0273-2289/97/6603—0263$9.50

Photosensitization of the Yeast *Phaffia rhodozyma* at a Low Temperature for Screening Carotenoid Hyperproducing Mutants

GIL-HWAN AN

Department of Biology, 68-370, Massachusetts Institute of Technology, 77 Massachusetts Ave. Cambridge, MA 02139

Received April 29, 1996; Accepted December 17, 1996

ABSTRACT

Phaffia rhodozyma strain Ant-1 produces more carotenoids, known as antioxidants, but it was more sensitive to light plus toluidine blue O (TBO), a superoxide producer, than wild strain 67-385 at 20°C. Carotenoid hyperproducing mutants (CHMs), Ant-1 and 2A2N, exhibited decreased activity of superoxide dismutase (SOD) compared to 67-385, and this is in part responsible for hypersensitivity of the mutants to photosensitization. Light plus TBO at 2°C allowed carotenoid hyperproducing mutants to produce higher colony-forming units than the wild-type. Photosensitization with limited cell metabolism by a low temperature, provides an idea of selective conditions for carotenoid hyperproducers of *P. rhodozyma*.

Index Entries: Photosensitization; carotenoid; superoxide dismutase; *Phaffia rhodozyma*.

INTRODUCTION

The red-pigmented fermenting yeast *Phaffia rhodozyma* synthesizes astaxanthin as its principal carotenoid *(1)*. There is presently of considerable interest in developing biological sources of carotenoids for use in the developing aquaculture industry. The commercial use of *P. rhodozyma* has been, however, limited because of the low astaxanthin content in wild strains.

Carotenoids, especially astaxanthin, have been shown to prevent oxidative injury to various organisms *(2, 3)*. Toluidine blue O (TBO) photoinactivates yeasts by generating O_2^- on the yeast cell surface, leading to

lethal membrane damage *(4)*. Tuveson et al. *(5)* reported the protection of the carotenoid-producing cells of *Escherichia coli* against inactivation was seen with TBO plus visible light.

A few screening methods for carotenoid hyperproducing mutants (CHMs) have been developed with antimycin *(6)*, β-ionone *(7)*, flow cytometry and cell sorting *(8)*, and light plus rose bengal *(9)*. However, development of screening methods for CHMs based on the protecting activity of carotenoids was not successful. CHMs are more sensitive to antimycin, H_2O_2, thenoyltrifluoroacetone, cyanide, UV *(6)*, and duroquinone *(10)*, which are related to oxygen radicals. It resulted in the lack of a screening method.

This study has shown that CHMs are more susceptible to photosensitization than wild-type yeast owing in part to low superoxide dismutase (SOD) activity. This study has also shown that photosensitization at a low temperature can be used as the selective agent of CHMs.

MATERIALS AND METHODS

Chemicals

SOD (from bovine erythrocytes, 15,000 u/mg), dimethylsulfoxide (DMSO), and TBO were purchased from Sigma Chemical Co. St Louis, (MO). Astaxanthin was kindly provided from Hoffman LaRoche Ltd. (Switzerland).

Yeast Strains and Growth

P. rhodozyma strains used were 67-385 (natural isolate), #18 (obtained from ethyl methane sulfonate mutagenesis), Ant-1 (antimycin-induced mutant), and 2A2N (obtained from serial nitrosoguanidine mutagenesis of Ant-1) *(6)*. Yeasts were maintained in a refrigerator or stored in a deep freezer (–70°C). *P. rhodozyma* was grown at 20°C in YM broth (Difco, MI). Growth in liquid media was measured by turbidity as previously described *(6)*.

Carotenoid Extraction and Analysis

Total yeast carotenoid was measured by the modified DMSO method of Sedmak et al. *(11)*. One-half to 1 mL of yeast culture was centrifuged at 1000*g* for 5 min with a desktop centrifuge (International Clinical Centrifuge, Model CL, International Equipment Co., Boston, MA), washed with distilled water, and dried with air for 1 min. One milliliter each of DMSO (55°C), acetone, petroleum ether, and sodium chloride 20% (w/v) solution was then added serially with vortex *(12)*. The upper petroleum ether layers containing the carotenoids were collected and analyzed by thin-layer chromatography and electronic absorbance spectrophotometry as previously described *(6)*.

Table 1
Effect of Light and TBO on Photosentization of *P. rhodozyma*

Strain	67-385	Ant-1
Condition	% Survival	
Control	100	100
Light	99	76
TBO 1 mM	95	105
Light + TBO 1 mM	88	19

SD was <⅒ of each value.

Superoxide Dismutase Assay

For the measurement of SOD activity, harvested cells were washed and resuspended in phosphate/sorbitol buffer (sodium phosphate 50 m*M* and sorbitol 1.2*M*, pH 7.0). Ten milliliters of cell suspension were passed three times through a French Press (French Pressure Cell Press, American Instrument Company, Division of Travenol Laboratories, Inc., Silver Spring, MD) at 150 bar. SOD was measured by observing the inhibition of cytochrome-*c* reduction, which was monitored with absorbance at 550 nm *(13)*. Samples were prepared by mixing the yeast homogenates with cytochrome-*c* and xanthine solution. Measurement was started with mixing xanthine oxidase with the sample solution. One unit of SOD was defined as the amount of enzyme that inhibited 50% of cytochrome-*c* reduction *(13)*.

