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Protective Effect of Enzymatic Extracts from *Sargassum coreanum* on H_2O_2-induced Cell Damage

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In our previous study, we preliminarily demonstrated that Celluclast and Neutrase extracts exhibited the strongest H_2O_2-scavenging activities among five carbohydrases (Viscozyme, Celluclast, Termamyl, Ultraflo and AMG) and five proteases (Kojizyme, Alcalse, Flavourzyme Protamex and Neutrase) extracts. Thus, Celluclast and Neutrase extracts were selected for use in further experiments and were separated into four different molecular weight fractions (<5, 5-10, 10-30 and >30 kDa). Among them, the 5-10 kDa fraction showed the highest H_2O_2-scavenging activity. The 5-10 kDa fraction also strongly enhanced cell viability against H_2O_2-induced oxidative damage. Furthermore, the fraction reduced the proportion of apoptotic cells induced by H_2O_2, as demonstrated by decreased sub-G_1 hypodiploid cells and decreased apoptotic body formation by flow cytometry. These results indicated that the 5-10 kDa fraction of the Celluclast and Neutrase extracts from *S. coreanum* exhibited strong antioxidant activity over H_2O_2-mediated cell damage *in vitro*.

Key words: *Sargassum coreanum*, Enzymatic extract, H_2O_2, Cell damage

Introduction

Reactive oxygen species (ROS) include free radicals, such as the peroxyl (ROO·), nitric oxide (NO·), hydroxyl (·OH), and superoxide radicals ($O_2^{\cdot-}$), and non free-radicals, such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), that are formed during normal metabolic processes (Heo et al., 2008). ROS can readily oxidize cellular components such as lipids, proteins, and lipoproteins (Lee et al., 2009). High levels of ROS are considered to be responsible for various pathological conditions including cancer, cardiovascular disease, and aging (Cox and Choen, 1996; Ames, 1998). Among the ROS, H_2O_2 plays a pivotal role because it is generated in nearly all types of oxidative stress and it can diffuse freely in and out of cells and tissues (Halliwell and Aruoma, 1991).

Synthetic antioxidants, such as butylated hydroxyanisol (BHA), butylated hydroxyltoluene (BHT), and tert-butylhydroquinone (TBHQ), have been used to reduce oxidative damage in the human body (Kim et al., 2008; Sherwin, 1990). However, the use of these antioxidants in human is also associated with some side effects, such as liver damage and carcinogenesis (Lindenschmidt et al., 1986). Thus, much attention has been focused on natural antioxidants in functional foods instead of synthetic antioxidants.

Seaweeds are known to provide an abundance of

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bioactive materials with valuable pharmaceutical and biomedical potential. According to the results of previous studies, the bioactive substances contained in seaweeds differ from those in land plants, and various of these constituents have been demonstrated to possess antioxidant, antitumor, anticoagulant, and antibacterial effects (Kashiwagi et al., 1980; Gonzalez et al., 1982; Kosovel et al., 1988). In particular, brown seaweeds possess a variety of biological compounds, including fucoxanthin, xanthophyll, and polyphenol-containing phlorotannins. Several studies on these kinds of compounds have pointed out a variety of biological benefits, including antioxidant, anticoagulant, anti-hypertensive, antibacterial, and antitumor activities (Athukorala and Jeon, 2005; Heo et al., 2005; Kotake-Nara et al., 2005; Mayer and Hmann, 2004; Nagayama et al., 2002).

In a previous study (Heo et al., 2005), a novel extraction technique using food-grade digestive enzymes, including proteases and carbohydrases, was used to extract products with a variety of bioactivities including antioxidant, anti-inflammatory, and anti-hypertensive effects (Kim et al., 2008; Athukorala and Jeon, 2005; Ahn et al., 2008). These seaweed-derived enzymatic extracts have been shown to have some important benefits, including higher yields, water-solubility, and safety, because this technique does not require the use of organic solvents or other toxic chemicals. In the present study, *S. coreanum*, a brown seaweed, was extracted enzymatically with different proteases and carbohydrases used in the food industry to prepare water-soluble extracts, and then the antioxidant effects of the resulting extracts were assessed with regard to their ability to scavenge H_2O_2 and their protective effects against H_2O_2-induced cell damage.

