Acta Biochimica Polonica

Vol. 52 No. 3/2005, 679–684

on-line at: www.actabp.pl

Review

Yeast as a biosensor for antioxidants: simple growth tests employing a *Saccharomyces cerevisiae* mutant defective in superoxide dismutase

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Received: 15 March, 2005; revised: 16 June, 2005; accepted: 06 July, 2005
available on-line: 04 August, 2005

Mutants of *Saccharomyces cerevisiae* devoid of Cu,Zn-superoxide dismutase are hypersensitive to a range of oxidants, hyperbaric oxygen and hyperosmotic media, show lysine and methionine auxotrophy when grown under the atmosphere of air and have a shortened replicative life span when compared to the wild-type strain. Ascorbate and other antioxidants can ameliorate these defects, which may be a basis of simple tests sensing the presence of antioxidants. In particular, tests of growth on solid medium (colony formation) in the absence of methionine and/or lysine, or in the presence of 0.8 M NaCl can be useful for detection and semiquantitative estimation of compounds of antioxidant properties. Hypoxic atmosphere was found to increase the sensitivity of detection of antioxidants. The test of abolishment of lysine auxotrophy showed a concentration dependence of the antioxidant effects of cysteine and *N*-acetylcysteine which, however, lost their protective action at high concentration, in contrast to glutathione which was effective also at higher concentrations.

Keywords: yeast, *Saccharomyces cerevisiae*, superoxide dismutase, antioxidants, ascorbate

For over two decades, we have been studying the effects of oxidative stress on the yeast *Saccharomyces cerevisiae*. We have isolated for the first time yeast mutants devoid of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (Bilinski *et al.,* 1985) and found that the mutants are hypersensitive to pure oxygen and to superoxide-generating agents, and show auxotrophy for lysine (Lys) and methionine (Met) when grown under air (Bilinski *et al.,* 1985; 1993; Chang *et al.,* 1991). We found subsequently that the lack of Cu,Zn-SOD leads to shortening of the replicative life span of the yeast while the lack of catalase is without effect (Wawryn *et al.,* 1999; Swiecilo *et al.,* 2000).

The increased sensitivity of Cu,Zn-SOD$^-$ mutants to oxidative stress prompted us to use this strain to get insight into the protective effects of antioxidants on cellular functions. *Saccharomyces cerevisiae* is an interesting model for studies of antioxidants. Living in oxidant-rich medium under natural conditions, it may be expected to require exogenous antioxidants. Being unable to synthesize polyunsaturated fatty acids, it apparently does not require protection by hydrophobic antioxidants, like tocopherols, against lipid peroxidation. It produces erythroascorbate instead of ascorbate, both compounds performing apparently identical functions.

The yeast seems to be a potentially useful eukaryotic model for studies of the effects of antioxidants at the cellular level, an attractive alternative to mammalian cell lines. Such studies can not only verify the results of test-tube experiments on the protection of macromolecules from the action of oxidants but also reveal possible side effects of antioxidants and products of their metabolism. Experimentation with yeast is easy, cheap and avoids ethical questions inherent to the use of animals. Yeast is a standard object of genetic manipulation and expression of proteins (including antioxidant proteins). Recently, the yeast *Saccharomyces cerevisiae*

*Presented at the International Review Conference on Biotechnology, Vienna, Austria, November 2004.
Abbreviation: SOD, superoxide dismutase; YNB, yeast nitrogen base medium.

has become a convenient model system for the studies of aging, since its replicative life span is limited and completed within 2–3 days.

The aim of this study was to examine the possibilities of use of the yeast for detection of antioxidants and to compare the potential usefulness of several tests employing *S. cerevisiae*. Since the mutant lacking Cu,Zn-SOD is much more sensitive to oxidative stress than the isogenic wild-type strain (Bilinski *et al.*, 1985), we decided to use this mutant as a tester.

MATERIAL AND METHODS

Yeast strains. Wild-type SP-4 strain (*MATα leu1 arg4*) (Bilinski *et al.*, 1978), the DSCD1-1C mutant devoid of Cu,Zn-SOD (*MATa leu1 arg4 sod1*), and a MS-2 disruptant (Koziol *et al.*, 2005), isogenic with SP-4, were used.

