Antioxidant Effect of the Constituents of Susabinori *(Porphyra yezoensis)*

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ABSTRACT: The antioxidant activities of several extracts from Susabinori *(Porphyra yezoensis)* were measured by the ferric thiocyanate method and the thiobarbituric acid method. The methanol, acetone, ethyl acetate, and hexane extracts, and the chloroform-soluble and water-soluble fractions from the chloroform–methanol extract exhibited higher activities than α-tocopherol. The hot water extract showed little activity. Thin-layer chromatography analysis of the active extracts suggested the existence of several antioxidants. The activity of the chloroform soluble fraction was due to chlorophyll analogs. A strong antioxidant was isolated from the methanol extract, accompanied by several amino acids such as leucine and phenylalanine. This compound was identified as usujilene, a kind of mycosporine-glycine like amino acid.

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Seaweeds, such as laver and kelp, are commonly eaten in Japan and their high dietary fiber and vitamin content have stimulated their evaluation as health foods. In addition, seaweeds contain high levels of polyunsaturated fatty acids (1). Although polyunsaturated fatty acids are very easily oxidized, seaweeds are known to resist oxidation during storage. Fujimoto *et al.* (2) reported the antioxidant effect of 21 species of marine algae and found that the phospholipid fraction in arame (*Eisenia bucyclis*) showed efficient activities. Tutour (3) described that brown algae had strong antioxidant activity and that they synergistically enhanced the effect of vitamin E. Furthermore, Nishibori *et al*. (4) identified pheophytin a, one of the chlorophyll analogs, as a major antioxidant in Aonori (*Enteromorpha* sp.). In this paper, we describe the antioxidant effect of several extracts from Susabinori (*Porphyra yezoensis*), one of laver, and the isolation of antioxidants from the methanol extract.

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EXPERIMENTAL PROCEDURES

Materials and extraction. Cultured *P. yezoensis* was purchased from Akashiura Fisherman's Union (Hyogo, Japan) in February 1995. A part (10 g each) of the ground freeze-dried material was extracted independently with 200 mL of *n*-hexane, ethyl acetate, acetone, chloroform/methanol (2:1), methanol, and hot water (90°C) under stirring. Each extract was filtered and concentrated *in vacuo*. Water was added to the chloroform/methanol extract and then partitioned with chloroform to afford a chloroform-soluble fraction and an aqueous fraction.

Antioxidative assay. (i) Ferric thiocyanate method (FTC). The method of Kikuzaki *et al.* (5) was slightly modified. To a mixture of 2 mg of the fractions of the Susabinori extract, α-tocopherol or isolated compounds in 2 mL of 99.5% ethanol in a vial (ϕ = 35, h = 80 mm), 2.05 mL of 2.51% linoleic acid in 99.5% ethanol, 4 ml of a 0.05 M phosphate buffer (pH 7.0), and 1.95 mL of water were added. The solution including linoleic acid without any antioxidant or extract was used as control. The vial was placed in an oven at 40°C in the dark. At each test time, to 0.1 mL of this sample solution were added 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the developed red color was measured at 500 nm.

(ii) Thiobarbituric acid method (TBA) (5). To 2 mL of the sample solution prepared as described above were added 2 mL of a 20% trichloroacetic acid aqueous solution and 1 mL of a 0.67% thiobarbituric acid aqueous solution. This mixture was placed in a boiling-water bath for 10 min and, after cooling, was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm.

Fractionation of the hexane extract. The hexane extract (50 mg) was subjected to column chromatography on Sephadex LH-20 (Pharmacia Biotec, Uppsala, Sweden) and eluted with isopropanol to give three fractions [Fraction 1 (32 mg), Fraction 2 (7.7 mg), and Fraction 3 (8.2 mg)]. Fraction 3 was analyzed by high-performance liquid chromatography (HPLC) (Shimadzu LC-7A, Kyoto, Japan) using Wakosil

$_5C_{18}$-200 column (4.6 × 150 mm; Wako Pure Chemical, Osaka, Japan). Chromatographic parameters were set as follows: Eluent, chloroform/hexane/methanol (7:3:10, vol/vol/vol); flow rate, 1 mL/min; detection, ultraviolet (UV) at 452 nm.

Separation of pigments from the chloroform-soluble fraction of the chloroform/methanol extract. The chloroform soluble-fraction (10 mg) was subjected to column chromatography on DEAE-Sepharose CL-6B (Pharmacia Biotec) according to the method of Murata *et al.* (6,7) to give four fractions. Fraction 1 [acetone eluate; 4.4 mg], Fraction 2 [acetone/methanol (10:1, vol/vol) eluate; 2.5 mg], Fraction 3 [0.3% ammonium acetate in acetone/methanol (7:3, vol/vol) eluate; 1.6 mg], and Fraction 4 [1% ammonium acetate in acetone/water (4:1, vol/vol) eluate; 0.7 mg].