Materials and Methods

Materials

S. coreanum was collected along the coast of Jeju Island in South Korea. The sample was washed three times with tap water to remove salt, epiphytes, and sand attached to the surface of the samples. Finally, the sample was carefully rinsed using fresh water and stored at -20°C. The frozen samples were then lyophilized and homogenized in a grinder prior to extraction. For enzymatic digestion, Celluclast 1.5 LFG, which catalyzes the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers, and Neutrase 0.8 L, an endoprotease, were used. Propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), and Hoechst 33342 were all purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Preparation of enzymatic extracts from *S. coreanum*

Enzymatic extraction of *S. coreanum* was conducted in accordance with the method described by Heo et al. (2005). *S. coreanum* was pulverized into powder with a grinder. Buffer solution (100 mL) was added to 1 g of the dried sample, and then 100 μL (or mg) of each enzyme was added. The enzymatic hydrolysis reaction was performed for 12 h to achieve optimal hydrolysis (Heo et al., 2003). After the reaction, the enzyme was inactivated by heating at 100°C for 10 min. The extracts were clarified by centrifugation (5,000×g, 20 min) to remove non-extracted residue. The resultant mixtures were filtered with Whatman No. 1 filter paper, and the pH of the filtrates was adjusted to pH 7 with 1 M HCl/NaOH. Finally, the enzymatic extracts of *S. coreanum* were lyophilized and maintained at -20°C until use.

Molecular weight fractionation of enzymatic extracts

Celluclast and Neutrase extracts were fractionated using Millipore's Labscale TFF system (Millipore, Billerica, MA, USA) with ultrafiltration membranes (5, 10 and 30 kDa). The resulting fractions were then collected according to molecular weight (<5, 5-10, 10-30 and >30 kDa) and evaluated with regard to H_2O_2-scavenging activity and effects on cell proliferation.

Total polyphenolic compounds

Total polyphenolic compounds were quantified according to a protocol similar to that described by Chandler and Dodds (1983). *S. coreanum* hydrolysate (1 mL) was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was then allowed to react for 5 min, after which 1 mL of 5% Na_2CO_3 was added. The mixture was then placed in a dark room for 1 h, and the absorbance was measured at a wavelength of 725 nm in a UV-vis spectrophotometer (Opron 3000 Hanson Tech. Co., Ltd., Korea). A gallic acid standard curve was prepared to calculate the polyphenolic content.

ABTS radical scavenging assay

Hydrogen peroxide scavenging activity was determined by a colorimetric assay according to the method of Müller (1985). Phosphate buffer (100 μL,

0.1 M, pH 5.0) and sample solution were mixed in a 96-microwell plate. Hydrogen peroxide (20 μL, 10 mM) was added to the mixture, which was then incubated for 5 min at 37°C. After incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, and it was then incubated for 10 min at 37°C. The absorbance was read with an ELISA plate reader at 405 nm.

Cell culture

Cells of a Chinese hamster lung fibroblast line (V79-4) were maintained at 37°C in an incubator in a humidified atmosphere at 5% CO_2. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, streptomycin (100 mg/mL), and penicillin (100 unit/mL).

Assessment of cell viability

Cell viability was then estimated using the MTT assay, a test of metabolic competence predicated on the assessment of mitochondrial performance. It is a colorimetric assay, dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/mL. After 16 h, the cells were treated with enzymatic extracts at different concentrations (25, 50 and 100 μg/mL). Then, 10 μL of H_2O_2 (1 mM) was added to the cell culture medium and incubated for 24 h at 37°C. MTT stock solution (50 μL; 2 mg/mL) was then added to each well to a total reaction volume of 200 μL. After 4 h of incubation, the plates were centrifuged (800×g, 5 min), and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μL of dimethylsulfoxide (DMSO), and the absorbance was measured with an ELISA plate reader at 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was deemed to represent 100% viability. The data are expressed as mean percentages of viable cells versus the respective control.

