

Protective Effects of *In Vitro* Gastrointestinal Digests of Abalone (*Haliotis discus hannai*) Intestines against Oxidative Stress in RAW264.7 Macrophage Cells

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Abalone (*Haliotis discus hannai*), mostly distributed and maricultured in southwestern coastal areas of South Korea, is recognized as an economically important species in the fishery industry. Abalone intestines are one of the by-products of abalone processing. To investigate abalone intestines as bioactive substances, abalone intestine gastrointestinal digests (AIGIDs) of various molecular weights (MWs) were prepared using *in vitro* gastrointestinal digestion and an ultrafiltration system, and tested for inhibitory effects against reactive oxygen species (ROS) and oxidative stress in macrophage cells treated with hydrogen peroxide (H₂O₂). In our results, among AIGIDs, AIGID-III (MW=5-10 kDa) showed potent inhibitory activities for lipid peroxidation and free radicals. Additionally, the results clearly indicated that AIGID-III treatment could prevent cytotoxic damage of macrophages by H₂O₂-induced oxidative stress due to its potent scavenging ability against cellular ROS. These results suggest that AIGIDs may have protective and therapeutic potential for oxidative stress syndromes and immune diseases through ROS inhibition in macrophage cells.

Key words: Abalone, *Haliotis discus hannai*, Intestine, *In vitro* gastrointestinal digestion, Antioxidant, ROS

Introduction

Oxidative stress by reactive oxygen species (ROS) is responsible for injuries to all the important cellular components and tissues. High levels of ROS, both free radicals and non-free radicals, have been implicated as the cause of many diseases such as cancer, diabetes, aging, and inflammatory diseases (Rackov et al., 2007). Free radicals consist of the peroxy (ROO·), nitric oxide (NO·), hydroxyl (·OH), and superoxide radicals (O₂^{·-}). Non-free radicals consist of singlet oxygen (¹O₂) and hydrogen peroxide (H₂O₂) (Lee et al., 2010). ROS produced during normal physiological events and by external conditions such as smoking, stress, and disease are removed by antioxidant-defensive mechanisms. These defensive mechanisms include anti-oxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, and small molecules, such as

glutathione, vitamin C, and vitamin E (Halliwell and Gutteridge, 1990). The endogenous and exogenous antioxidants play a crucial role controlling free radicals to reduce cell and tissue damage (Aruoma, 1994; Halliwell, 1994).

Many natural antioxidants have been identified as free radical or active oxygen scavengers (Duh, 1998; Pan et al., 2007). Recently, research interests have considerably increased in finding natural antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which have been restricted due to side effects such as carcinogenicity. These natural antioxidants can effectively protect the human body from free radicals and retard the progress of many chronic diseases as well as oxidative rancidity in foods or medicinal materials without any side effects (Kim et al., 2006; Jung et al., 2007; Qian et al., 2008).

Marine organism-derived proteins and peptides possess various bioactive compounds with many nutraceutical and pharmaceutical activities that are related to physiological functions such as anti-

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coagulation (Jo et al., 2008), antimicrobial effects (Liu et al., 2008), antihypertension (Byun and Kim, 2001), and cardiovascular disease (Mendis et al., 2005; Kim et al., 2006; Erdmann et al., 2008). Depending on the amino acid composition as well as molecular size, bioactive peptides can be involved in diverse biological functions (Elias et al., 2008). During gastrointestinal digestion, proteolytic digestion can generate absorbable and bioactive peptides in the stomach and small intestinal tracts (Roberts et al., 1999; Vermeirssen et al., 2004). With the advantage that the formed peptides will resist physiological digestion after oral intake, the digestion by gastrointestinal proteases can be useful as a production process for antioxidant peptides. Some recent studies have reported that *in vitro* gastrointestinal digests of marine organisms possessed biological activities as potent as that of other natural antioxidants (Jung et al., 2007; Qian et al., 2008).

Abalone is a marine gastropod, and one of the most commercially important fishery resources in Asian cultures (Zhu et al., 2008). To meet the current increasing demand of the Asian, American, Australian, and EU market, abalone culture is expanding in land- and sea-based systems worldwide and is expected to produce 7,580 metric tons of abalone in 2009 in South Korea (The Korea Maritime Institute, KMI). Among abalone species cultured, *Haliotis discus hannai* is the most commercially important species in South Korea. Although *H. discus hannai* is an important species among fishery resources, their biological activities have been rarely studied (Ekanayake et al., 2008; Wan et al., 2008; Sun et al., 2010).

