Food Sci. Biotechnol. Vol. 14, No. 5, pp. 614 ~ 620 (2005)

Food Science and Biotechnology

Antioxidative Effect of Proteolytic Hydrolysates from *Ecklonia cava* on Radical Scavenging Using ESR and H_2O_2-induced DNA Damage

Soo-Jin Heo[1], Pyo-Jam Park[2], Eun-Ju Park[3], Somi K. Cho[4], Se-Kwon Kim[5] and You-Jin Jeon[1,6,*]

[1]*Faculty of Applied Marine Science, Cheju National University, Jeju, Jeju 690-756, Korea*
[2]*Department of Biotechnology, Konkuk University, Chungju, Chungbuk 380-701, Korea*
[3]*Division of Life Sciences, Kyungnam University, Masan, Gyeongnam 631-701, Korea*
[4]*Faculty of Biotechnology, Cheju National University, Jeju, Jeju 690-756, Korea*
[5]*Department of Chemistry, Pukyong National University, Pusan 608-737, Korea*
[6]*Applied Radiological Science Research Institute, Cheju National University, Jeju, Jeju 690-756, Korea*

Abstract The antioxidative effect of *Ecklonia cava*, a brown marine alga, was investigated on radical scavenging, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydroxyl and alkyl radicals, using an electron spin resonance (ESR) technique, and on the inhibition of H_2O_2-induced DNA damage using comet assay. *E. cava* was enzymatically hydrolyzed with five food industrial proteases (Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex) to prepare water-soluble extracts. All the proteolytic hydrolysates exhibited strong dose-dependent radical scavenging activities (above 80%) at a concentration of 2.5 μg/mL. Kojizyme extract (obtained by proteolytic hydrolysation of *E. cava* with Kojizyme) showed the highest hydroxyl radical scavenging activity of around 98%. In addition, the H_2O_2-induced DNA damage was determined using a comet assay, which was quantified by measuring the tail length. Reduction of DNA damage increased with increasing concentrations of Kojizyme extract from *E. cava*. These results indicated that *E. cava* has a potential as a valuable natural antioxidative source.

Keywords: Antioxidant activity, *Ecklonia cava*, enzymatic extract, Electron spin resonance (ESR), DNA damage

Introduction

Oxidative stress by reactive oxygen species (ROS) not only causes disorders of DNA, proteins and other macromolecules, but also acts as a major factor of endogenous damage leading to aging. ROS generally involve free radicals, such as superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$), and non-free radicals, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS may be essential for many cellular functions such as killing phagocytes, bacterial ingestion and redox regulation of signal transduction. However, they also have harmful factors causing strong injuries to several cell components. Moreover, these active molecules can cause a number of pathological diseases, including atherosclerosis, arthritis, diabetes, cataractogenesis, muscular dystrophy, ischemia-reperfusion tissue damage and neurological disorders such as Alzheimer's disease (1, 2).

Antioxidants are widely used to protect oxidizable goods such as cosmetics and pharmaceuticals, and to preserve food quality mainly by preventing oxidative deterioration of lipid constituents. These antioxidative compounds scavenge ROS and inhibit the action of the enzyme, lipoxygenase, responsible for the catalysis of lipid peroxidation (3-5). The most commonly used synthetic antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ). However, the use of synthetic antioxidants in food products is strictly regulated due to potential health hazards caused by these compounds. In addition, several studies (6) have reported an inverse relationship between dietary intake of antioxidant-rich foods and the incidence of human diseases. Therefore, the development and utilization of more effective antioxidants from natural resources are desired for use in foods or medicinal materials to replace the synthetic antioxidants (7, 8).

Recently, phytochemicals in food materials and their effects on health have been intensively studied. Seaweeds contain many phytochemicals with various bioactivities, including antioxidant, antitumor, anticholesterolemic and antiinflammatory activities (9-13). Among them, intense research attention has been focused on the antioxidant activity due to the currently growing demand from the pharmaceutical industry for antiaging and anticarcinogenic, naturally bioactive compounds, which possess health benefits. Almost all photosynthesizing plants, including seaweeds, are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, yet they seldom suffer any serious photodynamic damage while alive. This fact implies that their cells have some protective antioxidative mechanisms and compounds (14). Moreover, seaweeds are rich in vitamins, minerals, natural bioactive compounds, and various functional polysaccharides. Therefore it is important to extract or isolate useful bioactive compounds from seaweeds.

Ecklonia cava has been widely used as a source of fucoidan, which is well known as an antitumor, anticoagulant and antithrombin polysaccharide (15-17). In our previous study (18), a novel extraction technique using digestive enzymes such as carbohydrases and proteases was employed in order to degrade seaweed tissues, thereby

*Corresponding author: Tel: 82-64-754-3475; Fax: 82-64-756-3493
E-mail: youjinj@cheju.ac.kr
Received May 6, 2005; accepted October 10, 2005

releasing a variety of bioactive compounds. These enzymatic extracts from seaweeds are water-soluble and safe, as this technique does not adapt any organic solvent or other toxic chemicals. Additionally, it has a high yield and high radical scavenging activity in comparison with organic extracts.

