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Potential Antioxidant Activites of Enzymatic Digests from Benthic Diatoms *Achnanthes longipes*, *Amphora coffeaeformis*, and *Navicula* sp. (Bacillariophyceae)

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Abstract

In this study, we focused on natural water-soluble antioxidants from the Jeju benthic diatoms, *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis*. 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 their potential antioxidant activity was assessed. Among the enzymatic digests, Neutrase digest from *A. coffeaeformis* exhibited the highest effect in DPPH radical scavenging. Flavourzyme (48.7%), Viscozyme (47.4%) and Celluclast (45.7%) digests from *Navicula* sp. exhibited higher O^{-}_{2} radical scavenging activity. Viscozyme digest from *A. coffeaeformis* (45.9%) possessed the highest effects in hydroxyl radical scavenging. Termamyl (89.3%) and Protamex (88.8%) digests from *A. coffeaeformis* had strong metal chelating activity. Lipid peroxidation was significantly inhibited in Termamyl and Kojizyme digests from *A. longipes*, AMG and Termamyl digests from *Navicula* sp. and Kojizyme digest from *A. coffeaeformisi*. These data suggest that enzymatic digests of the Jeju benthic diatoms might be valuable sources of antioxidant which can be applied in food and pharmaceutical industry.

Key words: benthic diatoms, *Achnanthes longipes*, *Navicula* sp., *Amphora coffeaeformis*, enzymatic digests, antioxidant activity

INTRODUCTION

Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and superoxide anions, which are produced because of exogenous factors (tobacco smoke, ionizing radiation and certain pollutants) and endogenous factors (normal aerobic respiration) have been implicated in cellular processes such as mutagenesis, carcinogenesis and premature aging. ROS have the ability to react with a large variety of easily oxidisable cellular components, such as proteins, lipids and lipoproteins (1). Oxidation is one of the major causes of food deterioration, resulting in the destruction of fat-soluble vitamins and development of off colors and toxicants (2,3). Besides, in the food industry lipid peroxidation is an important deteriorative reaction during processing and storage.

Commercial antioxidant supplements such as butylated hydroxyl anisole (BHA), butylated hydroxy tolouene (BHT), α-tocopherol and propyl gallate (PG) have been used in order to reduce oxidative damages in human body (4,5). However, it is suspected that those antioxidants are responsible for some side effects such as liver damage and carcinogenesis (6). Antioxidants are involved in the oxidation mechanism by scavenging free radicals, chelating catalytic metals and by acting as oxygen scavengers (7,8).

As a natural antioxidant source, plants have an ability to absorb the sun's radiation for generating high levels of oxygen as secondary metabolites of photosynthesis. Oxygen is easily activated by ultra violet (UV) radiation and heat from the sunlight to produce toxic ROS. Therefore, plants produce various antioxidative compounds to protect them from harmful effects of ROS (9). Although many studies regarding antioxidant effects from macroalgae are available, less attention has been paid to microalgae because of difficulties in the isolation and cultivation. Currently, microalgae are receiving more attention as nutraceuticals and health foods in the market. Several microalgae, such as *Chlorella* sp., *Spirulina* sp. and *Dunaliella* sp. are grown commercially for the production of algal products such as β-carotene, lutein and phycocyanin. In addition, the antioxidative activity of phycocyanobilin from *Spirulina platensis* was evaluated against oxidation of methyl linoleate in a hydrophobic system or with phosphatidylcholine liposomes (10).

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Phycocyanobilin effectively inhibited the peroxidation of methyl linoleate and produced a prolonged induction period. Another microalgae, *Aphanizomenon flos-aquae* (Cyanophyta) was reported to contain high amounts of phycocyanin, a photosynthetic pigment with antioxidant and anti-inflammatory properties (11).

Jeju is a volcanic island of Korea, which belongs to the subtropical region where the benthic diatoms are used in commercial hatcheries as a live feed for shellfish. The coastal water temperature and salinity of this island fluctuate widely and benthic diatoms are often found in high abundance in this coastal water throughout the year (12). In a previous study, it was found that the Jeju benthic diatom *Grammatophora marina*, which is commonly used as a live feed for shellfish in commercial hatcheries, possessed antioxidant activity (13).