In the present study, the objective of our research was to evaluate the antioxidant potential of *H. discus hannai* abalone intestine gastrointestinal digests (AIGIDs) and elucidate the protective effects against oxidative stress in macrophage cells.

Materials and Methods

Materials

Live adult abalone (*H. discus hannai*) were collected from Wando Island, Wando-gun, South Korea. Intestinal organs (guts) were separated from the washed abalone and lyophilized. RAW264.7 macrophage cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, penicillin/streptomycin/amphotericin (10,000 U/mL, 10,000 µg/mL, and 2,500 µg/mL, respectively), and fetal bovine serum

(FBS) were obtained from Gibco-BRL Life Technologies (Grand Island, NY, USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) reagent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), α -(4-pyridyl-1-oxide)-N-tertbutyl nitron (4-POBN), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and Folin-Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Other chemicals and reagents used were of analytical grade.

In vitro gastrointestinal digestion

The digestion process used the method described by Kapsokefalou and Miller (1991). One hundred milliliters of 4% (w/v) abalone intestine solution was brought to pH 2.2 in gastric digestion (phase I) using 1 M HCl and 10 M NaOH under vigorous mixing. Pepsin (EC 3.4.23.1., 400-800 U/mg protein; Sigma Chemical Co.) was added at an enzyme to substrate ratio of 1/100 (w/w), then incubated at 37°C on a shaker. After 2 h, the pH was set to 6.5 to obtain the conditions of small intestinal digestion (phase II). Similarly, trypsin (EC 3.4.21.4., 30 U/mg protein; Wako Pure Chemical Ltd., Osaka, Japan) and α -chymotrypsin (EC 3.4.21.1., 60 U/mg protein; ICN Biomedical Inc., Aurora, OH, USA) were both supplemented at an enzyme to substrate ratio of 1/100 (w/w). The solution was further incubated at 37°C for 2.5 h. When samples were taken at the beginning and end of digestion, the pH was adjusted to 6.5. Samples were centrifuged at 10,000 \times g for 15 min at 4°C, and the supernatant was lyophilized to obtain powdered AIGIDs.

Preparation of AIGIDs with various MWs using a UF membrane bioreactor system

AIGIDs were fractionated through ultrafiltration (UF) membranes with a range of molecular weight cutoffs (MWCOs) of 100, 10, 5 and 1 kDa, respectively. Fractions were designed as follows: AIGID-I with MW > 100 kDa, AIGID-II with MW of 100-10 kDa, AIGID-III with MW of 10-5 kDa, and AIGID-IV with MW of 5-1 kDa. AIGIDs I-IV were lyophilized and stored at -20°C.

Measurement of anti-oxidative activity in a linoleic acid model system

Inhibition of lipid peroxidation by AIGIDs was measured in a linoleic acid oxidation system according to the methods of Osawa and Namiki (1985). Briefly, to make a 1% sample solution in an emulsion of linoleic acid and ethanol, 1.3 mg of sample was

dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and added to a solution of 0.13 mL linoleic acid and 10 mL 99.5% ethanol. Then the total volume of the emulsion was adjusted to 25 mL with distilled water. The emulsion was incubated in a conical flask with a screw cap at $40 \pm 1^\circ\text{C}$ in darkness. The degree of oxidation was evaluated by measuring ferric thiocyanate values according to the method of Mitsuda et al. (1966). The emulsion (100 μL) incubated in the linoleic acid model system was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured based on the absorbance at 500 nm following color development with FeCl_2 and thiocyanate at different intervals during the incubation period at $40 \pm 1^\circ\text{C}$.

Amino acid composition analysis

For the determination of amino acid composition, the lyophilized AIGID-III (20 mg of powder) was hydrolyzed in 6 N HCl containing 0.1% thioglycolic acid at 110°C for 24 h under vacuum. Amino acids derived with phenylisothiocyanate were identified and quantified using an automatic amino acid analyzer (Biochrom 20; Pharmacia Biotech, Uppsala, Sweden).

