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Paraquat에 의해 유도된 Superoxide Dismutase 결핍 효모의 산소 독성

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Oxygen Toxicity of Superoxide Dismutase-Deficient *Saccharomyces cerevisiae* by Paraquat

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ABSTRACT

Using superoxide dismutase (SOD)-deficient mutants of *Saccharomyces cerevisiae*, the oxygen toxicity induced by paraquat was studied. In aerobic culture condition, yeasts lacking MnSOD (mitochondrial SOD) showed more significant growth retardation than CuZnSOD (cytoplasmic SOD)-deficient yeasts. However, not so big differences in growth pattern of those mutants compared with wild type were observed under anaerobic condition. When exposed to paraquat, the growth of yeasts lacking CuZnSOD was severely affected by higher than 0.01mM of paraquat in culture medium. By the analysis of several cellular components involved in free radical generating and scavenging system, it was found that, under aerobic condition, the content of lipid peroxides in cell membrane as well as cellular activity of glutathion peroxidase of CuZnSOD-deficient mutants was increased in the presence of paraquat, although significant decrease of catalase activity was observed in those strains. In MnSOD-deficient yeast, however, increment in cellular activity of glutathion peroxidase and catalase by paraquat was observed without any deterioration of membrane lipid. It implies that the lack of mitochondrial SOD could be compensated by both of glutathion peroxidase and catalase, but that only glutathion peroxidase might act for CuZnSOD in cytoplasm. In contrast, all of SOD-deficient mutants showed a significant decrease in catalase activity, but slight increase in the activities of glutathion peroxidase, when cultivated anaerobically in the medium containing paraquat. Nevertheless, any significant changes of lipid peroxides in cell membranes were not observed during anaerobic cultivation of SOD-deficient mutants. It suggests that a little amount of free radicals generated by paraquat under anaerobic condition could be sufficiently overcome by glutathion peroxidase but not by catalase.

INTRODUCTION

The free radical scavenging enzyme, superoxide dismutase(SOD), is one of the most important enzymes in most aerobic organisms and many anaerobic ones (1). This enzyme catalyzes the disproportion of the toxic superoxide radicals to give dioxygen and hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow O_2 + 2H_2O_2$), which is assumed to provide *in vivo*

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protection against superoxide radicals generated by oxidative metabolism. A lot of works have been focussed on its protective roles in eucaryotic cells from DNA damage, protein denaturation, lipid peroxidation, aging and carcinogenesis, particularly in mammalian animals (2). However, the *in vivo* experimental results did not give an accurate, consistent and quantitative data due to the complexicity of animal bodies.

As one of the simplest eucaryotic organism, yeast strains are also known to have 2 different SODs; one is CuZnSOD having Cu^{++} and Zn^{++} at an active site in the cytoplasmic fraction, the other is MnSOD having Mn^{++} in the mitochondrial part, as likely as other eucaryotes (3). Furthermore, yeasts are capable of growing both in aerobic and in anaerobic conditions. It means that yeast cells are able to be subjected to a model system in oxygen toxicity experiments instead of mammalian animals.

Recently, the genes for these 2 SODs have been cloned and characterized successfully in yeast systems by Bermingham-McDonogh *et al.* (4) and van Loon *et al.* (5-6). Following the gene cloning, SOD-deficient yeast strains have been constructed by insertional inactivation of the genes by 2 different groups (7-8), in order to elucidate the role of SOD genes in cellular metabolism and oxygen radical toxicity.

In this paper, we report the effect of paraquat, one of well-known free radical generating agent, on the cellular enzymes and metabolites in SOD-deficient yeasts, especially catalase, glutathion peroxidase, and lipid peroxides in cell membrane, which have been known to involve in free radical generating or scavenging processes.

MATERIALS AND METHODS

Microorganisms and Culture Condition

Throughout this work, four yeast strains were used; *Saccharomyces cerevisiae* EG103 (*MAT leu2-3, 11 his3△1 trp1-289a ura3-52 gal*$^+$) as wild type, EG110 (same except *sod2△::TRP1*) lacking mitochondrial MnSOD, EG118 (same except *sod1△A::URA3*) lacking cytoplasmic CuZnSOD, and EG133 (same except *sod1△A::URA* and *sod2△::TRP1*) deficient of both SOD enzymes, which were kindly provided by Dr. Edith B. Gralla at University of California, Los Angeles, CA, U. S. A. The yeast strains were maintained in YPD media, which consists of 1% yeast extract, 2% peptone, and 2% glucose.