Media and growth conditions. The cells were grown in minimal YNB medium (Difco), supplied with necessary additions in ribbed Erlenmeyer flasks at 50 ml of culture per 300 ml vessel on a rotary shaker (150 r.p.m.) at 28°C.

In assays on Petri dishes, the cells were inoculated on YNB medium (with appropriate additives) containing 2% agar. Antioxidants were added to the agar medium cooled down to about 50°C just before pouring of the plates. Atmosphere of lowered oxygen pressure was provided by placing plates in a desiccator flushed with nitrogen containing appropriate content (1%, 3% or 7%) of oxygen (Linde Gaz, Kraków, Poland).

Colony growth was monitored after 48 h. In order to quantify cell growth on solid medium, the number of inoculated cells was kept constant (four 50-μl drops of a suspension containing 10^6 cells/ml were applied for each experimental point). The obtained colonies were washed off with water, brought to 5 ml and optical density of the suspension was determined at 700 nm.

The results represent typical experiments, which were repeated at least thrice yielding reproducible effects, unless stated otherwise.

Determination of replicative life span. Life span of individual yeast cells was determined by a routine procedure on cells placed on agar plates. Briefly, a fresh bud was isolated by micromanipulation and formation of successive buds by such a virgin cell was monitored, each appearing bud being removed using a Narishige MO-202 hydraulic micromanipulator. The number of buds formed by each cell is referred to as its replicative life span. In contrast to the original protocol (Kim *et al.*, 1999), the plates were not placed in a refrigerator overnight, to avoid the stress connected with cooling and warming.

RESULTS AND DISCUSSION

Test based on the estimation of life span

We found that life span shortening evoked by the disruption of Cu,Zn-SOD was counteracted by the addition of ascorbate to the growth medium of the yeast. Relatively high concentrations (10–80 mM) of ascorbate were necessary to obtain a protective effect which, however, concerned only the mean, and not the maximal life span. This limited efficiency of ascorbate was seemingly due to its interaction with the complex complete medium used for culturing yeast cells, containing glucose (2%), bactopeptone (1%) and yeast extract (1%). Ascorbate undergoes autoxidation in this medium; periodical exchange of the medium with ascorbate during the experiment led to extension of not only mean but also maximal life span of the Cu,Zn-SOD$^-$ mutant. Ascorbate had no significant replicative-life-prolonging effect in the wild-type strain, so its effect on the mutant must have represented a partial compensation for the defect caused by the deficiency of an important antioxidant enzyme (Krzepilko *et al.*, 2004).

Test based on abolishment of auxotrophy

The effect on the life span of yeast mutants deficient in antioxidant enzymes seems to be a promising method for model studies of antioxidants; however, such studies are time-consuming, require a micromanipulator and a skilled experimenter and would be impractical as a cellular screening test for antioxidant activity. A much simpler method can be based on examination of the protection by antioxidants of the growth ability of oxidatively stressed yeast, by measurements of turbidity of liquid cultures of the yeast or inspection of growth of yeast colonies on a solid medium.

Using this approach we found that ascorbate and a range of other antioxidants were able to protect Cu,Zn-SOD$^-$ yeast against the action of such oxidant agents as hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide and menadione (Lewinska *et al.*, 2004). However, testing the interactions of antioxidants with oxidizing chemicals is prone to experimental artifacts and difficulties in interpretation, concerning, i.e., interaction of oxidants with the components of culture medium and direct oxidant–antioxidant reactions.

Cu,Zn-SOD$^-$ yeast, unlike the wild-type strain, are killed by exposure to 100% oxygen. Ascorbate was found to protect the mutant against the toxicity of pure oxygen in a dose-dependent manner (Krzepilko *et al.*, 2004). This test could be of potential value for testing of other antioxidants; however, it represents an unphysiological situation of exposure of the cells to hyperbaric oxygen.

