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Research Article

Anti-Inflammatory Effect of By-Products from *Haliotis discus hannai* in RAW 264.7 Cells

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Several reports promoted the potential of shellfish due to their ability to act as antioxidant, anti-inflammatory, and antimicrobial agents. Pacific abalone, *Haliotis discus hannai* viscera is, reported to possess bioactivities such as antioxidative stress and anti-inflammatory. In this study, anti-inflammatory potential of mucus-secreting glands from shell-shucking waste of *H. discus hannai* was evaluated using RAW 264.7 mouse macrophage cell model. Results indicated that presence of *H. discus hannai* mucosubstance by-products (AM) significantly lowered the nitric oxide (NO) production along the expressional suppression of inflammatory mediators such as cytokines TNF-α, IL-1β, and IL-6 and enzymes iNOS and COX-2. Also, AM was shown to increase expression of anti-inflammatory response mediator HO-1. Presence of AM also scavenged the free radicals *in vitro*. In conclusion, by-products of *H. discus hannai* are suggested to possess notable anti-inflammatory potential which promotes the possibility of utilization as functional food ingredient.

1. Introduction

In modern day, various chronic diseases and complications including aging, diabetes, and cancer are considered to be accompanied with an elevated oxidative stress in body [1, 2]. Recent studies evidently indicated a set of interactions between the pathways of oxidative stress and inflammation that defines these chronic diseases [3–5]. It is scientifically accepted that reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxyl radicals are generated in situation of oxidative stress. Although body has its own cellular mechanism to pacify these radicals through antioxidant enzymes and antioxidants, defense mechanisms can be overpowered by excessive oxidative stimulation [6]. Following the presence of untreated free radicals, vital cell damage occurs that can lead to inflammation as well as clinical diseases as a result [7]. Therefore, functional treatment is supposed and developed to focus on handling the underlying causes of clinical diseases. In this regard, studies turned their attention to attenuate severe inflammations instead of suppressing the symptoms. Therefore, regulating the shared biochemical pathway of oxidative stress and inflammatory response is aimed by several recent studies which are directed to developing natural antioxidant and anti-inflammation agents [8–10]. Reports of therapeutic agents suggest that being effective against both oxidative stress and inflammation might be significantly beneficial to human health by means of prevention and treatment of pathologic complications caused by ROS.

Past decades have witnessed discovery of numerous bioactive metabolites produced by natural sources that act as antioxidants and anti-inflammatory agents. In this regard, marine organisms including shellfish are of much interest in recent studies due to unique chemical composition of marine environment. Among shellfish, abalone is an important source to food industry throughout Asia, Africa, Australia, and America. Abalone is a marine gastropod and has been widely found as aquacultured in the coasts of Asian countries in order to meet the increasing demand of Asian food market [11]. Therefore, land- and sea-based farms are expanded and developed as well as production of abalone varieties such as dried, steamed, and spiced abalone. Unfortunately, elevated levels of abalone production bring along the organic waste which needs to be handled. Several by-products of marine aquaculture foods evidently are able to be utilized as functional food additives [12]. Studies credited these by-products which are regarded to be waste, to contain bioactive materials that act as health promoting substances. Also, it is well known that marine organisms possess several health beneficial effects due to presence of various bioactive materials. Most of these bioactive materials were already elucidated and their pharmaceutical and nutraceutical potential have been assessed [13]. Several compounds from shellfish are reported to have antioxidant, anti-inflammatory, antitumor, antimicrobial, and antiviral effects [14–16]. However, health beneficial effects of abalone have been reported vaguely and lack in detail action mechanism studies. Mainly, viscera of some abalone species were studied for their potential antioxidative and anti-inflammatory activities [17, 18]. Besides viscera, abalone shell-shucking leaves mucus and mucosubstance wastes of salivary gland. Mucus-secreting parts of abalone are considered to be a big part of processing by-product apart from viscera. To the best of our knowledge, there is not any reports regarding the nutraceutical potential of abalone mucosubstance by-products. Considering the high amounts of abalone production and elevated amounts of abalone by-products, studying the bioactivity of abalone-based substances holds great deal of potential regarding nutraceutical and pharmaceutical aspects.

