Screening for Antioxygenic Compounds in Marine Algae and Bromophenols as Effective Principles in a Red Alga *Polysiphonia ulceolata*

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As a result of screening for antioxygenic principles in marine algae, the effects of two types of red algae, *Polysiphonia urceolata* and *Gelidium divaricatum*, were found to be clearly superior to those of brown algae.

Bromophenols with 5-bromo-3,4-dihydroxy benzaldehyde (I) as the main constituent were identified as the effective principles in *P. urceolata*.

Two types of bromophenols found in *P. urceolata*, (I) and 5-bromo-3,4-dihydroxy benzyl-alcohol, were synthesized and their antioxidant activities compared with several natural and synthetic antioxidants by the following methods: (1) the oven test, (2) oxygen absorption in emulsion, (3) the Active Oxygen Method, (4) the chemiluminescence method. Both of the bromophenols showed considerable antioxidant activity in these tests at low temperature, however, their effects at higher temperatures were poor.

Previously, one of the authors reported that the phospholipids contained in *Porphyra tenera* showed antioxidant activity.¹⁾ By successive screening tests for antioxygenic principles in marine algae, more than half of them showed those effects.²⁾ Especially, the chloroform-soluble fractions extracted from several species of brown algae, *Eisenia bicyclis* and *Undaria pinnatifida*, showed excellent antioxygenic activities.²⁾

We have continued the screening tests for antioxygenic principles in marine algae, and found that a red alga which belongs to genus *Polysiphonia*, shows a marked antioxidant activity. Further, we determined the chemical structures of the antioxygenic compounds in *Polysiphonia* and compared their activities under various conditions with several natural and synthetic antioxidants.

Materials and Methods

Materials

Nineteen species of marine algae (5 brown algae and 14 red algae) collected at Onagawa and Kitakami (Miyagi Prefecture, Japan) in May were examined. Algae were washed with fresh water, air-dried, pulverized and kept in a freezer.

As the substrate oil for the screening test, the methyl ester of safflower oil, free from unsaponifiables, was used. Its fatty acid composition was

as follows: linoleate 77%, oleate 14%, palmitate and stearate 9%.

Preparation of Algal Extracts for Stability Tests

Samples were extracted from dried algae (5 g) with 50 ml of chloroform/methanol (2:1) a total of three times. Extracts were combined and mixed with water, giving a chloroform-soluble fraction and an aqueous methanol fraction. The chloroform-soluble fraction was treated with acetone and fractionated into acetone-soluble and -insoluble fractions.

Screening for Antioxidant Activities of Algal Extracts

The antioxidant activity of each algal extract was assayed by the oven test according to the method of OLCOTT and EINSETT³⁾ with some modification. An aliquot (5 mg) of the chloroform-soluble fraction was put into a 10-ml beaker; 500 mg of substrate ester were added and well mixed with the sample. Beakers were stored at 45°C, and weighed daily until the induction period had ended and the substrate had attained a weight gain of 10 mg.

Fractionation of Antioxygenic Substances from *Polysiphonia urceolata*

The acetone-soluble fraction of *P. urceolata* was

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fractionated chromatographically on a Sephadex LH-20 (Pharmacia) column. The column was eluted with the mixture of chloroform/methanol, increasing the ratio of methanol in steps (Table 2). The fraction eluted with methanol was analyzed by TLC (Polyamide 11 F254, methanol/water 9:1 v/v) to detect the active principles using the method of SEINO *et al.*⁴¹ The active fraction was also analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) after trimethylsilylation with TMS-BA (Tokyo Kasei). Analyses of TMS derivatives were carried on a JEOL model 1100 equipped with flame ionization detectors and a 2 m, 4 mm i.d. glass column packed with 3% SE-30 on a 80/100 mesh Chromosorb W. Column temperature was programmed from 160–280°C at 3°C/min. Injector and detector temperature were held at 250 and 280°C respectively. The mass spectra (MS) were determined with a Hitachi RMU-6MG. Ionization voltage was 20 eV.

