0273-2289/97/6603—0239$10.00

# Enzymatic Screening of Microalgae as a Potential Source of Natural Antioxidants

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Received September 12, 1996; Accepted December 2, 1996

## ABSTRACT

Extracts of over 100 species of microalgae both from nature and from laboratory cultures were screened for antioxidant activity. As an assay for antioxidant function, we examined the inhibition of the activity of two oxidizing enzymes, lipoxygenase and tyrosinase. Water, ethanol, and methanol extracts of microalgae were used for the assay. It was found that lipoxygenase and tyrosinase activities were inhibited by the extract of several microalgae. Our results suggest that these species of microalgae have useful antioxidant activity, and they will be further examined for potential biotechnological exploitation. The aim of our screening was to identify potential sources of natural antioxidant compounds, which if found to be sufficiently potent and nontoxic, might eventually replace the chemicals that are currently used as food additives and cosmetics, in order to prevent food spoilage and their oxidation.

**Index Entries:** Lipoxygenase; tyrosinase; inhibition; antioxidant activity; screening; microalgae.

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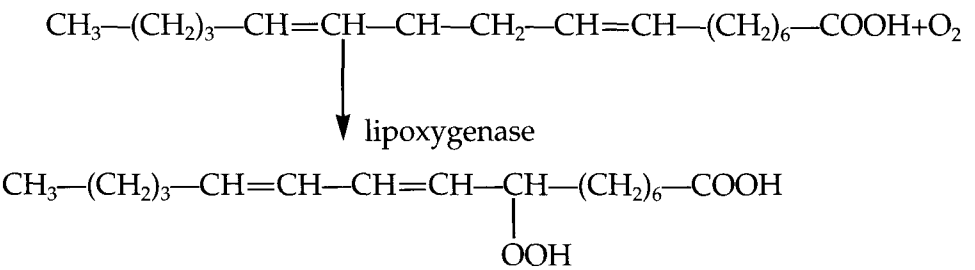
## INTRODUCTION

Like all plants, microalgae (phytoplankton) are capable, in the process of photosynthesis, of using the energy of sunlight to split water into protons, electrons, and oxygen. In that process, the photoactivated electrons and the protons reduce carbon dioxide ($CO_2$) to carbohydrate through a series of enzymatic reactions, whereas the oxygen is released. Energy is harvested through the absorption of light by arrays of photosynthetic pigments, such as the chlorophylls and carotenoids, and in the case of the Rhodophyta and the Cyanophyta, phycobiliproteins as well. These pigments, linked with light-harvesting proteins, form the light-harvesting "antennae." These, together with other components of the photosynthetic apparatus, such as the reaction centers of both Photosystem II (PSII) and Photosystem I (PSI), and the manganese-containing oxygen evolving complexes are embedded in the thylakoid membranes. The primary $CO_2$-assimilating enzyme RUBISCO, and other enzymes and electron carriers are located outside these membranes, either within the chloroplasts or, in the noncompartmentalized prokaryotic cyanophytes, in the cell lumen. $H_2O$ (in some cases $H_2S$) and oxygen serve as the electron donor and electron acceptor, respectively. The combination of high light intensity and high concentrations of oxygen is harmful, as evidenced by the photo-oxidative death of oxygen-producing cyanobacteria and algae during exposure to intense light *(1–3)*. This damage occurs via the in vivo oxygenation of a susceptible molecule by active oxygen, or other free radicals generated under such conditions. Such target molecules are the polyunsaturated fatty acids that are abundant structural components of the thylakoid membranes *(4)*, as well as the 32-kDa protein in PSII *(5)* involved in the onset of photoinhibition. In order to protect living cells from these potentially lethal oxidizing agents, different protective mechanisms have evolved in the course of the evolution of the atmosphere of our planet from its initial reducing composition to the present oxidizing one. It is well known that superoxide dismutase catalyzes superoxide degradation in aerobic bacteria *(6)* and other organisms *(7)*. The absence of such damage in microalgae, in spite of the proximity of the photosynthetically produced oxygen and the suitable targets within the photosynthetic apparatus, suggests that protective, antioxidative mechanisms and compounds exist in these cells. These maintain an acceptably low concentration of oxygen and other harmful agents, thereby preventing the deleterious oxygenation of target molecules. This reasoning has motivated studies on the occurrences of natural antioxidant compounds in microalgae. Borowitzka and Borowitzka *(8)* and Ben-Amots and Avron *(9)* have reported high light-induced β-carotene production by the halophilic green algae *Dunaliella salina*. That pigment has been shown to act both as an antioxidant, since it itself undergoes oxidation, thereby sparing other

sensitive and vital compounds. In addition, β-carotene acts as a shield, reducing the intensity of the short-wave components of the solar flux, including UV-A and blue light. This knowledge has been applied in the commercial production of β-carotene by that microalgae, as described by Grobbelaar *(10)* and Ben-Amotz *(11)*. Open pond production of the filamentous cyanobacterium *Spirulina platensis* for biomass *(12)* and of the chlorophyte *Haematococcus pluvialis* for the valuable red pigment astaxanthin *(13, 14)* has also been attained. All of these algae are cultured under conditions of intense light and oxygen supersaturation. It was this ability of microalgae to thrive under such extremely oxidizing conditions that led us to focus our investigation on identifying potential antioxidant activity in microalgae.