In the present study, we investigated the anti-inflammatory effects of mucus-based by-products of abalone, *Haliotis discus hannai*, *in vitro* in regard to relation between oxidative stress and inflammatory response so as to evaluate the health beneficial potential of abalone suggesting a possibility for functional foods substances.

2. Materials and Methods

2.1. Materials. Mucus-based by-products were obtained from the waste of shell-sucking processing of abalones. Mucous glands and mucus-based products left on shells after shucking were removed, processed to be introduced *in vitro* cell cultures. The samples from *Haliotis discus hannai* mucus-based by-products (AM) were collected in Wando on 2012, air-dried, and ground to a powder and obtained as samples to be used in further assays. Obtained samples were dissolved in 10% dimethyl sulfoxide (DMSO) and kept at 20°C until use.

2.2. Cell Culture and Cytotoxicity Determination Using MTT Assay. Murine RAW 264.7 macrophage cells were grown as monolayers at 5% CO_2 and 37°C humidified atmosphere using Dulbecco's modified eagle medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 μg/mL penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD, USA). The medium was changed twice or thrice each week. Cytotoxic levels of the *Sargassum* sp. on cultured cells were measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and were treated with control medium or the medium supplemented with *Sargassum* sp. After incubation for 24 h, cells were rewashed and 100 μL of MTT solution (1 mg/mL) was added and incubated for 4 h. Finally, 100 μL of DMSO was added to solubilize the formed formazan crystals and the amount of formazan crystal was determined by measuring the absorbance at 540 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan crystal. Viability of cells was quantified as a percentage compared to the control and dose response curves were developed.

2.3. Determination of Intracellular Formation of ROS Using DCF-DA Labeling. Intracellular formation of reactive oxygen species (ROS) was assessed using oxidation sensitive dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) as the substrate. RAW 264.7 cells growing in fluorescence microtiter 96-well plates were loaded with 20 μM DCF-DA in HBSS and incubated for 20 min in the dark. Nonfluorescent DCF-DA dye that freely penetrated into cells gets hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluororescein (DCFH) and tarps inside the cells. Cells were then treated with different concentrations of test compounds and incubated for 1 h. After washing the cells with PBS three times, 500 μM H_2O_2 dissolved in HBSS was added to the cells. The formation of 2′,7′-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of various ROS was read after every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 528 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). Dose-dependent and time-dependent effects were plotted and compared with fluorescence intensity of control and blank groups.

2.4. Measurement of Nitric Oxide Production. RAW 264.7 cells were seeded onto 96-well plates with 2×10^5 cells/well using DMEM without phenol red and allowed to adhere overnight with pretreated samples for 1 h. Cellular NO production was stimulated by adding 1 μg/mL final concentration of lipopolysaccharides (LPS) and further incubated for 24 h. After incubation, the production of NO was determined based on the Griess reaction. Briefly, 40 mL of 5 mM sulfanilamide, 10 mL of 2 M HCl, and 20 mL of 40 mM naphthylethylenediamine were added to 50 mL of culture medium.

Table 1: Sequences of primers used for RT-PCR.

Gene	Direction	Sequence
iNOS	Forward	5′-TTC-CAG-AAT-CCC-TGG-ACA-AG-3′
	Reverse	5′-TGG-TCA-AAC-TCT-TGG-GGT-TC-3′
COX-2	Forward	5′-AGA-AGG-AAA-TGG-CTG-CAG-AA-3′
	Reverse	5′-GCT-CGG-CTT-CCA-GTA-TTG-AG-3′
TNF-α	Forward	5′-GGA-GCC-AGC-TCC-CTC-TAT-TT-3′
	Reverse	5′-GGC-TAC-ATG-GGA-ACA-GCC-TA-3′
IL-1β	Forward	5′-CTG-TCC-TGC-GTG-TTG-AAA-GA-3′
	Reverse	5′-TTC-TGC-TTG-AGA-GGT-GCT-GA-3′
IL-6	Forward	5′-AGT-TGC-CTT-CTT-GGGACT-GA-3′
	Reverse	5′-CAG-AAT-TGC-CAT-TGCACA-AC-3′
HO-1	Forward	5′-CAC-GCA-TAT-ACC-CGC-TAC-CT-3′
	Reverse	5′-AAG-GCG-GTC-TTA-GCC-TCT-TC-3′
β-actin	Forward	5′-CCA-CAG-CTG-AGA-GGG-AAA-TC-3′
	Reverse	5′-AAG-GAA-GGC-TGG-AAA-AGA-GC-3′