In our opinion, the most promising method for testing the cellular action of antioxidants using the Cu,Zn-SOD$^-$ yeast may be based on the abolishment of their auxotrophy for lysine and methionine. This phenomenon can be easily observed in Cu,Zn-SOD$^-$ yeast grown on a minimal medium (Yeast Nitrogen Base with necessary supplements) and does not occur in wild-type yeast. The reasons for the Met and Lys auxotrophy of Cu,Zn-SOD-deficient yeast are not completely clear. In *Escherichia coli*, lack of SOD leads to oxygen-dependent auxotrophies for branched-chain, sulfur and aromatic amino acids. The auxotrophy for branched-chain amino acids is due to oxidative inactivation of dihydroxy acid dehydratase, an enzyme catalyzing the penultimate step in the biosynthetic pathway, by superoxide (Kuo *et al.,* 1987; Benov & Fridovich, 1999; Benov, 2001); that for sulfur-containing amino acids is a result of leakage of sulfite from cells (Benov *et al.,* 1996), and that for aromatic amino acids is due to oxidation by superoxide of 1,2-dihydroxyethyl thiamine pyrophosphate, an intermediate of transketolase, leading to inactivation of the enzyme (Benov & Fridovich, 1999). In *S. cerevisiae*, the auxotrophy for methionine has been attributed to the oxygen-dependent toxicity of sulfite, an intermediate in the reductive assimilation of sulfate (Bilinski & Litwinska, 1987; Chang & Kosman, 1990; Chang *et al.,* 1991), perhaps *via* inhibition of glyceraldehyde 3-phosphate dehydrogenase (Schimz, 1980; Hinze & Holzer, 1986). The lysine auxotrophy has been ascribed to superoxide-dependent damage to α-amino adipate transaminase (although it is not a Fe-S cluster protein) or its regulator (Liu *et al.,* 1992). Recently, the lysine auxotrophy has been suggested to be a result of oxidative inactivation of another enzyme of the biosynthetic pathway of this amino acid, homoaconitase (Lys4p), a 4Fe-4S enzyme (Wallace *et al.,* 2004). However, the enzyme is located in the mitochondrial matrix; one could therefore expect occurrence of lysine auxotrophy in mutants lacking the mitochondrially located Mn-SOD rather than in mutants lacking Cu,Zn-SOD which is located in the cytosol. However, no lysine auxotrophy could be detected in disruptants lacking Mn-SOD (unpublished).

Irrespective of its exact mechanism, the auxotrophy for lysine and methionine is a feature which can be observed under normal air atmosphere and is due to oxidative stress since it is ameliorated by hypoxic atmosphere. Growth (colony formation) in the presence of lysine, with no methionine (i.e., methionine prototrophy) could be partly restored under the atmosphere of 5% oxygen, growth in the presence of methionine but in the absence of lysine (i.e., lysine prototrophy) under the atmosphere of 1% oxygen, while growth of the Cu,Zn-SOD$^-$ mutant in the absence of both lysine and methionine was possible only under completely anoxic atmosphere (Zyracka *et al.,* 2005).

Addition of ascorbate to the growth medium also abolished the lysine and methionine auxotrophy of the Cu,Zn-SOD$^-$ mutant, in a dose-dependent manner. Like in the case of hypoxic atmosphere, methionine prototrophy was easier to achieve: partial restoration of growth in the presence of lysine but with no methionine was observed for 3 mM ascorbate while complete growth was obtained for 7 mM ascorbate. On media supplemented with methionine but not lysine, partial and complete restoration of growth was seen for 10 and 20 mM ascorbate, respectively.

The similarity of action of hypoxic atmosphere and ascorbate in the abolishment of methionine and lysine auxotrophy of Cu,Zn-SOD$^-$ yeast might lead to a suspicion that this phenomenon is due to a trivial effect of depletion of oxygen in the growth medium by ascorbate and creation of hypoxic conditions. In order to eliminate such a possibility, we examined the growth of yeast inoculated sparsely on solid medium where accessibility of oxygen could not be a problem. Under the conditions of growth on a solid medium, only up to 20% of the cell surface is in contact with the medium while majority of the surface is exposed to air. The Petri dishes with solid medium and inoculated yeast were incubated for 48 h in a sterile chamber and growth of colonies was inspected (Fig. 1). There was a clear-cut effect of ascorbate rescuing the growth ability of the yeast, precluding the effect of consumption of oxygen in the medium by ascorbate.