In aerobic cultivation, 1% of seed cultures (OD_{600} =4~5) were inoculated in YPD media, and cultivated for 16 hrs at 30℃ with vigorous shaking at 150rpm on a rotary shaker. In order to generate the oxygen toxicity in yeasts, 0.001mM of paraquat was added into culture medium.

On the other hand, the tightly closed capped Erlenmeyer flasks with side arm of spectrophotometric cuvette were employed for the anaerobic cultivation. In order to keep the culture media anaerobically, 0.03% sodium thioglycollate was supplemented in YPD medium with 1 ppm of resazurin as an indicator. After inoculating 1% of seed culture (OD_{600}=4~5), yeasts strains were cultivated for 16 hrs at 30℃ with shaking at 100rpm on a rotary shaker.

Analytical Procedures

The yeast cells were harvested by centrifugation at 5,000 rpm after 16 hrs-cultivation, washed twice and resuspended in 50mM phosphate buffer (pH 7.5) containing 0.1mM ethylenediamine tetraacetate (EDTA), and then disrupted by sonication for 5 min using Ultrasonic Processor (Model No. XL2010, Heat Systems, NY, U. S. A.). After centrifuging the resulting suspension at 15,000 rpm, the supernatant was used as enzyme sources for SOD, catalase and glutathion peroxidase. A part of sonicated suspension (0.4ml) was extracted with 1-butanol:pyridine (15:1) for the analysis of the degree of lipid peroxidation.

The activity of SOD was examined by staining the gel with 2.4mM nitroblue tetrazolium (NBT) and 28μM riboflavin after running 10% nondenaturated polyacrylamide gel electrophoresis, following the procedure of Beauchamp and

Fridovich (9). The catalase activity was assayed by the method of Aebi (10), and expressed as μ mole of hydrogen peroxide decomposed/min/mg of protein. The activity of glutathion peroxidase was analyzed by the procedure of Paglia and Valentine (11), and described as nmole of NADPH consumed in the reduction of oxidized glutathion/min/mg of protein. As an indicator for cellular deterioration by oxygen radical, the amount of lipid peroxides in cell membrane was determined from 1-butanol/pyridine(15:1) extracts by measuring malondialdehyde content according to the procedure of Ohkawa *et al.* (12).

The cell mass of yeasts was measured spectrophotometrically at 600nm, and converted to dry cell weight using standard curve. The protein content in samples was analyzed by Folin-Lowry method (13) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Growth Behavior of SOD-deficient Yeasts

At first, the growth kinetics of yeast strains lacking SOD genes were investigated under aerobic and anaerobic conditions, and compared with wild yeast strain. As seen in Fig. 1, SOD-deficient yeast strains showed the growth retardation compared to wild strain (EG103) during aerobic cultivation, whereas not so big differences in growth kinetics were observed in anaerobic culture. Especially in aerobic condition, the retarded growth patterns as well as low achievement of cell masses of SOD-deficient strains imply that these strains could not overcome the oxygen toxicity due to their loss of SOD activity, the most important enzyme in oxygen radical scavenging system. From the fact that yeasts lacking MnSOD gene (EG110 and EG133) were more severely affected than CuZnSOD-deficient strain (EG118) in aerobic condition, it could be also assumed that MnSOD (mitochondrial SOD) could play more important roles than cytoplasmic CuZnSOD in protecting cells from oxygen radical. In contrast, a little oxygen radical could be generated in anaerobic condition, and this amount of free radicals was likely not to give any severe effects on cell growth of SOD-deficient yeasts.

