Algae
Volume 24(3): 169-177, 2009

Potential Antioxidant Activities of Enzymatic Digests from Fresh Water Microalgae, *Pediastrum duplex* and *Dactylococcopsis fascicularis*

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In this study, we focused on natural water-soluble antioxidants from fresh water microalgae, *Pediastrum duplex* and *Dactylococcopsis fascicularis* from Jeju Island, Korea. They were prepared by enzymatic digestion using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase), and the potential antioxidant activity of each was assessed. All enzymatic digests from *P. duplex* showed significant DPPH scavenging effects. Termamyl (60.6%) digest from *P. duplex* possessed the highest effects on hydrogen peroxide scavenging. Celluclast (58.1%) and Kojizyme digests (56.9%) from *D. fascicularis* possessed higher effects on superoxide anion radical scavenging. All enzymatic digests exhibited significant effects on both NO· scavenging and metal chelating. Lipid peroxidation was significantly in inhibited Viscozyme, Termamyl and Kojizyme digests from *P. duplex* and Ultraflo, Protamex, Kojizyme and Alcalase digests from *D. fascicularis*. These data suggest that enzymatic digests of the fresh water microalgae, *P. duplex* and *D. fascicularis* might be valuable sources of antioxidant which can be applied in food and pharmaceutical industry

Key Words: antioxidant activity, *Dactylococcopsis fascicularis*, enzymatic digests, *Pediastrum duplex*

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide are physiological metabolites formed during aerobic life as a result of the metabolism of oxygen. High ROS levels induce oxidative stress, which can result in a variety of pathological conditions, including cardiovascular disease, cancer, and aging (Cox and Cohen 1996; Ames 1998).

As a natural antioxidant source, plants including algae have an ability to absorb the solar radiation for generating high levels of oxygen as secondary metabolites of photosynthesis. Oxygen is easily activated by ultraviolet (UV) radiation and heat from the sunlight to produce toxic ROS. Therefore, plants produce diverse antioxidative compounds to neutralize these ROS for its survival (Lu and Foo 1995). Although many studies regarding antioxidant effects from macroalgae are available, less attention has been paid for microalgae because of difficulties in the isolation and cultivation. Currently, microalgae are being paid more attention as nutraceutical and health food in the market. In the studies about biological composition influencing the nutritional value of microalgae, researchers have studied the levels of proteins that could be used as health food or animal feed (Brown and Jeffrey 1992; Fuentes *et al*. 2000), carbohydrates that could be used as stabilizers and emulsifiers in food and bioactive materials having potential medicinal value (Chu *et al*. 1982; Brown 1991; Brown and Jeffrey 1992). In addition, pigments that could be used as natural food dyes (Brown and Jeffrey 1992; Campo *et al*. 2000) and mineral (Fuentes *et al*. 2000) as well as the other natural bioactive compounds have been studied. So it is thought that microalgae would be very useful in functional materials.

Synthetic antioxidant supplements such as butylated hydroxyanisol (BHA), butylated hydroxytolouene (BHT), α-tocopherol and propyl gallate (PG) have been used in order to reduce oxidative damages in human body (Sherwin 1990; Gülçin *et al*. 2002). However, it is suspect-

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ed that those antioxidants are responsible for some side effects such as liver damage and carcinogenesis (Lindenschmidt *et al*. 1986). Antioxidants can involve with the oxidation process by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers (Shahidi and Wanasundara 1992; Büyükokuroğlu *et al*. 2001).

Recently many researchers are interested in finding any natural antioxidants having safety and effectiveness, which can be substituted for current and commercial synthetic antioxidants, BHA and BHT. Microalgae have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies (Hirata *et al*. 2000; Benedettia *et al.* 2004; Karawita *et al*. 2007; Lee *et al*. 2008). Thus, the objective of this study was to investigate the antioxidant properties of enzymatic digests using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) from fresh water microalgae *Pediastrum duplex* and *Dactylococcopsis fascicularis* from Jeju Island, Korea in order to understand the usefulness of these algae in food and pharmaceutical industry.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium nitroprusside, sulphanilic acid, naphthylethylenediamine dihydrochloride, xanthine, xanthine oxidase from butter milk, nitro blue tetrazolium salt (NBT), butylated hydroxytoluene (BHT), α-tocopherol, 3-(2-Pyridyl)-5,6-di (p-sulfophenyl)-1, 2, 4-triazine disodium salt (ferrozine), potassium ferricyanide ($K_3Fe(CN)_6$), Folin-Ciocalteu reagent and linoleic acid were purchased from Sigma Co. (St Louis, USA). 2, 2-Azino-bis (3-ethylbenz-thiazolin)-6-sulfonic acid (ABTS), peroxidase and 2-deoxyribose were purchased from Fluka Chemie (Buchs, Switzerland). Digestive enzymes of food grades such as Viscozyme, Celluclast, AMG, Termamyl, Ultraflo, Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase were purchased form Novo Co. (Novozyme Nordisk, Bagsvaerd Denmark). All the other chemicals used were of analytical grades.