Photosensitization of *P. rhodozyma*

Photosensitization of the yeast was performed with TBO and light (two 20-W fluorescent tubes at a distance of 20 cm). Yeasts were grown for 5 d in YM broth at 20°C. Yeasts were washed twice with 0.1*M* sodium phosphate buffer (pH 7.0). *P. rhodozyma* was incubated in the phosphate buffer for 1 d at 4°C. Then, cells were resuspended in fresh phosphate buffer containing 1 m*M* TBO, and they were incubated at 2–3°C with shaking at 150 rpm under illumination. For the measurement of colony-forming unit, duplicate or triplicate samples were used.

RESULTS AND DISCUSSION

β-carotene and its related carotenoids protect cells against oxygen radicals *(2,3)*. Light plus TBO was effective for the decomposition of astaxanthin. Fifty and 8% of astaxanthin (1 mg/mL) was decomposed by incubation with light plus TBO (0.1 m*M*) for 9.5 and 17.5 h, respectively. This

Table 2
SOD Activity of *P. rhodozyma*

Strain	Carotenoid (μg/g yeast)	Superoxide Dismutase (unit/g yeast)[a]
67-385	300	1830
Ant-1	960	1100
2A2N	2070	1350

[a]One unit: the amount of SOD inhibits 50 % of cytochrome-*c* reduction.

result suggested that astaxanthin protects the yeast cells against TBO plus light.

Mutant strain Ant-1 produced more carotenoids, but it was more susceptible to photosensitization than wild strain 67-385 (Table 1). CHMs are more susceptible to O_2^- than wild strain *(10)*. Since TBO + light produces O_2^- *(4)*, activity of SOD from different strains was measured. The assay of SOD indicated that the wild-type yeast 67-385 contained higher activity of SOD than CHMs (Table 2). Therefore, hypersensitivity of CHMs to photosensitization (Table 1) was owing in part to a low SOD activity (Table 2).

Since the product of SOD is H_2O_2, catalase activity of the yeasts was also measured. The higher the carotenoid contents of the yeasts, the more was the catalase activity (data not shown), which is the same as the previous observation *(10)*. However, CHMs are more susceptible to H_2O_2 than their parents *(6)*. Synthesis of certain enzymes in other fungi has been shown to be induced by O_2^-, including SOD in *Saccharomyces cerevisiae (14)* and catalase in *Neurospora crassa (15)*. Increased catalase activity in CHMs compared to 67-385 and increased SOD activity in 2A2N compared to Ant-1 might be owing to induction of those enzymes by O_2^-.

P. rhodozyma does not contain Fe- or Zn/Cu-SOD, but it contains Mn-SOD located in mitochondria *(10)*. The mitochondrial size and number of *P. rhodozyma* CHM 2F-1 are different from those of wild strain 67-385 *(16)*. CHMs grow less with succinate as the main carbon source *(6, 16)*, and they are more sensitive to antimycin than their parent *(6)*. Lowered activity of SOD in CHMs was therefore the result of the mitochondrial mutation decreasing Mn-SOD. Decreases in SOD activity may significantly affect the resistance of *P. rhodozyma* to O_2^- and cause increased sensitivity of CHMs to O_2^- (Table 1).

Carotenes were shown to prevent injury to the yeast *Rhodotorula mucilaginosa* on exposure to reactive oxygen species *(17)*. The apparent lack of Fe-SOD or Cu/Zn-SOD in *R. mucilaginosa* may make the antioxidant activity of β-carotene very critical in this yeast *(17)*. *P. rhodozyma* also contains only Mn-SOD *(10)*. If the activity of SOD and other cell metabolisms

