J Appl Phycol (2008) 20:705–711
DOI 10.1007/s10811-007-9254-8

Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae

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Received: 23 April 2007 / Revised and Accepted: 5 September 2007 / Published online: 6 October 2007

Abstract The antioxidant activities of brown algal phlorotannins were evaluated using the inhibition of phospholipid peroxidation in the liposome system, and by determining radical scavenging activities against the superoxide anion and 1,1-diphenyl-2-picrylhydrazyl (DPPH). Oligomers of phloroglucinol (1,3,5-trihydroxybenzene), eckol (a trimer), phlorofucofuroeckol A (a pentamer), dieckol and 8,8′-bieckol (hexamers), isolated from the Laminarian brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome*, showed potent inhibition of phospholipid peroxidation at 1 μM in the liposome system. The phlorotannins had significant radical scavenging activities against the superoxide anion (50% effective concentration values: 6.5–8.4 μM) and DPPH (50% effective concentration values: 12–26 μM), and were more effective compared to ascorbic acid and α-tocopherol. For the purpose of using phlorotannins as functional food ingredients, the antioxidant activity of a complex of crude phlorotannins and soybean protein was examined. The complex had a pronounced DPPH-radical scavenging activity. These results suggest that phlorotannins are potent anti-inflammatory substances, and that the Laminariaceous brown algae, which are abundant in phlorotannins, may be useful as a new functional foodstuff or supplement with anti-inflammatory activity.

Keywords Brown algae · DPPH-radical · Liposome · Phlorotannin–soy bean protein complex · Phaeophyta · Polyphenols · Superoxide anion radical

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Introduction

It is well-known that the oxidative damage to cell membranes, DNA and/or proteins induced by free radicals and reactive oxygen species may play a causative role in ageing and lifestyle diseases. Therefore, it is believed that inhibiting such damage by taking antioxidant supplements could be an effective therapy for such diseases. Recent epidemiological and clinical studies have indicated that consumption of plant foods and drinks (e.g., tea, red wine and soybean products) moderate the risk of lifestyle diseases

(Messina et al. 1994; Sasazuki et al. 2000; Wu et al. 2001). The beneficial effects of such foods and drinks are thought to be due to the presence of polyphenols with antioxidant activity, and many active substances (e.g., catechins, resveratrol and isoflavones) have been isolated (Jang et al. 1997; Esaki et al. 1999a, b; Zhao et al 2001; Havsteen 2002; Aggarwal and Shishodia 2006). Polyphenols—common secondary metabolites—are found universally in plants (Haslam 1989; Herbert 1989). However, in stark contrast to polyphenols in terrestrial plants, the literature on the properties of marine algal polyphenols from a human physiological viewpoint is very sparse. Marine algal polyphenols—phlorotannins—which have been found to exist only within brown algae, are formed by the polymerization of phloroglucinol (Ragan and Glombitza 1986; Nakamura et al. 1996). Previously, we isolated the phlorotannins eckol (a phloroglucinol trimer), phlorofucofuroeckol A (a pentamer), dieckol and 8,8′-bieckol (hexamers) from the Japanese Laminariaceous brown algae *Eisenia bicyclis* (Kjellman) Setchell, *Ecklonia cava* Kjellman and *Ecklonia kurome* Okamura, and reported their distribution (Shibata et al. 2004) and physiological properties (Shibata et al. 2002a, b, 2003). However, the effectiveness of phlorotannins against free radicals is still obscure.

The aim of this study was to evaluate the antioxidant and free radical scavenging activities of phlorotannins isolated from Laminariaceous brown algae. As a first step, the activities of the phlorotannins were determined using the inhibition of phospholipid peroxidation in the liposome system, and their scavenging activities against the superoxide anion and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Thereafter, a phlorotannin–soybean protein complex was prepared and its function as an antioxidant foodstuff assessed.

Materials and methods

The brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* were collected from along the coasts of the Itoshima Peninsula (33°37′N, 130°10′E) in Fukuoka Prefecture and the Miura peninsula (35°08′N, 130°36′E) in Kanagawa prefecture, Japan. The algae were washed with filtrated seawater, air-dried and pulverized, and the algal powders were then stored at −40°C until use.