Nuclear staining with Hoechst 33342

Nuclear morphology of cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was considered indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The V79-4 cells were placed in 24-well plates at a concentration of 1.0×10^5 cells/mL. At 16 h after plating, the cells were treated with various concentrations of the sample fractions and further incubated for 1 h prior to exposure to H_2O_2 (1 mM). After 24 h, 1.5 μL of Hoechst 33342 (stock, 10 mg/mL), a DNA-specific fluorescent dye, was added to each well, followed by a 10 min incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

Comet assay analysis

The cell suspension was mixed with 75 μL of 0.5% low-melting-point agarose (LMPA) and added to slides precoated with 1% normal-melting-point agarose (NMPA). The slides were kept at 4°C for 10 min, then covered with another 75 μL of 0.5% LMPA and kept at 4°C for 40 min to solidify the agarose. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, 1% Triton X-100) at 4°C for 1 h. The slides were next placed in an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied at 4°C for 20 min. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) at 4°C for 5 min and then treated with ethanol for another 5 min before staining with 50 μL of ethidium bromide (20 μg/mL). Measurements were made with an image analysis system (Kinetic Imaging, Komet 5.0, UK) and a 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).

Flow cytometry analysis

Flow cytometry analyses were conducted to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The V79-4 cells were placed in 6-well plates at a concentration of 1.0×10^5 cells/mL, and 16 h after plating, the cells were treated with various concentrations of the sample fraction (100 μg/mL). After a further 1 h incubation, H_2O_2 (1 mM) was added to the culture. After 24 h, the cells were harvested at the indicated times and fixed for 30 min in 1 mL of 70% ethanol at 4°C. The cells were then washed twice with PBS and incubated for 30 min in the dark in 1 mL of PBS containing 100 μg PI and 100 μg RNase A at 37°C.

Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs (Wang et al., 1999).

Results and Discussion

Recently, due to the relatively limited resources of the land, many researchers have focused on natural marine resources with various biological activities. Reactive oxygen species (ROS) form as natural by-products from normal oxygen metabolism and perform important functions in cell signaling. Cells are normally able to defend themselves against ROS damage by the use of antioxidant enzymes, including superoxide dismutases and catalases (Heo and Jeon, 2008). However, an imbalance between ROS and antioxidant defense mechanisms can result in oxidative modification of the cellular membrane or intracellular molecules (Halliwell et al., 1992; El-Habit et al., 2000). Recently, interest has been increasing in the therapeutic potential of natural plants as sources of antioxidants for the reduction of such free radical-induced tissue injuries. It has been suggested that many plants may possess therapeutically useful antioxidant activities (Kim et al., 2006). In this study, we focused on natural water-soluble antioxidants isolated from *S. coreanum*, which were prepared via enzymatic hydrolysis using different carbohydrate-degrading enzymes and proteases. We then investigated the antioxidant effects of these *S. coreanum* enzymatic extracts after the administration of H_2O_2 treatment.

In a previous study, we preliminarily demonstrated that Celluclast and Neutrase extracts exhibited the strongest H_2O_2-scavenging activities among five carbohydrases (Viscozyme, Celluclast, Termamyl, Ultraflo and AMG) and five proteases (Kojizyme, Alcalse, Flavourzyme Protamex and Neutrase) extracts (Athukorala et al., 2003). Thus, the Celluclast and Neutrase extracts were selected for further study. To characterize the molecular weight of the active com-pounds, the extracts were passed through ultrafiltration membranes. Then, the fractions (unfractionated sample, <5, 5-10, 10-30 and >30 kDa) at different con-centrations (0.1, 0.25 and 0.5 mg/mL) were investigated separately with regard to their H_2O_2 scavenging activities. The 5-10 kDa fraction of the Celluclast and Neutrase extracts showed the most potent H_2O_2-scavenging activity among the fractions, with IC_{50} values of 0.177 and 0.182 mg/mL, respectively (Table 1). Previous reports have examined high H_2O_2 scavenging ability in seaweeds (Athukorala et al., 2003; Siriwardhana et al., 2004). In particular, brown seaweeds has been shown to exhibit profound H_2O_2-scavenging and DPPH radical-scavenging activities (Heo et al., 2005; Heo and Jeon, 2008; Kim et al., 2006).

