Antioxidant role of carotenoids in *Phaffia rhodozyma*

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The role of carotenoids in protecting the yeast *Phaffia rhodozyma* against reactive oxygen species was studied. Addition of the O_2^- -generator duroquinone (DQ) to yeast–malt broth increased total carotenoid content as well as the relative amounts of xanthophylls present, while the reactive oxygen scavenger mannitol reversed this effect. Resistance to DQ increased in the stationary phase, particularly in the carotenoid-hyperproducing strain studied. Assay of superoxide dismutase (SOD) in *P. rhodozyma* indicated the presence of Mn-SOD and the complete absence of Fe-SOD and Cu/Zn-SOD. Catalase activity in *P. rhodozyma* was significantly lower than in *Saccharomyces cerevisiae*, particularly in stationary cultures. In young cells, H_2O_2 resistance was directly related to carotenoid levels, and selection of cultures based on peroxide resistance resulted in slightly increased carotenoid production. These results indicate that carotenoids play an antioxidant role during ageing in *P. rhodozyma*.

Introduction

The red fermenting yeast, *Phaffia rhodozyma* (Miller *et al.*, 1976), has received considerable attention in recent years as a potential biological source of the carotenoid astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) (Andrewes *et al.*, 1976). This interest is due to the unique role of astaxanthin in providing pigmentation to aquacultured salmonids and red sea bream. Currently, astaxanthin is produced by total chemical synthesis and its market has been estimated as 60–100 million US dollars per year (Johnson & An, 1991). Astaxanthin synthesized by *P. rhodozyma* could provide an alternative source if it is produced economically and satisfactorily incorporated by salmonids. Astaxanthin produced by *P. rhodozyma* is deposited in the flesh of salmonids (Johnson *et al.*, 1980; Torrisen *et al.*, 1989); however, wild-type isolates of the yeast are uneconomical because of their low astaxanthin production. We have attempted to increase productivity by understanding the function of carotenoids in *P. rhodozyma* and the development of selective procedures for increased pigmentation. Selections with antimycin A have produced yeast strains with significantly enhanced pigmentation (An *et al.*, 1989), which may be due to a blockade of the primary

respiratory chain at the bc_1 complex, producing activated oxygen species such as O_2^- , H_2O_2 and $OH^\cdot$ (Fee & Teitelbaum, 1972) which stimulated carotenoid formation. These species have been reported to be involved in ageing in fungi (Munkres *et al.*, 1984). Since carotenoids can be sensitive antioxidants (Burton & Ingold, 1984; Terao, 1989), we felt that carotenoids might protect *P. rhodozyma* from oxidative injury, and this hypothesis was investigated in this study.

Methods

Chemicals. The following chemicals were obtained from Sigma: hydrogen peroxide (30% solution, v/v), superoxide dismutase (SOD) (from bovine erythrocytes, 15000 units mg^{-1}), catalase (from bovine liver, 2500 units mg^{-1}), 2,3,5,6-tetramethyl-2,5-cyclohexadiene-1,4-dione (duroquinone; DQ), nitro blue tetrazolium (NBT), DMSO, acriflavine, ethidium bromide, 2,3,5-triphenyl-2H-tetrazolium chloride, succinic acid, potassium hydrogen phthalate, and all sugars except for glucose. Glucose and acetone (ACS grade) were from Fisher Scientific. Petroleum ether (residue analysis grade; boiling range 30–60 °C) was from EM Industries. Ethanol (200 proof; USP grade) was from USI Chemicals. BCA protein assay reagent was from Pierce Chemical. All other chemicals used were of analytical grade or the best grade commercially available.

Yeast strains and growth. The *P. rhodozyma* natural isolate (67-385) was kindly provided by Mary Miranda, University of California, Davis. AF-1 (a white mutant blocked in carotenoid biosynthesis), and the hyperproducing strains 2A2N, MI-1, and anF1-P were derived from 67-385 by mutagenesis (An, 1991; An *et al.*, 1989). Isolate 67-385 was chosen as the parent strain, owing to its relatively high pigmentation and growth. Yeasts were maintained on slants of yeast extract/malt extract/peptone/glucose medium (YM broth, Difco) with 2% (v/v) agar (YM agar) and refrigerated at 4 °C. Yeast strains were also stored in 40% glycerol/60% YM broth (v/v) at –70 °C.

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Abbreviations: SOD, superoxide dismutase; HDCO, 3-hydroxy-3', 4'-didehydro- β,ψ -carotene-4-one; DQ, duroquinone.