Catechin and epigallocatechin gallate (EGCG) were kindly donated by Kurita Water Industries (Tokyo, Japan).

Extraction of phlorotannins from algal powders was carried out according to the modified method of Folch et al. (1957) described in previous reports (Nagayama et al. 2002, 2003; Shibata et al. 2006b). Each of the phlorotannins in the crude extracts was purified on a column (1.5 cm i.d.×150 cm) of Wakogel C-300HG (Wako, Tokyo, Japan) with $CHCl_3$:methanol (MeOH):water (80:20:2, v/v) as the eluent (Shibata et al. 2002b, 2003). The purity of the phlorotannins was checked by thin-layer chromatography (TLC) and reversed-phase three-dimensional high-performance liquid chromatography (RP-3D-HPLC). TLC plates (Silica Gel 60 F_{254}, 0.25 mm; Merck, Darmstadt, Germany), which had been activated at 120°C for 5 min before use, were developed with $CHCl_3$:MeOH:water:acetic acid (65:25:4:3, v/v) (Shibata et al. 2002a, 2004, 2006b). Fifty percent H_2SO_4 (Krebs et al. 1969), vanillin-H_2SO_4 (Krebs et al. 1969), and paprika pigment (Nakamura et al. 1991), were used as the detecting agents for organic compounds, for phenolic compounds, and for antioxidant substances, respectively. The HPLC system consisted of a Waters 600 pump (Waters, Milford, MA), a MCPD-3600 UV detector (Otsuka Electronics, Osaka, Japan) and an Inertsil ODS-3 column (4.6 mm i.d.×

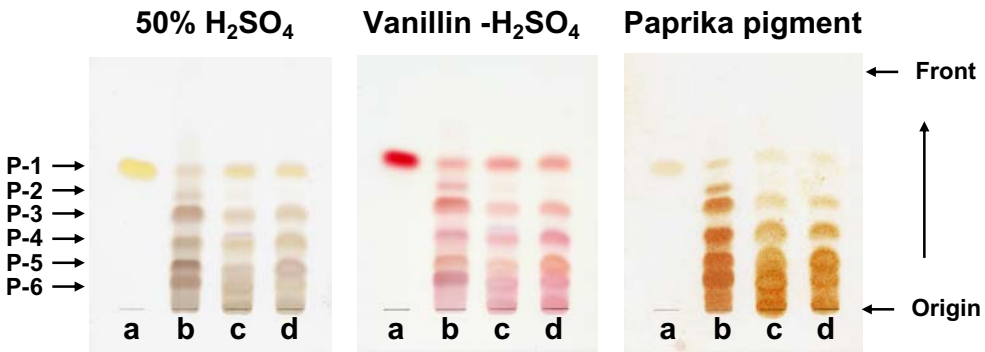

Fig. 1 Thin-layer chromatography (TLC) of phlorotannins from the Laminariaceous brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome*. *P-1* Phloroglucinol, *P-2* phloroglucinol tetramer, *P-3* eckol, *P-4* phlorofucofuroeckol A, *P-5* dieckol, *P-6* 8,8′-bieckol. *a* Phloroglucinol, *b* crude phlorotannins from *Eisenia bicyclis*, *c* crude phlorotannins from *Ecklonia cava*, *d* crude phlorotannins from *Ecklonia kurome*

250 mm, GL Science, Tokyo, Japan). Elution was performed at a flow-rate of 1.0 mL min^{-1} with a linear gradient from 30% MeOH/water (eluent A) to 100% MeOH (eluent B) for 20 min, and was maintained for 25 min (Shibata et al. 2002a, b, 2003, 2004, 2006b). The photodiode array detector was set to a wavelength range of 190–400 nm.