Fractionation and purification of the methanol extract. The methanol extract (19.8 g) from Susabinori (100 g) was mixed with chloroform (500 mL) and water (500 mL) to give a chloroform-soluble fraction (2.2 g) and an aqueous fraction (16.4 g). The aqueous fraction was subjected to DIAION HP-20 (Mitsubishi Kasei Corp., Tokyo, Japan) column chromatography to give a water-eluted fraction (15.5 g), 30% methanol-eluted fraction (783 mg), 60% methanol-eluted fraction (26 mg), methanol-eluted fraction (15 mg), and acetone-eluted fraction (22 mg). The 30% methanol fraction was further purified by Chromatorex-ODS DM1020T (Fuji Silysia Chem. Ltd., Kasugai, Japan) column chromatography to afford five fractions (Fraction 1: 267 mg; Fraction 2: 135 mg; Fraction 3: 47 mg; Fraction 4: 39 mg; Fraction 5: 12 mg). Fractions 1 and 2 were analyzed with an Amino Acid Autoanalyzer (Hitachi L-8500, Tokyo, Japan). Fractions 4 and 5 were further purified by Sephadex LH-20 (water/methanol, 2:1, vol/vol) and octadecyl silane (water/methanol, 4:1, vol/vol) column chromatography to furnish three compounds (compound **I**: 12 mg; compound **II**: 3 mg; compound **III**: 4 mg).

*Instruments used for structure determination of compound **I**.* UV absorbance was measured with a Beckman DU-640 spectrophotometer (Palo Alto, Ca), λ_{max} (methanol) 355 nm (log ε 4.28). High-resolution fast atom bombardment (HR-FAB) mass spectrometry was determined with a Jeol SX-102 (Tokyo, Japan), *m/z* 285.1440 (calculated for $C_{13}H_{20}O_5N_2 + H^+$, 285.1441). Infrared (IR) spectrum was recorded with a Perkin-Elmer 1720X apparatus (Norwalk, CT), ν_{max} (film) 3300, 1665(s), 1611, 1554, 1276, 1160, 1131, 1051, 969 cm^{-1}. ^1H nuclear magnetic resonance (NMR) (500 MHz) and ^{13}C NMR (125 MHz) spectra were determined with a Varian Unity 500 instrument (Palo Alto, CA), using tetramethylsilene as an internal standard (Table 1).

TABLE 1
^1H Nuclear Magnetic Resonance (NMR) and ^{13}C NMR Data of Compound I Isolated from Methanol Extract of Susabinori

	δ_C	δ_H
1	155.6	
2	127.9	
3	164.1	
4	34.9	2.74 (1H, *d*, *J* = 17.6 Hz), 2.92 (1H, *d*, *J* = 17.6 Hz)
5	72.2	
6	34.6	2.74 (1H, *d*, *J* = 17.6 Hz), 2.91 (1H, *d*, *J* = 17.6 Hz)
7	69.4	3.47 (2H, *s*)
8	59.9	3.68 (3H, *s*)
9	48.2	3.99 (2H, *s*)
10	173.4	
11	123.6	6.41 (1H, br. *d*, *J* = 7.6 Hz)
12	116.5	5.29 (1H, *dq*, *J* = 7.6, 7.1 Hz)
13	11.4	1.78 (3H, *dd*, *J* = 1.7, 7.1 Hz)

RESULTS AND DISCUSSION

Antioxidant activities of the extracts from Susabinori. The antioxidant activity of each extract from Susabiori *(P. yezoensis)* measured by the FTC method and the TBA method is shown in Figure 1A and 1B, respectively. Except for the hot water extract, the six tested samples—methanol, acetone, ethyl acetate, and hexane extracts and chloroform-soluble and water-soluble fractions—exhibited higher activities than α-tocopherol. The hot water extract showed little activity. Thin-layer chromatography (TLC) analysis of five of the active extracts on SiO_2 revealed different constituent patterns among them, as shown in Figure 2.