Sample collection and isolation

Fresh water microalgae sample were directly collected into one liter plastic bottles from the different places of the Mu-Soo Cheon in Jeju Island, Korea. Environmental factors such as water temperature and pH were measured at the sampling spot. Thereafter, recommended applying concentration of F/2 (Aquacenter Ltd. USA) media was added to the natural samples and the bottles were immediately transferred into the plant growth chamber (Vision, VS-3D, Korea) with the temperature of 20, 25 and 30°C and then it was kept for three days. The incubated samples were taken out and the pH was again measured and 1 mL sample was transferred to the S-R chamber for the observation of abundance and community changes of phytoplankton under an inverted microscope. Finally the single cell of *P. duplex* and *D. fascicularis* was picked up from the S-R chamber by using micropipette and transferred into the 12 multi well cell culture plate which contained culture media of F/2 nutrients, soil extract and distilled water. Subculture was carried out until getting the mono-strain of those phytoplankton.

Mass culture

The mass culture of microalgae was done with F/2 nutrients media, 10% soil extract and distilled water. Culture condition was maintained with the temperature, pH, light intensity and L:D cycle of 25°C, 8.0, 180 μE m^{-1} S^{-1} and 12:12 for *P. duplex* and 30°C, 8.5, 180 μE m^{-1} S^{-1} and 12:12 for *D. fascicularis* respectively. Microalgae culture was done in 10 L media carrying capacity bottles (Transparent Polycarbonate Containers, Nalgene, USA). After two weeks of culture, the microalgae biomass was separated with the process of vacuum filtration and freeze-dried, then weighed.

Preparation of enzymatic digests

Freeze-dried fresh water microalgae sample were ground into a fine powder and one gram was mixed with 100 mL of distilled water. The optimum pH of the each reaction mixtures were adjusted with 1 M HCl/NaOH. Optimum pH and temperature conditions for the respective enzymes were similar as described by Heo *et al*. (2003). In this study, food grade enzymes consisting of five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) were used. Enzymes were added at the ratio of 1% to the sample and incubated for 24 h. After the incubation period, reaction mixture was filtered and the enzyme was inactivated by heat (100°C for 10 min). Finally, the pH of the each hydrolysate was adjusted to

pH 7 with 1 M HCl/NaOH. Each digest was adjusted to a concentration of 2 mg mL^{-1} and all activities of enzymatic digests were compared with commercial antioxidants (BHT and α-tocopherol) dissolved in methanol at same concentration.

Proximate composition

Proximate chemical composition of freeze-dried fresh water microalgae sample were determined according to the AOAC methods (1995). Crude lipid content was determined by Soxhlet method and crude protein content was determined by Kjeldhal method. Ash content was determined by calcinations in furnace at 550°C and the moisture content was determined keeping in a dry oven at 105°C for 24 h. In enzymatic digests, the crude protein content was determined by the Lowry method and the polysaccharide content was determined by phenol-sulfuric method.

DPPH free radical scavenging assay

The DPPH free radical scavenging activity of digests was measured by DPPH using the modified method of Brand-Williams *et al*. (1995). The sample (2 mL) was mixed thoroughly with 2 mL of freshly prepared DPPH solution (3×10^{-5} M). The reaction mixture was incubated for 30 min and the absorbance was measured at 517 nm using UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea).

Hydrogen peroxide scavenging assay

The ability of the fresh water microalgae to scavenge H_2O_2 was determined according to the method of Muller (1995). Sample (80 μL) and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37°C for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase (1 U mL^{-1}) were mixed and incubated at 37°C for 10 min and the absorbance was measured at 405 nm.