The growth test on a solid medium could be useful for the detection and quantification of ascorbate. Its main advantage is the low cost, however, its sensitivity is far from striking. We found that lowering the oxygen content in the atmosphere makes the test more sensitive. Partial restoration of growth in the presence of methionine was observed for 9 mM ascorbate under the atmosphere of air (21% oxygen), 6 mM ascorbate under 7% oxygen, and 3 mM ascorbate under 3% oxygen (Zyracka *et al.,* 2005). However, working with hypoxic atmosphere is troublesome.

Other antioxidants (glutatione, cysteine and *N*-acetylcysteine) were also able to restore methionine and lysine prototrophy in a concentration-dependent manner. Hypoxic atmosphere makes the test more sensitive to antioxidants, making possible the detection of lower concentrations of the antioxidants (Figs. 2–4). Interestingly, higher concentrations of cysteine and *N*-acetylcysteine proved less effective in restoring the prototrophy of the Cu,Zn-SOD$^-$ mutant, due to adverse effects of an excess of these antioxidants. This result may support the view that glutathione has been evolutionarily selected as the main thiol antioxidant over cysteine due to the

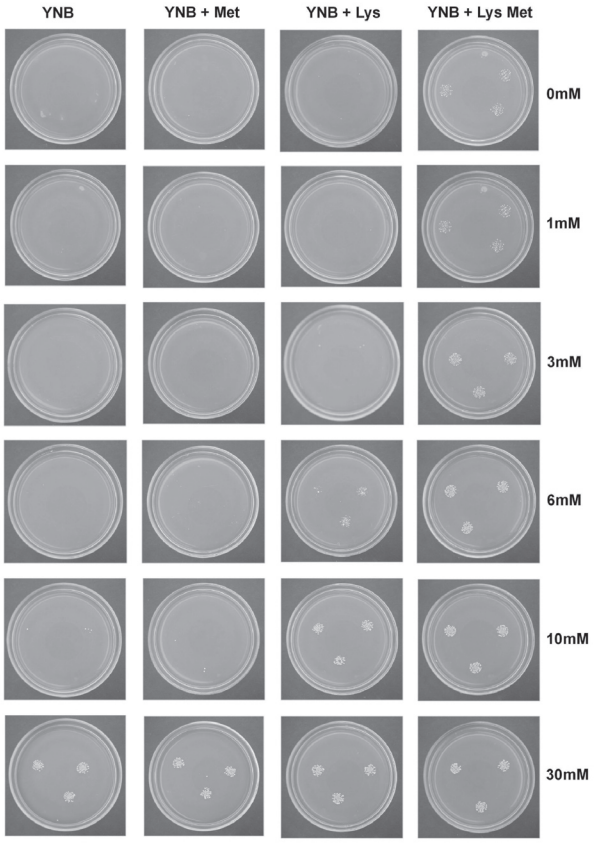

Figure 1. Effect of ascorbate on the Lys and Met auxotrophy of Cu,Zn-SOD⁻ strain (1C) of *S. cerevisiae*.
Yeast was inoculated sparsely to minimize cell to cell contacts on open agar plates in a sterile chamber on YNB (Difco) medium supplemented with either Lys (to study Met auxotrophy) or Met (to study Lys auxotrophy). The plates were photographed after 48 h. Heterogeneity of the colonies is due to sparse seeding (each subcolony originates from a single cell).

