Torularhodin as a Potent Scavenger against Peroxyl Radicals Isolated from a Soil Yeast, *Rhodotorula glutinis*

Hideyuki SAKAKI,1,* Tatsuya NAKANISHI,1 Sadao KOMEMUSHI,1
Koshi NAMIKAWA,2 and Wataru MIKI3

1Department of Agricultural Chemistry, Faculty of Agriculture, Kinki University, Nara 631–8505, Japan
2Institute for Fundamental Research and 3Research Institute for New Product Development, Suntory Limited, Wakayamadai, Shimamato-cho, Osaka 618–8503, Japan

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**Summary** A soil yeast *Rhodotorula glutinis* increased torularhodin, that is one of the final products of carotenoid biosynthesis, when loading oxygen stress. In addition, a mutant of this yeast, which produces torularhodin more than β-carotene, gained resistance to oxygen stress. In this study, changes in carotenoids biosynthesis due to addition of 2,2’-azobis(2-amidinopropane)-dihydrochloride (AAPH) as a peroxyl radicals generator were examined, and scavenging peroxyl radicals by torularhodin was also compared with that by β-carotene. When 0.01 mM AAPH was added to the culture medium, the amount of carotenoids in yeast was increased. With increase in load by AAPH addition, torularhodin was consumed faster than β-carotene. After torularhodin decreased to the same concentration as AAPH free condition, β-carotene began to decrease. These results suggest that torularhodin was important in defense against oxidation loading by peroxyl radicals in *R. glutinis*. Scavenging of peroxyl radicals by torularhodin was evaluated using electron spin resonance. In the reaction of cumene hydroperoxide and 5,10,15,20-tetraphenyl-21H, 23H-prophine iron (III) [TPP-Fe(III)], torularhodin exhibited concentration-dependent inhibition and its activity reached to 60% at 2.5 mM. On the other hand, α-tocopherol exhibited strong activity at low concentrations with a maximum at 0.25 mM, but its activity decreased above this concentration. Scavenging activity by torularhodin was greater than that of α-tocopherol at 2.5 mM. In the pyrolysis of 2,2’-azobisisobutyronitrile (AIBN), both torularhodin and α-tocopherol exhibited concentration-dependent inhibition, but the scavenging activity...
of \(\alpha\)-tocopherol was stronger. In addition, torularhodin inhibited lipid peroxide formation of rat brain homogenate in a concentration-dependent manner, and its inhibitory effect was stronger than that of \(\alpha\)-tocopherol at concentration of 1 \(\mu\)M and above. Torularhodin thus appears to be an effective scavenger of peroxyl radicals and to have strong antiperoxidative activity. The strong inhibition of lipid peroxidation by torularhodin at low concentration suggests that torularhodin has other activities in addition to peroxyl radical scavenging. \(R.\ glutinis\) tends to increase the production of torularhodin by load oxygen stress, but this was because of its strong peroxyl radicals scavenging.

**Key Words:** torularhodin, *Rhodotorula glutinis*, peroxyl radicals, lipid peroxidation

Carotenoids, classified as terpenoids, are mainly C\(_{40}\) tetraterpen, and it mainly distributes to the body of fish and vegetable, and microorganism. Carotenoids take color with prolongation of conjugated polyene chain, and \(\beta\)-carotene, representative carotenoid, shows a yellow color. Over 700 kinds of carotenoids have been detected in nature besides \(\beta\)-carotene. It became clear that various functions are in carotenoids biologically. Effect of decreasing risk of tumor formation has been found in epidemiologic study \([1]\). Much attention has therefore been focused on research on the biological activity of \(\beta\)-carotene, but a large-scale clinical study revealed no evidence of antitumor activity of \(\beta\)-carotene \([2]\). This indicated that \(\beta\)-carotene is not an epoch-making drug for human health. On the other hand, \(\alpha\)-carotene was shown to more effectively prevent carcinogenesis than \(\beta\)-carotene in an *in vivo* study \([3]\), and strong \(\alpha\)-tocopherol like activity was reported in astaxanthin that classified into highly oxidized xanthophyll \([4]\). Thus, active research has been carried out on carotenoids.

Major functions have been indicated in fungi and non-photosynthetic bacteria, which clearly lack the antenna function of carotenoids. Schrott \([5]\) proposed that carotenoids protect against active oxygen species, mainly singlet oxygen ('\(O_2^*\)') produced under irradiation of strong sunlight. Several studies have reported on the role of carotenoids in yeast in the past few years \([6, 7]\). Prevention of oxygen stress was clearly demonstrated by results showing increase in carotenoid content by oxygen stress loading \([6]\). This study, however, did not evaluate the fluctuation of individual carotenoid although yeast accumulated multiple carotenoids as final products. Thus, difference of contribution of each carotenoid was not defined. We sought to examine the role of a characteristic carotenoid carboxylate, torularhodin, which is a chemical indicator of *Rhodotorula* sp.