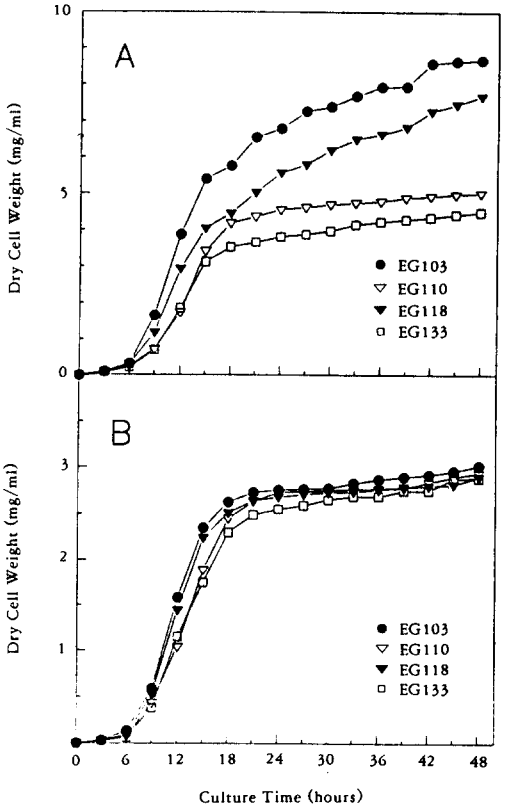

Fig. 1. Growth profiles of SOD-deficient yeasts under aerobic or anaerobic cuture condition
A; aerobic cultivation, B; anaerobic cultivation.

Effect of Paraquat Concentrations on Cell Growth

In order to investigate the effect of paraquat, one of well-known free radical generating agents, the growth patterns of SOD-deficient strains were examined on YPD media added with the different concentrations of paraquat under aerobic cultivation condition. Although the wild strain (EG103) could grow at the concentration above 0.5mM, as reported by Lee and Hassan (14), the growth of SOD-deficient strains could not be observed at higher than 0.1mM, as seen in