Measurement of free radical scavenging activity by electron spin resonance (ESR) spectroscopy

Diverse radicals were generated according to the following procedures, and spin adducts were recorded using a JES-FA electron spin resonance (ESR) spectrometer (JEOL Ltd., Tokyo, Japan). Radical scavenging ability was calculated with following equation in which H and H_0 were the relative peak height of the radical signals with and without sample, respectively.

$$\text{Radical scavenging activity} = \left[\frac{1-H}{H_0} \right] \times 100$$

Scavenging effect on the DPPH radical

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1995). A 30 μL sample solution (or ethanol as a blank) was added to 30 μL of DPPH (60 μM) in ethanol. After mixing vigorously for 10 s, the solution was then transferred to a 100 μL quartz capillary tube, and the scavenging activity was measured using the ESR spectrometer. The spin adduct was measured on the ESR spectrometer exactly 2 min later. Experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz;

amplitude, 1×1000 ; sweep time, 30 s. DPPH radical scavenging ability was calculated with the previous equation in which H and H_0 were the relative peak height of the radical signals with and without sample, respectively.

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber-Weiss reaction, and the generated hydroxyl radicals were rapidly reacted with nitron spin traps (DMPO) (Rosen and Rauckman, 1984). The resultant DMPO-OH adducts were detectable with an ESR spectrometer. The sample solution (20 μL) was mixed with DMPO (0.3 M, 20 μL), FeSO_4 (10 mM, 20 μL), and H_2O_2 (10 mM, 20 μL) in a phosphate buffer solution (pH 7.4), and then transferred to a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The experimental conditions employed were as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1×200 ; sweep time, 4 min. Hydroxyl radical scavenging ability was calculated as the previous equation in which H and H_0 were the relative peak height of the radical signals with and without sample, respectively.

Superoxide anion radical scavenging activity

Superoxide anion radicals were generated by a UV-irradiated riboflavin/EDTA system (Guo et al., 1999). The reaction mixture containing 0.3 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO, and sample was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was transferred to a 100 μL ESR quartz capillary tube for measurement. The experimental conditions were as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000 ; sweep time, 1 min. Superoxide radical scavenging ability was calculated as the previous equation in which H and H_0 were the relative peak height of the radical signals with and without sample, respectively.

Peroxy radical scavenging activity

Alkyl radicals were generated according to the method of Hiramoto et al. (1993). Briefly, 20 μL of 40 mM 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH) was mixed with 20 μL of phosphate buffered-saline (PBS), 20 μL of 40 mM a-(4-pyridyl-1-oxide)-N-tert-butyl nitron (4-POBN), and 20 μL of sample solution. The mixture was incubated at 37°C for 30 min, and then transferred to a 100 μL ESR quartz capillary tube for measurement. The experimental conditions were as follows: modulation fre-

quency, 100 kHz; microwave power, 10 mW; microwave frequency, 9441 MHz; magnetic field, 336.5 ± 5 mT; and sweep time, 30 s. Peroxyl radical scavenging ability was calculated as the previous equation in which H and H₀ were the relative peak height of the radical signals with and without sample, respectively.

Cell culture and viability determination

RAW264.7 macrophages were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified air environment. Cell viability was measured based on the formation of blue formazan metabolized from colorless MTT by mitochondrial dehydrogenases, which are active only in live cells. RAW264.7 macrophages were plated in 96-well plates at a density of 4×10^3 cells per well for 24 h, and then washed. Cells incubated with various concentrations of samples were incubated in 0.5 mg/mL MTT solution. Three hours later, the supernatant was removed and the formation of formazan was measured at 540 nm using a microplate reader (PowerWave XS model; BioTek Instruments, Inc., Winooski, VT, USA).

Measurement for cytotoxicity on H₂O₂-induced RAW264.7 cells

Cells were treated with various concentrations of the AIGID-III and incubated for 10 h. Cellular oxidation was accelerated by exposing cells to 200 µM H₂O₂. After 6 h, cell viability was measured using the MTT method. Data were expressed as the mean percentages of viable cells compared to the respective control culture.

Cellular ROS determination by DCFH-DA

Intracellular formation of ROS was assessed using oxidation sensitive dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA), as the substrate (Okimoto et al., 2000). RAW264.7 cells cultured in fluorescence microtiter 96-well plates were loaded with 20 µM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated for 20 min in darkness. Non-fluorescent DCFH-DA dye freely penetrates into cells and is hydrolyzed by intracellular esterases to 2',7'-dichlorofluorescein (DCFH). Cells were then treated with different concentrations of test extracts and incubated for 1 h. After washing the cells with PBS three times, 300 µM H₂O₂ dissolved in HBSS was added to the cells. The formation of DCF due to the oxidation of DCFH in the presence of various ROS was measured every 30 min at an excitation wavelength (Ex) of 485 nm and an emission wavelength

(Em) of 535 nm using the microplate reader. Dose-dependent and time-dependent effects of AIGID-III treatment groups were plotted and compared to the fluorescence intensity of the control and blank groups.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean (n=3). Student's t test was used to determine the level of significance ($P < 0.05$).