It has been suggested that \(R.\ glutinis\), a soil yeast that accumulates torularhodin and \(\beta\)-carotene as final products \([8]\), regulates biosynthesis of carotenoids by increasing production of torularhodin based on increase in dissolved oxygen of

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cultivative medium [9]. In addition, a mutant strain with increased torularhodin productivity exhibited resistance against oxygen loading caused by addition of methylene blue, a singlet oxygen generator, to culture medium and proliferated better than the mother strain or another mutant with increased $\beta$-carotene productivity [10]. These results suggested that R. glutinis produced more torularhodin against oxygen loading than $\beta$-carotene. It can therefore be presumed that torularhodin defenses against active oxygen species in yeast cells. In this study, carotenoid composition was analyzed after addition of peroxyl radicals generator to culture medium. In addition, peroxyl radicals, main chain carrying species in lipid peroxidation, scavenging activity of torularhodin was examined. Inhibitory effect on lipid peroxidation was also compared with that of $\beta$-carotene, which was reported to be a strong peroxyl radical scavenger [11-13]. Finally biological significance of torularhodin in red yeast R. glutinis was estimated based on the results obtained.

MATERIALS AND METHODS

Changes in carotenoid content in yeast with oxygen stress loading. R. glutinis No. 21, which was isolated from soil [14], was used. A glucose peptone broth (Nihonseiyaku Co., Ltd., Tokyo, GP medium) was used for yeast cultivation. A 500-ml flask containing 100 ml of GP medium was inoculated with yeast cells washed with phosphate-buffered saline (pH 5.6) and then incubated for 3 to 5 days at 30°C with reciprocal shaking at 120 rpm with a 5 cm span. 2,2'-Azobis(2-amidinopropane)-dihydrochloride (AAPH, Wako Pure Chemical Industries Ltd., Osaka) was added as a generator of peroxyl radicals at various concentrations from 0.01 to 1 mM to the GP medium. Yeast growth was monitored by optical density at 610 nm. Carotenoids were isolated and analyzed quantitatively as described previously [9].

Electron spin resonance (ESR) spectrometry. Two reaction systems were used for generation of peroxyl radicals [15]. The first system used cumene hydroperoxide (CHP) with 5,10,15,20-tetraphenyl-21$H$, 23$H$-prophine iron (III) chloride [TPP-Fe (III)] (exp. 1). Fifty microliters of 0.1 mM TPP-Fe(III), 50 $\mu$L of 0.2 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap reagent, 50 $\mu$L of test substances dissolved in dimethyl sulfoxide (DMSO) and 0.4 mM CHP were placed in a test tube and mixed using an automatic mixer. Exactly 7 min after, the spin adducts formed by the reaction of peroxyl radicals were recorded by ESR spectrometry (JES FR-30, JOEL, Tokyo). The second system used pyrolysis of 2,2'-azobisobutyronitrile (AIBN) (exp. 2). Fifty microliters of 0.2 M AIBN, 50 $\mu$L of 0.2 M N-tert-butyl-$\alpha$-phenylnitrone (PBN) as a spin trap reagent, and 50 $\mu$L of test substances dissolved in DMSO were placed in a test tube and mixed using an automatic mixer. After heating at 50°C for 10 min, recording of spin adducts was started using ESR spectrometry. Conditions for ESR spectrometry were as follows: magnetic field, 335.5 mT; power, 8.0 mW; response time, 0.10 s; modulation, 0.2
mT; temperature, 298 K; sweep time, 1.0 min. Manganese oxide was used as an internal standard. Scavenging activity was calculated as the ratio to blank sample that did not contain test compound.

**Lipid peroxidation of rat brain homogenate.** The brain was isolated from euthanized Sprague Dawley rats at 4 weeks age and stripped of its meninges. Five grams of brain was homogenized in 30 ml phosphate buffered saline (pH 7.4) and supernatant was obtained by centrifugation (750 x g, 10 min). Supernatant was diluted to one-fifth with the same buffer. Ten microliters of each test substance such as carotenoid and α-tocopherol in DMSO were added to 990 μl of diluted supernatant in container. Containers were transferred to a 37°C water bath and incubated continuously for 1 h after addition of 0.02 mM FeCl₂, (final concentration). Malondialdehyde (MDA) was quantified using the calibration curve from absorbance at 532 nm after thiobarbituric acid reaction [16].