In the present study, the brown seaweed, *E. cava* was enzymatically hydrolyzed with five different proteases widely used in the food industry to prepare water-soluble extracts. The antioxidant effects of the resultant extracts were investigated on the radical scavenging activities of 1,1, diphenyl-2-picrylhydrazyl (DPPH), and hydroxyl and alkyl radicals using electron spin resonance (ESR), and on H_2O_2-induced DNA damage using comet assay.

Materials and Methods

Materials The brown seaweed *Ecklonia cava* was collected along the coast of Jeju Island, Korea, during the period from October 2002 to March 2003. The samples were washed three times using tap water to remove salt, epiphytes and sand attached to the surface. Finally the samples were rinsed carefully using freshwater and stored in a medical refrigerator at -20°C. The frozen samples were lyophilized and homogenized with a grinder before extraction. Proteases including Alcalase 2.4L FG (an endoprotease), Flavourzyme 500 MG (containing both endoprotease and exopeptidase), Kojizyme 500 MG (boosting of the soy sauce fermentation), Neutrase 0.8L (an endoprotease) and Protamex (hydrolysis of food proteins) were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). 5,5-Dimethyl-1-pyrrolin *N*-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN), DPPH and Histopaque 1077 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

Preparation of enzymatic hydrolysates from *E. cava* The hydrolysis of *E. cava* was performed according to the method described by Heo *et al.* (18). *E. cava* was pulverized into powder using a grinder. The buffer solution (100 mL) was added to a dried sample (1 g), to which 100 μL (or mg) of each enzyme was mixed. The enzymatic hydrolysis reactions were performed for 12 hr to achieve an optimum hydrolytic level (19). The hydrolysates were clarified by centrifugation at 5,000 × g for 20 min to remove the unhydrolyzed residue. Finally, the enzymatic extracts of *E. cava* were obtained after filtering and the supernatant was lyophilized and then stored at -20°C until use.

Hydroxyl radical scavenging assay Hydroxyl radicals were generated by Fenton reaction, and reacted rapidly with nitrone spin trap DMPO; the resultant DMPO-OH adducts was detectable with an ESR spectrometer (20). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (PBS; pH 7.4) with 0.3 M DMPO 0.2 mL, 10mM $FeSO_4$ 0.2 mL and 10 mM H_2O_2 0.2 mL using an ESR spectrometer set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, and temperature 25°C.

DPPH radical scavenging assay DPPH radical scavenging activity was measured using the method described by Nanjo *et al.* (21). An ethanol solution of 60 μL of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μmol/L) in ethanol. After mixing vigorously for 10 seconds, the solutions were transferred into a 100 μL Teflon capillary tube and fitted into the cavity of the ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, and temperature 25°C.

Alkyl radical scavenging assay Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures, containing 10 mmol/L AAPH, 10 mmol/L 4-POBN and indicated concentrations of tested samples, were incubated at 37°C in a water bath for 30 min (22), and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on JES-FA ESR spectrometer. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, temperature 25°C.

Isolation of rat lymphocytes Blood samples were obtained from the inferior vena cava of five male Sprague-Dawley rats aged 15 weeks (Samtako, Korea). For each treatment, 100 μL of fresh whole blood was added to 900 μL of PBS and layered onto 100 μL of Histopaque 1077. After centrifugation for 5 min at 2000 rpm at room temperature, the lymphocytes were collected from just above the boundary with the Histopaque 1077, washed with 1 ml PBS, and centrifuged under the same condition.

Incubation of lymphocytes Each lyophilized extract was dissolved in PBS and diluted into concentrations of 0, 1, 10, 25 and 50 μg/mL. The lymphocytes containing 2×10^5 cells/mL were incubated with each diluted extract for 60 min at 37°C in a dark incubator together with untreated control sample. After preincubation, samples were centrifuged at 2000 rpm for 5 min at 4°C. The incubated cells were resuspended in PBS with 50 μM H_2O_2 for 5 min on ice. The untreated control sample was resuspended only in PBS without H_2O_2. Cells were centrifuged as described above and then washed with 1 mL PBS. Lymphocytes were checked for viability by trypan blue exclusion. All the experiments were repeated three times on alternate days.

Determination of DNA damage (Comet assay) The alkaline comet assay was conducted according to Singh *et al* (23)'s method with a little modification. The cell suspension was mixed with 75 μL of 0.5% low melting agarose (LMA), and added to slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 75 μL of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium

laurylsarcosine, 1% Triton X-100 and 10% DMSO) for 1 hr at 4°C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For DNA electrophoresis, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), to determine the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

Statistical analysis Data were analysed using the SPSS package for Windows (Version 10). Values were expressed as mean±standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A *P*-value of less than 0.05 was considered significant.