Antioxidant activity in the liposome system

A large unilamellar vesicle was prepared by the extrusion method described by MacDonald et al. (1991) with some modifications. Briefly, egg yolk lecithin (Wako, Tokyo, Japan) in chloroform (10 mg mL^{-1}) was dried under nitrogen gas and kept under high vacuum for 1 h. The lipid was suspended in 10 mM phosphate buffer (pH 7.4) and freeze-thawed 5 times in a MeOH bath. The multilamellar vesicle suspension was transferred into a Lipofast Apparatus (Avestin, Ottawa, Canada) and passed thorough a polycarbonate membrane (pore size 100 nm) 19 times to obtain unilamellar liposomes (10 mg mL^{-1}). The antioxidant activity of the sample to be tested in the liposome system was measured by the method of Esaki et al. (1997). The test samples were dissolved in 100 μL ethanol (EtOH). In the control test, 100 μL EtOH was used instead of the sample solution. The amount of thiobarbituric acid-reacting substance was determined by measuring the absorbance at 535 nm.

Radical scavenging activities against DPPH and superoxide anion

The DPPH-radical scavenging activity was measured using the modified method of Yamaguchi et al. (1998) described in a previous report (Shibata et al. 2006a). The samples were dissolved in 100 μL EtOH, and the solution was mixed with 100 μL 300 mM DPPH (Wako) in EtOH. In controls, 100 μL EtOH was used instead of the sample solution. After the reaction mixture had been stored at room temperature for 30 min, its absorbance was measured at 520 nm. The superoxide anion radical scavenging activity was determined with the SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan) (Ukeda et al. 1999). Samples were dissolved in 20 μL EtOH. In controls, 20 μL EtOH was used instead of the sample solution. The production of water-soluble tetrazolium salt was compared with that of controls.

The 50% effective concentration (EC_{50}) value was defined as the amount of sample required to scavenge 50% DPPH radical or superoxide anion radical. Terrestrial polyphenols (resveratrol, catechin and EGCG) and antioxidant vitamins (ascorbic acid and α-tocopherol) were used as positive controls.

Preparation of phlorotannin–soybean protein complex

A phlorotannin–soybean protein complex was prepared by the method of Ishimaru and Nonaka (2001). Briefly, defatted soybean (200 g) was steeped in water (1 L) and