Superoxide anion scavenging assay

Measurement of superoxide anion scavenging activity of microalgae digests was based on the method of Nagai *et al*. (2003). A mixture of 0.48 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 mL of 3 mM xanthine, 0.02 mL of 3 mM EDTA (ethylenediaminetetraacetic acid), 0.02 mL of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT and 0.02 mL of sample was incubated at 25°C for 10 min. Thereafter the reaction was started by adding 6 mU XOD and kept at 25°C for 20 min. The reaction was stopped by adding 0.02 mL of 6 mM CuCl. The absorbance was measured in microplate reader (Sunrise; Tecan Co. Ltd., Austria) at 560 nm.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined as described by Chung *et al*. (1977). The Fenton reaction mixture (200 μL of 10 mM $FeSO_4.7H_2O$, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose) was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) and with 200 μL of sample. Thereafter, 200 μL of 10 mM H_2O_2 was added and incubated (37°C for 4 h). After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were mixed and placed in a boiling water bath (10 min). After cooling, the mixture was centrifuged (5 min, 395 x g) and absorbance was measured at 532 nm.

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging effect was determined according to the method reported by Garrat (1964). Two milliliter of 10 mM sodium nitroprusside in 0.5 mL of phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of sample and incubated at 25°C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL sulphanilic acid reagent (0.33% in 20% glacial acetic acid) then incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min and absorbance was measured at 540 nm.

Ferrous ion chelating effect

The chelating of ferrous ions by the digests was estimated by the method of Decker and Welch (1990). Sample (5 mL) was added to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine solution and reaction mixture was incubated for 10 min at a room temperature in a shaking incubator. After incubation, the absorbance of reaction mixture was measured at 562 nm.

Determination of lipid peroxidation inhibitory effect with the ferric thiocyanate (FTC) method

The lipid peroxidation inhibitory effect of fresh water microalgae was determined according to the ferric FTC method (Kikuzaki and Nakatani, 1993). Two milliliter of Sample (100 mg L^{-1}) was mixed with 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.05 M of phosphate

buffer (pH 7) and 2 mL of distilled water and kept at 40°C in the dark. A total of 0.1 mL of the above mixture was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate and after 5 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was mixed. The absorbance was measured every 24 h for 7 days.

Total phenolic contents

Total phenolic compounds in the digests were determined according to the method of Chandler and Dodds (1993) using gallic acid as a standard phenolic compound. Sample (1 mL) was mixed with 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. After mixing thoroughly, the mixture was placed in the dark for 1 h then absorbance was measured at 725 nm.

Statistical analysis

Statistical analyses were conducted with the SPSS 11.5 version software package on the triplicate (n = 3) test data. The mean values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Turkey's test. A p-value of less than 0.05 was considered as significant.

RESULTS

The temperature and pH varied from 25 to 30°C and from 7.6 to 8.2, respectively in the natural condition. Incubated samples showed higher abundance of *P. duplex* at 25°C with the pH of 8.1 and the abundance of *D. fascicularis* was higher in the temperature of 30°C with pH 8.5 than other phytoplankton community of several bottles. The biomass production of *P. duplex* and *D. fascicularis* was 0.8 g L^{-1} and 0.6 g L^{-1}, respectively on dry weight basis.

Proximate composition of freeze-dried *P. duplex* and *D. fascicularis* are shown in Table 1. Moisture content of *P. duplex* and *D. fascicularis* were 6.1% and 5.5%, respectively. Carbohydrate, lipid and ash content for *P. duplex* were 30%, 2.4% and 15.2%, respectively, while those for *D. fascicularis* were 33.7%, 4.5% and 8.9%, respectively. In case of protein, *P. duplex* and *D. fascicularis* had 46.3% and 47.4%, respectively. Protein was the most major component in approximate compositions of the microalgae. Total phenolic, polysaccharide and protein content of enzymatic digests are depicted in Table 2. Significant differences in the total phenolic, polysaccharide and protein content among different digests were observed.