Result and Discussion

ROS are considered to be important causative factors in the development of diseases of aging such as neurodegenerative disease, cancer and cardiovascular disease. In the present study, we used the ESR technique. Spin trapping is the most direct method to detect highly reactive free radicals. With this ESR technique, a higher steady-state concentration of free radicals (as radical adducts) is achieved, which can overcome the sensitivity problem inherent in the detection of endogenous radicals in biological systems (24-26). The brown seaweed, *E. cava*, was successfully hydrolyzed by five types of proteases, Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex, to produce enzymatic hydrolysates according to the method of the previous study (18). The enzymatic hydrolysates were investigated for their effect on the scavenging activities of three radicals, hydroxyl, DPPH and alkyl, and for their inhibitory effect against H_2O_2-induced DNA damage.

The proximate composition of *E. cava* shown in Fig. 1 presents values comparing the protein and carbohydrate compositions of the raw material (10.55 and 68.42%, respectively) and proteolytic hydrolysates. For the hydrolysates, the protein composition was a little higher and the carbohydrate composition was a little lower than those of the raw material. It seems that the protein compounds were easily released due to the enzymatic digestion and contributed to the change of composition.

Hydroxyl radicals generated in the Fenton system (Fe^{2+} /H_2O_2) were trapped by DMPO, forming a spin adduct as detected by the ESR spectrometer. All the tested extracts scavenged more than 90%, with the values being 90.68, 90.09, 98.20, 93.99 and 91.89% at 2.5 μg/mL for Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex, respectively (Fig. 2). Moreover, even the lowest concentration (0.625 μg/mL) exhibited over 50% activity. In addition, the decrease of the amount of DMPO-OH adduct was shown by ESR spectra after the addition of Kojizyme hydrolysate

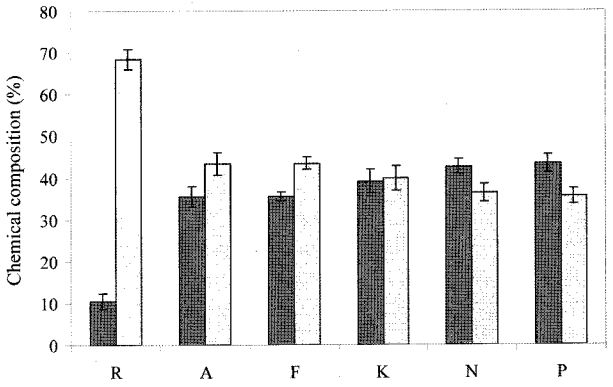

Fig. 1. Proximate composition of raw materials and five proteolytic hydrolysates of *E. cava*. R, raw material; A, Alcalase extract; F, Flavourzyme extract; K, Kojizyme extract; N, Neutrase extract; P, Protamex extract. (▨ : protein, ▩ : carbohydrate)

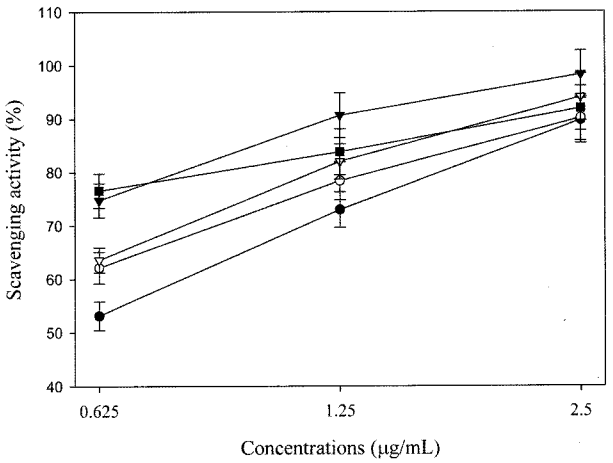

Fig. 2. Hydroxyl radical scavenging activity of the extracts hydrolyzed from *E. cava* by various proteases. Extracts hydrolyzed by: ●, Alcalase (Alcalase extract); ○, Flavourzyme extract; ▼, Kojizyme extract; ▽, Neutrase extract; ■, Protamex extract. Mean ± SE of determinations were made in triplicate experiments.

from *E. cava* (Fig. 3). Among the ROS, the hydroxyl radical is the most reactive and it induces severe damage to the adjacent biomolecules. In this ESR technique, all the extracts exhibited strongly scavenged hydroxyl radicals in a dose-dependent manner. Several studies have reported the effects of this parameter on the Fenton reaction with ESR method. Park *et al.* (27) reported that the efficiency for generation of DMPO-OH gradually decreased when the enzymatic extracts of *Sargassum horneri* increased from 6.25 to 2 μg/mL. Zhang *et al.* (28) also observed that 0.25% ginseng extract completely counteracted the DMPO-OH adduct formation generated by a ferrous ion-mediated, Fenton reaction system.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, and has often been used as a substrate to evaluate the antioxidative activity of natural compounds (29). *E. cava* enzymatic hydrolysates exhibited a positive effect on DPPH radical scavenging (Fig. 4), with all values being over 80%; namely 87.85, 83.50, 89.08, 85.74 and 87.67% at 2.5 μg/mL for the five types of protease (Alcalase, Flavourzyme, Kojizyme, Neutrase and Protamex,