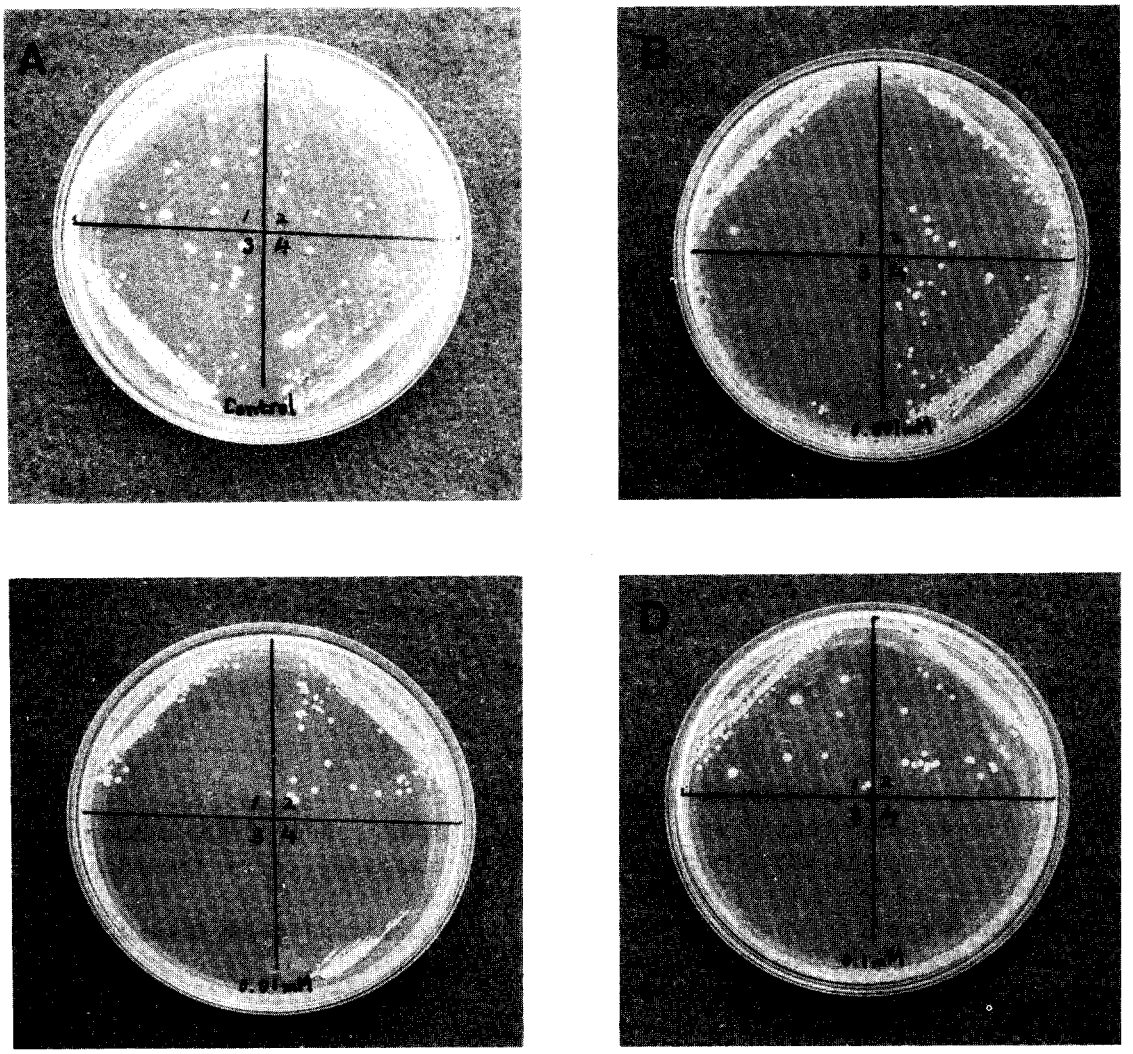

Fig. 2. Growth inhibition of SOD-deficient yeasts by different concentrations of paraquat.
Yeast strains were cultivated on YPD media without paraquat (A), or supplemented with 0.001mM (B), 0.01mM (C) or 0.1mM of paraquat (D). 1; *Saccharomyces cerevisiae* EG103, 2; *S. cerevisiae* EG103, 3; *S. cerevisiae* EG118, 4; *S. cerevisiae* EG110.

Fig. 2. The result that EG118 and EG133 strains responded more significantly to the paraquat concentration (Fig. 3) suggests that paraquat might generate free radicals mainly in cytoplasmic fraction, but not in mitochondrial fraction. In the other word, the yeast strains lacking CuZnSOD enzyme (EG118 and EG133) could get more severely damaged than MnSOD-deficient strain (EG110) by free radicals generated by paraquat.

Oxygen Toxicity in SOD-deficient Yeasts

The changes of several enzyme activities and lipid peroxidation were carefully investigated to examine the effects of SOD deficiency in yeasts on intracellular adaptation to free radicals generated by paraquat.

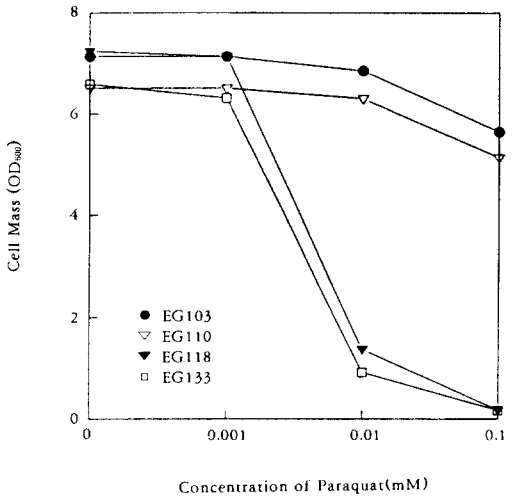

Fig. 3. Cell growth of SOD-deficient yeasts in the presence of paraquat during aerobic cultivation.

First of all, the activity of SOD of yeast mutants was examined by activity staining on 10% nondenaturated polyacrylamide gels, as shown in Fig. 4. By supplementing paraquat in culture media, CuZnSOD activities in wild yeast (EG103) and MnSOD-deficient yeast (EG110) were not only significantly increased under aerobic culture condition, but also slight increment of activities was found under anaerobic condition. However, CuZnSOD-deficient yeasts (EG113 and EG118) did not show any remarkable changes in MnSOD activities.

In case of catalase converting hydrogen peroxide to water, its cellular activity was significanly decreased in CuZnSOD-deficient mutants, whereas slightly higher activity was observed in MnSOD-deficient strains, when exposed to

