiae*. As the generation of oxygen radicals in these organelles could have important effects on cellular metabolism, the identification of superoxide dismutase in peroxisomes suggested that this is an important mechanism for protection of yeast cells from free radical damage.

In silico analysis of amino acid sequence of SOD enzymes and subcellular distribution

In order to identify which of the two SOD enzymes (Cu/Zn or Mn SOD) was responsible for the observed superoxide dismutase activity in yeast peroxisomes, *in silico* analysis of their amino acid sequence was performed. The protein sequences of *S. cerevisiae* Sod1p and Sod2p were retrieved from *Saccharomyces* Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces/). Next, the data were analyzed through PSORT II Prediction software (http://psort.ims.u-tokyo.ac.jp/form2.html), which applies McGeoch's (25) and Heijne's (37) methods of signal sequence recognition. The obtained bioinformatic data were presented in **Table 1**.

When examined the amino acid sequence of Cu/Zn SOD enzyme in *S. cerevisiae* neither N-terminal nor C-terminal signal sequence was detected and the intracellular localization of this protein was predicted to be cytoplasmic with high probability of 65.2%. However, in the last decade it was already proven that yeast Cu/Zn SOD besides its typical cytoplasmic localization could be also targeted to the mitochondrial intermembrane space (27, 34). Sturtz et al. (34) have shown that a fraction of yeast Cu/Zn-superoxide dismutase (Sod1p), although lacking a typical mitochondrial import signal, could be directed to this organelle though co-localization with its copper chaperone CCS. However, no peroxisomal targeting signal (PTS1 or PTS2) was detected in the *SOD1* protein sequence.

TABLE 1

In silico analysis of amino acid sequences of Cu/Zn and Mn SOD enzymes in *S. cerevisiae* and deduction of their most probable intracellular localization. The typical peroxisomal targeting signal (PTS1) was indicated with continuous frame. Dashed frame enclosed the cleavage site for mitochondrial presequence

Protein name [gene]	Amino acid sequence					Possible localization
Cu/Zn SOD [SOD1]	MVQAVAVLKG FGDATNGCVS KDSLIKLIGP GLTN	DAGVSGVVKF AGPHFNPFKK TSVVGRSVVI	EQASESEPTT THGAPTDEVR HAGQDDLGKG	VSYEIAGNSP HVGDMGNVKT DTEESLKTGN	NAERGFHIHE DENGVAKGSF AGPRPACGVI	**Cytosol localization**
Mn SOD [SOD2]	MFAKTAAANL LHYTKHHQTY GGGFTNHCLF LAGVQGSGWA YLQYQNKKAD	TKKGGLSLLS VNGFNTAVDQ WENLAPESQG FIVKNLSNGG YFKAIWNVVN	TTARRTKVTL FQELSDLLAK GGEPPTGALA KLDVVQTYNQ WKEASRRFDA	PDLKWDFGAL EPSPANARKM KAIDEQFGSL DTVTGPLVPL GKI	EPYISGQINE IAIQQNIKFH DELIKLTNTK VAIDAWEHAY	**Mitochondrial and peroxisomal localization**

Furthermore, the amino acid sequence of Mn SOD enzyme (Sod2p) was analyzed and two possible subcellular localizations were identified – mitochondrial and peroxisomal. The *in silico* study of Sod2p revealed that at the N-terminal 20 residues of the protein there is a positively charged region and at 35 position a R-2 motif RRT|KV was found ("Gavel" cleavage site), which suggested the mitochondrial localization of the protein. These results coincided with already well known data about mitochondrial matrix localization of this enzyme and its key role in protecting these organelles from free radicals damage (11, 17, 27).

However, besides the well known target place of Mn SOD, a new signal sequence was also discovered – C-terminally located GKI sequence. It was already proven that in some yeast species like *Candida albicans* the alternation of PTS1 - AKI signal sequence into AQI or GKI could still successively target the proteins to cellular peroxisomes (30). Further divergence in permissible tripeptide sequences is also found in other yeasts like *Hansenula polymorpha* (15).

Based on the performed biochemical study, *in silico* data analysis and available literature data it could be concluded that in yeast *Saccharomyces cerevisiae* Mn SOD besides its typical subcellular localization – mitochondrial matrix, could be also successively targeted to yeast peroxisomes. In these organelles the enzyme most probably plays important role in protecting them from oxidative damages through its cooperative action with the catalase enzyme localized there.

Conclusions

The data obtained from our investigations allow us to propose a possible mechanism for detoxification of ROS in yeast peroxisomes. Mn SOD catalyzes the dismutation of $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$ to protect cellular peroxisomes from superoxide radical damage. The hydrogen peroxide (H_2O_2) in peroxisomes is then degraded by catalase (17) and the steady state levels of $O_2^{\cdot-}$ and H_2O_2 are maintained. In this way the production of highly reactive hydroxyl radical ($OH^{\cdot}$) is prevented.

Alongside our investigations, many different studies provided strong evidence that $O_2^{\cdot-}$anion generated during peroxisomal metabolic processes may lead to irreversible inhibition of catalase and β – oxidation enzymes (8). This on its turn may provoke in higher eukaryotic cells severe ischemia-reperfusion injuries (10). In this respect it is reasonable to suggest that future investigation with this model system will provide not only clear vision for the processes of oxidation in eukaryotic cell but will help for better understanding of the mechanisms of cellular aging and apoptosis.

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