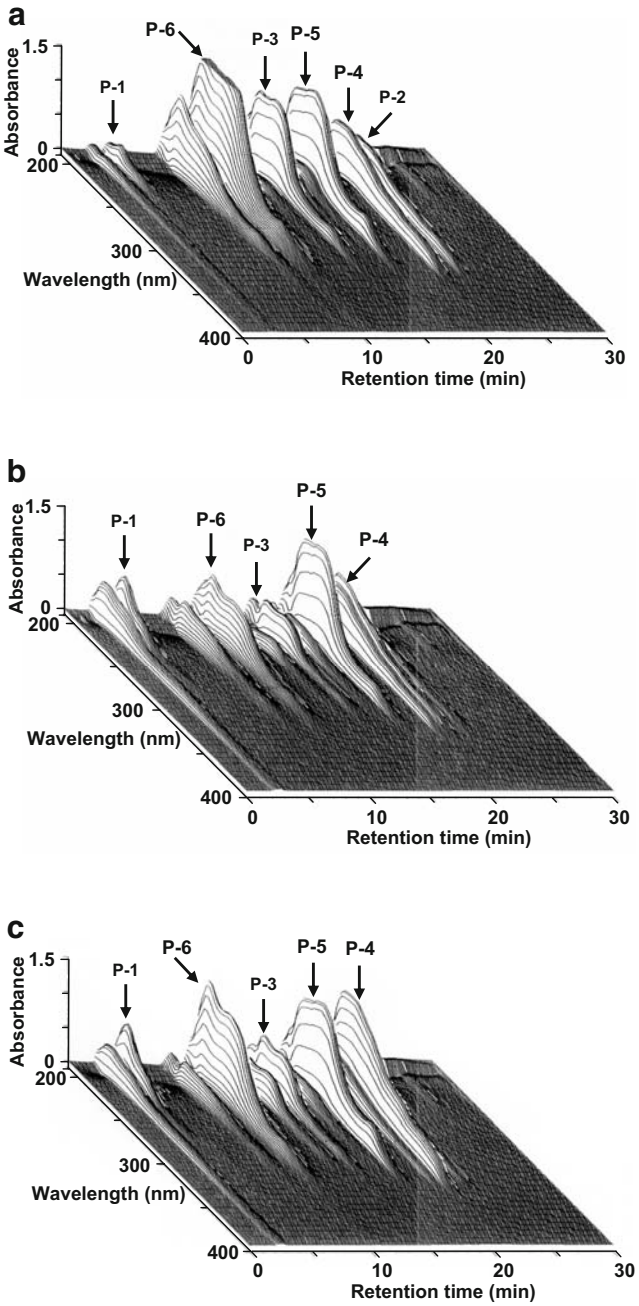

Fig. 2 Reversed-phase three-dimensional high-performance liquid chromatography (RP-3D-HPLC) of crude phlorotannins from the Laminariaceous brown algae *Eisenia bicyclis* (**a**), *Ecklonia cava* (**b**) and *Ecklonia kurome* (**c**). *P-1* Phloroglucinol, *P-2* phloroglucinol tetramer, *P-3* eckol, *P-4* phlorofucofuroeckol A, *P-5* dieckol, *P-6* 8,8′-bieckol

autoclaved at 121°C for 20 min. After filtration, the soybean protein extract (500 mL) was mixed with 100 mL of the crude phlorotannins from *Eisenia bicyclis* in water (10 mg/mL), and the pH of the mixture was adjusted to 4.5 with 2 *N* HCl. The phlorotannin–soybean protein complex that precipitated in the acidic solution was lyophilized. The amount of phlorotannins conjugated to soybean protein was determined by RP-3D-HPLC. The conjugated amount (%) was calculated using the following formula:

Conjugation ratio (%) = (1− the amount of phlorotannins detected in the supernatant of the phlorotannin–soybean protein complex / the initial content of each compounds in the crude phlorotannins) × 100.

The DPPH-radical scavenging activity of the complex was measured using the method of Huang et al. (2004). In the control test, lyophilized soybean protein extract was used instead of the sample.

Results

The yield of crude phlorotannins from *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* was about 3% of the algal powder (dry weight) for all three algae. The crude phlorotannins from the brown algae were analyzed by TLC and RP-3D-HPLC. As shown in Fig. 1, five to six spots detected in all three algal fractions were stained by paprika pigment, indicating the presence of antioxidant substances. Judging from previous reports (Nakamura et al. 1996; Shibata et al. 2004, 2006b), the spots can be identified as follows: phloroglucionl (P-1), unknown phloroglucinol tetramer (P-2), eckol (P-3), phlorofucofuroeckol A (P-4), dieckol (P-5) and 8,8′-bieckol (P-6). In RP-3D-HPLC analysis, peaks in the fractions of crude phlorotannins were identified by comparing their retention times with those of purified phlorotannins and their on-line ultraviolet spectra. Within 30 min, a distinct separation of each of the components in the brown algal extracts was obtained by a linear gradient system of 30–100% MeOH (Fig. 2). Each individual phlorotannin was purified by silicic acid column chromatography. The purity of each oligomer in this experiment exceeded 90%.

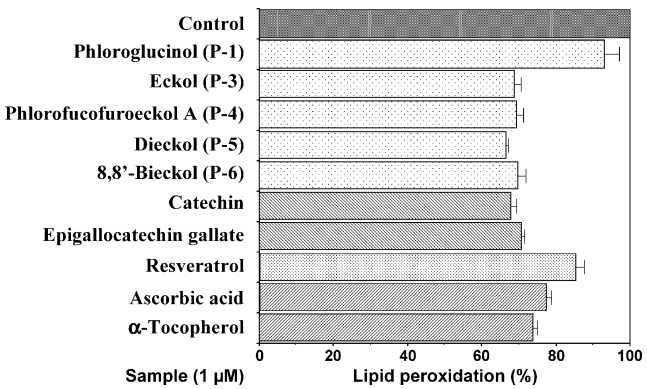

Fig. 3 Antioxidant activities of phlorotannins, terrestrial polyphenols and antioxidant vitamins in the liposome system. Lipid peroxidation was induced by 4 mM 2, 2′-azobis (2-amidinopropane) dihydrochloride. The analytical data are presented as mean ± SD (n=5)