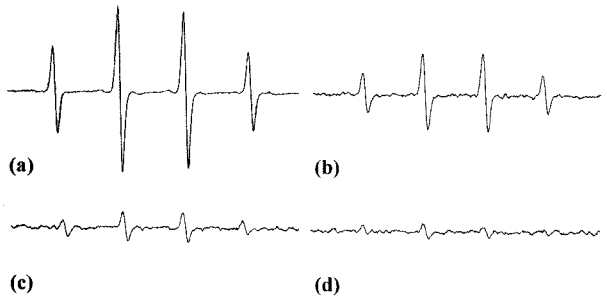

Fig. 3. ESR spectrum obtained in Fenton reaction system at various concentrations of the extracts hydrolyzed from *E. cava* by Kojizyme. (a) control; (b) 0.625 μg/mL; (c) 1.25 μg/mL; (d) 2.5 μg/mL.

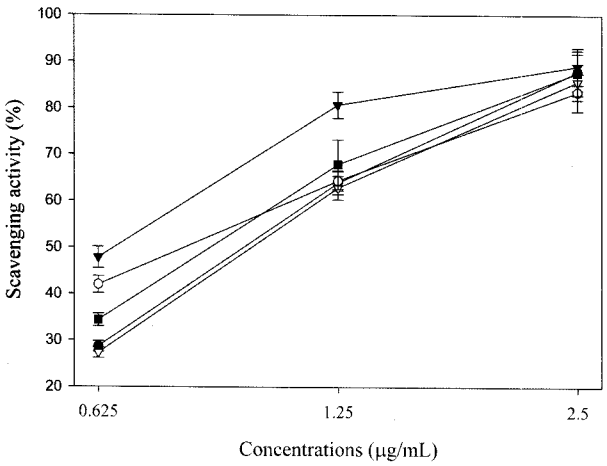

Fig. 4. DPPH radical scavenging activity of the extracts hydrolyzed from *E. cava* by various proteases. Extracts hydrolyzed by: ●, Alcalase (Alcalase extract); ○, Flavourzyme extract; ▼, Kojizyme extract; ▽, Neutrase extract; ■, Protamex extract. Mean ± SE of determinations were made in triplicate experiments.

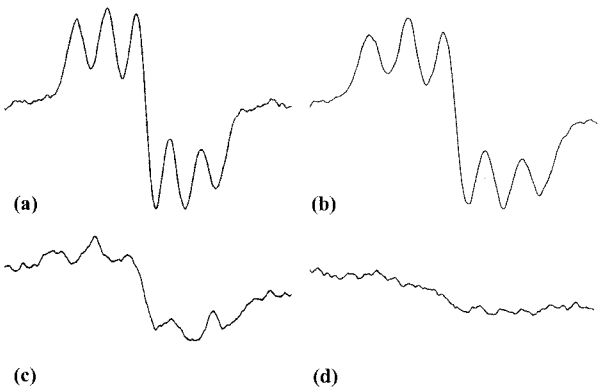

Fig. 5. ESR spectrum obtained in an ethanol solution of 30 μmol/L DPPH at various concentrations of the extracts hydrolyzed from *E. cava* by Kojizyme. (a) control; (b) 0.625 mg/mL; (c) 1.25 μg/mL; (d) 2.5 μg/mL.

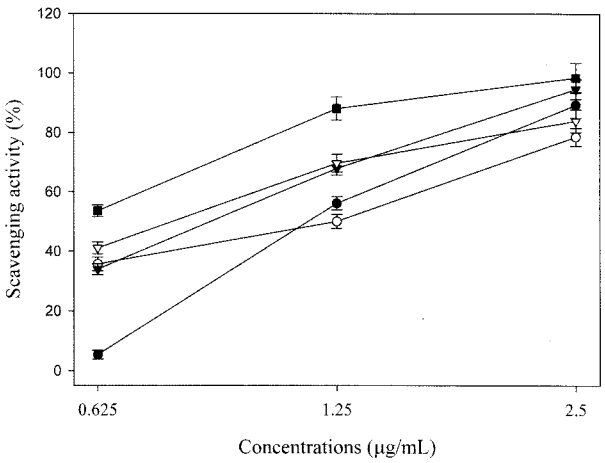

Fig. 6. Alkyl radical scavenging activity of the extracts hydrolyzed from *E. cava* by various proteases. Extracts hydrolyzed by: ●, Alcalase (Alcalase extract); ○, Flavourzyme extract; ▼, Kojizyme extract; ▽, Neutrase extract; ■, Protamex extract. Mean ± SE of determinations were made in triplicate experiments.

respectively). In addition, the scavenging activity of those hydrolysates increased as their concentrations were increased from 0.625 μg/mL to 2.5 μg/mL, while the ESR signal was decreased with the dose increment of Kojizyme hydrolysate (Fig. 5). It is well known that antioxidant properties are correlated with high contents of phenolic compound (21, 30, 31). Nagai and Yukimoto (32) reported that a high content of polyphenols is correlated with high antioxidant ability using a linoleic acid model system with high scavenging abilities against DPPH radicals. In our previous study (18), *E. cava* enzymatic hydrolysates also exhibited a positive correlation between DPPH radical scavenging activities and total polyphenolics.