In this study, we used selected carbohydrate degrading enzymes and proteases derived from microorganisms. These enzymes can convert water-insoluble microalgae into water-soluble materials. Enzymatic digests from algae possess water-solubility and safety, as this method does not utilize any organic solvent or other toxic chemicals. Additionally it has a high yield and high radical scavenging activity in comparison with organic extracts, as shown in the previous report (14).

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 benthic diatoms, *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis* 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). Food grade digestive enzymes: 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.

MATERIALS

Sample collection

Natural samples of benthic diatoms were collected from National Fisheries Research and Development Institute (NFRDI), Jeju Island, Korea. Benthic diatoms attached to wavy plastic plate (called Papan) are used for abalone larval live food. Environmental conditions, especially temperature, pH and salinity were measured on the sampling spot and were 25°C, 8.02 and 30 psu, respectively. The attached benthic diatoms were recovered from the papan and diluted with the same seawater. The sample was again diluted and 1 ml was transferred to the S-R chamber. A single cell of the benthic diatoms was picked up from the S-R chamber by using micropipette under inverted microscope (Olympus, IX71). The single cell was transferred into multi-well for subculture. Subculture of the isolated species was done with autoclaved seawater which was filtered through 0.4 μm filter membrane (Millipore Co., Bedford, MA) and enriched with F/2 nutrients media (Aquacenter Ltd., USA), trace metals and metasilicate anhydrous crystals (Na_2SiO_3). The isolation process was carried out until a mono-strain of the benthic diatoms was obtained. The cultured benthic diatoms were observed under the phase-contrast microscope (Zeiss Axioplan) and the identification was done as described by Shim (15).

Isolation of axenic strain

Bacteria were found to grow with the diatom mono-strain during culture which decomposes the microalgae. Further, it was suspected that the bacteria's own cell compounds might play a vital role in the determination of antioxidant properties of the phytoplankton. Thus, axenic strains of those benthic diatoms were prepared according to the method described by Affan et al. (13). In brief, the mono-strain of each species was streaked on agar plate that was prepared with 2% agar (w/v) and 0.04% F/2 (v/v) nutrients media and autoclaved seawater. Mono-strain colony was transferred from the agar plate into the 250 mL flask which contained 100 mL of F/2 enriched culture media and antibiotics cocktail (Penicillin, Streptomycin and Neomycin) (SIGMA P 4083). Seven different dosages of an antibiotic cocktail (penicillin 100 ~250 unit/mL, streptomycin 100~250 μg/mL and neomycin 200~500 μg/mL) with interval of 25 unit-penicillin/mL, 25 μg-streptomycin/mL and 50 μg-neomycin/mL were used. The cultured sample was again streaked

on the bacto-agar media for the observation of the presence of bacteria. Finally axenic strain of those benthic diatoms was obtained for succeeding studies.

Mass culture

The mass culture of benthic diatoms was done for 2 weeks in 10 liter high-density polypropylene bottles (Nalgene, USA) with autoclaved artificial seawater media, which was enriched with F/2 nutrients media, sodium silicate (Na_2SiO_3), and trace metals solution and with aeration of 0.03 kgf/cm^2 which provided 10 liter of air per minute (Btech, Korea). Mass culture condition was maintained with a salinity of 30 psu, temperature of 25°C, pH 8, 12L: 12D cycle and fluorescent light intensity of 30 180 μE m^{-1} s^{-1}, respectively. The standing crop was separated from the culture media by filtering and finally freeze-dried at -70°C.

Preparation of enzymatic digests

Freeze-dried benthic diatom samples were ground into a fine powder and one gram was mixed with 100 mL of distilled water. The optimum pH of each reaction mixtures were adjusted with 1 M HCl/NaOH. Optimum pH and temperature conditions for the respective enzymes were as described by Heo et al. (14). 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 hr. After the incubation period, reaction mixture was filtered and the enzyme was inactivated by heat (100°C for 10 min). Finally, the pH of each hydrolysate was adjusted to pH 7 with 1 M HCl/NaOH. All activities of digests were compared with commercial antioxidants (BHT and α-tocopherol) dissolved in methanol.

Proximate composition

Proximate chemical composition of freeze-dried benthic diatom samples were determined according to the AOAC methods (16). 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 by keeping in a dry oven at 105°C for 24 hr. In extracts, the crude protein content was determined by the Lowry spectrophotometric method and the polysaccharide content was determined by the phenol-sulfuric method.