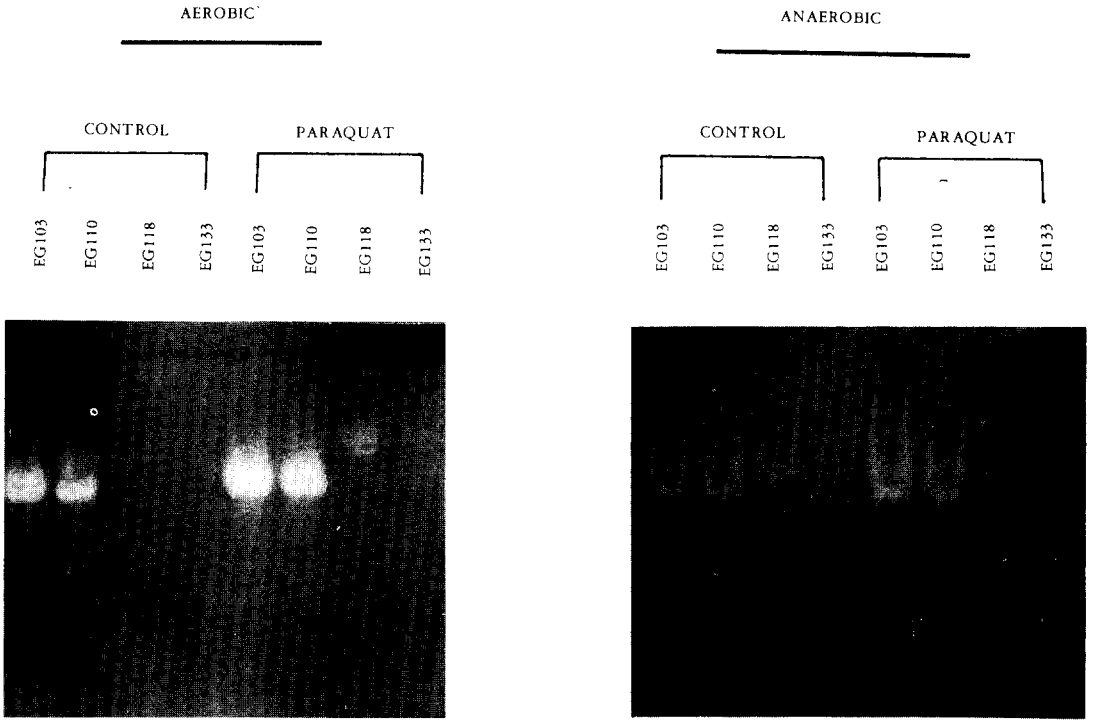

Fig. 4. Total cellular activities of SOD in wild yeast strain and its SOD-deficient mutants during aerobic or anaerobic cultivation in the medium containing paraquat.
After sonication of yeast cells, the supernatants were subjected to 10% nondenaturated polyacrylamide gel electrophoresis, and the activities of SOD was stained as described in Materials and Methods.

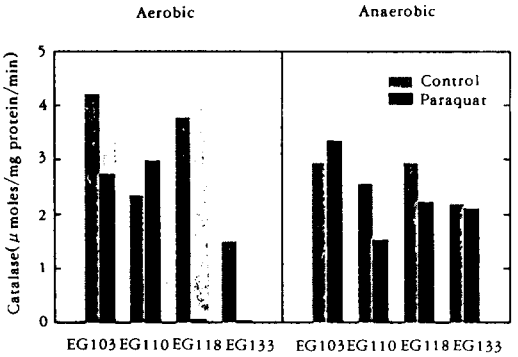

Fig. 5. Total cellular activities of catalase in wild yeast strain and its SOD-deficient mutants when cultivated aerobically or anaerobically in the presence of paraquat.

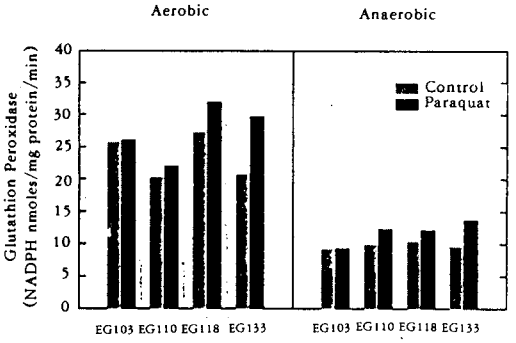

Fig. 6. Total cellular activities of glutathion peroxidase in wild yeast strain and its SOD-deficient mutants when exposed to paraquat under aerobic or anaerobic culture condition.

paraquat during aerobic cultivation (Fig. 5). On the other hand, the cellular activity of glutathion peroxidase, one of the alternative radical scavenging systems, was significantly increased in yeast strains lacking CuZnSOD in the presence of paraquat when cultivated aerobically (Fig. 6). It strongly suggests that the free radical scavenging system might shift to glutathion peroxidase rather than catalase when cytoplasmic CuZnSOD is inactivated. The same phenomenon was also obtained during anaerobic cultivation. In that case, all of SOD-deficient yeasts showed decrease profiles of catalase activity, but an increase pattern