The alkyl radical has been found to be a primary intermediate in many hydrocarbon reactions. These radicals were easily detected with ESR, a technique which has been found to be very useful in the characterization of solid surfaces and in the elucidation of active surface sites as well as surface reactions (33). The alkyl radical spin adduct was observed when AAPH was incubated with the spin trap 4-POBN at 37°C for 30 min. The results in Fig. 6 showed that the hydrolysates prepared by the five proteases, Alcalase, Flavourzyme, Kojizyme, Neurease and Protamex, scavenged 89.29, 78.57, 94.64, 83.93 and 98.43% at 2.5 μg/mL, respectively. Among them, the Protamex hydrolysate exhibited highly protective effects on alkyl radicals even at 1.25 μg/mL (around 90%). The ESR signals were decreased with the dose increment in the Kojizyme hydrolysate (Fig. 7). In this result, we obtained a predominantly protective effect on the alkyl radical. Park *et al.* (34) also studied the scavenging effect of reactive radicals and observed that 90% chitosan could scavenge 70% of alkyl radicals at the concentration of 5 mg/mL.

The comet assay, which measures the breaking of the DNA strand at the level of single cells, is very easily applied to lymphocytes and therefore lends itself to human bio-monitoring studies. It has become a standard technology for the measurement of oxidative DNA damage both *in vitro* and *in vivo* (35, 36). H_2O_2, an oxidative stress agent, causes a concentration-dependent increase in DNA strand breakage. Our previous study reported that *E. cava* carbohydrases hydrolysates could strongly scavenge H_2O_2 *in vitro* (37). It can therefore be inferred that the *E. cava* enzymatic hydrolysates can be used to protect cells as confirmed by comet assay.

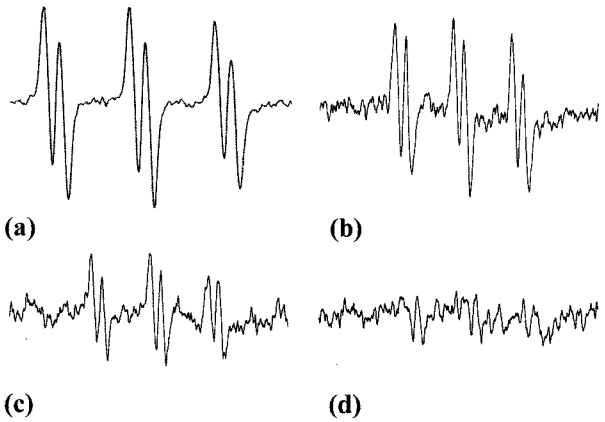

Fig. 7. ESR spectrum obtained during incubation of AAPH with 4-POBN at various concentrations of the extracts hydrolyzed from *E. cava* by Kojizyme. (a) control; (b) 0.625 μg/mL; (c) 1.25 μg/mL; (d) 2.5 μg/mL. The incubation was done in a water bath containing 0.05 mol/L PBS, 10 mmol/L AAPH and 0.1 mmol/L 4-POBN.

Fig. 8 shows the effect of proteolytic hydrolysates from *E. cava* on H_2O_2-induced DNA damage in human lymphocytes. All the tested hydrolysates showed positive effects against DNA damage in a dose-dependent manner, not only at high concentrations (50 μg/mL), but also at a low concentration of 1 μg/mL. Especially Kojizyme hydrolysate exhibited the highest inhibitory effect (82.32 %) at 50 μg/mL and Alcalase hydrolysate showed more than 50% even at the lowest concentration (1 μg/mL) tested.

Fig. 9 shows photomicrographs of different DNA migration profiles obtained from human lymphocytes, when treated with different concentrations of hydrolysate. In the group treated with only H_2O_2, the DNA was completely damaged but the addition of *E. cava* proteolytic hydrolysates with H_2O_2 reduced the damage caused by H_2O_2. In the application of samples at different concentrations (0 to 50 μg/mL) the DNA migration changed with the increment of the concentrations. DNA damage can be enhanced by exposure to various