Table 1. Proximate composition of *P. duplex* and *D. fascicularis*

Nutrient	*P. duplex*	*D. fascicularis*
Moisture (%)	6.1 ± 0.3	5.5 ± 0.3
Carbohydrate (%)	30.4 ± 0.2	33.7 ± 0.1
Protein (%)	46.3 ± 0.1	47.4 ± 0.2
Lipid (%)	2.4 ± 0.2	4.5 ± 0.3
Ash (%)	15.2 ± 0.4	8.9 ± 0.4

Values are means of three replicates ± SD

The percentage scavenging activity of enzymatic digests against DPPH are shown in Table 3. All enzymatic digests from *P. duplex* exhibited remarkable DPPH scavenging effects and among them, activities displayed by Ultraflo (94.2%), Flavourzyme (94.1%), and Neutrase digest (93.7%) even statistically similar with commercial antioxidants ($P < 0.05$). Further, enzymatic digests from *D. fascicularis* did not exhibit significant effect on DPPH radical scavenging compared to the commercial antioxidants (Table 3). However, digests of Protamex, Viscozyme and Neutrase exhibited moderate effects as 67.9%, 58.4% and 57.4%, respectively.

The H_2O_2 scavenging activities on enzymatic digests are shown in Table 3. Among the recorded results, Termamyl digest (60.6%) from *P. duplex* exhibited ($P < 0.05$) similar H_2O_2 scavenging effects, compared to the commercial antioxidants. In addition, enzymatic digests by Alcalase (48.3%) and Neutrase (40.6%) also displayed considerable effect on H_2O_2 scavenging. Among the enzymatic digests from *D. fascicularis*, Kojizyme (50.2%) and Protamex digest (48.6%) exhibited considerable H_2O_2 scavenging effects.

According to the Table 3, among all the enzymatic digests from *P. duplex*, Termamyl digest (52.8%) seemed significantly effective in superoxide anion scavenging ($P < 0.05$). In addition, all the rest of digests displayed activities more than 40%. Of the enzymatic digests from *D. fascicularis*, Kojizyme, Celluclast, Termamyl, and Ultraflo digests indicated remarkable superoxide anion scavenging effects (56.9%, 58.1%, 54.65, and 50.1%, respectively).

As shown in Table 3, enzymatic digests by Neutrase (46.3%), Celluclast (31.1%) from *P. duplex* and Viscozyme (33.8%) from *D. fascicularis* exhibited higher hydroxyl radical scavenging effects. However, rest of digests did not demonstrate considerable effect.

Nitric oxide scavenging effect of enzymatic digests is shown in Table 3, almost all the enzymatic digests have shown significantly higher activities compared to the

Table 2. Total phenolic, polysaccharide and protein content of different enzymatic digests from *P. duplex* and *D. fascicularis*

Microalgae	Digest	Yield (g/100 g)	Total phenolic[a] (mg/100 g)	Polysaccharide[b] (g/100 g)	Protein[c] (g/100 g)
P. duplex	Viscozyme	45.7	1101.5 ± 52	7.5 ± 0.2	6.7 ± 0.2
	Celluclast	46.2	1214.8 ± 64	2.3 ± 0.1	5.5 ± 0.1
	AMG	44.3	1077.9 ± 58	8.8 ± 0.2	8.6 ± 0.2
	Termamyl	46.5	1167.6 ± 61	7.8 ± 0.1	6.1 ± 0.3
	Ultraflo	43.1	1229.0 ± 72	1.9 ± 0.2	5.7 ± 0.1
	Protamex	32.3	1092.1 ± 68	2.1 ± 0.3	4.7 ± 0.1
	Alcalase	26.4	1082.6 ± 69	2.3 ± 0.3	4.9 ± 0.1
	Flavourzyme	33.1	1247.9 ± 61	9.0 ± 0.1	7.5 ± 0.2
	Neutrase	46.6	1337.6 ± 84	7.5 ± 0.2	6.7 ± 0.1
	Kojizyme	24.2	1082.6 ± 69	2.0 ± 0.2	5.5 ± 0.3
D. fascicularis	Viscozyme	21.6	978.8 ± 32	3.2 ± 0.3	7.4 ± 0.3
	Celluclast	42.4	941.0 ± 34	9.7 ± 0.2	5.1 ± 0.5
	AMG	36.8	922.1 ± 48	2.7 ± 0.4	5.1 ± 0.3
	Termamyl	28.1	931.5 ± 31	6.1 ± 0.1	10.1 ± 0.4
	Ultraflo	30.2	870.2 ± 32	6.8 ± 0.1	10.1 ± 0.2
	Protamex	28.8	1384.8 ± 28	8.4 ± 0.1	9.8 ± 0.7
	Alcalase	31.2	1342.3 ± 39	9.8 ± 0.2	7.8 ± 0.3
	Flavourzyme	38.4	1092.1 ± 21	8.6 ± 0.2	1.3 ± 0.1
	Neutrase	26.4	1068.5 ± 24	7.1 ± 0.4	8.8 ± 0.1
	Kojizyme	28.1	983.5 ± 19	5.3 ± 0.3	8.2 ± 0.4