The fraction that had the lowest IC_{50} of the tested extracts was selected for use in the next set of experiments. Meanwhile, all of the molecular weight fractions were subjected to total polyphenolic assays to determine the polyphenolic contents of each extract. The total polyphenolic amount in each extract is shown in Table 1. The highest polyphenolic content was found in the 5-10 kDa fraction of Celluclast (13.98 mg/100 mL) and Neutrase (15.58 mg/100 mL), whereas the lowest content was seen in the <5 kDa fraction of Celluclast (4.2 mg/100 mL) and Neutrase (3.38 mg/100 mL). As reported previously (Siriwardhana et al., 2004), a correlation exists between total polyphenolic content and radical-scavenging activity of seaweed extracts; this was confirmed in the present study. Many researchers have reported a positive correlation between ROS-scavenging activity and total phenolic compound content (Kim et al., 2006; Ahn et al., 2007, Oki et al., 2002). As the 5-10 kDa fraction of the Celluclast and Neutrase extracts generated in this study showed such high H_2O_2-scavenging activity, this fraction was further evaluated with regard to its protective effects against the cellular damage induced by H_2O_2. The protective effect of the 5-10 kDa fraction of Celluclast and Neutrase extracts on H_2O_2-induced cellular damage to the V79-4 cell line is shown in Fig. 1. In this study, both of the tested extracts controlled H_2O_2-induced

Table 1. IC_{50} values of H_2O_2 scavenging activity and total phenolic contents of the different molecular weight fractions of *S. coreanum* Celluclast and Neutrase extract

Treated enzymes	Molecular weight fractions	IC_{50} values (mg/mL)	Total phenolic contents (mg/100 mL)
Celluclast	Original extract	0.545	8.69
	>30 kDa	0.379	11.80
	10-30 kDa	0.367	9.82
	5-10 kDa	0.182	13.98
	<5 kDa	0.932	3.38
Neutrase	Original extract	0.536	9.45
	>30 kDa	0.275	13.62
	10-30 kDa	0.251	15.38
	5-10 kDa	0.177	15.58
	<5 kDa	0.876	4.20

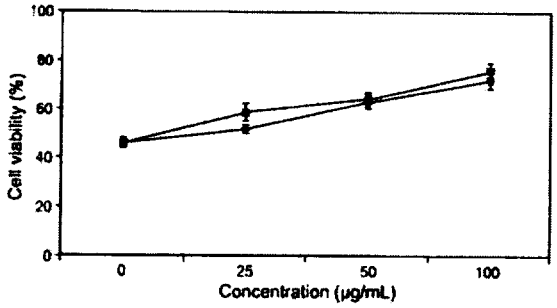

Fig. 1. Protective effects of 5-10 kDa fractions of the Celluclast and Neutrase extracts from *S. coreanum* against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were conducted in triplicate, and data are expressed as the average percentage change from control ± S.D. (◆) 5-10 kDa fractions of Celluclast extract and (■) 5-10 kDa fractions of Neutrase extract.

cellular damage in a dose-dependent manner. The addition of H_2O_2 to the cell culture medium without enzymatic extracts resulted in a 46% cell survival rate, but the addition of extracts coupled with exposure to H_2O_2 resulted in a dose-dependent increase in cell survival rates. In particular, the highest cell viability, 72% and 76%, was recorded with the 5-10 kDa fraction of Celluclast and Neutrase extract, respectively, at concentrations of 100 μg/mL.