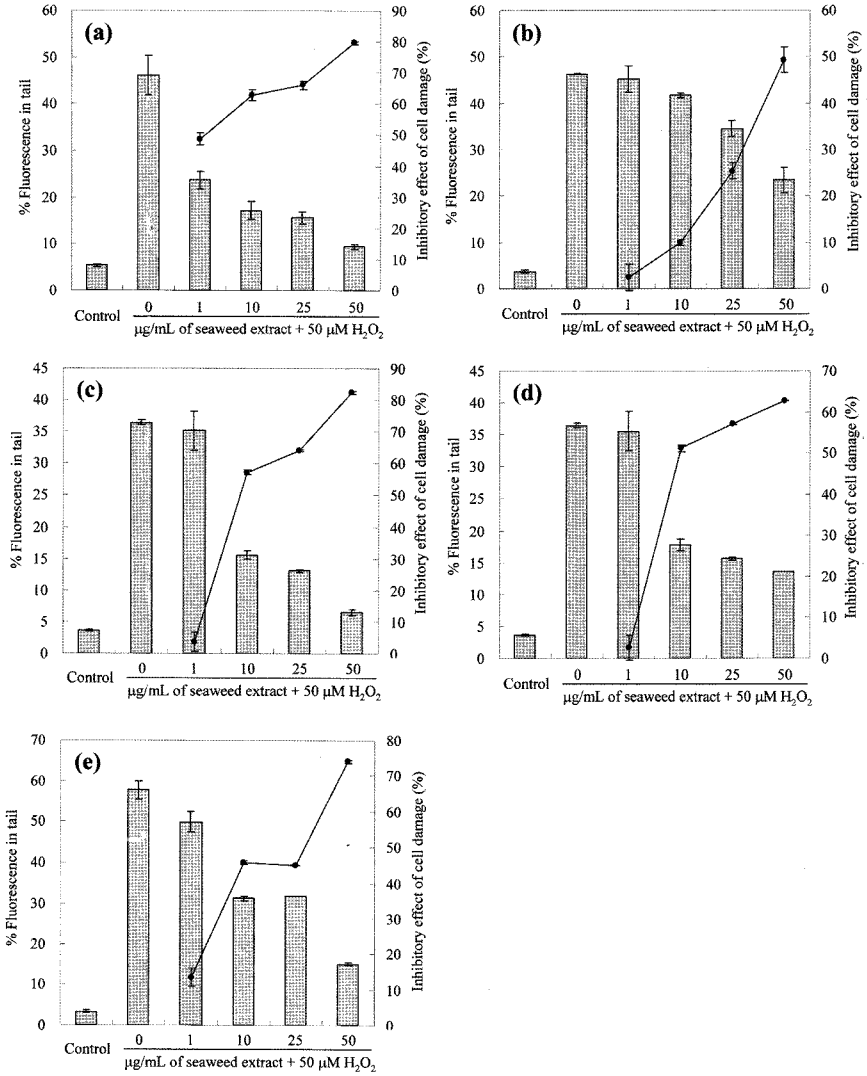

Fig. 8. Inhibitory effects of different concentrations of *E. cava* proteases extracts on H_2O_2-induced, human lymphocyte, DNA damages. (a) Alcalase extract, (b) Flavourzyme extract, (c) Kojizyme extract, (d) Neutrase extract, (e) Protamex extract. Values are mean with standard error of triplicate experiments with lymphocytes from each of two different donors. (▒ : % Fluorescence in tail, – ● –: Inhibitory effect of cell damage)

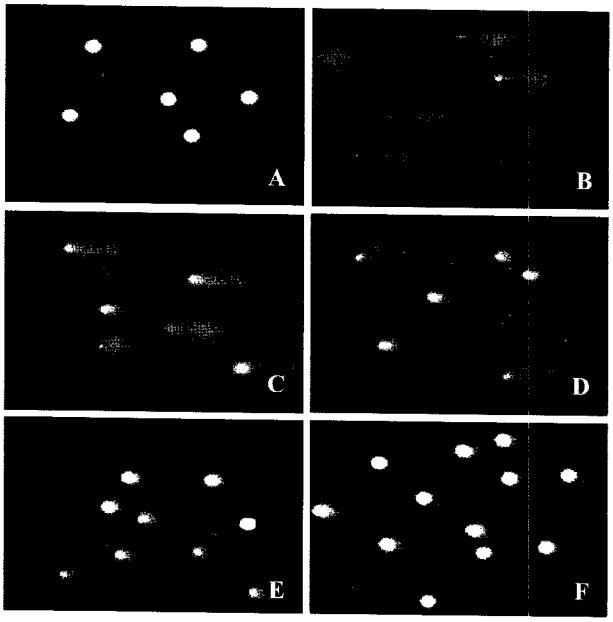

Fig. 9. Photomicrographs of DNA damage and migration observed under Kojizyme extract treatment where the tail moments were decreased. (A) negative control; (B) lymphocytes treated with 50 μM H_2O_2; (C) lymphocytes treated with 1 μg/mL *E. cava* Kojizyme ext. + 50 μM H_2O_2; (D) lymphocytes treated with 10 μg/mL *E. cava* Kojizyme ext. + 50 μM H_2O_2; (E) lymphocytes treated with 25 μg/mL *E. cava* Kojizyme ext. + 50 μM H_2O_2; (F) lymphocytes treated with 50 μg/mL *E. cava* Kojizyme ext. + 50 μM H_2O_2

chemicals, environmental pollutants, steroid hormones and radiation (23, 38, 39) which can be associated with infections, disease, dietary habits, lifestyle and advancing age (40-42). If these DNA lesions are not repaired, they can initiate a cascade of biological consequences in the population and can also promote cancer development via several mechanisms (43). In this result, the enzymatic hydrolysates from *E. cava* showed an inhibitory effect on H_2O_2-induced DNA damage in human lymphocytes. Therefore, the enzymatic hydrolysates from *E. cava* have the potential to inhibit the DNA damage induced by H_2O_2.