Values are means of three replicates ± SD.
[a] As equivalent gallic acid, [b] As equivalent to glucose, [c] As equivalent to bovine serum albumin

Table 3. Antioxidant activities of enzymatic digests from *P. duplex* and *D. fascicularis*

Microalgae	Digest	DPPH[a]	H_2O_2[b]	$O^{\cdot-}_2$[c]	OH·[d]	NO·[e]	FC[f]
P. duplex	Viscozyme	81.5d ± 5.4	32.5e ± 1.3	41.1g ± 1.6	28.4e ± 0.9	39.1c ± 1.1	71.4d ± 4.3
	Celluclast	88.5b ± 5.9	33.2e ± 1.6	43.2g ± 2.4	31.1d ± 1.1	44.8a ± 2.2	24.8h ± 1.2
	AMG	77.3e ± 4.7	33.1e ± 1.7	42.1g ± 1.8	22.7e ± 0.3	42.6a ± 2.4	77.1b ± 4.2
	Termamyl	90.5a ± 6.2	60.6a ± 3.7	52.8d ± 2.1	21.4e ± 0.7	41.8a ± 2.5	45.7g ± 1.1
	Ultraflo	94.2a ± 6.4	30.3f ± 1.4	40.3g ± 1.9	28.5e ± 0.6	40.1b ± 1.7	73.6d ± 4.7
	Protamex	91.2a ± 6.5	34.1d ± 2.1	40.3g ± 2.3	19.3e ± 0.4	39.6c ± 1.1	75.2c ± 5.1
	Alcalase	89.4b ± 6.2	48.3b ± 1.9	46.2f ± 2.4	18.7f ± 0.9	20.7g ± 0.8	64.8e ± 3.4
	Flavozyme	94.1a ± 6.3	37.1d ± 1.3	43.9g ± 1.1	29.6e ± 1.3	38.7c ± 1.1	55.5f ± 2.7
	Neutrase	93.7a ± 6.1	42.2c ± 1.5	42.1g ± 2.1	46.3c ± 1.7	37.9d ± 1.5	71.7d ± 5.7
	Kojizyme	83.1c ± 5.3	27.2h ± 0.9	41.2g ± 1.4	22.4e ± 1.3	40.4b ± 1.8	79.1b ± 6.1
D. fascicularis	Viscozyme	58.4g ± 2.3	32.4e ± 1.7	49.6f ± 2.1	33.8d ± 1.3	39.8c ± 1.3	65.4e ± 3.2
	Celluclast	41.2i ± 1.6	24.8h ± 1.3	58.1b ± 2.3	23.2e ± 0.9	32.2e ± 1.6	86.8a ± 5.7
	AMG	38.3e ± 1.4	34.6d ± 1.3	47.1f ± 3.1	17.1g ± 0.4	40.4b ± 0.9	58.6f ± 3.3
	Termamyl	46.7h ± 2.4	32.5e ± 1.5	54.6c ± 2.4	18.2f ± 0.3	35.2d ± 0.7	77.7b ± 4.5
	Ultraflo	43.2i ± 2.1	31.3e ± 1.2	50.1e ± 2.9	19.3f ± 0.7	42.8a ± 0.5	77.1b ± 5.7
	Protamex	67.9f ± 3.7	48.6b ± 2.2	34.3h ± 1.7	20.9e ± 1.1	39.8c ± 0.7	75.6c ± 6.2
	Alcalase	49.5h ± 2.4	27.6g ± 0.7	39.5h ± 1.4	15.7g ± 0.3	36.8c ± 0.3	76.6c ± 5.1
	Flavozyme	42.7i ± 1.2	24.9h ± 0.4	47.6f ± 2.4	23.2e ± 0.4	43.9a ± 1.2	53.3f ± 3.7
	Neutrase	57.4g ± 2.4	40.6c ± 1.4	41.9g ± 2.3	23.2e ± 0.9	41.5a ± 1.6	80.1b ± 6.2
	Kojizyme	41.4i ± 1.4	50.2b ± 2.1	56.9b ± 2.1	26.1e ± 0.3	41.2a ± 1.2	74.2d ± 4.9
	BHT	94.6 a ± 6.4	60.1a ± 4.2	63.2a ± 4.3	76.6b ± 4.6	26.1f ± 0.9	11.5i ± 0.1
	Tocopherol	94.3a ± 7.1	62.5a ± 4.9	61.5a ± 4.7	79.5a ± 4.7	25.2f ± 0.6	10.3i ± 0.1