Due to the profound antioxidant activity shown by the 5-10 kDa fraction, the fraction was evaluated further with regard to its ability to protect against DNA damage. DNA damage is one of the most sensitive biological markers for the evaluation of oxidative stress and illustrates the imbalance between free radical generation and the efficiency of the antioxidant system (Gutteridge, 1995; Kassie et al., 2000). Hoechst 33342 dye specifically stains DNA

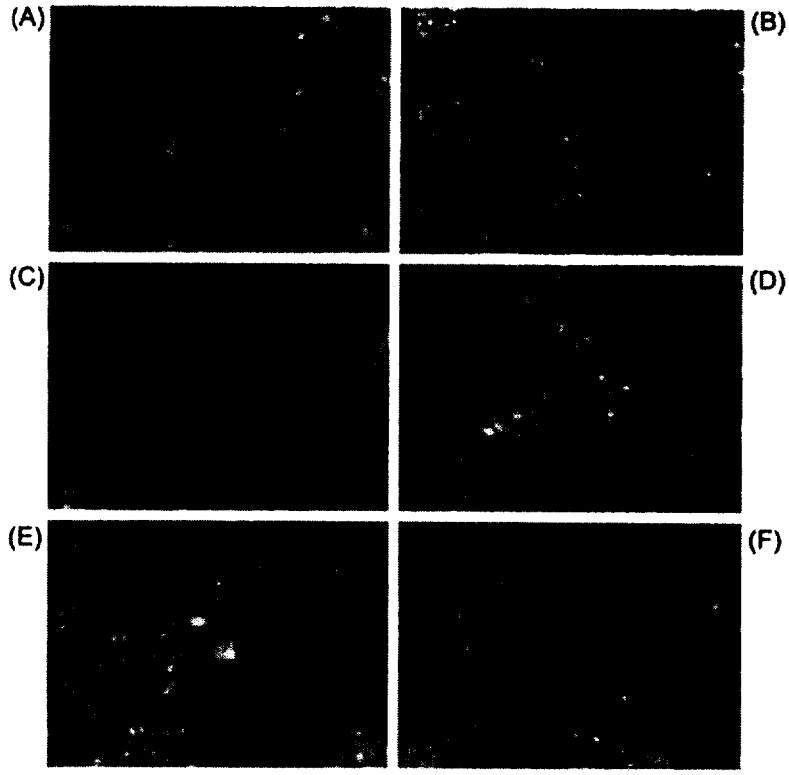

Fig. 2. Protective effect of 5-10 kDa fractions of the Celluclast and Neutrase extracts from *S. coreanum* against H_2O_2-induced apoptosis in V79-4 cells. The cells were treated with 5-10 kDa fractions of the Celluclast and Neutrase extracts at concentrations of 50 and 100 μg/mL. After 1 h, 1 mM of H_2O_2 was added to the plate. After incubation of 24 h, the apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining. (A) Negative control; (B) positive control (cells treated with 1 mM H_2O_2); (C) cells treated with 50 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (D) cells treated with 100 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (E) cells treated with 50 μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2; (F) cells treated with 100 μg/mL μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2.