DPPH free radical scavenging assay

The DPPH free radical scavenging activities of digests were measured by DPPH using the modified method of Brand-Williams et al. (17). The sample (2 mL) was mixed thoroughly with 2 mL of freshly prepared DPPH solution (3×10^{-5} M). The reaction mixture was incubated in the room temperature 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 diatom to scavenge H_2O_2 was determined according to the method of Muller (18). 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) 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 digests was based on the method described by Nagai et al. (19). 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 according to the method of Chung et al. (20). The Fenton reaction mixture (200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 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, $395\times g$) and absorbance was measured at 532 nm.

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging was determined according to the method reported by Garrat (21). 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 a 0.5 mL aliquot was removed 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 (22). 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 the benthic diatom was determined according to the FTC method (23). Two milliliter of Sample (100 mg/L) 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 hr for 7 days.

Total polyphenolic content

Total phenolic compounds in the digests were determined with Folin-Ciocalteu reagent according to the method of Chandler and Dodds (24) using gallic acid as a standard phenolic compound. Sample (1 mL) was mixed with 1mL 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 hr and 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 Tukey's test. A p-value less than 0.05 was considered significant.

RESULTS

The benthic diatoms were found as an axenic strain with the dosages of 200 units-penicillin/mL, 200 μg-streptomycin/mL and 400 μg-neomycin/mL among different dosages of antibiotic cocktails. The mass culture of the isolated benthic diatoms was done according to the natural environmental conditions. The yield of *Achnanthes longipes*, *Navicula* sp. and *Amphora coffeaeformis* was 1.25, 0.94, and 1.08 g/L on dry weight basis respectively.

Proximate composition

Proximate compositions of freeze dried *A. longipes Navicula* sp. and *A. coffeaeformis* are shown in Table 1 and total polyphenolic, polysaccharide and protein content in the enzymatic digests are depicted in Table 2.

DPPH free radical scavenging effect

The percentage scavenging activity of enzymatic digests against DPPH are shown in Table 3. Significant differences in the activities ($p<0.05$) among the different diatom species and among different digests were observed. The enzymatic digests exhibited less significant ($p<0.05$) effects on DPPH radical scavenging compared to commercial antioxidants (Table 3). However, Neutrase digest from *A. coffeaeformis* (75.9%) and *Navicula* sp. (72.6%) and Flavourzyme digest from *A. coffeaeformis* (65.6%) exhibited relatively higher effects.

Hydrogen peroxide scavenging effect

As depicted in Table 3, enzymatic digests from the diatoms exhibited less significant ($p<0.05$) effects on hydrogen peroxide scavenging. Ultraflo (24.9%) and Neutrase (23.4%) digests from *Navicula* sp. showed higher activities among those enzymatic digests and no significant activity ($p<0.05$) was observed from the rest of the digests.

Superoxide anion scavenging effect

As shown in Table 3, the enzymatic digests from *A. longipes* by AMG (48.3%), Viscozyme (44.6%) and Celluclast (43.7%) and the digests from *Navicula* sp. by Flavourzyme (48.7%), Celluclast (45.7%) and Viscozyme (47.4%) indicated remarkable superoxide anion scavenging activity. However, *A. coffeaeformis* showed less effect in superoxide anion scavenging.

Table 1. Proximate composition of *A. longipes*, *Navicula* sp. and *A. coffeaeformis*

Nutrient	*A. longipes*	*Navicula* sp.	*A coffeaeformis*
Moisture (%)	8.1±0.3	3.6±0.3	5.9±0.3
Carbohydrate (%)	16.4±0.2	13.5±0.1	15.8±0.4
Protein (%)	6.5±0.1	16.9±0.2	15.6±0.3
Lipid (%)	1.1±0.2	2.1±0.3	6.9±0.3
Ash (%)	67.9±0.4	63.9±0.4	55.8±0.3

Values are means of three replicates±SD.