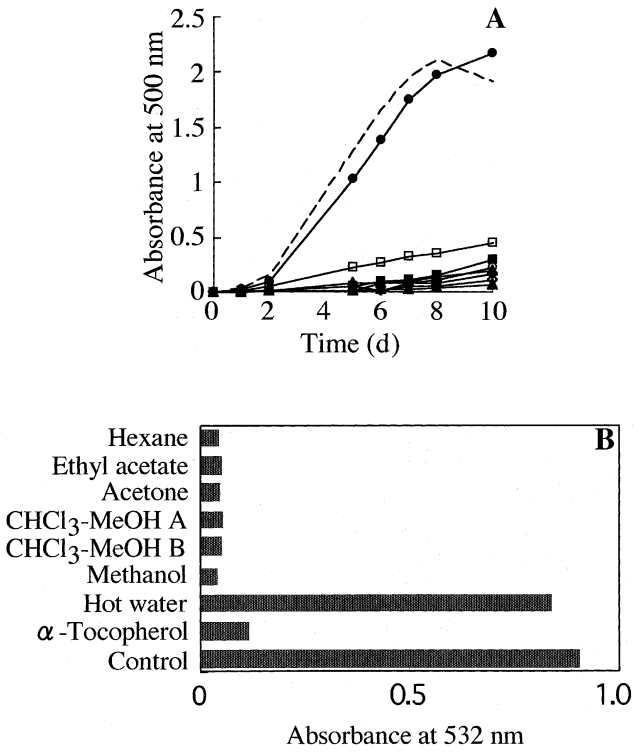

FIG. 1. Antioxidant activities of the extracts from *Porphyra yezoensis*. (A) Ferric thiocyanate (FTC) method: – – –, control (without additives); □, α-tocopherol; ◇, methanol extract; ◆, chloroform-soluble fraction of chloroform/methanol (2:1) extract; ■, water-soluble fraction of chloroform/methanol (2:1) extract; △, ethyl acetate extract; ▲, *n*-hexane extract; ●, hot water extract; ○, acetone extract. (B) Thiobarbituric acid (TBA) method: The TBA values were measured on the 10th day of the tested sample solution kept at 40°C in the dark. $CHCl_3$-MeOH A, chloroform-soluble fraction of chloroform/methanol (2:1) extract; $CHCl_3$-MeOH B, water-soluble fraction of chloroform/methanol (2:1) extract.

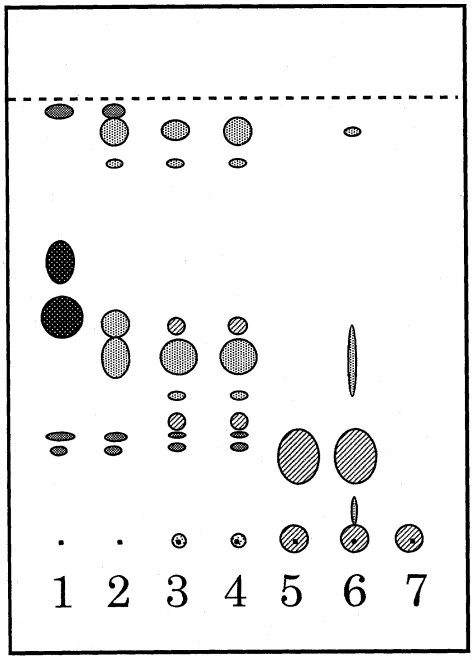

FIG. 2. Thin-layer chromatography (TLC) patterns of extracts from Susabinori. The TLC on SiO_2 was developed using the solvent of benzene/methanol (9:1). The solvent front is indicated by a dotted line. 1, *n*-Hexane extract; 2, ethyl acetate extract; 3, acetone extract; 4, chloroform-soluble fraction of chloroform/methanol (2:1) extract; 5, water-soluble fraction of chloroform/methanol (2:1) extract; 6, methanol extract; 7, hot water extract.

Antioxidant activities of fractions from the hexane extract. The activities of the three fractions from the hexane extract were measured by the FTC method. As shown in Figure 3, only Fraction 3 showed strong antioxidant activity compared to α-tocopherol. Some spots in Fraction 3 were detected by TLC, and the main compound was identified as β-carotene by HPLC analysis. It is well known that β-carotene is an effective singlet oxygen quencher. However, Heinonen *et al*. (8) reported that the antioxidant activity of β-carotene depends on other antioxidants present, the oxidation model used, and the oxygen tension. Therefore, the strong activity of Fraction 3 may suggest the existence of some minor components, which might have antioxidant ability or act as a synergist.