and has been used extensively for the detection of nuclear shrinkage, including chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies, all of which are indicative of apoptosis (Heo and Jeon 2009). As shown in Fig. 2, the negative control, which contained no extracts or H_2O_2, harbored intact nuclei (Fig. 2A), and the positive control (cells treated with 1 mM H_2O_2) showed a significant level of nuclear fragmentation, indicative of apoptosis (Fig. 2B). However, addition of the 5-10 kDa fractions with H_2O_2 reduced the number of apoptotic bodies (Fig. 2C-F), suggesting the ability of the 5-10 kDa fractions to protect against nuclear fragmentation in H_2O_2 challenge and indicating that the cells were protected against oxidative stress-associated cellular injury. The protective effects of 5-10 kDa fractions on cell damage were also confirmed via a comet assay, which is a rapid and sensitive fluorescent microscopic method for the detection of primary DNA damage at the individual cellular level and is used to evaluate the genotoxicity of test substances (Bagchi et al., 2000). The percentage of the fluorescence tail DNA intensity of V79-4 cells subjected to 30 min of treatment with the negative control differed significantly from that of the positive control (H_2O_2 preincubated in PBS). This increase in H_2O_2-induced cell damage was inhibited significantly, in a dose-dependent fashion, by preincubating the H_2O_2 and 5-10 kDa fractions together with the negative cells (Fig. 3). The inhibitory activities of the 5-10 kDa fraction of the Celluclast and Neutrase extracts on DNA damage were 67 and 68%, res-pectively, at concentrations of 100 μg/mL. We also examined photomicrographs of different DNA migration profiles after treatment with different concentrations of the 5-10 kDa fractions of Celluclast and Neutrase extracts (Fig. 4). In the positive control (cells treated only with H_2O_2), the DNA was severely and systemically damaged, and the quantities of tail DNA were significantly increased, compared with those of the negative control. However, when we added the 5-10 kDa fraction of Celluclast and Neutrase extracts to the cells, we determined that the amounts of tail DNA were increasingly reduced with increasing concentrations of the 5-10 kDa fractions of the Celluclast and Neutrase extracts. Reactive oxygen species such as H_2O_2 are highly reactive and are capable of inflicting damage to a variety of biological macromolecules, including DNA, RNA, proteins, and lipids (Yan et al., 1999). In addition to our morphological evaluations, the protective effects of the 5-10 kDa fraction were confirmed by flow cytometry. DNA content analyses conducted following the H_2O_2

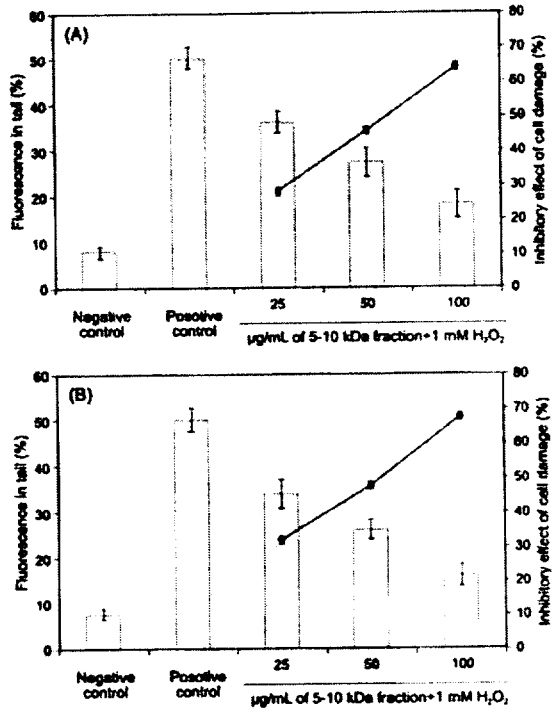

Fig. 3. Inhibitory effect of 5-10 kDa fractions of the Celluclast and Neutrase extracts from *S. coreanum* on H_2O_2-induced DNA damage. Cells were treated with 5-10 kDa fractions at concentrations of 25, 50, and 100 μg/mL. After 30 min, 1 mM H_2O_2 was added and incubated for 15 min. The percentage of cellular DNA damage was detected by comet assay. (A) Inhibitory effect of 5-10 kDa fraction of the Celluclast extract on H_2O_2-induced DNA damage. (B) Inhibitory effect of 5-10 kDa fraction of the Neutrase extract on H_2O_2-induced DNA damage. (□) % Fluorescence in tail; (●) inhibitory effect of cell damage.