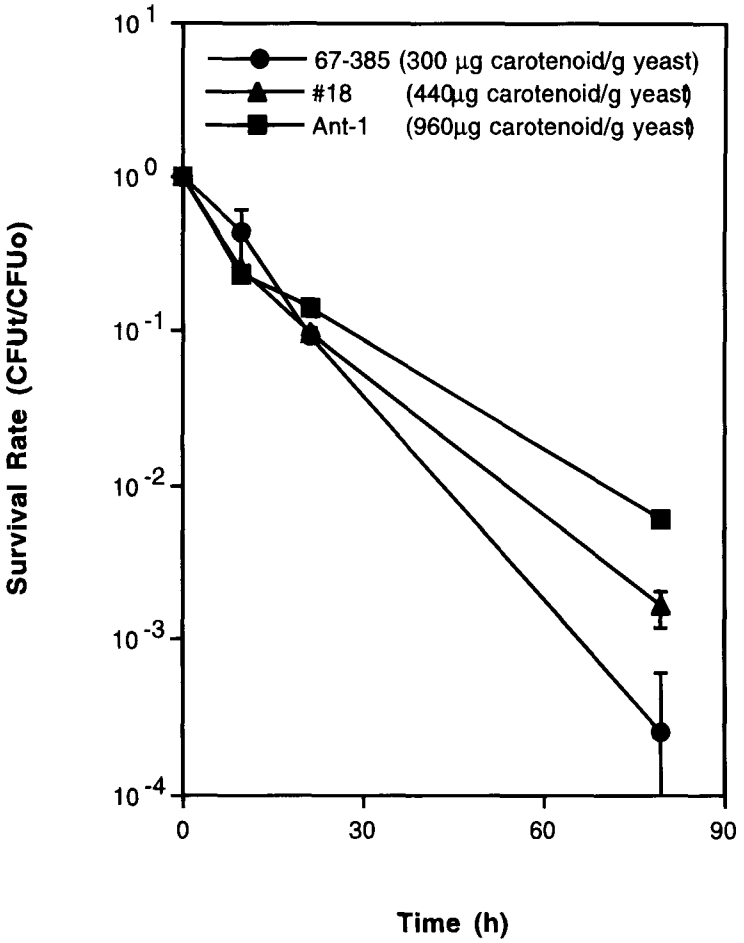

Fig. 1. Photosensitization associated with carotenoid contents of *P. rhodozyma*. Yeasts were photosensitized by light (20 cm apart from two 20-W fluorescence tubes) plus TBO (1 m*M*). Bars indicate SDs. CFUt, colony-forming unit at the time t; CFUo, colony-forming unit without photosensitization.

are limited, it is expected that the nondisturbed protecting effect of carotenoids from O_2^- can be observed. Therefore, activity of SOD was limited by starving cells and incubating them at 2°C. The resistance of the yeasts to light plus TBO was proportional to carotenoid contents (Fig. 1). By light plus TBO for 80 h at 2°C, the mutant Ant-1 (960 µg carotenoid/g yeast) produced a 25-fold increased colony-forming unit compared to the wild-type 67-385 (300 µg carotenoid/g yeast) (Fig. 1). This method can be used for screening CHMs of *P. rhodozyma*.

Many light-derepressible genes regulating carotenogenesis in fungi require molecular oxygen and a photosensitive pigment in addition to blue light for activation *(18)*. These gene products are induced when conditions favor O_2^- formation. Carotenoid production increases when *P. rhodozyma* is

grown in media containing duroquinone *(10)*, a redox-cycling quinone that generates intracellular O_2^- *(17)*. CHMs of *P. rhodozyma* contained decreased SOD (Table 2), and this might cause a high level of O_2^- concentration in CHMs. If carotenogenesis of *P. rhodozyma* is regulated by O_2^-, lowered SOD activity in CHMs promotes carotenogenesis. This also means that carotenogenesis of *P. rhodozyma* is a protecting activity against harmful O_2^-.

ACKNOWLEDGMENTS

This work was supported by G7 grant (contract # HS310M) from the Korean Ministry of Science and Technology.

REFERENCES

1. Andrewes, A. G., Phaff, H. J., and Starr, M. P. (1976), *Phytochemistry* **15,** 1003–1007.
2. Burton, G. W. and Ingold, K. U. (1984), *Science* **224,** 569–573.
3. Miki, W. (1991), *Pure Appl. Chem.* **63,** 141–148.
4. Ito, T. (1978), *Photochem. Photobiol.* **28,** 493–508.
5. Tuveson, R. W., Larson, R. A., and Kagan, J. (1988), *J. Bacteriol.* **170,** 4675–4680.
6. An, G.-H., Schuman, D. B., and Johnson, E. A. (1989), *Appl. Environ. Microbiol.* **55,** 116–124.
7. Lewis, M. J., Ragot, N., Berlant, M. C., and Miranda, M. (1990), *Appl. Environ. Microbiol.* **56,** 1944.
8. An, G.-H., Bielich, J., Auerbach, R., and Johnson, E. A. (1991), *Bio/Technology* **9,** 70–73.
9. Schroeder, W. A. and Johnson, E. A. (1995), *J. Ind. Microbiol.* **14,** 502–507.
10. Schroeder, W. A. and Johnson, E. A. (1993), *J. Gen. Microbiol.* **139,** 907–912.
11. Sedmak, J. J., Weerasinghe, D. K., and Jolly, S. O. (1990), *Biotech. Tech.* **4,** 107–112.
12. An, G.-H., Kim, C.-H., Choi, E.-S., and Rhee, S.-K. (1996), *J. Ferment. Bioeng.* **82**: Nov.
13. Flohé, L. and Otting, F. (1984), *Methods Enzymol.* **105,** 93–104.
14. Westerbeek-Marres, C. A. M., Moore, M. M., and Autor, A. P. (1988), *Eur. J. Biochem.* **174,** 611–620.
15. Chary, P. and Natvig, D. O. (1989), *J. Bacteriol.* **171,** 2646–2652.
16. An, G.-H. (1991), Ph.D. thesis, University of Wisconsin-Madison, Madison, WI.
17. Moore, M. M., Breedveld, M. W., and Autor, A. P. (1989), *Arch. Biochem. Biophys.* **270,** 419–431.
18. Hoober, J. K. and Phinney, D. G. (1988), *Trends Biochem. Sci.* **13,** 371–374.