The torularhodin used in these studies was isolated and purified from *R. glutinis* No. 21 [10]. d,l-α-Tocopherol and β-carotene were purchased from Wako Pure Chemical Industries as comparative controls. Addition of DMSO without test substances was performed as a negative control.

**RESULTS**

*Changes in carotenoid content in yeast with oxygen stress loading*

The growth of *R. glutinis* in AAPH was similar to that in the control group.

![Graph showing changes in carotenoid content in yeast with oxygen stress loading](image)

**Fig. 1.** Growth of *R. glutinis* No. 21 and change in content of major carotenoids depending upon AAPH concentrations. The yeast cells were harvested at 72 h after inoculation, washed and then disrupted in a supersonic generator. HPLC system was used for isolation and quantitative analysis of carotenoids. Growth was represented by optical density at 48 h after inoculation.

up to a concentration of 0.1 mM, but low cell density at 48 h after inoculation was observed at 0.5 mM or higher (Fig. 1). And remarkable growth inhibition was noted at 1 mM. When AAPH was added to the culture medium at the concentration of 0.01 mM, the amount of carotenoids in yeast was increased. With increase in load by AAPH addition, torularhodin was consumed faster than \( \beta \)-carotene. After torularhodin decreased to the same concentration as AAPH free condition, \( \beta \)-carotene began to decrease.

Peroxyl radical scavenging activity

The radical scavenging activities of each compound obtained at different concentrations in exp. 1 are shown in Fig. 2. Torularhodin in the range of 0.0025 to 2.5 mM exhibited concentration-dependent scavenging activity and it reached to 60% at 2.5 mM. In respect of \( \beta \)-carotene, the elevation of the activity was observed only at 2.5 mM. The activity of torularhodin was stronger than that of \( \beta \)-carotene at all concentrations tested. On the other hand, \( \alpha \)-tocopherol exhibited concentration-dependent scavenging activity up to 0.25 mM, and its activity was clearly stronger than that of \( \beta \)-carotene or torularhodin. However, at concentration above 0.25 mM, the activity of \( \alpha \)-tocopherol was decreased, and it was weaker than that of torularhodin at 2.5 mM.

In exp. 2 (Fig. 3), \( \alpha \)-tocopherol exhibited concentration-dependent scavenging activity. At concentrations above 0.25 mM, scavenging activity of torularhodin was clearly elevated. However, \( \alpha \)-tocopherol exhibited stronger scavenging activity than did torularhodin at the same concentration. Scavenging by \( \beta \)-carotene against
peroxyl radicals was not found in the tested concentration range in exp. 2.

The results of these two experiments indicated that torularhodin exhibited peroxyl radicals scavenging activity regardless of the generating system used, and its activity was stronger than that of \( \alpha \)-tocopherol at high concentration particularly in exp. 1.

**Inhibition of lipid peroxidation of rat brain homogenate**

Each compound exhibited concentration-dependent inhibition of lipid perox-

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idation in the range of 0.1–10 μM (Table 1). Of the compounds tested, torularhodin exhibited the strongest inhibition, and its inhibitory activity was stronger than those of α-tocopherol and β-carotene at concentrations above 1 μM. At 0.1 μM, no significant difference was observed among 3 compounds. At 1 μM or lower, β-carotene had almost the same inhibitory activity as α-tocopherol, whereas at 10 μM its activity was weaker than that of α-tocopherol. Of the 3 compounds tested in this study, the inhibitory activity of β-carotene was weakest.

DISCUSSION

The biological function of carotenoid was actively researched after it became clear that there is strong antioxidative activity [3, 4, 17–19]. Yeast carotenoids also turned out to play an important role against oxidative stress [6]. However, an individual evaluation was not examined though yeast synthesizes plural kind of carotenoids as final products. Torularhodin is a carotenoid that is classified to the carboxyl group, and is isolated mainly from the fungi and a part of bacteria. R. glutinis, a yeast accumulates torularhodin abundantly, varied system of carotenoid biosynthesis and increased torularhodin productivity by oxygen stress [9, 10]. These findings suggested that torularhodin plays a more important role in defensive against oxygen stress than other carotenoids in yeast. Hence, the antioxidative properties of torularhodin were compared with β-carotene. And we sought to estimate the biological function in yeast of torularhodin.