Results and Discussion

Preparation of AIGIDs and antioxidant activity in linoleic acid model systems

To generate anti-oxidative digests from abalone intestine, an *in vitro* gastrointestinal (GI) digestion system was applied. The GI digestion consisted of hydrolysis processes that simulated physiological digestions: gastric digestion (phase 1) corresponded to a pepsin-hydrolysis, and intestine digestion (phase 2) corresponded to proteolysis by two enzymes (trypsin and α -chymotrypsin). The results of the lipid peroxidation inhibitory assay using a linoleic acid model (Table 1), the anti-oxidative activity of gastrointestinal digests (phase 2; $88.2 \pm 7.2\%$) was significantly elevated after GI digestion of abalone intestines. The activity of gastric digests (phase 1) showed an inhibition value of $69.8 \pm 5.7\%$. The results indicated that the constituent proteins or peptides of AIGIDs are the precursors of antioxidant peptides via the action of GI enzymes.

Table 1. Inhibitory effects of gastrointestinal digests of abalone intestine against lipid peroxidation

Digestion phase	Inhibition of lipid peroxidation (%)
Gastric digests (phase 1)	69.8 ± 7.7
Gastrointestinal digests (phase 2)	88.2 ± 7.2

AIGIDs with potent antioxidant activity in the linoleic acid model system were further separated into four MW groups: AIGID-I (<100 kDa), AIGID-II (10-100 kDa), AIGID-III (5-10 kDa), and AIGID-IV (1-5 kDa) using a UF membrane system (MWCOs: 100, 10, 5, and 1 kDa). The direct free radical scavenging effects of AIGIDs (AIGID-I to IV) were investigated using an ESR spin-trapping technique. DPPH is a stable free radical and acceptor for an electron or hydrogen to become a stable diamagnetic molecule. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity. An ESR spectrometer can visualize hydroxyl radicals generated by the Fenton reaction. The ESR signals

become weak in the presence of $\cdot\text{OH}$ scavengers, which compete with DMPO for $\cdot\text{OH}$. Superoxide radicals were generated by UV irradiation of a riboflavin/EDTA solution. AAPH can decompose to form carbon-centered radicals that can react swiftly with O_2 to yield peroxy radicals to stimulate lipid peroxidation (Halliwell and Gutteridge, 1990). Table 2 shows the free radical scavenging activities of the four groups against different radical sources. The results showed that AIGID-III exhibited higher free radical scavenging activity than the other groups. At 1 mg/mL, AIGID-III exhibited considerable scavenging activity of 84.3%, 81.9%, 72.6%, and 60.9% of DPPH, hydroxyl, superoxide, and peroxy radicals, respectively. Additionally, AIGID-III exhibited ROS scavenging activity in dose-dependent manner (Fig. 1).

Table 2. Free radical scavenging activity of AIGIDs

AIGIDs (1 mg/mL)	Radical scavenging activity (%)			
	DPPH	Hydroxyl	superoxide	peroxyl
AIGID I	86.2±1.5*	68.6±1.7	70.8±1.6	60.8±1.7*
AIGID II	75.3±1.2	70.6±2.6	69.4±2.0	56.2±2.5
AIGID III	84.3±0.8*	81.9±2.3*	72.6±3.0*	60.9±1.6*
AIGID IV	59.6±2.2	48.2±1.8	46.2±1.8	30.6±1.9

The asterisk (*) indicated a significant difference among the tested groups ($P < 0.05$). Values are mean \pm SD ($n = 3$).

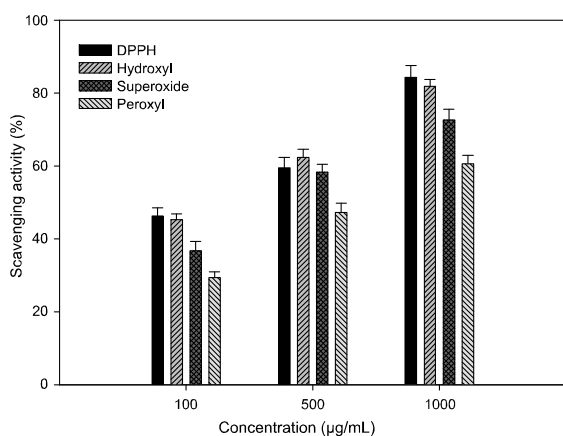


Fig. 1. Free radical scavenging activity in the presence of various concentrations of AIGID-III using ESR spectrometer.