In the present study, we used enzymatic hydrolysates from *E. cava* as a potential source of natural, water-soluble antioxidants and examined their radical scavenging effect using ESR spectrophotometry, and the H_2O_2 scavenging effect using comet assay in human lymphocytes. Enzymatic hydrolysates exhibited a positive effect on reactive radicals, such as hydroxyl, alkyl and DPPH free radicals, and an inhibitory effect against DNA damage. Therefore, enzymatic hydrolysates from *E. cava* can be used in the food and pharmaceutical industries. Further studies are required in order to identify their structure, as well as the functional properties of the antioxidant compounds from enzymatic hydrolysates of *E. cava*.

Acknowledgments

This research was supported by a grant (p-2004-02) from the Marine Bioprocess Research Center of the Marine Bio 21 Center, funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea and Taerim Trading Co., Ltd.

References

1. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. Oxidative damage to DNA during aging: 8-hydroxyl-2'-deoxyguanosine in rat organ DNA and urine. Natl. Acad. Sci. USA. 87: 4533-4537 (1990)
2. Frlich I, Riederer P. Free radical mechanisms in dementia of Alzheimer type and the potential for antioxidative treatment. Drug Res. 45: 443-449 (1995)
3. Schewe T, Rapoport SM, Kuhn H. Enzymology and physiology of reticulocyte lipoxygenase: comparison with other lipoxygenases. Adv. Enzymol. Relat. Areas Mol. Biol. 58: 192-272 (1986)
4. Yamamoto Y, Niki E. Role of antioxidants in lipid peroxidation. In: Membrane Lipid Oxidation, Volume I. CRC Press, Boca Raton, Florida, pp. 285-301 (1990)
5. Lee EJ, Jang HD. Antioxidant activity and protective effect of five edible mushrooms on oxidative DNA damage. Food Sci. Biotechnol. 13: 443-449 (2004)
6. Lu Y, Foo Y. Antioxidant radical scavenging activities of polyphenols from apple pomace. Food Chem. 68: 81-85 (2000)
7. Hettiarachy NS, Glenn KC, Gnanasambandam R, Johnson MG. Natural antioxidant extract from fenugreek (Trigonella foenum-graecum) for ground beef patties. J. Food Sci. 61: 516-519 (1996)
8. Kim JA, Lee JM, Shin DB, Lee NH. The antioxidant activity and tyrosinase inhibitory activity of phloro-tannins in *Ecklonia cava*. Food Sci. Biotechnol. 13: 476-480 (2004)
9. Athukorala Y, Lee KW, Song CB, Ahn CB, Shin TS, Cha YJ, Shahidi F, Jeon YJ. Potential antioxidant activity of marine red alga *Grateloupia filicina* extracts. J. Food Lipids 10: 251-265 (2003)
10. Hiqashi-Okaj K, Otani S, Okai Y. Potent suppressive effect of a Japanese edible seaweed, Enteromorpha prolifera (Sujiao-nori) on initiation and promotion phases of chemically induced mouse skin tumorigenesis. Cancer Lett. 140: 21-25 (1999)
11. Read TA, Stensvaag V, Vindenes H, Ulvestad E, Bjerkvig R, Thorsen F. Cells encapsulated in alginate: a potential system for delivery of recombinant proteins to malignant brain tumours. Int. J. Dev. Neurosci. 17: 653-663 (1999)
12. Siriwardhana N, Lee KW, Kim SH, Ha JW, Jeon YJ. Anti-oxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Sci. Tech. Int. 9: 339-346 (2003)
13. Wong KH, Sam SW, Cheung PCK, Ang Jr. PO. Changes in lipid profiles of rats fed with seaweed-based diets. Nutr. Res. 19: 1519-1527 (1999)
14. Matsukawa R, Dubinsky Z, Kishimoto E, Masak K, Masuda Y, Takeuchi T, Chihara M, Yamamoto Y, Niki E, Karube I. A comparison of screening methods for antioxidant activity in seaweeds. J. Appl. Phycol. 9: 29-35 (1997)
15. Satoru K, Noboru T, Hiroo N, Shinji S, Hiroshi S. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. Biochem. Pharmacol. 65: 173-179 (2003)
16. Takashi N, Akihiro F, Terukazu N, Michio F, Eisuke K. Inhibition of the generation of thrombin and factor Xa by a fucoidan from the brown seaweed *Ecklonia kurome*. Thromb. Res. 96: 37-49 (1999)
17. Takashi N, Tomoko Y, Mariko H, Terukazu N, Haruo S. Effects of a fucoidan on the activation of plasminogen by u-PA and t-PA. Thromb. Res. 99: 623-634 (2000)
18. Heo SJ, Lee KW, Song CB, Jeon YJ. Antioxidant activity of enzymatic extracts from brown seaweeds. Algae 18: 71-81 (2003a)
19. Heo SJ, Jeon YJ, Lee JH, Kim HT, Lee KW. Antioxidant effect of enzymatic hydrolysate from a Kelp, *Ecklonia cava*. Algae 18: 341-347 (2003b)
20. Rosen GM, Rauckman EJ. Spin trapping of superoxide and hydroxyl radicals. In L. Packer (Ed.), Methods in enzymology (Vol. 105, pp. 198-209). Orlando, FL: Academic Press (1984)
21. Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylydrazyl radical. Free Radical Bio. Med. 21: 895-902 (1996)
22. Hiramoto K, Johkoh H, Sako KI, Kikugawa K. DNA breaking activity of the carbon-centered radical generated from 2,2'-