Table 2. Total polyphenolic, polysaccharide and protein content of different enzymatic digests from *A. longipes*, *Navicula* sp. and *A. coffeaeformis*

Diatom	Extract	Yield (g/100 g)	Total polyphenol[1)] (mg/100 g)	Polysaccharide[2)] (g/100 g)	Protein[3)] (g/100 g)
A. longipes	Viscozyme	45.1	334.9±26	1.9±0.05	3.3374±0.02
	Celluclast	47.8	343.7±24	2.5±0.12	3.9196±0.02
	AMG	47.4	331.8±22	3.1±0.14	5.3562±0.01
	Termamyl	43.8	310.8±19	3.1±0.16	3.8544±0.02
	Ultraflo	27.1	193.6±13	2.1±0.11	2.1138±0.03
	Protamex	36.9	272.9±15	2.4±0.14	4.1697±0.03
	Alcalase	48.6	367.8±29	3.4±0.18	4.2768±0.01
	Flavourzyme	40.2	304.9±24	2.6±0.12	4.5426±0.04
	Neutrase	46.2	336.5±27	4.4±0.24	2.3562±0.02
	Kojizyme	26.3	204.9±16	1.7±0.09	3.9187±0.05
Navicula sp.	Viscozyme	30.2	185.5±11	1.4±0.07	2.5368±0.04
	Celluclast	34.3	160.4±11	1.6±0.08	2.6411±0.05
	AMG	43.2	158.8±08	1.7±0.09	2.6352±0.01
	Termamyl	32.1	160.7±11	3.8±0.16	2.9211±0.02
	Ultraflo	20.6	152.8±07	1.5±0.06	2.9046±0.03
	Protamex	32.6	212.7±14	1.8±0.07	0.6846±0.02
	Alcalase	36.4	215.5±16	1.8±0.07	0.6916±0.06
	Flavourzyme	32.1	186.6±07	1.6±0.08	0.6741±0.05
	Neutrase	28.3	269.1±18	2.6±0.12	3.9903±0.03
	Kojizyme	24.6	217.9±17	0.7±0.02	2.4108±0.02
A. coffeaeformis	Viscozyme	54.6	227.1±13	6.1±0.31	5.2962±0.04
	Celluclast	44.3	190.3±09	7.4±0.42	4.7401±0.05
	AMG	40.2	123.1±08	4.6±0.21	1.9698±0.02
	Termamyl	53.6	195.5±11	5.2±0.32	5.5744±0.04
	Ultraflo	48.3	288.4±17	4.4±0.24	5.6994±0.05
	Protamex	40.1	263.7±19	1.2±0.01	7.8195±0.03
	Alcalase	40.6	333.4±27	3.7±0.16	1.8676±0.02
	Flavourzyme	38.3	159.1±11	2.4±0.14	3.6002±0.04
	Neutrase	49.9	125.7±07	2.1±0.11	3.9421±0.02
	Kojizyme	54.3	151.8±11	2.2±0.15	4.2354±0.01

Values are means of three replicates±SD.
[1)]As gallic acid equivalents. [2)]As glucose equivalents. [3)]As bovine serum albumin equivalents.

Hydroxyl radical scavenging effect

According to Table 3, enzymatic digests of *A. longipes* exhibited higher scavenging activity. Out of them, Flavourzyme (43.9%), Ultraflo (42.8%) Neutrase (41.5%) and Kojizyme (41.2%) digests gave remarkable results (Table 3). However, *Navicula* sp. and *A. coffeaeformis* (except Viscozyme digest; 45.9%) showed less significant ($p<0.05$) effects in hydroxyl radical scavenging.

Nitric oxide radical scavenging effect

Nitric oxide scavenging effect of enzymatic digests is shown in Table 3, Kojizyme (29.9%) and Viscozyme (28.4%) digests from *A. coffeaeformis* exhibited significant ($p<0.05$) inhibitory effects, as compared to the commercial antioxidants. Among the other digests, Ultraflo (24.9%) and Termamyl (23.8%) digests possessed considerable nitric oxide scavenging inhibition. All enzymatic digests from *Navicula* sp. and *A. coffeaeformis* showed significant nitric oxide inhibition activities ($p<0.05$) and among them, Viscozyme (46.4%), Celluclast (44.6%) and AMG (43.6%) digests from *A. coffeaeformis* were most outstanding.

Ferrous ion chelating effect

According to Table 3, all the enzymatic digests except Viscozyme, Celluclast, and AMG from *A. longipes* revealed significantly higher activities ($p<0.05$), as compared to the commercial antioxidants. The enzymatic digests from *A. coffeaeformis* by Termamyl (89.3%), Protamex (88.8%), Alcalase (86.2%) and AMG (60.7%), and which from *A. longipes* by Ultraflo (75.8%) and Kojizyme (73.5%) exhibited strong chelating effect.