P. rhodozyma was usually grown in YM broth or on YM agar plates at 20 °C. In determining sensitivity of yeast strains to H₂O₂ or DQ, cultures were grown in YM broth, serially diluted in 0.85% NaCl and plated onto 1% YM agar supplemented with either DQ (dissolved in 0.1% DMSO/YM agar) or H₂O₂. All chemical additions were made to molten YM agar cooled to 39 °C and immediately poured into plates. Survival was measured by determining the number of colonies recovered on YM agar with inhibitor (DQ or H₂O₂) compared to numbers recovered on YM agar without inhibitor. Growth in liquid media was measured by cell dry mass: 5 ml liquid culture was pipetted into baked, tared 13 × 100 mm disposable borosilicate tubes, which were centrifuged for 1 min; the pellet was washed once with distilled water, and dried overnight at 115 °C.

Measurement of catalase and superoxide dismutase. Cells were harvested by centrifugation, washed and resuspended in ice cold 100 mM-phosphate buffer (pH 7.0) (catalase assays) or ice cold distilled water (SOD assays). The cells were disrupted by passage through a French press at 2000 p.s.i. at 0 °C. Samples were observed by microscopy and estimated to be greater than 95% disrupted. Total cellular protein was assayed by the BCA protein assay method (Pierce). Cell-free extracts were obtained by centrifugation at 10000 g for 10 min at 0 °C. For assay of catalase (Beers & Sizer, 1951), samples were added to a solution containing a final concentration of 10 mM-H₂O₂ in 100 mM-phosphate buffer (pH 7.0), and the decrease in absorbance was recorded over 30 s at 240 nm in a Gilford Response Spectrophotometer. One unit of catalase was defined as the amount of enzyme which broke down 1 µmol H₂O₂ min⁻¹. For SOD activity stains, 350 µg total cell protein in 30 µl was mixed 1:1 with loading buffer (20% (v/v) glycerol; 0.01% bromophenol blue loading buffer), and applied to a 6.5% (w/v) polyacrylamide/bisacrylamide (30:0.8) non-denaturing gel (pH 8.8) containing a 5% (w/v) stacking gel (pH 6.8). The gel was run at 40 mA constant current for 3–4 h. The gels were then developed by NBT staining as described by Flohe & Otting (1984).

Carotenoid extraction and analysis. For analysis of yeast carotenoid content, *P. rhodozyma* was grown for 5 d in 30 ml liquid YM or YNB medium in 300 ml baffled flasks shaken at 150 r.p.m. on an orbital shaker controlled at 20 °C. Liquid culture (25 ml) was harvested and centrifuged, resuspended in about 25 ml water, and 1 ml of the suspension was centrifuged at 15000 r.p.m. for 5 min and extracted by the DMSO method (Sedmak *et al.*, 1990). Cells were washed twice in a 5 ml test tube with 2 ml deionized water, and once with 1 ml acetone; the pellets were air-dried for about 1 min and 1 ml DMSO preheated to 55 °C was added to each tube. After the addition of glass beads (0.5 mm), the tubes were vortexed for 20–30 s. Acetone (1 ml) was added to extract the carotenoid, and 1 ml petroleum ether and 1 ml 20% (w/v) NaCl in water were added. The phases were separated by centrifugation for 2 min. The petrol phase was removed and the carotenoid content determined by the A₄₇₄. Total carotenoids were calculated using A_{1cm}^{1%} = 2100. Individual carotenoids were analysed by TLC and absorption spectra as previously described by An *et al.* (1989).

All experiments were performed in duplicate or triplicate and replicated at least once. The error estimates presented in the text and figures for yeast mass, carotenoid content, and enzyme activities represent one standard deviation from the mean.

Results

Influence of duroquinone on survival and carotenogenesis in growing and stationary cultures

Previous studies showed that astaxanthin synthesis continued in *P. rhodozyma* after growth had terminated

(Johnson & Lewis, 1979). The sensitivity to duroquinone, a redox-cycling quinone that generates intracellular O₂⁻ (Moore *et al.*, 1989), was determined in actively growing (2 d) and mature (5 d) cultures. Two-d-old cultures were quite sensitive to DQ, while 5-d-old cultures were more resistant (Fig. 1). The carotenoid hyperproducing strain 2A2N showed the greatest increase in resistance to DQ during aging. DQ growth inhibition was relieved by addition of SOD to the medium (data not shown), suggesting that O₂⁻ led to the growth inhibition. These results indicated a relationship between carotenoid levels and O₂⁻ sensitivity in *P. rhodozyma*.