As shown in Table 3, the amino acid composition of AIGID-III explains the antioxidant activity by its being composed of hydrophobic and hydrogen-donating amino acids such as Glu/n (355 mg/g, w/w), Asp/n (89 mg/g, w/w), Gly (66.3 mg/g, w/w), Lys (54.7 mg/g, w/w), Leu (47 mg/g, w/w), Thr (43.1 mg/g, w/w), Ser (44.7 mg/g, w/w), Arg (41.6 mg/g), and Ala (40.8 mg/g, w/w).

Table 3. Contents of compositional amino acids in AIGID-III (mg/g)

Amino acids	Intestine
Asp/n	89.4
Thr	43.1
Ser	44.7
Glu/n	355.9
Gly	66.3
Ala	40.8
Cys	14.6
Val	39.3
Met	16.9
Ile	26.2
Leu	47
Tyr	30.8
Phe	31.6
Lys	54.7
Trp	1.5
His	23.9
Arg	41.6
Pro	31.6
Total	1,000

Bioactive peptides usually contain less than 50 amino acid residues per molecule, and the lower molecular weight of these molecules can give a higher probability of crossing the intestinal barrier and exert biological effects (Roberts et al., 1999). Lipid peroxidation is thought to proceed via radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids. Since the hydrophobicity of antioxidants is important for accessibility to the hydrophobic targets (Chen et al., 1995), the presence of hydrophobic amino acids in the active peptides may have contributed to lipid peroxidation inhibitory activity by increasing the solubility of peptides in lipid and thereby facilitating better interaction with radical species. Additionally, the presence of the hydrophobic amino acid Leu in the AIGID-III could be important for antioxidant activity (Chen et al., 1995) because Leu is assumed to increase the interaction between peptides and fatty acids. Additionally, the presence of Asp seems to play a vital role irrespective of its position as observed in several anti-oxidative peptide sequences. The chemical activity of the hydroxyl radical is the strongest among ROS. It easily reacts with biomolecules, such as amino acids, proteins, and DNA (Cacciuttolo et al., 1993). Therefore, the removal of hydroxyl radicals is one of the most effective defenses of a living body against various diseases. As a whole, the potent antioxidant activity of AIGID-III could be attributable to the presence of specific amino acids and the distribution of molecular size.

MTT assay and protective effects of AIGID-III against H₂O₂-induced cytotoxicity

Cytotoxic effects of AIGID-III were evaluated on mouse macrophages (RAW264.7). The results showed that AIGID-III had no cytotoxic effects on RAW264.7 cells at the tested concentrations (Fig. 2). Therefore, nontoxic concentrations of AIGID-III (100-1,000 µg/mL) were used for this experiment.

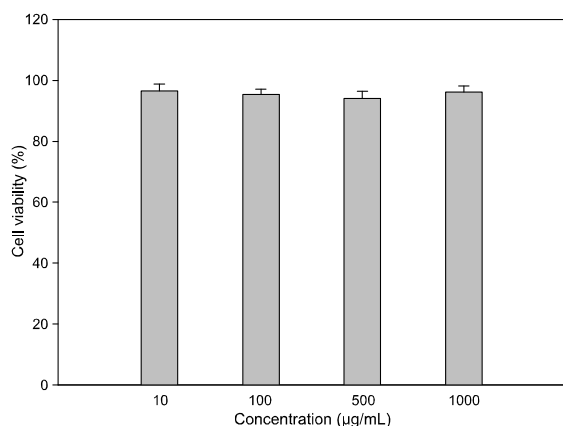


Fig. 2. Cytotoxic effects of AIGID-III on RAW264.7 macrophage cell lines. Different concentrations of samples were applied to the cells for 24 h and cell viability was assessed by MTT assay as described in the text. Results are means \pm standard error of three independent experiments.

Oxidative stress induced by treatment of H₂O₂ is a useful model to study oxidative injury mechanisms. Cells were exposed to H₂O₂ for 2 h, and cell viability was determined using the MTT assay. As shown in Fig. 3, RAW264.7 cell viability increased with treatment of AIGID-III in a dose-dependent manner, and the results showed that AIGID-III treatment could enhance the viability of macrophages up to 95% at a concentration of 1,000 µg/mL after exposure to H₂O₂.