Sample concentration is 2 mg mL^{-1}; Data are Means ± SE (n = 3); values in each column followed by different letters denote significant difference at $p < 0.05$.
[a] DPPH free radical scavenging activity, [b] hydrogen peroxide scavenging activity, [c] superoxide anion scavenging activity, [d] hydroxyl radical scavenging activity, [e] nitric oxide scavenging activity, [f] ferrous ion chelating effect

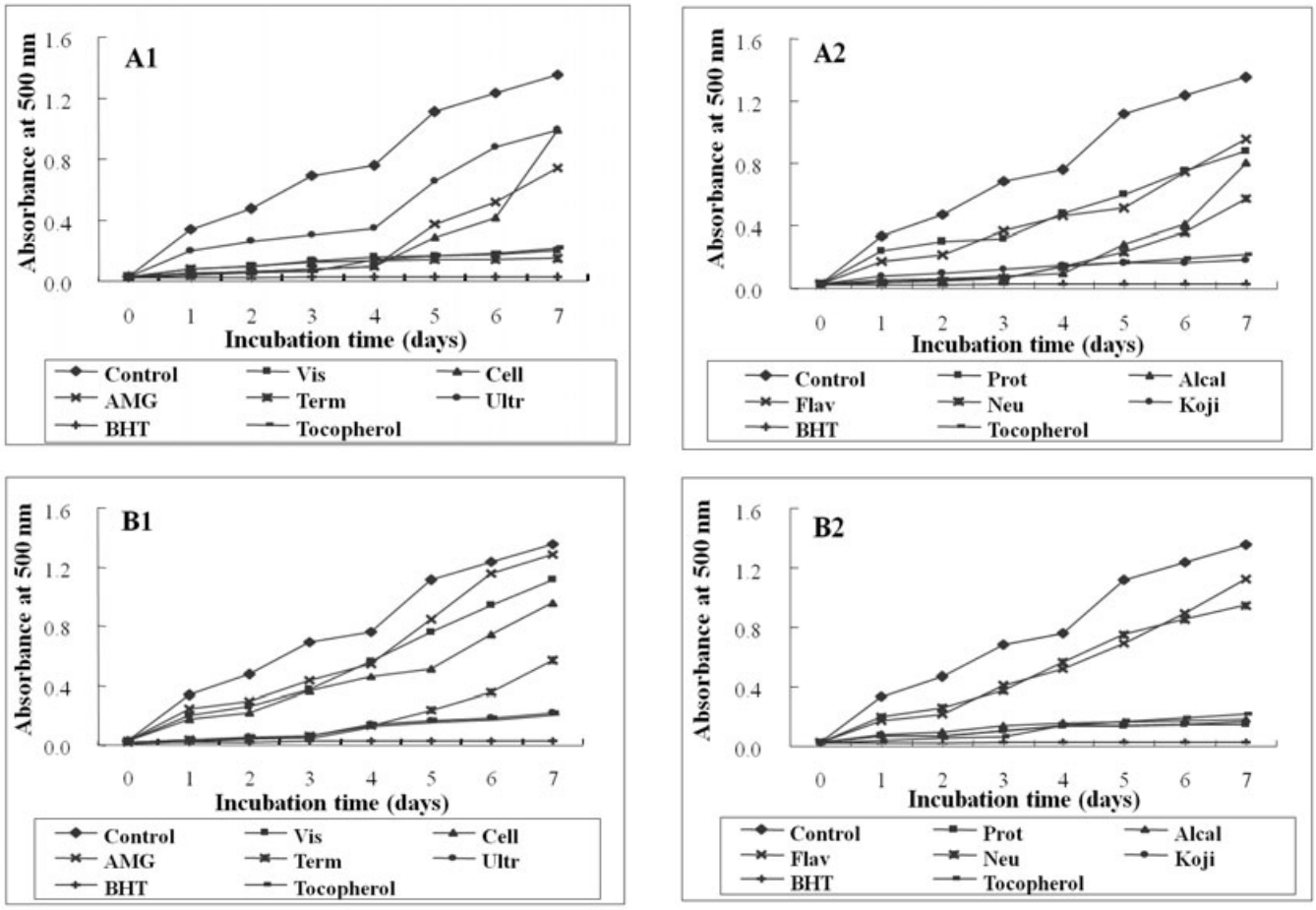

Fig. 1. Lipid peroxidation inhibitory activity of different enzymatic digests from *P. duplex* (A_1: Carbohydrases; A_2: Proteases) and *D. fascicularis* (B_1: Carbohydrases; B_2: Proteases) compared to BHT and α - Tocopherol at 1 mg mL^{-1} of concentration of ethanol as assessed by linoleic acid (Vis: Viscozyme, Cell: Celluclast, Term: Termamyl, Ultr: Ultraflo, Prot: Protamex, Koji: Kojizyme, Neu: Neutrase, Flav: Flavourzyme, Alcal: Alcalase).

commercial antioxidants ($P < 0.05$). Among these digests, Celluclast (44.8%), AMG (42.6%), Termamyl (41.8%), Kojizyme (40.4%) and Ultraflo digest (40.1%) from *P. duplex* and Flavourzyme (43.9%), Ultraflo (42.8%), Neutrase (41.5%), Kojizyme (41.2%), and AMG digest (40.4%) from *D. fascicularis* were notable (Table 3).

According to the Table 3, all the enzymatic digests have exhibited strong ferrous ion chelating effects even significantly ($P < 0.05$) higher than the effects in the commercial antioxidants. Among them Kojizyme (79.1%), AMG (77.1%), Protamex (75.2%), Ultraflo (73.6%), Neutrase (71.7%) and Viscozyme digest (71.4%) from *P. duplex* and Celluclast (86.8%), Neutrase (80.1%), Termamyl (77.7%), Ultraflo (77.1%) and Alcalase digest (76.6%) from *D. fascicularis* exhibited strong chelating effects.

As shown in Fig. 1, the absorbance of linoleic acid emulsion without the addition of any digest was increased. Enzymatic digests from *P. duplex* by Viscozyme, Termamyl, and Kojizyme as well as the enzymatic digests from *D. fascicularis* by Ultraflo, Protamex, Kojizyme and Alcalase had strong antioxidant effects statistically similar with α- tocopherol (Fig. 1). No significant antioxidant activity was presented with other digests compared to the commercial antioxidants.

DISCUSSION