The influence of DQ on carotenogenesis was determined in a hyperproducer and the wild type. Carotenoid

Fig. 1. Susceptibility of *P. rhodozyma* strains 67-385 (●) and 2A2N (□) to duroquinone on YM agar. (a) Two-d-old cultures; (b) 5-d-old cultures.

Table 1. Effects of duroquinone and mannitol on growth and pigmentation of *P. rhodozyma* 67-385 grown in YM broth for 5 d

Addition to YM broth	Growth (mg ml ⁻¹)	Carotenoid [$\mu\text{g (g yeast)}^{-1}$]
None	4.43	314
Duroquinone (100 μM)	4.56	425
Duroquinone (150 μM)	4.52	492
Duroquinone (100 μM) + mannitol (20 mM)	5.17	331
Duroquinone (100 μM) + mannitol (30 mM)	5.14	302

Table 2. Effects of duroquinone and mannitol on the carotenoid composition of *P. rhodozyma* 67-385

	No treatment	Duroquinone (100 μM)	Duroquinone (100 μM) + mannitol (30 mM)
Carotenoid [$\mu\text{g (g yeast)}^{-1}$]			
DCD*	3	ND	ND
<i>cis</i> -Astaxanthin	10	23	14
<i>trans</i> -Astaxanthin	177	380	273
Phoenicoxanthin	6	ND	ND
HDCO	16	69	64
Torulene	22	57	27
3-Hydroxy-echinenone	19	29	32
γ -Carotene	16	6	23
β -Zeaxanthin	3	ND	ND
β -Carotene	35	6	14
Others	10	6	9
Total [$\mu\text{g (g yeast)}^{-1}$]	317	576	456
Growth (mg ml ⁻¹)	2.8	3.2	3.5

* DCD, 3,3'-dihydroxy- β - γ -carotene-4,4'-dione (An, 1991).
ND, not determined, because of low content (< 1%).

formation in the hyperproducing mutant anF-1P increased by 50% from 1100 $\mu\text{g g}^{-1}$ to 1650 $\mu\text{g g}^{-1}$ in YM broth containing 100 μM -DQ added during the exponential phase (data not shown). Growth in the wild-type strain 67-385 was unaffected by 150 μM -DQ but carotenoid production increased by about 60% (Table 1). Carotenoid production was significantly decreased when mannitol was added to DQ incubation (Tables 1 and 2). These results suggested that activated oxygen species stimulated carotenoid synthesis in the yeast.

Yeasts treated with DQ (100 μM) or DQ (100 μM) plus mannitol (30 mM) were analysed for carotenoid composition. DQ increased the proportions of the major xanthophylls [astaxanthin, HDCO (3-hydroxy-3'-4'-didehydro- β - ψ -carotene-4-one), and 3-hydroxy-

Fig. 2. Polyacrylamide electrophoresis gel stained for SOD activity from *P. rhodozyma* and *S. cerevisiae* grown for 5 d in YM broth. (a) Gel stained in the absence of KCN; (b) gel stained in the presence of KCN. Lanes: 1, *P. rhodozyma* hyperproducing strain MI-1; 2, *S. cerevisiae*; 3, wild-type *P. rhodozyma* strain 67-385; 4, commercial bovine Cu/Zn-SOD (0.6 μg ; 3.2 U μg^{-1}); 5, albino *P. rhodozyma* strain AF-1; 6, hyperproducing *P. rhodozyma* strain 2A2N; 7, *S. cerevisiae* (duplicate); 8, wild-type strain 67-385 (duplicate); 9, hyperproducing strain MI-1 (duplicate).

echinenone] but decreased the proportions of major carotenenes (β -carotene and γ -carotene (Table 2). The addition of mannitol together with DQ resulted in a decreased proportion of xanthophylls and an increased proportion of carotenenes (Table 2).

Superoxide dismutase (SOD) activity in *P. rhodozyma*

Since resistance to duroquinone varied between wild-type and mutant *P. rhodozyma* strains, SOD activity was assayed in four strains (the albino strain AF-1, 67-385, 2A2N and a second hyperproducing strain MI-1) and in

Fig. 3. Susceptibility of *P. rhodozyma* strains 67-385 (●) and 2A2N (□) and *S. cerevisiae* (○) to H₂O₂ on YM agar. (a) Two-d-old cultures; (b) 5-d-old cultures.