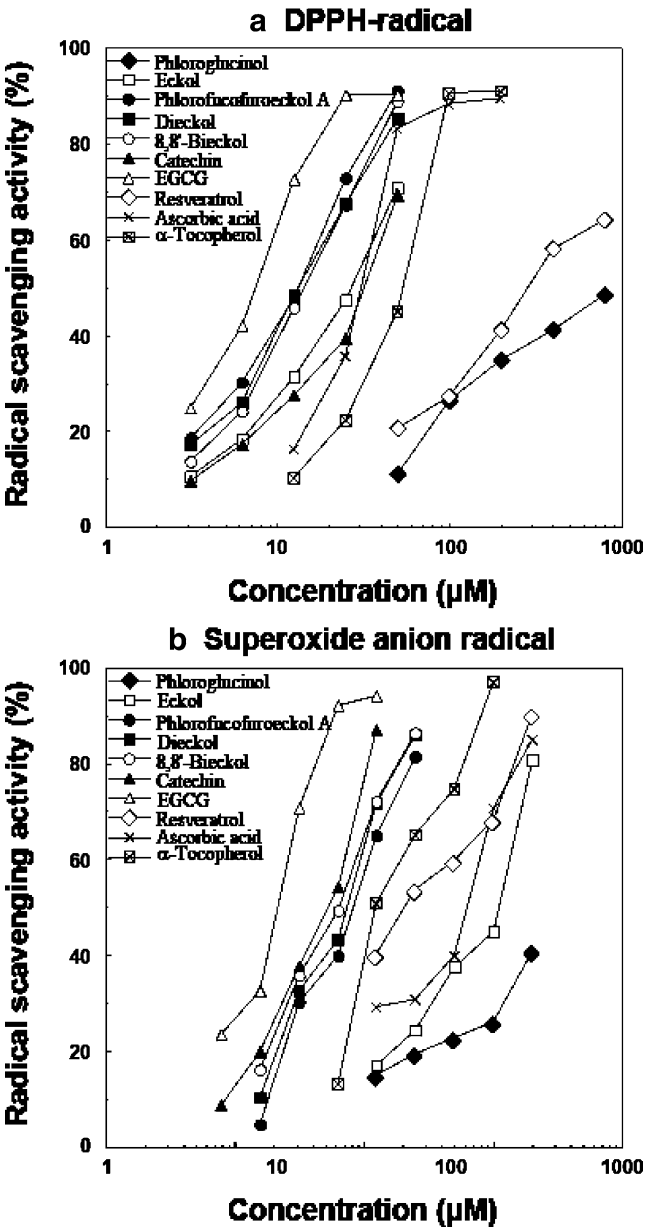

Fig. 4a,b Radical scavenging activities of phlorotannins, terrestrial polyphenols and antioxidant vitamins. **a** 1-Diphenyl-2-picrylhydrazyl (DPPH)-radical, **b** superoxide anion radical. All analytical data are the means of three determinations

Table 1 Conjugation of phlorotannins to soybean protein. Reversed-phase three-dimensional high-performance liquid chromatography (RP-3D-HPLC) conditions are described in Materials and methods. All analytical data in the table are the mean of three determinations

Compound	Molecular weight	Conjugation (%)
Eckol (P-3)	372	27.5
Unknown phloroglucinol tetramer (P-2)	478	98.2
Phlorofucofuroeckol A (P-4)	602	100
Dieckol (P-5)	742	100
8,8′-Bieckol (P-6)	742	100

Antioxidant activity of phlorotannins in the liposome system

Figure 3 shows the inhibitory effects of phlorotannins, terrestrial polyphenols and antioxidant vitamins on phospholipid peroxidation in a liposome system initiated by 2,2′-azobis (2-amidinopropane) dihydrochloride. At a final concentration of 1 μM, eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol each showed about 30% inhibition of phospholipid peroxidation. Thus, the oligomeric phloroglucinols were found to possess potent antioxidant activity, comparable in magnitude to that of well-known antioxidants such as catechins and vitamins.