Lipid peroxidation inhibitory effect

As shown in Fig. 1, the absorbance of linoleic acid

Table 3. Antioxidant activities of enzymatic digests from *A. longipes*, *Navicula* sp. and *A. coffeaeformis* (tested concentration is 2 mg/mL)

Diatom	Digest	DPPH[1)]	H_2O_2[2)]	$O^{\cdot-}_2$[3)]	OH·[4)]	NO·[5)]	FC[6)]
A. longipes	Viscozyme	24.3±0.3^{h}	10.8±0.2^{g}	44.6±1.1^{c}	39.8±1.2^{c}	28.4±0.7^{f}	8.8±0.4^{i}
	Celluclast	23.6±1.3^{h}	4.5±0.1^{h}	43.7±0.9^{c}	32.2±1.6^{d}	17.1±0.5^{h}	10.2±0.2^{i}
	AMG	21.8±1.6^{h}	5.3±0.3^{h}	48.3±0.6^{b}	40.4±2.1^{c}	16.1±0.6^{h}	9.4±0.2^{i}
	Termamyl	23.6±1.3^{h}	7.1±0.4^{h}	36.8±1.3^{e}	35.2±1.1^{c}	23.8±0.8^{f}	15.1±0.4^{i}
	Ultraflo	36.4±1.6^{f}	10.1±0.7^{g}	40.5±1.6^{d}	42.8±2.4^{b}	24.9±1.2^{f}	75.8±3.7^{b}
	Protamex	31.1±1.4^{f}	10.9±0.7^{g}	41.8±1.4^{c}	39.8±1.6^{c}	14.6±0.4^{h}	49.3±1.3^{c}
	Alcalase	30.8±1.3^{g}	8.2±0.6^{h}	36.1±1.7^{e}	36.8±1.7^{c}	17.7±1.1^{h}	51.5±2.7^{c}
	Flavourzyme	26.1±0.3^{g}	11.3±0.7^{g}	35.4±0.6^{f}	43.9±2.4^{b}	22.1±0.9^{g}	27.3±1.6^{g}
	Neutrase	28.8±1.4^{g}	12.1±0.9^{g}	28.6±1.4^{g}	41.5±1.7^{b}	17.1±0.9^{h}	17.3±1.4^{h}
	Kojizyme	34.5±1.6^{f}	7.7±0.2^{h}	29.1±0.4^{g}	41.2±1.4^{b}	29.9±1.1^{e}	73.5±4.9^{a}
Navicula sp.	Viscozyme	32.1±1.3^{f}	16.2±0.6^{f}	47.4±1.2^{b}	24.3±1.1^{f}	30.1±1.6^{e}	22.1±0.6^{h}
	Celluclast	37.5±1.4^{f}	15.1±0.4^{f}	45.7±1.8^{b}	19.3±0.6^{g}	27.4±1.3^{f}	25.3±0.7^{g}
	AMG	30.3±1.4^{f}	19.4±0.5^{e}	43.1±1.7^{c}	5.3±0.4^{f}	32.7±1.1^{e}	25.3±0.7^{g}
	Termamyl	38.8±1.1^{f}	16.4±0.7^{f}	40.9±1.6^{d}	2.4±0.1^{h}	29.3±1.6^{e}	40.8±1.9^{f}
	Ultraflo	44.6±1.2^{d}	24.9±0.5^{c}	34.3±1.4^{f}	13.8±0.7^{h}	40.6±1.7^{c}	34.3±1.7^{g}
	Protamex	41.3±1.8^{e}	12.4±0.2^{g}	38.1±0.7^{e}	11.1±0.4^{h}	24.3±0.6^{f}	28.6±1.3^{g}
	Alcalase	42.9±2.1^{e}	15.4±0.9^{f}	36.4±1.6^{e}	12.1±0.7^{h}	32.9±1.4^{e}	27.3±0.9^{g}
	Flavourzyme	41.1±1.7^{e}	11.6±0.1^{g}	48.7±1.7^{b}	9.5±0.7^{g}	39.5±1.8^{c}	44.7±0.7^{e}
	Neutrase	72.6±5.4^{b}	23.4±0.2^{c}	24.3±0.7^{g}	16.6±0.7^{g}	31.3±1.3^{e}	45.2±2.1^{d}
	Kojizyme	46.7±1.2^{d}	18.9±0.8^{e}	23.2±0.6^{g}	12.4±0.7^{e}	41.7±2.4^{b}	26.9±0.9^{g}
A. coffeaeformis	Viscozyme	31.8±1.4^{f}	19.4±0.3^{e}	8.9±0.6^{j}	45.9±2.9^{b}	46.4±2.1^{a}	56.6±2.3^{c}
	Celluclast	24.3±1.9^{h}	17.8±0.6^{e}	4.2±0.1^{k}	38.2±1.9^{c}	44.6±2.2^{a}	24.8±1.2^{g}
	AMG	29.7±1.7^{g}	10.5±0.7^{g}	6.5±0.1^{j}	33.6±1.3^{c}	43.6±2.4^{b}	60.7±4.2^{c}
	Termamyl	24.3±1.2^{h}	16.7±0.7^{f}	4.2±0.1^{k}	29.1±1.3^{e}	28.2±0.5^{f}	89.3±4.1^{a}
	Ultraflo	27.5±1.4^{g}	15.4±0.4^{f}	7.1±0.2^{j}	26.7±1.6^{e}	35.7±1.7^{d}	55.1±2.7^{c}
	Protamex	25.6±1.5^{g}	17.3±0.8^{e}	6.8±0.3^{j}	20.5±1.4^{g}	37.5±2.1^{c}	88.8±5.1^{a}
	Alcalase	41.8±2.2^{e}	21.1±1.1^{d}	8.9±0.4^{j}	29.8±1.9^{e}	31.1±1.8^{e}	86.2±3.4^{a}
	Flavourzyme	65.6±3.3^{c}	21.1±1.2^{d}	10.6±0.7^{i}	29.3±2.1^{e}	31.1±1.9^{e}	51.2±2.7^{c}
	Neutrase	75.9±4.1^{b}	22.1±1.3^{d}	11.1±0.3^{h}	38.5±1.7^{c}	31.4±1.3^{e}	45.4±2.7^{d}
	Kojizyme	46.2±2.3^{d}	13.9±0.9^{g}	17.8±0.8^{h}	36.4±2.3^{c}	23.2±1.8^{g}	52.2±3.1^{c}
	BHT	94.6±6.4^{a}	60.1±4.2^{b}	63.2±4.3^{a}	76.6±4.6^{a}	26.1±0.9^{f}	11.5±0.1^{i}
	Tocopherol	94.3±7.1^{a}	62.5±4.9^{a}	61.5±4.7^{a}	79.5±4.7^{a}	25.2±0.6^{f}	10.3±0.1^{i}