We presently focused on natural water-soluble antioxidants from two species of the Jeju freshwater microalgae, which was prepared by enzymatic digestion using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) and potential antioxidant activities of the resultant enzymatic digests were evaluated using different reactive oxygen species (ROS) scavenging assays, ferrous ion chelating effect and lipid peroxidation inhibitory activity.

DPPH is a free radical generating compound and has been widely used to evaluate the free radical scavenging ability of various antioxidative compounds. DPPH free radical scavenging activity of all enzymatic digests from *P. duplex* was relatively higher than the digests from *D. fascicularis*. Especially Ultraflo, Flavourzyme and Neutrase digests of *P. duplex* exhibited strong scavenging activity (above 90%). Many researchers have reported a positive correlation between free radical scavenging activity and total phenolic compound. Oki *et al*. (2002) observed that the radical scavenging activity of sweet potato increased with the increase of phenolic compound content. The two studies conducted by Lu and Foo (2000) and Siriwardhana *et al*. (2003) reported a high correlation between DPPH radical scavenging activities and total polyphenolics. In this study, some enzymatic digests of *D. fascicularis* did not possess antioxidant activity, although they contained as much polyphenolic compounds as the other digests. It is thought that other bioactive components, such as proteins, polysaccharides and different kinds of pigments were present in the digestion of microalgae. For example, it was found that oligosaccharides, sulfate and glycoprotein components in red microalga *Porphyridium* sp. have exhibited antioxidant activities (Spitz *et al*. 2005). Thus, it can be concluded that all those factors influenced the antioxidant activity.

Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals. Thus, removing H_2O_2 is very important for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent HO. mediated oxidative DNA damage (Halliweill 1991). According to the H_2O_2 scavenging activity results, all the enzymatic digests exhibited activities indicating the efficiency of scavenging of H_2O_2 by both microalgae.