S. cerevisiae by activity staining of electrophoresis gels. SOD levels did not appear to differ among the four strains (Fig. 2), and its activity was not changed in cells grown in the presence of duroquinone, nor did it change as a function of culture age (data not shown). Interestingly, SOD activity in *P. rhodozyma* was resistant to both KCN (10 mM) and H₂O₂ (5 mM). This indicated a lack of Cu/Zn-SOD (CN⁻-sensitive) and Fe-SOD (H₂O₂-sensitive) and the presence of only Mn-SOD (insensitive to H₂O₂ and CN⁻). Multiple bands of activity were observed, possibly indicating aggregates or isozymes of Mn-SOD in *P. rhodozyma*. Since it has been reported that Mn-SOD is present only in the mitochondria (Moore *et al.*, 1989), carotenoids may be important in protecting against extramitochondrial oxidative stress. The related carotenogenic yeast, *Rhodotorula muci-*

Fig. 4. Effect of culture age on susceptibility of *P. rhodozyma* strains 67-385 (●) and 2A2N (□) and *S. cerevisiae* (○) to 2 mM-H₂O₂ on YM agar.

laginosa, also has been reported to contain only Mn-SOD (Moore *et al.*, 1989).

H₂O₂ resistance and catalase activity in *P. rhodozyma*

Since O₂⁻ can be converted *in vitro* to H₂O₂ by superoxide dismutase:

we examined whether strains of *P. rhodozyma* that differed in sensitivity to duroquinone showed the same pattern of sensitivity to H₂O₂. Unexpectedly, the pattern was opposite and the hyperproducing strain 2A2N was most resistant in exponential-phase growth and yet more sensitive in stationary phase compared to the wild-type strain 67-385 (Fig. 3). The drop in resistance to H₂O₂ at the onset of stationary phase was particularly large in the hyperproducing strain 2A2N (Fig. 3). Because of the decreased resistance of 2A2N to H₂O₂ in older cultures, sensitivity to 2 mM-H₂O₂ was followed throughout the course of 5 d fermentation with 2A2N, 67-385 and *S. cerevisiae* (Fig. 4). Although the resistance of 67-385 was relatively constant, the resistance of 2A2N dropped significantly on entering the stationary phase.

We examined whether catalase activity differed in 67-385 and 2A2N as a function of culture age. In both strains, an age dependence was found in which catalase activity increased from approximately 5 U (mg protein)⁻¹ during the exponential phase to approximately 15 U mg⁻¹ in the stationary phase (Fig. 5). The de-repression of catalase activity was relatively small compared to that observed in *S. cerevisiae* (Fig. 5).

Fig. 5. Effect of culture age on catalase activity in *P. rhodozyma* strains 67-385 (●) and 2A2N (□) and *S. cerevisiae* (○) grown in YM broth.

Table 3. Effect of H_2O_2 on carotenogenesis and growth in *P. rhodozyma* 67-835

Results are means $\pm$ SD.

Time(s) of addition of 2 mM- H_2O_2 (h)	Carotenoid ($\mu g\ g^{-1}$)	Growth ($mg\ ml^{-1}$)	Productivity* ($\mu g\ ml^{-1}$)
Not added	428 $\pm$ 3	4.9 $\pm$ 0.1	2.11 $\pm$ 0.04
0	518 $\pm$ 39	4.7 $\pm$ 0.4	2.41 $\pm$ 0.04
24	482 $\pm$ 29	4.8 $\pm$ 0.3	2.31 $\pm$ 0.03
0 + 24	461 $\pm$ 5	5.5 $\pm$ 0.2	2.55 $\pm$ 0.11

*Productivity = Growth $\times$ Carotenoid

S. cerevisiae was also found to have significantly higher catalase activity than *P. rhodozyma* throughout growth, particularly after 5 d (Fig. 5). The sensitivity of *S. cerevisiae* to H_2O_2 was intermediate relative to the *P. rhodozyma* strains examined. These data suggest that catalase activity could not explain the pattern of sensitivity to H_2O_2 in *P. rhodozyma*. In contrast, H_2O_2 sensitivity in *S. cerevisiae* correlated with catalase activity and is probably attributable to the activity of this enzyme.

Since 2A2N had higher resistance to H_2O_2 than 67-385 in young cultures, a positive selection for increased H_2O_2 resistance was attempted with 67-385. This protocol involved successively subculturing 67-385 every 48 h in YM media and exposing them to various H_2O_2 challenges. This protocol gave colonies with increased pigmentation (10–20% increase in productivity after 5 passages) (Table 3). These results support the concept that carotenoids are providing a protective antioxidant role in *P. rhodozyma*.