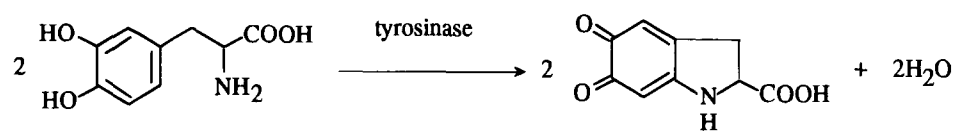
In this study, we report the screening of microalgae for antioxidant activity. As an assay for antioxidant function, we examined the inhibition of the activity of two oxidizing enzymes, lipoxygenase (EC 1.13.1.12) and tyrosinase (EC 1.14.18.1). Lipoxygenase is known to catalyze the oxidation of unsaturated fatty acids and their esters to produce hydroperoxides (15). The enzymatic reactions of lipoxigenase is shown in Eq. (1).

$$\text{linoleic acid} + O_2 \xrightarrow{\text{lipoxygenase}} \text{hydroperoxides} \qquad (1)$$




Tyrosinase catalyzes reactions involved in the synthesis of melanin through dopachrome from tyrosine. The enzymatic reactions of tyrosinase are shown in Eq. 2.

$$2\ \text{dopa} + O_2 \xrightarrow{\text{tyrosinase}} 2\ \text{dopachrome} + 2\ H_2O \qquad (2)$$




The antioxidant activity of microalgae extracts was screened using the inhibition of the activities of these two enzymes.

## MATERIALS AND METHODS

### Enzyme and Chemicals

Soybean lipoxygenase was obtained from Funakoshi Co. (Tokyo, Japan). Tyrosinase, linoleic acid, and DOPA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

### Preparation of Microalgal Extract

Over 100 species of microalgae were collected from hot springs and ponds in Japan. From the collected species, some were isolated and brought into culture in suitable media. The microalgae were separated from the medium by centrifugation at 3000 rpm for 10 min. An equal volume of distilled water with culture medium was added to wash the solid algal pellets. The algae were resuspended and centrifuged at the same conditions. A volume of solvent weighing 5–10 times the sample weight was added to the pellets. The sample was then homogenized in a sonicator (type UCD-200T, Tosho Denki Co., LTD. Yokohama, Japan) for 30 min at 30-s intervals. The homogenate was then centrifuged at 3000 rpm for 10 min, and the supernatant was used as the algal extract. Extracts in water, ethanol, and methanol solvents were collected, providing water, ethanol, and methanol extracts, respectively. These were prepared by extracting microalgae first with water and then ethanol and last methanol. These extracts of microalgae were used for assay of both lipoxygenase and tyrosinase activities.

### Determination of Lipoxygenase Activity

The assay of lipoxygenase activity was carried out using the method of Ben-Aziz et al. *(16)*. The reaction mixture for the assay of lipoxygenase activity contained 0.2*M* citrate-phosphate buffer, pH 9.0, 0.25% Tween 20, 0.125 m*M* linoleic acid, an enzyme solution (57 μg protein), and 10 μL of microalgal extract. As a reference, the same volume of water, ethanol, or methanol was used instead of the extract from microalgae in 1 mL of total volume. The enzyme reaction was carried out in the cuvet of a Hitachi U-3210 spectrophotometer at 234 nm, and recorded until the reaction rate reached a steady-state plateau. That wavelength is the peak of absorption for the hydroperoxides generated by the action of the lipoxygenase on linoleic acid, with the uptake of oxygen. The inhibition was defined as the ratio of the rate of increase of $OD_{234}$ in the absence of algal extract in the control, to that measured with the sample. The results given here are mean values of two separate experiments.