treatment of V79-4 cells revealed an increase in the proportion of cells with sub-G_1 DNA content to 18.5% (Fig. 5). This result indicated that apoptosis was induced by the added H_2O_2. However, the cells that were pretreated with the 5-10 kDa fraction of the Celluclast and Neutrase extracts showed significantly reduced sub-G1 DNA contents (2.68 and 1.8%, respectively). Cells in the human body are basically under continuous attack by physical agents such as solar radiation, a variety of chemical compounds, and reactive oxygen species, which are natural by-products of metabolic processes (Kim et al., 2006). These substances can all induce DNA damage. If this DNA damage remains unrepaired, a cascade of biological consequences can be initiated within the cell population. Unresolved DNA damage can also

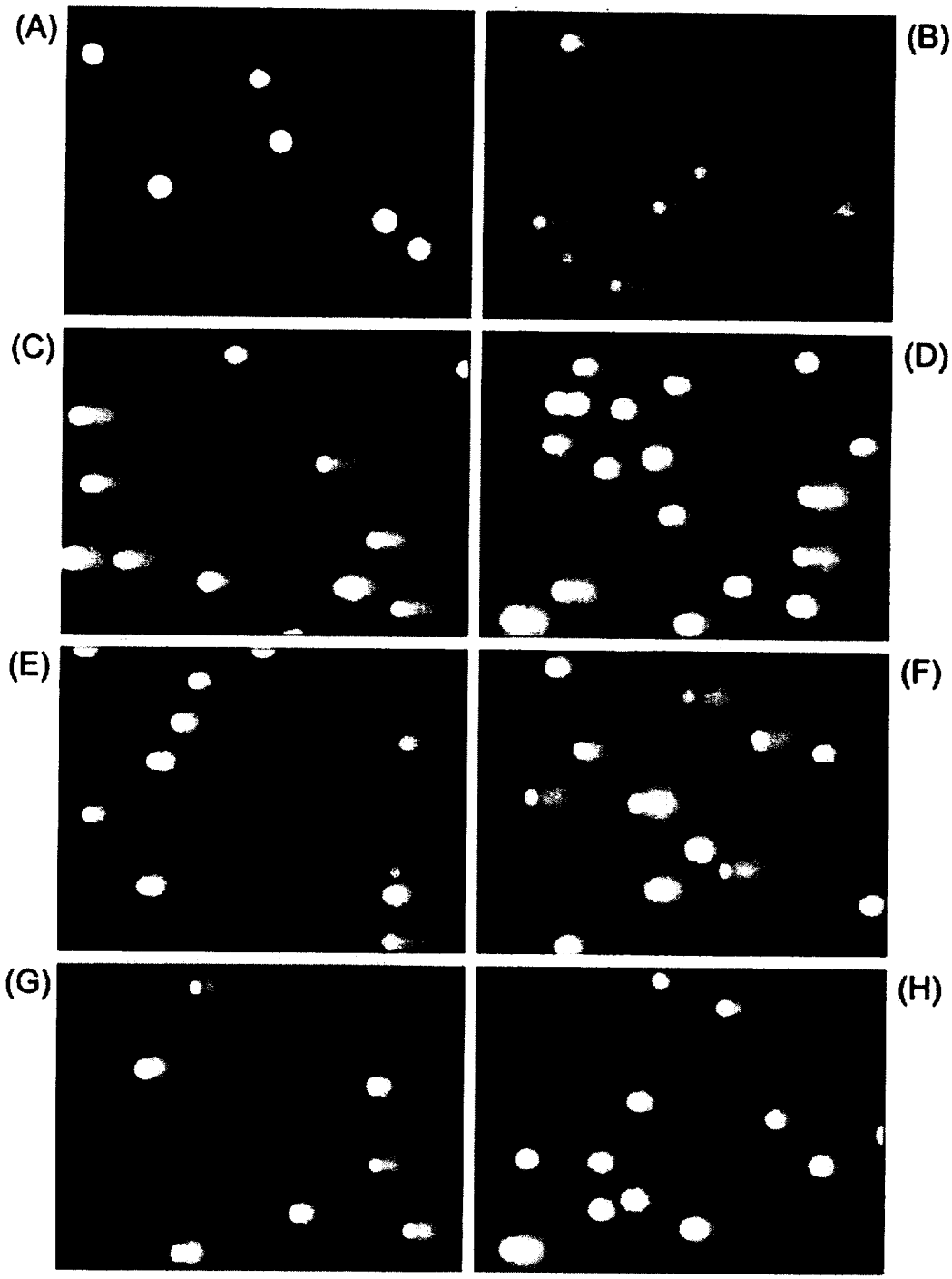

Fig. 4. Photomicrographs of DNA damage and migration observed under 5-10 kDa fractions of the Celluclast and Neutrase extracts from *S. coreanum* where the tail moments were decreased. (A) Negative control; (B) positive control (cells treated with 1 mM H_2O_2); (C) cells treated with 25 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (D) cells treated with 50 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (E) cells treated with 100 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (F) cells treated with 25 μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2; (G) cells treated with 50 μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2; (H) cells treated with 100 μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2.

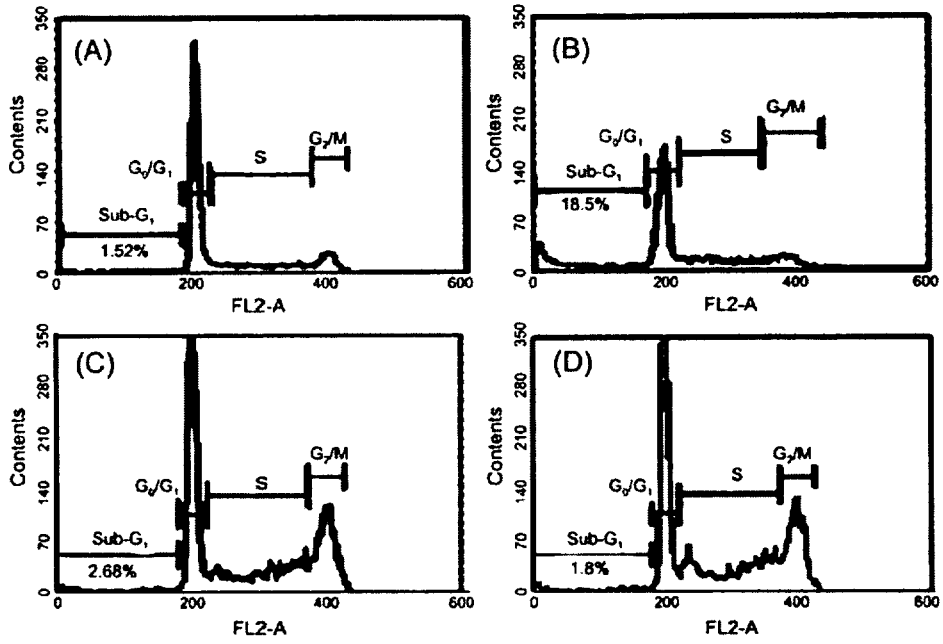

Fig. 5. Effects of the 5-10 kDa fraction on cell cycle pattern and apoptotic cell proportion in V79-4 cells with H_2O_2 (1 mM) via flow cytometric analysis. (A) Control; (B) H_2O_2-treated; (C) cells treated with 100 μg/mL 5-10 kDa fraction of Celluclast extract + 1 mM H_2O_2; (D) cells treated with 100 μg/mL 5-10 kDa fraction of Neutrase extract + 1 mM H_2O_2.

promote cancer development via several mechanisms (Nardella et al., 1996). The antioxidative activities of *E. cava*, as with other brown seaweeds, may be primarily attributable to carotenoid pigments (fucoxanthin), polyphenols (phlorotannins), vitamins (vitamins C, E), sulfated polysaccharides (laminarin, fucoidans), or their breakdown products, as well as proteins or their breakdown products (Yan et al., 1999; Nardella et al., 1996; Peña-Ramos and Xiong, 2002). In this enzymatic hydrolysis, polyphenolics, vitamin C, sulfated polysaccharides, and proteins, among the many materials with antioxidative potential, may be considered among the most effective due to their native water-soluble nature.

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