Radical scavenging activities of phlorotannins

A further experiment was carried out using the DPPH-radical and the superoxide anion radical scavenging assays to determine the antioxidant activity of the phlorotannins. With the exception of phloroglucinol and resveratrol, the phlorotannins, terrestrial polyphenols and antioxidant vitamins scavenged the DPPH-radical in a dose-dependent manner (Fig. 4a). The EC_{50} values of the phlorotannins eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were 26, 12, 13 and 15 μM, respectively. In contrast, those of catechin, EGCG, ascorbic acid and α-tocopherol were 32, 7.4, 30, and 52 μM, respectively. The phlorotannins were potent free radical scavengers and about twice as effective as catechin, ascorbic acid and α-tocopherol. Figure 4b shows the superoxide anion scavenging activities of the phlorotannins, terrestrial polyphenols and antioxidant vitamins. The EC_{50} values of eckol, phlorofucofuroeckol A, dieckol, 8,8′-bieckol, catechin, EGCG, resveratrol, ascorbic acid and α-tocopherol were 107, 8.4, 7.6, 6.5, 5.2, 2.1, 21, 62, and 12 μM, respectively. With the exception of eckol, the phlorotannins were found to have very potent superoxide anion radical scavenging activity, being about 2–10 times more active than ascorbic acid and α-tocopherol.

Phlorotannin–soybean protein complex

In order to use phlorotannins as functional food ingredient, a complex of crude phlorotannins and soybean protein was prepared. As shown in Table 1, phlorotannins of high molecular weight—the pentamer and hexamers of phloroglucinol—showed pronounced affinity for the soybean protein. About 5 g phlorotannin–soybean protein complex was obtained from 1 g crude phlorotannins and 200 g defatted soybean. The DPPH-radical scavenging activity of the complex was about four times stronger than that of the lyophilized soybean protein extract alone (Fig. 5).

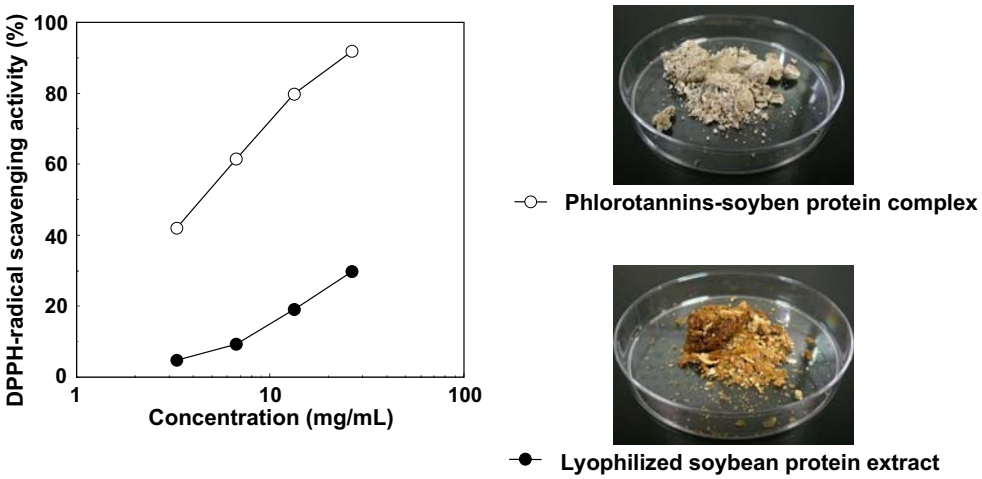

Fig. 5 DPPH-radical scavenging activity of phlorotannin–soybean protein complex. The complex was steeped in acetate buffer (pH 4.5) overnight at room temperature before being homogenized and centrifuged. The supernatant was mixed with the DPPH in EtOH. All analytical data are the means of three determinations