Superoxide anion and hydroxyl radicals are the two most effective representative free radicals. In cellular oxidation reactions, superoxide anion radical is normally formed first and its effects can be magnified because it produces other kinds of cell damaging free radicals and oxidizing agents (Liu and Ng 2000). In this study, the superoxide anion scavenging activities of carbohydrase digests of *P. duplex* and *D. fascicularis* exhibited higher effects than proteases digests. Therefore, it could be assumed that carbohydrases have more ability to liberate potential antioxidant compounds in superoxide anion scavenging. Although superoxide anion is itself a weak oxidant, it can be converted into the powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Dahl and Richardson 1978).

Hydroxyl radical scavenging activity of the enzymatic digests from fresh water microalgae was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. According to the hydroxyl radical scavenging activity results, all the enzymatic digests of *P. duplex* and *D. fascicularis* had significantly low scavenging activity for hydroxyl radical, as compared to the other reactive oxygen species scavenging activities. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization and fragmentation (Liu and Ng 2000).

Nitric oxide is a gaseous free radical, which has important roles in physiological and pathological conditions. Enzymatic digests exhibited considerable effects which could be attributed to their hydrophilic properties. The reactivities of the NO· and $O\cdot_2^-$ were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induce toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Radi *et al*. 1991; Moncada *et al*. 1991). Therefore, the scavenging ability of NO· from the microalgae digests may help to interrupt the chain reactions initiated by excessive production of NO· that are harmful to human health.

Ferrous is known as the most important lipid oxidizing prooxidant among the transition metals due to its high reactivity. Ferrozine can make red color complexes with ferrous ions. In presence of chelating agents, complex formation is interrupted and as a result, the red color of the complex is decreased. All the enzymatic digests from fresh water microalgae indicating significant chelating ability, which further support the idea that the hydrophilic components are accountable for ion chelating. Ferrous ions accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliweill 1991; Fridovich 1995). In this study, different digests of fresh water microalgae demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protector, which relates to their ferrous binding capacity (Gülçin *et al*. 2004).

To evaluate the lipid peroxidation inhibition effect of microalgae digests, their lipid peroxidation were compared with the commercial antioxidants using FTC method by determining the amount of peroxide formed in emulsion during incubation period. High absorbance is an indication associated with high concentration of formed peroxides. In this experiment, the enzymatic digests from *P. duplex* by Kojizyme, Termamyl and Viscozyme, and Kojizyme, Alcalase, Protamex, and Ultraflo digests from *D. fascicularis* provided higher antioxidant effects and were able to suppress the formation of peroxide. It suggests that according to enzyme digestion, different antioxidant components were released from the inside of microalgae cells. These digests contained high levels of polysaccharides, proteins and polyphenols and it could be assumed that those components are responsible for inhibition of lipid peroxidation.

The cell wall of *D. fascicularis* consisted of murein, a peptidoglycan with peptide side chains attached to linked, alternating residues of N-acetylglucosamine and N-acetylmuramic acid. The cell wall layers lying outside the murein layer consist for the most part of lipopolysaccharides. The mucilage sheaths are composed predominantly of complex polysaccharides. The cell wall of *P. duplex* consists of a fibrillar fraction embedded in an amorphous matrix. The fibrillar fraction gives the cell wall its strength and usually consists predominately cellulose. In this study, enzymes (carbohydrases and proteases) were used for the extraction of antioxidant compounds. Materials of the fresh water microalgae cell wall may interface with the proper extraction of bioactive compounds. In order to overcome such barriers, enzymes can be applied to efficiently extract the desired compounds with higher yields. Enzymatic digests of algae have reported significant yields of desired compounds in the previous studies (Heo *et al*. 2003; Siriwardhana *et al*. 2004). The breakdown and releasing of high molecular weight polysaccharides and proteins themselves may contribute to the enhance antioxidative activities (Ramos and Xiong 2002; Ruperez *et al*. 2002). Additionally, enzymatic digests possesses innovative advantages and characters over conventional extraction procedures. Of the several advantages, water solubility, higher extraction efficacy, greater variation of constituents, minimized environmental pollution and comparative inexpensiveness are obvious. In conclusion, Jeju fresh water microalgae exhibited antioxidant potential against ROS and it would be an excellent candidate as a natural antioxidant source, which can be applied in food and pharmaceutical industry.

ACKNOWLEDGMENTS

This research was supported by a grant from the Jeju Sea Grant Program, funded by the Ministry of Land, Transport and Maritime Affairs, Republic of Korea.

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Received 30 July 2009
Accepted 18 August 2009