### Determination of Tyrosinase Activity

The assay of tyrosinase activity was carried out by microplate method. The reaction mixture for the assay of tyrosinase activity con-

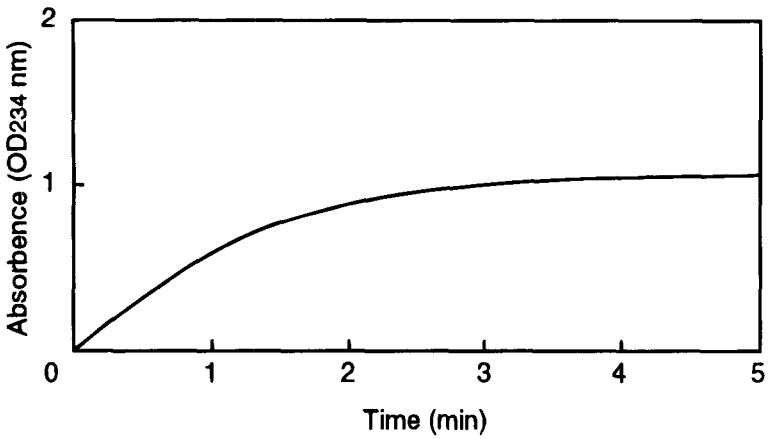



Fig. 1. Time-course of lipoxygenase activity. Assay method of the enzyme reaction was described in Materials and Methods.

tained 7 m*M* Tris-HCl buffer, pH 7.5, 1 m*M* L-DOPA, an enzyme solution (67 μg protein), and 10 μL of microalgal extract in each well in 150 μL total volume. The reaction was carried out for 5 min in the well on the microplate of a Toso MPR-A4i. Activity was determined from absorbence at the wavelength of 475 nm. That wavelength is the peak of absorption for the adrenochrome generated by the action of the tyrosinase on DOPA. As a reference, 10 μL of water, ethanol, or methanol were used instead of the extract from microalgae. The inhibition was defined as the ratio of the rate of increase of $OD_{475}$ in the absence of algal extract to that measured with the sample. The results given here are mean values of three separate experiments.

## RESULTS AND DISCUSSION

### Inhibition of Lipoxygenase Activity

The antioxidant activity of the microalgal extracts was examined by systematically adding the extract of about 100 species of microalgae to the lipoxygenase-catalyzed linoleic acid reaction mixture. Figure 1 shows the time-course of lipoxygenase activity. The reaction rate in the enzyme activities reached a steady-state plateau after an elapse time of 3–5 min. Therefore, the enzyme reaction was recorded for 5 min of incubation time.

Figure 2 shows the percentage inhibition of lipoxygenase activity of various microalgae compared with solvent as the 100% control. Several microalgae samples from hot springs and ponds showed lipoxygenase inhibition activity in both water and ethanol extracts. Hkh3 and Hkh5 showed 68 and 26% inhibition in lipoxygenase activity in water extract, respectively. Both also showed lipoxygenase inhibition, 99 and 72%, in the

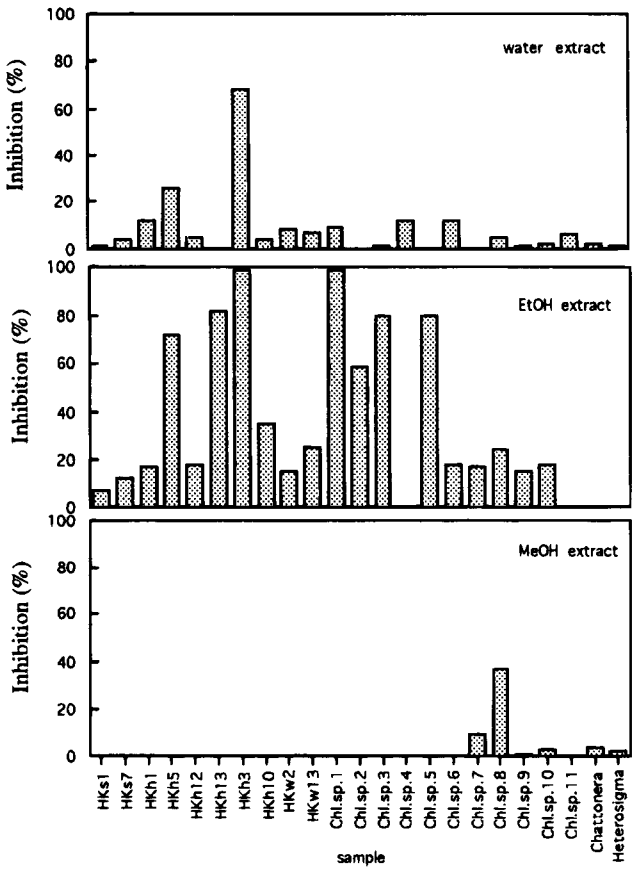



Fig. 2. Inhibition of lipoxygenase by extracts from microalgae. Enzyme activity was assayed as described under Materials and Methods.

ethanol extract. In the case of *Chlorella* sp., the ethanol extracts were observed to be more effective compared to water extracts in inhibiting lipoxygenase activity. Seven samples extracted in ethanol showed more than 50% inhibition in lipoxygenase activity. The most effective microalgae were *Chlorella* sp. 1 and Hkh3 in ethanol extract. *Chlorella* sp. 8 showed lipoxygenase inhibition in the methanol extract. It has been reported that lipoxygenase was inhibited by several flavonoids, such as quercetin *(17)*. It is unclear whether any of the microalgae examined by us contains flavonoids. Inhibition of lipoxygenase by the extract of microalgae suggested the existence, in these algae, of a cellular mechanisms protecting them from oxidation in vivo.