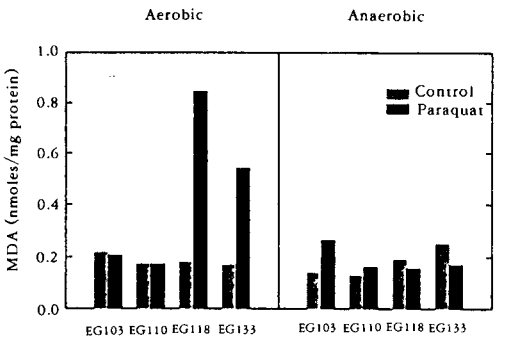

Fig. 7. The content of lipid peroxides, determined as malondialdehyde, in wild yeast strain and its SOD-deficient mutants cultured aerobically or anaerobically in the presence of paraquat.

of the activity of glutathion peroxidase in culture media containing paraquat.

In contrast, the significant increment in the content of lipid peroxides as malondialdehyde was observed in CuZnSOD-deficient mutants (EG118 and EG133) when exposed to paraquat, whereas the cell membrane lipids of wild type (EG103) and MnSOD-deficient mutant (EG110) were not severely deteriorated by paraquat under aerobic condition (Fig. 7). It implies that the glutathion peroxidase activated in the absence of CuZnSOD could not remove perfectly the free radicals generated in cytoplasm to cause membrane damage, whereas MnSOD could be compensated by slightly increased amount of catalase and glutathion peroxidase. However, not so significant effects appear in anaerobic culture, although slight increase of malondialdehyde content in wild yeast strain was recognized. It means that small amount of free radicals generated under anaerobic condition could not give the severe damage on membrane lipids by detoxication through glutathion peroxidase, in spite of the absence of SOD enzymes.

It could be concluded that paraquat could induce oxygen toxicity in SOD-deficient yeasts, especially CuZnSOD-deficient yeasts. It was also an interesting finding that glutathion peroxidase could be activated for scavenging radicals from

cytoplasm rather than catalase even though this enzyme could not replace of SOD and catalase activities perfectly.

요 약

활성산소 소거 효소인 superoxide dismutase (SOD)가 결핍된 효소 변이주들을 대상으로 하여, 활성산소 유발물질인 paraquat을 배지에 첨가하여 배양하면서 산소 독성을 관찰하였다. 호기 상태에서는 MnSOD (mitochondria SOD) 결핍 효모는 CuZnSOD (세포질 SOD) 결핍 효모보다 성장이 많이 저하되었지만, 혐기 상태에서는 이들 SOD 결핍 효모 모두 성장 속도에서 야생 효모와 큰 차이를 보이지 않았다. Paraquat으로 처리한 결과, 호기 배양에서 CuZnSOD 결핍 효모는 0.01mM 이상에서 성장하지 않음을 알 수 있었다. 따라서 0.001mM paraquat을 배지에 첨가한 후 호기적으로 배양하면서 세포내 성분의 변화를 관찰하였더니, CuZnSOD 결핍 효모에서는 catalase 역가가 떨어진 반면 glutathion peroxidase 역가와 세포막 지질의 과산화물이 증가하였으며, MnSOD 결핍 효모에서는 catalase 역가와 glutathion peroxide 역가가 모두 조금씩 증가하면서 세포막 지질의 과산화물은 그다지 변화하지 않았다. 이러한 사실로부터 CuZnSOD가 없는 경우 활성산소 소거계로써 catalase 보다 glutathion peroxidase가 훨씬 활성화되어지지만, 이렇게 활성화된 glutathion peroxidase로는 세포질내 산소 radical을 완벽히 제거하는데에는 다소 불충분하다는 사실을 알 수 있었다. 한편, 혐기적 배양에서는 SOD 결핍 효모들의 catalase 역가는 모두 감소한 반면, glutathion peroxidase 역가는 다소 증가하였고, 또한 세포막 지질의 과산화물은 다소 감소하는 추세를 보였다. 이는 혐기 상태에서 산소 radical이 소량밖에 생기지 않으므로, 활성화된 glutathion peroxidase에 의해 어느 정도 극복되어지기 때문인 것으로 사료된다.

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