Data are Means±SE (n=3); values in each column followed by different letters denote significant difference at p=0.05. [1)]DPPH free radical scavenging activity. [2)]hydrogen peroxide scavenging activity. [3)]superoxide anion scavenging activity. [4)]hydroxyl radical scavenging activity. [5)]nitric oxide scavenging activity. [6)]ferrous ion chelating effect.

emulsion without the addition of any digest increased. Enzymatic digests from *A. longipes* by Termamyl and Kojizyme as well as the enzymatic digests from *Navicula* sp. by AMG and Termamyl had strong antioxidant effects statistically similar with α-tocopherol. Interestingly, Kojizyme digest from *A. coffeaeformis* exhibited remarkable antioxidant activity which was similar to BHT.

DISCUSSION

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. Benthic diatoms have become good candidates for sources of natural antioxidants, as revealed by a number of recent studies (10,11,13).

In the present study, we focused on natural water-soluble antioxidants from three species of the Jeju benthic diatoms, which were 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 donor, which has been widely used to evaluate the free radical scavenging effect of natural antioxidants (25,26). In this study, DPPH free radical scavenging activity of Proteases digests from *Navicula sp.* and *A. coffeaeformis* was relatively higher than the digests by treated with carbohydrases. Especially Neutrase digests of *Navicula sp.* and *A. coffeaeformis* indicated the highest scavenging activity (above 70%). Many researchers have reported a positive correlation