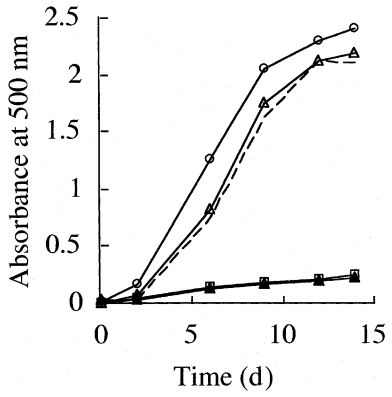

FIG. 3. Antioxidant activities of the fractions from hexane extract measured by the FTC method. – – –, control (without additives); □, α-tocopherol; △, fraction 1; ○, fraction 2; ▲, fraction 3. See Figure 1 for abbreviation.

Antioxidant activity of the chloroform-soluble fraction from the chloroform/methanol extract. The antioxidant activities of Fractions 1 and 2 obtained from the fractionation of the chloroform-soluble fraction of the chloroform/methanol extract were measured by the FTC method. As shown in Figure 4A, these two fractions exhibited strong activities. Fractions 1 and 2 were identified as pheophytin a and chlorophyll a, respectively, by comparison of their UV spectra with reported data (7). The antioxidant activity of pheophytin a and chlorophyll a were reported by Nishibori *et al*. (4) and Endo *et al*. (9). From this result, the activity of the chloroform soluble fraction from chloroform/methanol extract was due to chlorophyll analogs.

Antioxidant activity of the methanol extract. The activities of the five fractions from the aqueous fraction of the methanol extract are shown in Figure 4B. The water fraction showed only a slight activity, but the 30, 50, and 100% methanol frac-

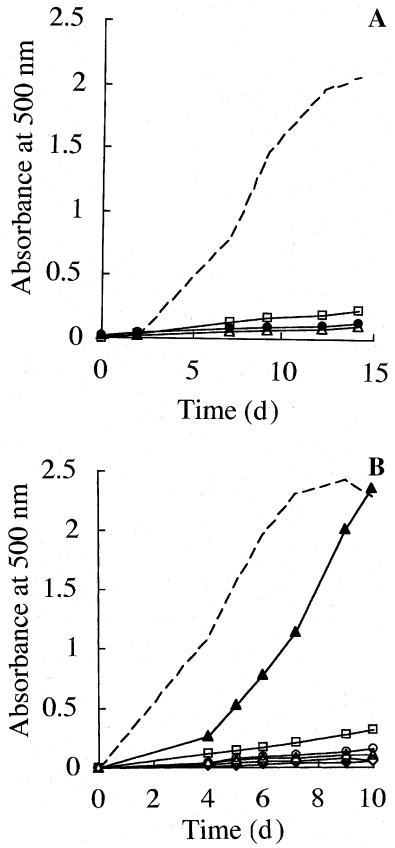

FIG. 4. (A) Antioxidant activities of fractions from the chloroform-soluble fraction of chloroform/methanol (2:1) extract measured by the FTC method. – – –, control (without additives); □, α-tocopherol; △, fraction 1; ●, fraction 2. (B) Antioxidant activities of the aqueous fractions of the methanol extract measured by the FTC method. – – –, control (without additives); □, α-tocopherol; ▲, water fraction; ◆, 30% methanol fraction, ◇, 60% methanol fraction; △, 100% methanol fraction; ○, acetone fraction. See Figure 1 for abbreviation.

tions exhibited very strong activities. The main compounds in Fractions 1 and 2 from the 30% methanol fraction were identified as taurine and alanine, respectively, by Amino Acid Autoanalyzer. Three compounds (**I**, **II**, and **III**) were isolated from Fractions 4 and 5. Compounds **II** and **III** were identified as leucine and phenylalanine by comparison of authentic samples. Compound **I** was not a simple amino acid, so we determined the structure of **I** by instrumental analysis.

Structure determination of compound I. Compound **I** was soluble in methanol and water, and its UV spectrum showed a strong absorption at 355 nm. The HR-FAB mass spectrum of **I** exhibited the highest peak at *m/z* 285.1440 $(M + H)^+$, indicating its molecular formula to be $C_{13}H_{20}O_5N_2$. The IR spectrum showed absorption bands assignable to hydroxyl and amino groups and conjugated double bonds at 3300 and 1611 cm^{-1}, respectively. In the color tests on silica gel TLC (10), this compound turned yellow against ninhydrin reagent, but the results for ferric chloride, 2,4-dinitrophenylhydrazine, orcinol-sulfuric acid, and bromocresol green reagents were negative. The ^1H and ^{13}C NMR spectral data of **I** are shown in Table 1. The ^1H and ^{13}C NMR spectra of **I**, in conjunction with ^1H-Detected Multiple Quantum Coherence spectrum (HMQC) (11) experiment indicated the presence of a *cis*-propenyl group, a methoxyl group, four methylenes, and a carboxyl group together with four quaternary carbons. The connectivities of these partial structures were confirmed by ^1H-Detected Heteronuclear Multiple-Bond Coherence spectrum (HMBC) experiment (Fig. 5) (12). Consequently, compound **I** was identified as usujilene (13), a kind of mycosporine-glycine like amino acid. Usujilene has already been isolated from *Palmaria palmata* (13), but not from any *Porphyra* species. Sivalingam *et al.* (14,15) isolated characteristic UV-absorbing substances (334, 337 nm) from *P. yezoensis* and reported on their physicochemical properties, but they did not assign the structures. These substances were presumed to have closely similar structures with usujilene by comparison of their spectral data.