In the cellular system, we employed H₂O₂ to generate lipid-derived radicals. H₂O₂ rapidly penetrates into mammalian cells. The proposed mechanisms of H₂O₂-induced toxicity include the finding that cytochrome P450 catalyzes the hemolytic scission of H₂O₂ to the reactive hydroxyl radical, and as a result of catalysis, initiates peroxidation of membrane lipids (Veerman et al., 2004). These results demonstrate the ability of AIGID-III to overcome H₂O₂-induced cytotoxicity.

Cellular ROS determination by DCFH-DA

To identify preventive effects of AIGID-III against H₂O₂-induced cytotoxicity by cellular ROS, we

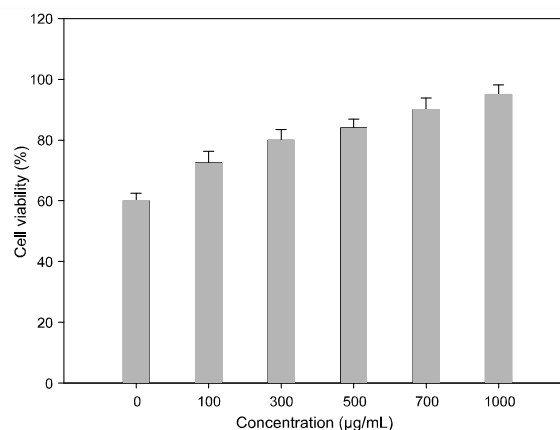


Fig. 3. Effect of AIGID-III on H₂O₂-induced cytotoxicity to RAW264.7 cells. RAW264.7 cells were cultured in DMEM medium, and cellular oxidative stress was artificially induced. Viable cells were determined by MTT assay (n=3).

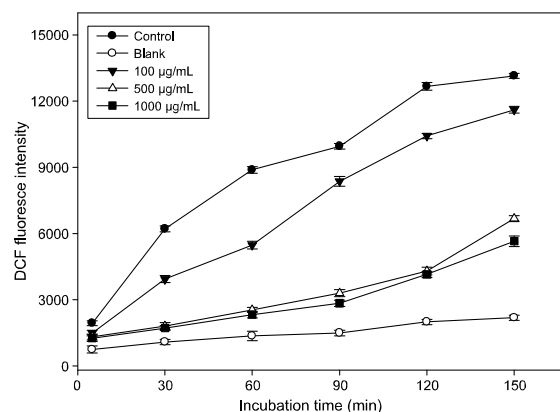


Fig. 4. Cellular radical scavenging activity of AIGID-III at 1,000 µg/mL, 500 µg/mL, and 100 µg/mL. RAW264.7 cells were labeled with non-toxic fluorescence dye, DCFH-DA, and treated with different concentrations of AIGID-III. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H₂O₂) were detected time-dependently ($\lambda_{excitation}=485$ nm and $\lambda_{emission}=528$ nm). Effects of extracts of SH on the scavenging of cellular ROS were compared with H₂O₂ non-stimulated blank and sample non-treated group (control) in three independent experiments.

further examined the direct effects of AIGID-III to scavenge cellular radicals; RAW264.7 cells were labeled with the fluorescent probe DCFH-DA. DCFH-DA is hydrolyzed by esterases to DCFH, which is trapped within the cells. This non-fluorescent molecule is then oxidized to DCF via the action of cellular oxidants (Robinson, 1988). As shown in Fig. 4, fluorescence emitted by DCF

following ROS-mediated oxidation of DCFH followed a time-dependent course up to 150 min. Pretreatment with AIGID-III decreased DCF fluorescence dose- and time-dependently. AIGID-III exerted a considerable radical scavenging effect at 100 µg/mL concentration after 30 min. More clearly, at a concentration of 1,000 µg/mL, AIGID-III could scavenge radicals significantly throughout the incubation time. These results suggest that AIGID-III can protect macrophage cells from oxidative damage by ROS. It could be developed into a potential biomolecular candidate to inhibit ROS formation of cellular damage. Furthermore, AIGID-III was shown to contain potent antioxidant activity, and inhibit radical-mediated oxidation and protect macrophage cells.

In this study, we determined that the AIGIDs of *H. discus hannai* possess high antioxidant activity and exhibit protective effects against oxidative stress in macrophage cells. In conclusion, these results suggest that AIGID-III contains bioactive compounds regarding immune and oxidative diseases, and abalone *H. discus hannai* may be a valuable invertebrate animal as a potential marine pharmaceutical resource.

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