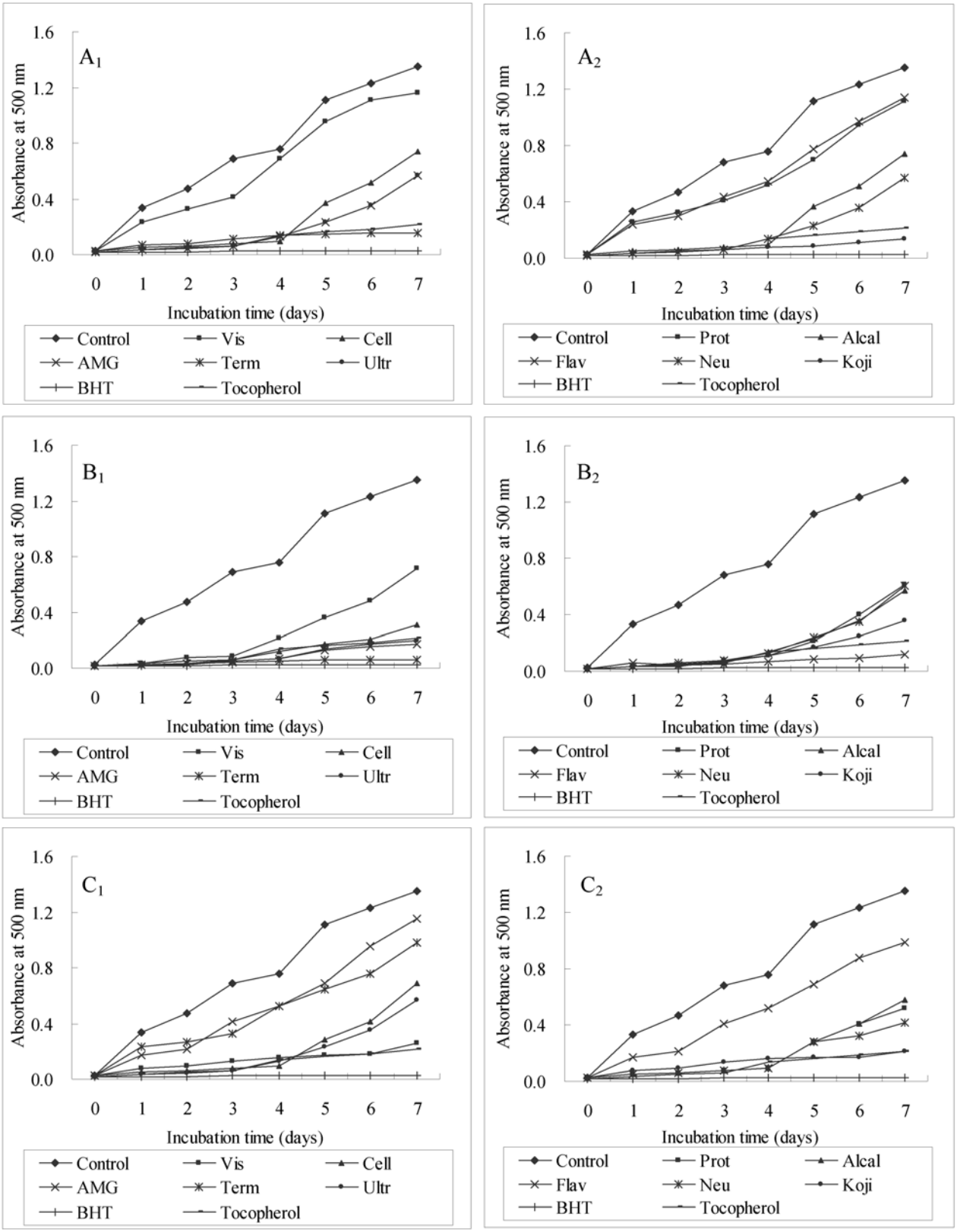

Fig. 1. Lipid peroxidation inhibitory activity of different enzymatic digests from *A. longipes* (A_1: Carbohydrases, A_2: Proteases), *Navicula* sp. (B_1: Carbohydrases, B_2: Proteases) and *A. coffeaeformis* (C_1: Carbohydrases, C_2: Proteases) compared to BHT and α-Tocopherol at 1 mg/mL of concentration of ethanol as assessed by linoleic acid peroxidation. Vis: Viscozyme, Cell: Celluclast, Term: Termamyl, Ultr: Ultraflo, Prot: Protamex, Koji: Kojizyme, Neu: Neutrase, Flav: Flavourzyme, Alcal: Alcalase.