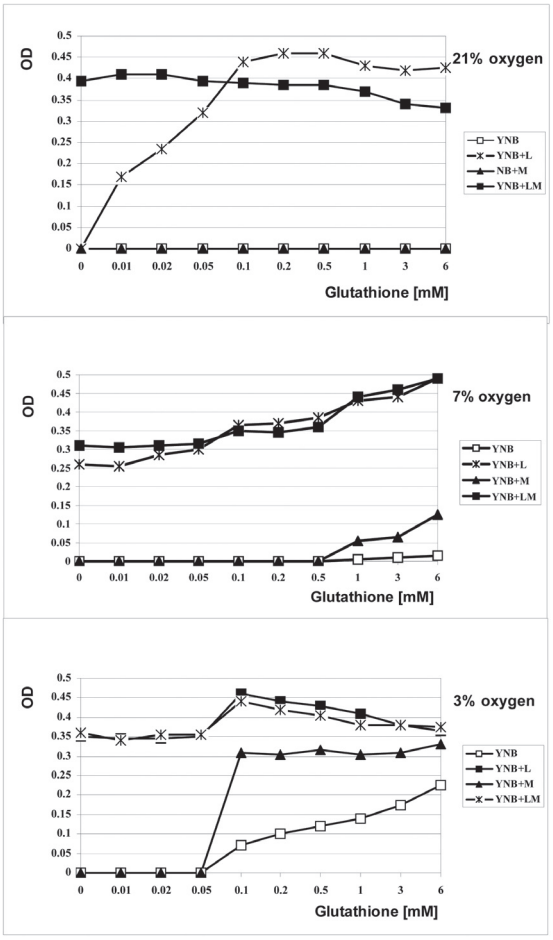

Figure 2. Effect of glutathione on the lysine and methionine auxotrophy of the Cu,Zn-SOD⁻ strain (1C) of *S. cerevisiae*.
The yeast was grown on solid YNB medium without Lys and Met, and on medium supplemented with Lys or/and Met under atmosphere of various partial oxygen pressures for 48 h. Then the colonies were suspended in 5 ml of water and optical density of the suspensions was determined at 700 nm. Results represent mean ± S.D. from 4 independent experiments. Note: the scaling of the concentration axis is not linear.

lack of toxic effects even at relatively high concentrations.

TEST BASED ON GROWTH IN HYPERTONIC MEDIUM

Another similar test can be based on the ability of antioxidants to restore the growth of yeast in a hypertonic medium. The osmotic stress imposed by high-osmolarity medium (e.g., one containing 0.8 M NaCl) hampers the growth of yeast cells (Garay-Arroyo *et al.*, 2003). This effect is mediated by oxidative stress since the Cu,Zn-SOD⁻ mutant is oversensitive to hypertonic medium, compared to wild-type yeast (Garay-Arroyo *et al.*, 2003), increased formation of reactive oxygen species is observed in yeast exposed to hypertonic media, and anoxic atmosphere and antioxidants alleviate the growth inhibition (Koziol *et al.*, 2005). Also in this test, ascorbate, glutathione and a range of antioxidants were protective while the effects of some oxidants (e.g., nitroxides) were limited to a concentration window (Koziol *et al.*, 2005).

Therefore, a simple plate test based on the abolishment of leucine and methionine auxotrophy of SOD-deficient *S. cerevisiae* (strain available on request) or restoration of growth on hypertonic medium can be useful for the detection and semi-quantitative estimation of concentrations of antioxidants. Obviously, the test based on restoration of amino-acid prototrophy would not be suitable for analysis of complex biological material which may contain lysine and/or methionine. Reversion of the mutant can also be expected under the influence of mutagenic compounds which may be present in the material tested. The test of growth on a hypertonic medium is free from these limitations (Table 1). Both tests can also be performed in liquid medium