Antioxidant activity of the isolated compounds. The activities of the isolated compounds are shown in Figure 6. Alanine, phenylalanine, and leucine showed weak activity, while taurine had no activity. Compound **I**, however, exhibited very strong activity.

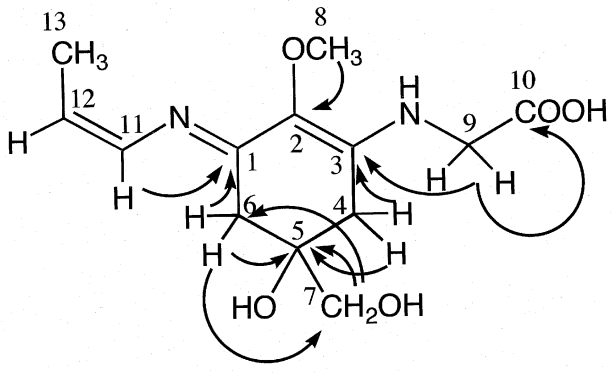

FIG. 5. Heteronuclear multiple-bond coherence spectrum correlations observed for compound **I.**

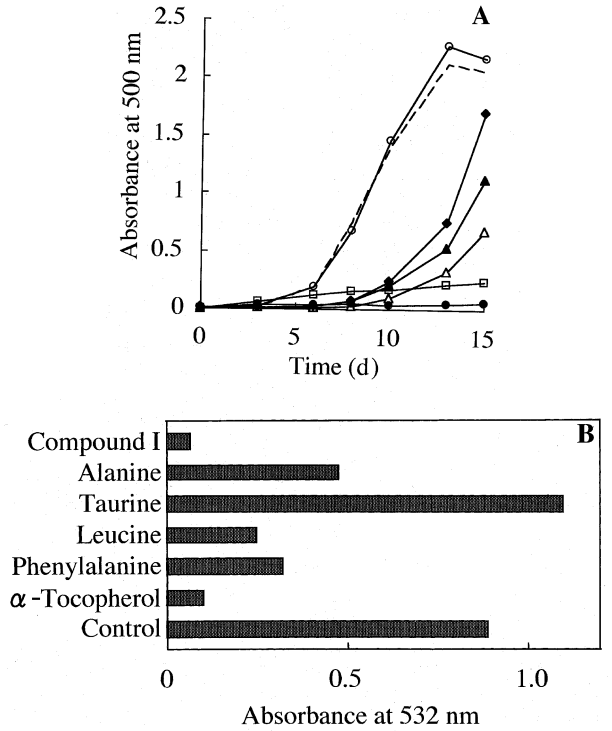

FIG. 6. Antioxidant activities of constituents of 30% methanol fraction. (A) FTC method: – – –, control (without additives); □, α-tocopherol; ▲, phenylalanine; △, leucine; ○, taurine; ◆, alanine; ●, compound **I**. (B) TBA method: The TBA values were measured on the 15th day of the tested sample solution kept at 40°C in the dark. See Figure 1 for abbreviations.

Some mycosporine-glycine like amino acids are found in many marine plants and animals (16–19). Several reports have suggested that these compounds function as biological antioxidants for protection against UV radiation (20–22). Our results indicated that usujilene has also an antioxidant effect on autoxidation of linoleic acid. The activity of usujilene was remarkably stronger than those of simple amino acids such as alanine, phenylalanine, and leucine.

The high antioxidant activity of usujilene might be explained by the fact that usujilene seems to easily donate a hydrogen atom from C-4, C-6, or C-9 methylene to a free radical of a lipid such as LOO·, because the free radical formed in the molecule is stabilized by resonance in conjugation with double bonds as seen in the antioxidant mechanism of α-tocopherol (23).

This is the first report of the isolation of usujilene, one of the mycosporine-glycine like amino acids, from *P. yezoensis*, and of the high antioxidant activity of usujilene.

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