## Inhibition of Tyrosinase Activity

Figure 3 shows inhibition of tyrosinase activity by extracts from microalgae. A few extracts in water and ethanol showed inhibition of

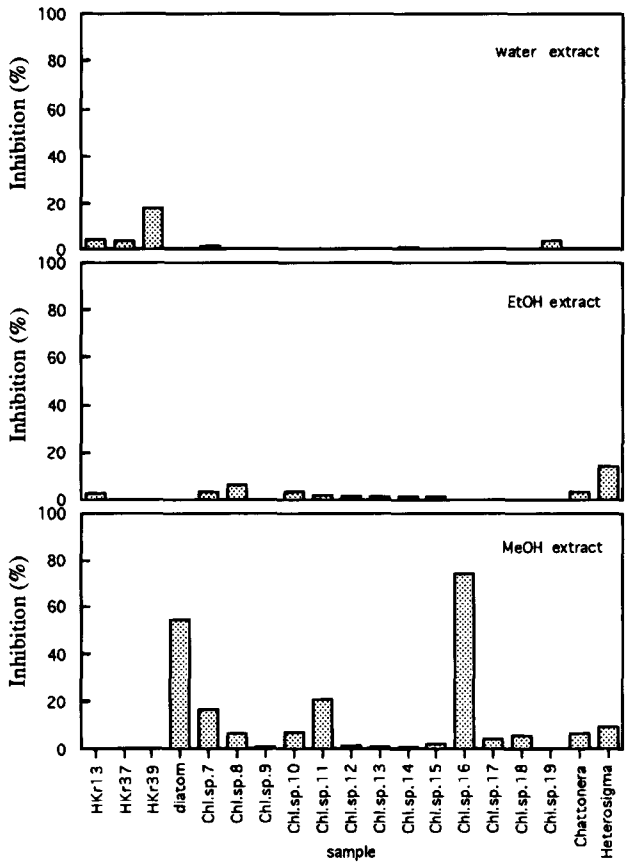



Fig. 3. Inhibition of tyrosinase by extracts from microalgae. Enzyme activity was assayed as described under Materials and Methods.

tyrosinase at low activity. Water extract of HKr39 showed about 20% inhibition of tyrosinase. This species showed no activity of tyrosinase inhibition by either ethanol or methanol extract. This fresh water strain was identified as *Synechococcus* sp. Wachi et al. *(18)* have reported that an aqueous extract of marine *Synechococcus* also showed tyrosinase inhibition. Two samples of methanol extract showed high activity of the enzyme inhibition. *Chlorella* sp. 16 and diatom showed 75 and 55% inhibition in tyrosinase activity, respectively. Methanol extracts of other *Chlorella* sp. slightly inhibited tyrosinase activity. These results suggest that the methanol extract of *Chlorella* sp. contains active antioxidant components.

*Chlorella* sp. is well known to contain carotenoids, which are β-carotene, astaxanthin, and lutein. Those are proposed to have antioxidant activity. We have found the presence of those carotenoids in *Chlorella* sp, which had the activity of the enzyme inhibition. Lutein and α- and β-carotenes are monitored at 450 nm and tocopherol at 290 nm in methanol extracts in some *Chlorella* species. We also measured the UV

absorption spectra of the water extract of algal species. Maximum absorbance of UV in the water extract of some algae, which showed activity for lipoxygenase inhibition, is shown at 280 and 340 nm. These results suggest the possibility of the existence of water-soluble antioxidants, except carotenoids.

We are now conducting detailed studies on the algal species that showed significant antioxidant activity.

## CONCLUSIONS

The ethanol extract was superior to those of water and methanol extracts in the screening of microalgae for antioxidant activity by inhibition of lipoxygenase activity. The methanol extract showed the highest activity of tyrosinase inhibition. These results suggested that ethanol and methanol are useful solvents to screen the microalgae for antioxidant by the inhibition of lipoxygenase and tyrosinase activity, respectively. Several species of microalgae have been identified to have useful antioxidant activity. These species of microalgae may be potential sources of natural antioxidant compounds, which could be used as food additives and cosmetics in order to prevent food spoilage and their oxidation.

## ACKNOWLEDGMENTS

This work was supported by Toyo Suisan Kaisha, Japan and a part of a grant from the Esso Gas Foundation in Japan. The authors thank T. Nishida of the University of Florida for critical reading of the manuscript and helpful suggestions.

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