After 15 min incubation at room temperature, absorbance was measured at 550 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). The concentrations of nitrite were calculated from regression analysis, using serial dilutions of sodium nitrite as a standard.

2.5. RNA Extraction and Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was isolated using Trizol reagent (Invitrogen Co., CA, USA). For synthesis of cDNA, RNA (2 μg) was added to RNase-free water and oligo (dT), denaturated at 70°C for 5 min, and cooled immediately. RNA was reverse-transcribed in a master mix containing 1X RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reserve transcriptase, and 40 U RNase inhibitor at 42°C for 60 min and at 72°C for 5 min using an automatic T100 Thermal Cycler (Bio-Rad, UK). The target cDNA was amplified using the sense and antisense primers shown in Table 1. The amplification cycles were carried out at 95°C for 45 sec, 60°C for 1 min, and 72°C for 45 sec. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide visualized by UV light using Davinch-Chemi imager (CAS-400SM, Wako Co., Japan).

2.6. Western Blot Analysis. Western blotting was performed according to standard procedures. Briefly, cells were lysed in RIPA lysis buffer (Sigma-Aldrich Corp., St. Louis, USA) at 4°C for 30 min. Cell lysates (25 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, UK), blocked with 5% skim milk, and hybridized with primary antibodies (diluted 1 : 1000) against iNOS, COX2, and β-actin (Cell Signaling Technology, Inc., USA). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescece ECL assay kit (Amersham Pharmacia Biosciences, UK) according to the manufacturer's instructions. Western blot bands were visualized using a Davinch-Chemi imager (CAS-400SM, Wako Co., Japan).

2.7. Statistical Analysis. The data were presented as mean ± SEM. Differences between the mean of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 (SAS Institute, Cary, NC, USA) with Duncan's multiple range tests. The significance of differences was defined at the $P < 0.05$ level.

3. Results and Discussion

3.1. Free Radical Scavenging Activity of H. discus hannai. Prior to performing RAW 264.7 mouse macrophage-based *in vitro* oxidative stress assay, cytotoxicity of AM was evaluated by MTT assay. At all concentrations (0.05~2 mg/mL) cytotoxicity was not observed in RAW 264.7 mouse macrophages (Figure 1(a)). According to results, cell viability was not affected by concentration-wise treatment of *H. discus hannai*. Hence, further *in vitro* assays were performed accordingly. Following confirmation of nontoxic presence of AM in cells, antioxidant potential of AM was assayed through DCFH-DA based experiment. Ability of ROS scavenging was calculated through fluorescent intensity of DCFH oxidation to result in DCF as an indicator of ROS activity in H_2O_2-exposed RAW 264.7 cells. Supposedly, AM acted on cells to protect cells from oxidative stress caused by H_2O_2 inducement, by means of data interpretation, lowering the fluorescence intensity which indicates the ROS scavenging activity. All treated concentrations starting from 0.05 mg/mL to 1 mg/mL were able to lower the intracellular oxidative stress in RAW 264.7 cells in comparison to H_2O_2-induced control cells in the absence of AM (Figure 1(b)), indicated as DCF fluorescence intensity. Treatment of 1 mg/mL lowered the DCFH oxidation the most, suggesting a significant free radical scavenging towards H_2O_2 inducement, approximately 75% compared to relation between untreated control cells with H_2O_2 and blank cells without H_2O_2 exposure. At the lowest concentration