azobis(2-amidinopropane) hydrochloride (AAPH). Free Radical Res. Com. 19: 323-332 (1993)
23. Singh NP, Graham MM, Singh V, Khan A. Induction of DNA single-strand breaks in human lymphocytes by low doses of γ-rays. Int. J. Rodiat. Biol. 68: 563-569 (1995)
24. Auddy B, Ferreira M, Blasina F, Lafon L, Arredondo F, Dajas F, Tripathi PC, Seal T, Mukherjee B. Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. J. Ethnopharmacol. 84: 131-138 (2003)
25. Janzen EG, Towner RA, Haire DL. Detection of free radicals generated from the in vitro metabolism of carbon tetrachloride using improved ESR spin trapping techniques. Free Radical Res. Com. 3: 357-364 (1987)
26. Kadiiska MB, Mason RP. In vivo copper-mediated free radical production: an ESR spin-trapping study. Spectrochim. Acta A 58: 1227-1239 (2002)
27. Park PJ, Shahidi F, Jeon YJ. Antioxidant activities of enzymatic extracts from an edible seaweed *Sargassum horneri* using ESR spectrometry. J. Food Lipids 11: 15-27 (2004)
28. Zhang D, Yasuda T, Yu Y, Zheng P, Kawabata T, Ma Y, Okada S. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. Free Radical Bio. Med. 20: 145-150 (1996)
29. Soares JR, Dins TCP, Cunha AP, Ameida LM. Antioxidant activity of some extracts of Thymus zygis. Free Radical Res. 26: 469-478 (1997)
30. Oki T, Masuda M, Furuta S, Nishibia Y, Terahara N, Suda I. Involvement of anthocyanins and other phenolic compounds in radical-scavenging activity of purple-fleshed sweet potato cultivars. Food Chem. Toxicol. 67: 1752-1756 (2002)
31. Lee EJ, Kwon YI, Shetty K, Jang HD. Antioxidant activity of Rhodiola rosea extract on human low-density lipoprotein oxidation and DNA strand scission. Food Sci. Biotechnol. 13: 814-820 (2004)
32. Nagai T, Yukimoto T. Preparation and functional properties of beverages made from sea algae. Food Chem. 81: 327-332 (2003)
33. Adebajo MO, Gesser HD. ESR study of alkyl radicals adsorbed on porous Vycor glass I. Build-up of methyl and ethyl radicals. Appl. Surf. Sci. 171: 120-124 (2001)
34. Park PJ, Je JY, Kim SK. Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. Carbohydr. Polym. 55: 17-22 (2004)
35. Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N. Comet assay in human biomonitoring studies: Reliability, validation, and applications. Environ. Mol. Mutagen. 30: 139-146 (1997)
36. Senthilmohan ST, Zhang J, Stanley RA. Effects of flavonoid extract Enzogenol with vitamin C on protein oxidation and DNA damage in older human subjects. Nutr. Res. 23: 1999-1210 (2003)
37. Heo SJ, Park EJ, Lee KW, Jeon YJ. Antioxidant activities of enzymatic extracts from brown seaweeds. Bioresource Technol. 96: 1613-1623 (2005)
38. Hartmann A, Herkommer K, Gluck M, Speit G. DNA damaging effect of cyclophosphamide on human blood cells in vivo and in vitro studied with the single cell gel test (Comet assay). Environ. Mol. Mutagen. 25: 180-187 (1995)
39. Valverde M, Lopez MDC, Sanchez I, Fortoul TI, Ostrosky-Wegman P, Rojas E. DNA damage in leucocytes and buccal and nasal epithelial cells of individuals exposed to air pollution in Mexico city. Mutat. Res. 30: 147-152 (1997)
40. Betancourt M, Ortiz R, Gonzalez C, Perez P, Cortes L, Rodriguez L, Villasenor L. Assessment of DNA damage in leukocytes from infected and malnourished children by single cell gel electrophoresis/Comet assay. Mutat. Res. 331: 65-77 (1995)
41. Betii C, Davini T, Giannessi L, Loprieno N, Barale R. Comparative studies by Comet test and SCE analysis in human lymphocytes from 200 healthy subjects. Mutat. Res. 307: 201-207 (1995)
42. Singh NP, Danner DB, Tice RR, Brant L, Schneider EL. DNA damage and repair with age in individual human lymphocytes. Mutat. Res. 237: 123-130 (1990)
43. Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 148: 187-197 (2000)