Peroxyl radical is most dominants radical in the process of lipid peroxide formation. And the chain reaction ends with formation of non-radicals owing to collision of peroxyl radicals with one another or scavenging by antioxidants. Therefore, evaluation of the potency of scavenging of peroxyl radicals is very important in evaluation of lipid oxidation suppression.

The total carotenoids increased at the concentration of the AAPH, peroxyl radical generator, with which yeast growth is not inhibited. R. glutinis consumed torularhodin than β-carotene with increase in loading by AAPH. But it cannot be declared that peroxyl radicals are main factor. Therefore, we examine scavenging of peroxyl radicals by carotenoids biosynthesized by R. glutinis. Although scavenging activity was not measured directly so far, Namikawa et al. developed a novel procedure using ESR for detection of peroxyl radicals generated by the CHP-TPP-Fe(III) reaction and pyrolysis of AIBN [15]. This has enabled determination of the peroxyl radicals scavenging activity of highly lipophilic antioxidants. In this method of direct measurement, torularhodin exhibited scavenging activity equivalent or superior to that of α-tocopherol, although the activity measured differed depending on the peroxyl radicals generator system used. The activity of torularhodin never surpassed that of α-tocopherol at any concentration in the system with pyrolysis of AIBN. This may be attributed to the heat stability of carotenoids. In the reaction of CHP and the TPP-Fe(III) system, activity of α-tocopherol decreases at high concentration. It was reported that α-tocopherol became proox-
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There are a few reports on scavenging of peroxyl radicals by β-carotene, and partial oxygen pressure strongly affected this [17]. Scavenging activity of β-carotene was not detected under the present experimental conditions.

When torularhodin was added to rat brain homogenate, concentration-dependent suppression of MDA formation was observed, and the effect of torularhodin was stronger than those of α-tocopherol and β-carotene. It is thought that scavenging of peroxyl radicals by β-carotene is the main cause of suppression of the lipid peroxidation [17], and strong activity is related to stabilizing of hydroperoxide in α-tocopherol [21]. Torularhodin showed strong activity compared with these compounds against lipid peroxide formation. The fact that torularhodin inhibited peroxide formation more effectively than α-tocopherol or β-carotene is very interesting, considering the deep involvement of peroxyl radicals in the chain reaction of lipid peroxidation [22].

Although torularhodin exhibited potency in scavenging peroxyl radicals similar to that of α-tocopherol, it exhibited marked difference from it in suppression of lipid peroxide formation in rat brain homogenate. This finding suggested that torularhodin may contribute to the suppression of peroxide formation by an activity in addition to scavenging of peroxyl radicals. To explain the above results, more examination is needed.

It has been demonstrated that antioxidative activities are due to capturing or scavenging of active oxygen species by carotenoids, and their relation to structure has been discussed [23-25]. There are two pathways of biosynthesis of carotenoids by yeast in Rhodotorula sp., which are known to produce carotenoids. One is from γ-carotene to β-carotene accompanying cyclization of β-ionone ring, and the other is to torularhodin formation through desaturation and carboxylation [8]. Since torularhodin is not cyclized in the process of biosynthesis from β-carotene, the carbon chain that contributes to the stability of radicals appears to be long. It can therefore be presumed that the difference in activity between torularhodin and β-carotene was related to the difference between them in length of carbon chain.

*R. glutinis* increased production of torularhodin upon excessive aeration of culture medium [9]. In addition, a high-torularhodin-producing mutant (TL/21) showed resistance to oxygen stress higher than another strain [10]. Lactate dehydrogenase activity in medium of low resistance strain was high as compared with that of medium cultured with TL/21. This indicated that the yeast membrane has received the damage by oxygen stress. Aeration and singlet oxygen generated by methylene blue addition are considered to be an initiator on the process of damage of yeast cells. It is reasonable to assume that a dominant factor is peroxyl radicals because cell membrane is rich in lipid. Although it is important to remove initiator to prevent growth inhibition of yeast, the deletion of peroxyl radicals becomes important in the stop of the chain reaction begun. As a result of examination which used ESR, torularhodin was found to be a strong scavenger of peroxyl radicals than β-carotene at all concentrations tested. It thus appears that torula-

rhodin was biosynthesized to preserve yeast from oxygen stress. Further, the results that cannot be explained only by difference of the scavenging activity of peroxyl radicals was obtained from experiment of lipid peroxide formation. This result suggested that torularhodin have another effective activity or biological property such as affinity for the membrane.

In conclusion, torularhodin is an effective scavenger against peroxyl radicals, and appears to play an important role in protecting against injury by oxygen stress in R. glutinis. Further investigation of this carotenoid is expected.

REFERENCES