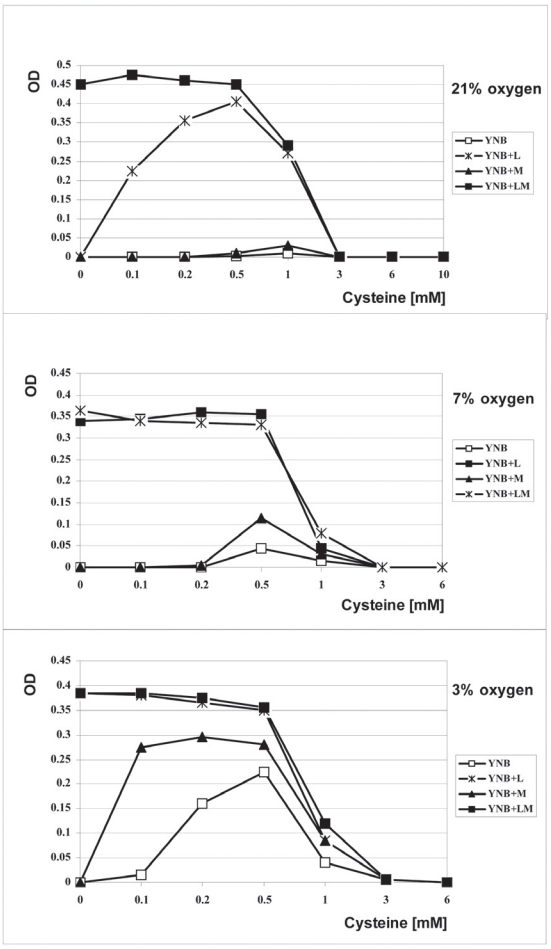

Figure 3. Effect of cysteine on the lysine and methionine auxotrophy of the Cu,Zn-SOD⁻ strain (1C) of *S. cerevisiae*.
Conditions as in the legend to Fig. 2.

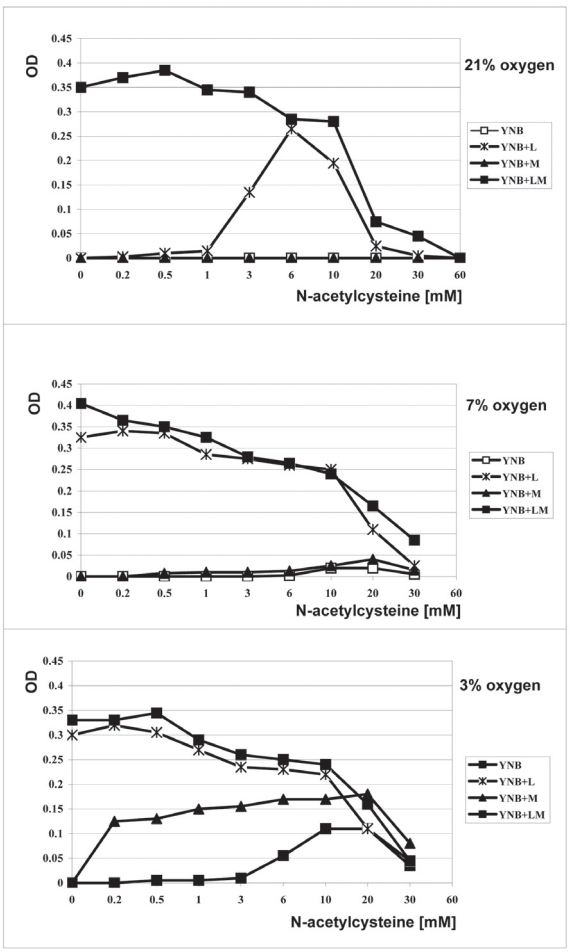

Figure 4. Effect of *N*-acetylcysteine on the lysine and methionine auxotrophy of the Cu,Zn-SOD⁻ strain (1C) of *S. cerevisiae*.
Conditions as in the legend to Fig. 2.

and automatized. Although the tests are not less complicated than commercial tests based on *in vitro* reactions of antioxidants, their advantage lies in the sensitivity to the adverse and toxic action of the antioxidants and their metabolites at the cellular level; this facet escapes detection by the commercial tests. Moreover, the proposed assays can constitute an intermediate step between *in vitro* and animal studies.

Table 1. Comparison of various tests for detection and quantification of antioxidants using Cu,Zn-SOD-deficient yeast

Test based on	Measurement of replicative life span	Abolishment of auxotrophy	Restoration of growth in hypertonic medium
Equipment needed	Microscope, micromanipulator, skilled experimenter	Petri dishes (optional: spectrophotometer, microplate reader)	Petri dishes (optional: spectrophotometer, microplate reader)
Experimenter skill required	Micromanipulation, sterile work	Sterile work	Sterile work
Possibility of automatization	No	Yes	Yes
Time to perform analysis	2–3 days	2–3 days	2–3 days
Number of compounds which can be tested simultaneously	1	Unlimited	Unlimited
Analysis of an antioxidant in a complex material	Yes	No	Yes
Detection of adverse/toxic effects of antioxidants	Yes	Yes	Yes

Acknowledgements

This work was supported by Grants 6 P04A07920 and 3 P04B00622 from the State Committee for Scientific Research (KBN, Poland). The authors are also indebted to the Polish Science Foundation for supplying us with the scientific equipment indispensable in this research.

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