Discussion

A main target of free radical damage in the body is the cell membrane, which contains abundant unsaturated lipids. The liposome has been used extensively as a model of the cell membrane for in vitro biochemical research. Therefore, the unilamellar liposome lipid/aqueous system was used to evaluate the antioxidative properties of phlorotannins. In the present study, the oligomeric phloroglucinols were found to possess potent antioxidant activity in the liposome system. Catechins have been reported to show strong interactions with lipid bilayers and to exert various physiological activities (Kondo et al. 1999). Like catechins, it may be that phlorotannins have high affinity for lipid bilayers and inhibit phospholipid peroxidation. Most of the phlorotannins tested had more potent reducing activities toward the DPPH and superoxide anion radicals than antioxidant vitamins. Generally, it is believed that the radical scavenging activities of polyphenols such as flavonoids are ascribed to the phenolic hydroxy groups attached to the ring structure (Kondo et al. 1999). Although relationships between the structure of phlorotannins and their radical scavenging activities are unclear, it may be that phenolic hydroxy groups attached to the eckol skeleton play an important role in the radical scavenging activities. The phlorotannin–soybean protein complex had pronounced DPPH-radical scavenging activity; the activity was higher than that of soybean protein, which is rich in isoflavones. This complex may have a potential use as a new functional material in the food industry.

Previously, we described the inhibitory effect of phlorotannins on hyaluronidase (Shibata et al. 2002b) and lipoxygenases (Shibata et al. 2003). We also showed that phlorotannins had no toxicity following oral administration to mice (Nagayama et al. 2002). In addition, we reported that the yield of phlorotannins among *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* was about 3.0–4.0% of the algal powder (dry weight), with no clear seasonal variation in phlorotannin content being observed (Shibata et al. 2004). Recently, Sugiura et al. (2006) and Tsukui et al. (2006) isolated phlorotannins from the Laminarian brown alga *Eisenia arborea*. Laminariaceous brown algae are one of the most common algae in Japan. Thus, the evidence obtained in the previous and present studies suggests that phlorotannins are potent anti-inflammatory substances and that the Laminarian brown algae, which are rich in phlorotannins, may be useful as a novel functional foodstuff or supplement with anti-inflammatory activity.

Acknowledgments We thank grateful to Dr. Takashi Nakamura of the Graduate school of Kyushu University for his cooperation throughout this study. This work was supported in part by the Comprehensive Research Laboratory of Fukuoka Institute of Technology.

References

Aggarwal BB, Shishodia S (2006) Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 71:1397–1421

Esaki H, Onozaki H, Kawakishi S, Osawa T (1997) Antioxidant activity and isolation from soybeans fermented with *Aspergillus* spp. J Agric Food Chem 45:2020–2024

Esaki H, Watanabe R, Onozaki H, Kawakishi S, Osawa T (1999a) Formation mechanism for potent antioxidative *o*-dihydroxyisoflavones in soybeans fermented with *Aspergillus saitoi*. Biosci Biotechnol Biochem 63:851–858

Esaki H, Kawakishi S, Morimitsu Y, Osawa T (1999b) New potent antioxidative *o*-dihydroxyisoflavones in fermented Japanese soybean products. Biosci Biotechnol Biochem 63:1637–1639

Folch J, Less M, Sloan Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Haslam E (1989) Plant polyphenols, Cambridge University Press, Cambridge

Havsteen BH (2002) The biochemistry and medical significance of the flavonoids. Pharmacol Ther 96:67–202

Herbert RB (1989) The biosynthesis of secondary metabolites, 2nd edn. Chapman and Hall, New York

Huang S-M, Shigetomi N, Tanaka N, Terahara N, Fujioka T, Yoshida M, Ishimaru K (2004) Preparation and functional evaluation of plant polyphenol-bean protein complex. Nippon Shokuhin Kagaku Kogaku Kaishi 51:626–632

Ishimaru K, Nonaka G (2001) Rapid purification of catechins using soybean protein. Nippon Shokuhin Kagaku Kogaku Kaihi 48:664–670

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218–220

Kondo K, Kurihara M, Miyata N, Suzuki T, Toyoda M (1999) Mechanistic studies of catechins as antioxidants against radical oxidation. Arch Biochem Biophys 362:79–86

Krebs KG, Heusser D, Wimmer H (1969) Spray reagents. In: Stahl E (ed) Thin-layer chromatography. Springer, Heidelberg, pp 854–909

MacDonald RC, MacDonald RI, Menco BPhM, Takeshita K, Subbarao NK, Hu L-R (1991) Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim Biophys Acta 1061:297–303

Messina MJ, Persky V, Setchell KDR, Barnes S (1994) Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr Cancer 21:113–131