Preparation of 5-Bromo-3,4-dihydroxy Benzaldehyde (BrPhenolCHO), 5-Bromo-3,4-dihydroxy Benzylalcohol (BrPhenolCH₂OH), and N-Vanillyl Octadecanamide

BrPhenolCHO was synthesized from protocatechualdehyde (Tokyo Kasei) by the method described by PSCHORR,⁵¹ and recrystallized from 50% ethanol. BrPhenolCH₂OH was prepared from BrPhenolCHO by reduction with NaBH₄ according to the method of GLOMBITZA *et al.*⁶¹ The synthesized bromophenols gave a single spot on TLC, and their purities were also confirmed by elementary analysis.

N-Vanillyl octadecanamide was synthesized as described previously.⁷¹

Comparison of Antioxidant Activities of Synthetic Bromophenols

Antioxidant activities of synthetic bromophenols were assayed by the following methods, with comparison to butyl hydroxyanisole (BHA), protocatechualdehyde and N-vanillyl octadecanamide.

1) Oven test. As described for the screening test, the length of the induction period of the substrate oil was determined by the method of OLCOTT and EINSTETT.³¹ In this experiment, the stripped corn oil (Eastman Kodak), free from tocopherols, was used in place of the methyl ester of safflower oil used in the screenings.

2) Active Oxygen Method (AOM) The stabilities of lard samples to which antioxidant was added were measured at 97.8°C with constant aeration according to the Tentative Method of the American Oil Chemists' Society (Cd 12-57).

3) Antioxidant activities in emulsion were evaluated by the method described by YAGI⁸¹ with some modification.

One hundred g of stripped corn oil, described above, was emulsified with 300 ml of distilled water and 5 g of Tween 40. Fifty g of the emulsion were put into a 50 ml-erlenmeyer flask with a stopper, and well shaken with an aliquot of antioxidant sample. After addition of 0.5 ml of 1% FeSO₄ solution, the oxygen content in the emulsion kept at 30°C was measured polarographically with a Beckmann Monitor System (model 12301).

4) Chemiluminescence method According to the method of ENDO *et al.*,^{*} antioxidant activity for photosensitized oxidation was evaluated using a chemiluminescence analyzer. Five g of the methyl ester of safflower oil were put into a cell and mixed with 1 mg of antioxidant and 0.5 ml of chlorophyll a solution (3.2 mg/25 ml in acetone/hexane (1:1)). After irradiation with a 500W tungsten lamp at 0°C for 15 min, the emission intensity of the ester was measured for 30 s using an ultra weak chemiluminescence analyzer OX-7 (Tohoku Electronic Industries).

Results and Discussion

Antioxygenic Activities of Marine Algae

In this screening test, only the chloroform-soluble fractions were assayed for antioxidant activity because the activities of the other fractions (aqueous-methanol and residues) had been shown to be very weak.²¹ As a result, contrary to the previous screening,²¹ in which the extracts of brown algae had been more effective than those of red algae, the effects of two species of red algae, *Polysiphonia urceolata* and *Gelidium divaricatum*, were found to be superior. By addition of the extracts of these algae at a 1% level, the induction periods of the substrate was extended to 73 days with *P. urceolata* and 31 days with *G. divaricatum*. Under the same conditions, the induction period of substrate without addition of the extract was 3 days, while to which the extracts of other algae were within the range of 6 to 16 days. The effect of *P. urceolata* was the strongest of all marine

* Y. ENDO, R. USUKI, and T. KANEDA: Abstracts of 28th Annual Meeting of the Japan. Soc. Food Technol., Fukuoka, p. 33, 1981.

algae ever examined.

Purification of the Antioxygenic Principles in Polysiphonia urceolata

Polysiphonia urceolata is widely distributed along the intertidal zone of the Japanese coast, but is not commonly used for food. As shown in Table 1, the antioxygenic substances were found in the acetone-soluble fraction. It was proven that the active principles were not phospholipids. The acetone-soluble fraction was further fractionated by chromatography on a Sephadex LH-20 column (Table 2). The most pronounced antioxidant activity was found in the fraction eluted with methanol. On TLC, this fraction gave three antioxygenic spots,⁴⁾ with R_f values = 0.09-0.14, 0.25 and 0.37 all of which absorbed UV light. Two major spots with higher R_f values reacted positively with ferric chloride reagent⁹⁾ and in the