Discussion

Carotenoid formation in *P. rhodozyma* is stimulated by antimycin and light (An & Johnson, 1990). Many light-derepressible genes, including those regulating carotenogenesis in fungi, require molecular oxygen and a photosensitive pigment in addition to blue light for activation. Generally, these genes are induced when conditions favour the formation of activated oxygen species (Hoover & Phinney, 1988). From the results of the present study, it seems plausible that light-regulated carotenoid biosynthesis in *P. rhodozyma* could be induced by a mechanism involving activated oxygen species.

We have found that carotenoid content in *P. rhodozyma* changes in conditions that would alter the level of intracellular activated oxygen species. The mechanism by which these species affect carotenoid levels is not presently known. It is possible that active oxygen species inhibit or destroy biosynthetic enzymes that utilize acetyl-CoA or other common precursors which are needed for carotenoid synthesis. Alternatively, they may induce, activate, or serve as substrates for enzymes involved in carotenoid formation. *P. rhodozyma* produces several oxygenated carotenoids, including DCD and HDCO, and these were postulated as intermediates in the biosynthesis of astaxanthin by two pathways (Johnson & An, 1991). However, we have recently found that incubating washed *P. rhodozyma* cells in buffer with H_2O_2 resulted in the formation of DCD and an unidentified oxygenated carotenoid, and a corresponding decrease in astaxanthin and HDCO (data not shown). These results suggest that certain carotenoids, including some of those previously postulated as biosynthetic intermediates, may actually be oxidative degradation products. Our laboratory is investigating this further.

In contrast to certain other yeasts including *S. cerevisiae*, *P. rhodozyma* appears to contain only Mn-SOD and lacks cytosolic Cu/Zn-SOD and Fe-SOD. The related yeast *Rhodotorula mucilaginosa* also possesses only Mn-SOD, which is located exclusively in the mitochondria (Moore *et al.*, 1989). In *S. cerevisiae*, deletion of a gene encoding SOD made the yeast hypersensitive to oxygen (Van Loon *et al.*, 1986). Owing to the absence of cytosolic SOD in *P. rhodozyma*, carotenoids may compensate for Cu/Zn-SOD and Fe-SOD in providing antioxidant protection.

Carotenoids may also serve their antioxidant role by reacting directly with peroxyl radicals (Kennedy & Liebler, 1992; Krinsky, 1992), providing antioxidant protection in the cytoplasm and in lipid membranes. We have found that *P. rhodozyma* contains relatively low catalase activity compared to *S. cerevisiae*, particularly

in the stationary phase. *P. rhodozyma* underwent marked changes in resistance to O_2^- and H_2O_2 during transition to stationary phase, the period when astaxanthin production is at its highest. These aging cells may have a high demand for antioxidants to sustain viability and astaxanthin has been postulated to be a more effective antioxidant than β -carotene (Terao, 1989). Studies in our laboratory have shown that respiration shifts from cyanide-sensitive to cyanide-insensitive as *P. rhodozyma* enters into stationary phase. Cyanide-insensitive respiration has been associated with the production of superoxide radicals (Sohal *et al.*, 1988). In *Nicotiana tabacum*, it has been suggested that the intracellular ratio of O_2^- to H_2O_2 is the major determinant of the degree of intracellular oxidative damage. The presence of the two oxygen species will allow the Haber–Weiss reaction to occur in the presence of transition metals such as iron or copper with the consequent formation of hydroxyl radicals (Bowler *et al.*, 1991):

Control of the balance of the two oxygen species in the various compartments of the eukaryotic cell appears to be particularly important in preventing oxidative damage.

Carotenoids and O_2^- do not directly react with each other without the involvement of a more reactive oxygen intermediate such as hydroxyl radical (Krinsky, 1992). In the present study, the intracellular levels of carotenoids were increased by the O_2^- -generator duroquinone. Preliminary data indicate that H_2O_2 reacts with carotenoids giving oxidative degradation products. This suggests a mechanism for antioxidant protection by carotenoids in *P. rhodozyma*. O_2^- induces the formation of carotenoids which are then available to react with H_2O_2 , altering the ratio of O_2^- to H_2O_2 and minimizing the production of $OH^\cdot$. In summary, carotenoids produced in *P. rhodozyma* may function, together with SOD and catalase, to preserve viability in the face of continued production of oxygen radicals in ageing cells.

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