between free radical scavenging activity and total polyphenolic compounds. Oki et al. (27) observed that the radical scavenging activity of sweet potato increased with the increase of polyphenolic compound content. The two studies conducted by Lu and Foo (28) and Siriwardhana et al. (29) reported a high correlation between DPPH radical scavenging activities and total polyphenolics. In this study, some enzymatic digests of benthic diatoms (especially *A. longipes*) did not possess antioxidant activity, although they contained as much polyphenolic compounds as the other digests of benthic diatoms. It is thought that other bioactive components, such as proteins, polysaccharides and different kinds of pigments were in the digestion of benthic diatoms. For example, it was found that oligosaccharides, sulfate and glycoprotein components in red microalga *Porphyridium sp*. exhibited antioxidant activities (30). 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. Therefore, it is very important to remove H_2O_2 for the protection of living systems. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $HO^{\cdot}$ mediated oxidative DNA damage (31). According to the H_2O_2 scavenging activity results, all the enzymatic digests of the three benthic diatoms had significantly low scavenging activity ($p<0.05$) for H_2O_2, as compared to the other reactive oxygen species scavenging activities.

Superoxide anion radical generally form first and its effects can be exaggerated as it produces other kinds of cell damage inducing free radicals and oxidizing agents (32,33). In this study, the superoxide anion scavenging activities of carbohydrase digests of *A. longipes* and *Navicula* sp. 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 (34).

Hydroxyl radical scavenging activity of the enzymatic digests from the benthic diatoms was determined as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Most of the enzymatic digests from the benthic diatoms exhibited activities of less than 50%, and protease-treated digests exhibited greater effects than carbohydrase-treated digests, suggesting that proteases have more capability to liberate hydroxyl radical scavenging compounds. Hydroxyl radical is the most reactive oxygen species among all ROS due to its strong ability to react with various biomolecules. Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from phospholipid membranes bringing about peroxidic reactions of lipids (35). 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 (33).

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 $NO\cdot$ and $O^{\cdot-}_2$ were found to be relatively low, but their metabolite ONOO- (peroxynitrite) is extremely reactive and directly induces toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (36,37). Therefore, the scavenging ability of $NO\cdot$ from the diatom extracts may help to interrupt the chain reactions initiated by excessive production of $NO\cdot$ that are harmful to human health.

Ferrozine can make red color complexes with ferrous ions. In the presence of chelating agents, complex formation is interrupted and as a result, the red color of the complex is decreased. Enzymatic digests (*A. coffeaeformis* by Termamyl, Protamex, Alcalase, and *A. longipes* by Ultraflo and Kojizyme) showed higher iron chelating activities, which further support the idea that the hydrophilic components are accountable for ion chelating. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as by accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (1,31). In this study, different digests of the benthic diatoms demonstrated a noticeable capacity for iron binding, suggesting their ability as peroxidation protectors, which relates to their ferrous binding capacity (38).

The FTC method was used to determine the amount of peroxide generated at the initial stage of lipid peroxidation. During linoleic acid oxidation, peroxides are formed and these compounds oxidize Fe^{2+} to Fe^{3+} and form complexes with SCN^{-}. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Thus, high absorbance is an indication of high concentration of formed peroxides. Enzymatic digests from *A. longipes* by Kojizyme and Termamyl, and Termamyl and AMG digests from *Navicula* sp., and Kojizyme digest from *A. coffeaeformis* provided higher antioxidant effects and were able to decrease the formation of peroxide. It is suggested that according to en-

zyme digestion, different antioxidant components are released from the inside of diatom cells. These digests contain high level of polysaccharides, proteins and polyphenols and it could be assumed that these components are responsible for the decreased lipid peroxidation.

In this study, enzymes (carbohydrases and proteases) were used for extraction of antioxidant compounds. Materials of the diatom cell wall may interfere 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 from algae have reported significant yields of desired compounds in the previous studies (14,39). The breakdown and release of high molecular weight polysaccharides and proteins themselves may contribute to enhanced antioxidative activities (40,41). Additionally, enzymatic digests possesses innovative advantages and characteristics over conventional extraction procedures such as water solubility, higher extraction efficacy, greater variation of constituents, comparative inexpensiveness and minimizes environmental pollution (14,42,43).

CONCLUSION

The study has shown that Jeju benthic diatoms have different antioxidative activities. Enzymatic digests exhibited appreciable antioxidative activities. In conclusion, Jeju benthic diatoms exhibited antioxidant potential against ROS and it would be a potential candidate as a natural water-soluble antioxidative source, which can be applied in food or food related industries.

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