Table 1. Antioxidant activities of the lipid fractions extracted from *Polysiphonia urceolata* at a 1% concentration

Fraction	Induction period (days) of the substrate at 45°C
Control (without addition)	3
Aqueous methanol	4
Chloroform	76
Acetone-soluble	80
Acetone-insoluble	10

Table 2. Fractionation of the acetone-soluble fraction on a Sephadex LH-20 column, and evaluation of antioxidant activities

Solvent	Yield (%)	Antioxidant activity*
Chloroform-1	tr.	—
Chloroform-2	6.4	13
Chloroform-3	24.2	18
Chloroform/methanol (95:5)	37.5	17
Methanol	31.9	35
Control	—	4

* Length of the induction period of the substrate (days) at a 0.5% level, 45°C.

Fig. 1. Bromophenols found in the antioxygenic fraction of *Polysiphonia urceolata*.

Beilstein test. This suggests that the major antioxygenic compounds are similar to catechol and contained halogen. Because red algae of the family Rhodomelaceae, especially the genera *Rhodomela*, *Odonthalia* and *Polysiphonia* contain several kinds of bromophenols,^{8,10)} it was thought that the antioxygenic principles in *Polysiphonia* lipids might be bromophenols. Therefore, the antioxygenic fraction was analyzed by GC-MS.

As a result, four bromophenols, shown in Fig. 1, were identified in decreasing order of abundance by comparison with the data in the literatures.^{9,10)} The significant peaks (m/z) on MS are as follows:

(1) 362, 360, 347, 345, 273, 271.

(2) 428, 426, 424, 411, 409, 407, 347, 345, 337, 335, 333, 147.

(3) 436, 434, 424, 411, 409, 347, 345, 337, 345, 333, 331, 259, 257.

(4) 348, 346, 267, 259, 257, 147.

As the peaks in (2) and (3) on GC were heavily overlapped, the exact composition of each bromophenols is not known, but the main component, (1), accounted for approximately 75% of the total content.

The bromophenol composition found in this work is exactly equal to that reported by GLOMBITZA *et al.*⁹⁾ However, PEDERSEN *et al.*⁹⁾ found dibromo analogues of (1) and (3) as minor constituents besides major ones we found.

A wide variety of brominated organic compounds, including phenols, terpenoids and others¹⁰⁾ are known to be the characteristic constituents in red algae of the order Ceramiales. In order to clarify the correlation between the antioxidant activity and occurrence of bromophenols in these algae, several algae of the family Rhodomelaceae were analyzed for antioxidant activity and qualitatively tested for bromophenols (TLC with ferric chloride reagent). Although the number of samples was limited, as shown in Table 3, two

Table 3. Antioxidant activities of the chloroform-soluble fractions of red algae in the family Rhodomelaceae

Species	Antioxidant activity*	
	Acetone-soluble fr.	Acetone-insoluble fr.
<i>Polysiphonia morrowii</i>	50	5
<i>Polysiphonia urceolata</i>	53	3
<i>Chondria crassicaulis</i>	3	3
<i>Laurencia glandulifera</i>	6	3

* Length of the induction period of the substrate (days) at a 1% level, 45°C.

algae which contain bromophenols showed distinct antioxidant activities, while others which are known to contain terpenoids in place of phenols¹⁰⁾ were ineffective.

Although the antibiotic or growth regulating activities of bromophenols are well known,^{6,9,10)} their excellent antioxidant activities are first noted in this work.

Antioxidant Activities of Synthetic Bromophenols

Two major bromophenols, BrPhenolCHO(1) and BrPhenolCH₂OH(3), were synthesized and their antioxidant activities were compared by four different methods with the following natural and synthetic antioxidants: α -tocopherol, protocatechualdehyde, BHA and *N*-vanillyl octadecanamide (a synthetic tasteless homolog of capsaicin, the pungent principle in red pepper^{7,11)}).

In the oven test, both of the bromophenols were considerably effective and their activities were equivalent to that of BHA in dilute solutions. However, at 200 ppm, they were equal to that of vanillylamide but inferior to those of BHA and α -tocopherol (Table 4).