Nagayama K, Iwamura Y, Shibata T, Hirayama I, Nakamura T (2002) Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. J Antimicrob Chemother 50:889–893

Nagayama K, Shibata T, Fujimoto K, Honjo T, Nakamura T (2003) Algicidal effect of phlorotannins from the brown alga *Ecklonia kurome* on red tide microalgae. Aquaculture 218:601–611

Nakamura T, Mukaiyama T, Nagayama K (1991) A rapid and simple method to detect antioxidant substances on a thin-layer chromatography plate. J Fac Agric Kyushu Univ 36:93–98

Nakamura T, Nagayama K, Uchida K, Tanaka R (1996) Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. Fisheries Sci 62:923–926

Ragan MA, Glombitza K-W (1986) Phlorotannins, brown algal polyphenols. Progr Phycol Res 4:129–241

Sasazuki S, Kodama H, Yoshimasu K, Liu Y, Washio M, Tanaka K, Tokunaga S, Kono S, Arai H, Doi Y, Kawano T, Nakagaki O, Takada K, Koyanagi S, Hiyamuta K, Nii T, Shirai K, Ideishi M, Arakawa K, Mohri M, Takeshita A (2000) Relation between green

tea consumption and the severity of coronary atherosclerosis among Japanese men and women. Ann Epidemiol 10:401–408

Shibata T, Yamaguchi K, Nagayama K, Kawaguchi S, Nakamura T (2002a) Inhibitory activity of brown algal phlorotannins against glycosidases from the viscera of the turban shell *Turbo cornutus*. Eur J Phycol 37:493–500

Shibata T, Fujimoto K, Nagayama K Yamaguchi K, Nakamura T (2002b) Inhibitory activity of brown algal phlorotannins against hyaluronidase. Int J Food Sci Technol 37:703–709

Shibata T, Nagayama K, Tanaka R, Yamaguchi K, Nakamura T (2003) Inhibitory effects of brown algal phlorotannins on secretory phospholipase A_2s, lipoxygenases and cyclooxygenases. J Appl Phycol 15:61–66

Shibata T, Kawaguchi S, Hama Y, Inagaki M, Yamaguchi K, Nakamura T (2004) Local and chemical distribution of phlorotannins in brown algae. J Appl Phycol 16:291–296

Shibata T, Nagayama K, Kawaguchi S, Hama Y (2006a) Antioxidant and radical scavenging activities of brown algal phlorotannins. ITE Lett Batter New Tecnol Med 7:69–72

Shibata T, Hama Y, Miyasaki T, Ito M, Nakamura T (2006b) Extracellular secretion of phenolic substances from living brown algae. J Appl Phycol 18:787–794

Sugiura Y, Matsuda K, Yamada Y, Nishikawa M, Shioya K, Katsuzaki H, Imai K, Amano H (2006) Isolation of a new anti-allergic phlorotannin, phlorofucofuroeckol-B, from an edible brown alga, *Eisenia arborea*. Biosci Biotechnol Biochem 70:2807–2811

Tsukui K, Kakinuma M, Amano H, Uchiyama Y, Shioya K, Katsuzaki H, Imai K (2006) Identification of elastase inhibitors from brown alga, *Eisenia arborea*. ITE Lett Batter New Tecnol Med 7:616–619

Ukeda H, Kawana D, Maeda S, Sawamura M (1999) Spectrophotometric assay for superoxide dismutase based on the reduction of highly water-soluble tetrazolium salts by xanthine–xanthine oxidase. Biosci Biotechnol Biochem 63:485–488

Wu JM, Wang ZR, Hsieh TC, Bruder JL, Zou JG, Huang YZ (2001) Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine. Int J Mol Med 8:3–17

Yamaguchi T, Takamura H, Matoba T, Terao J (1998) HPLC method for evaluation of the free radical-scavenging activity of foods by using 1, 1-diphenyl-2-picrylhydrazyl. Biosci Biotechnol Biochem 62:1201–1204

Zhao B-L, Guo Q, Xin W-J (2001) Free radical scavenging by green tea polyphenols In: Packer L (ed) Flavonoids and other polyphenols. Methods in enzymology, vol 335. Academic, New York, pp 217–231