Protocatechualdehyde, the non-brominated homolog of BrPhenolCHO, was also an excellent

Table 4. Comparison of antioxidant activities with the oven test

Compound	Concentration	Induction period of the substrate (days)	
		Me ester of safflower oil	Stripped corn oil
None (control)	—	3	7
BrPhenolCHO (1)	20 ppm	4	
	50 ppm	7	
	100 ppm	9	
	200 ppm	11	21
BrPhenolCH ₂ OH (3)	1 mM		22
	200 ppm		20
	1 mM		21
α -Tocopherol	200 ppm		28
	1 mM		31
Protocatechualdehyde	200 ppm		26
	1 mM		25
BHA	20 ppm	3	
	50 ppm	4	
	100 ppm	9	
	200 ppm	21	37
<i>N</i> -Vanillyl octadecanamide	1 mM		37
	200 ppm		21
	1 mM		18

Table 5. Comparison of antioxidant activities with oxygen absorption in emulsion

Compound	Concentration in emulsion (ppm)	Antioxidant activity*	
		Soybean oil	Stripped corn oil
None (control)	—	7.8	4.5
BrPhenolCHO (1)	0.02	16	
	0.1	32	
	0.2	74	7.9
	1.0	>100	8.1
BrPhenolCH ₂ OH (3)	0.2		5.5
	0.4		9.3
	1.0		12.2
α -Tocopherol	0.2		5.3
	0.4		5.8
Protocatechualdehyde	0.2		5.7
	0.4		6.0
	1.0		6.4
BHA	0.02	17	
	0.1	25	
	0.2		5.4
	0.4		7.5
<i>N</i> -Vanillyl octadecanamide	1.0	>100	8.6
	0.2		5.8
	0.4		7.5
	1.0		8.6

* Time in min until the dissolved oxygen in the emulsion decreases below 50% of the original.

Table 6. Comparison of antioxidant activities with the Active Oxygen Method

Compound	Antioxidant activity*
None (control)	1.0
BrPhenolCHO (1)	2.5
BrPhenolCH ₂ OH (3)	1.3
α -Tocopherol	7.0
Protocatechualdehyde	6.1
BHA	7.3
<i>N</i> -Vanillyl octadecanamide	1.0

* Time in h until the peroxide value of the substrate reaches 20.

Table 7. Comparison of antioxidant activities with the chemiluminescence method

Compound	Chemiluminescent intensity (counts/30 sec)
None (control)	6992
BrPhenolCHO (1)	5626
BrPhenolCH ₂ OH (3)	5777
α -Tocopherol	4074
Protocatechualdehyde	6279
BHA	6681
<i>N</i> -Vanillyl octadecanamide	6574

antioxidant and its effect was better than that of BrPhenolCHO. This phenolic aldehyde is known to be widely distributed as a minor constituent in plants, but its antioxidant activity has been overlooked.

The antioxidant activities in emulsion systems are shown in Table 5. In this condition, both of the bromophenols were very effective. Especially the effect of BrPhenolCHO was the most distinguished of all antioxidants examined. However, the problem with bromophenols of the catechol type in this system is the discoloration caused by reaction with iron.

Antioxidant activities evaluated with the AOM test are shown in Table 6. Bromophenols were less effective in this test because of their unstabilities at a high temperature (97.8°C) in accordance with the previous observation.¹²⁾ Vanillylamine was ineffective because of its volatility.

The antioxidant activities for photosensitized oxidation, assayed by chemiluminescence, are shown in Table 7.

In the case of photooxidation, singlet oxygen is known to be the active oxygen species. It has also been reported¹³⁾ that a weak light mainly attributable to singlet oxygen is generated in the oxidation of vegetable oils. As a result, the effect of α -tocopherol which has been known to be a quencher of singlet oxygen was the strongest of all samples. The activities of bromophenols were next, and better than that of BHA.

From these combined results, the properties of bromophenols as antioxidants have been clarified considerably. Bromophenols are excellent antioxidants at low temperature, especially in aqueous emulsion. However, their activities are very poor at higher temperatures. Although these bromophenols are natural compounds and the characteristic constituents in red algae, in order to apply

these bromophenols as food antioxidants, detailed toxicity study will be required.

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