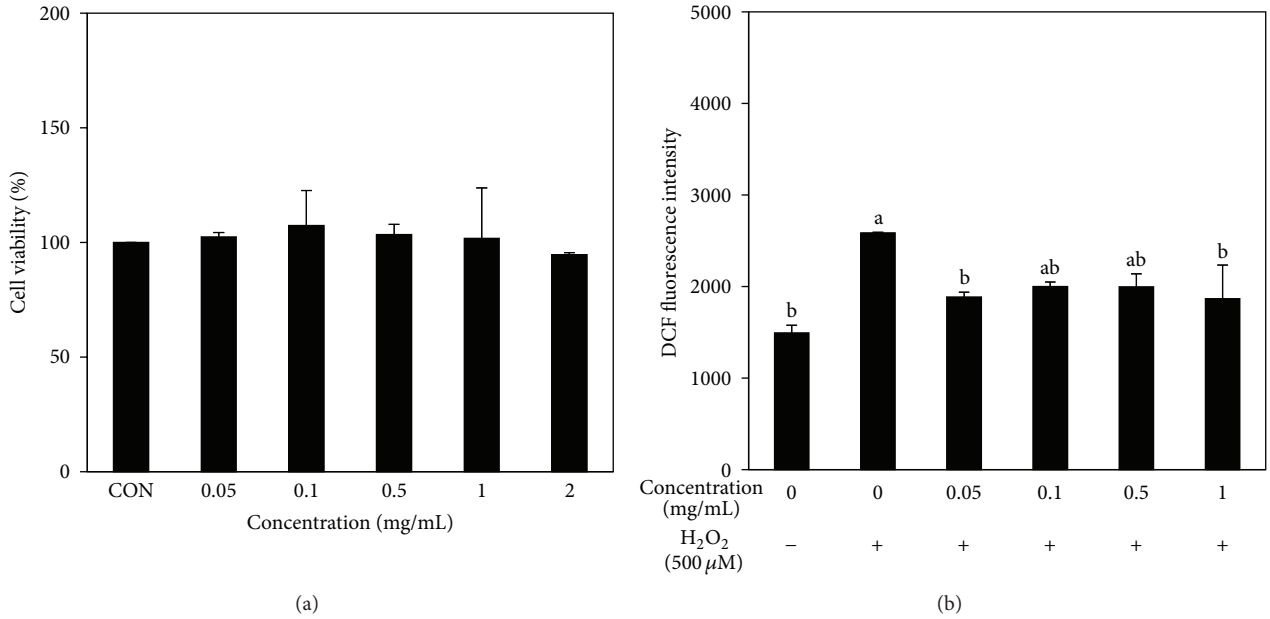

Figure 1: Cytotoxicity (a) and effect on intracellular ROS level induced by hydrogen peroxide (b) of AM in RAW 264.7 cells. After preincubation of the RAW 264.7 cells in 20 μM DCF-DA, the cells were treated with AM for 120 min. DCF fluorescence was measured following addition of 500 μM H_2O_2 at $\lambda_{excitation}$ 490 nm and $\lambda_{excitation}$ 620 nm. [a-b]Mean with the different letters is significantly different ($P < 0.05$) by Duncan's multiple range test.

of 0.05 mg/mL, surprisingly, this effect was around 70% of control cells. Free radical scavenging of samples also did not follow a linear fashion towards dose-dependency. Regardless of the dose of treatment, ability to scavenge free radicals was at the rate of 70% except concentration of 1 mg/mL at which rate was 75%. However, statistical analysis showed that the effects of the extract were not concentration-dependent. The main reason behind the non-dose-dependent efficiency was speculated as antagonism. The extract contains various types of molecules such as glycolipid and polysaccharide complexes, some of which could be antagonistic. Therefore, at low doses hindrance of these antagonistic compounds was not observed while they prevented the dose-dependent increase in effect at higher concentrations. A similar observation was reported by Dore et al. [19], on some antioxidant properties of polysaccharide-rich extracts. Nonetheless, AM were able to lower the intracellular oxidative stress and protect RAW 264.7 cells from oxidative stress induced cell damage. Enzymatic hydrolysates of viscera of AM were reported to include antioxidant properties due to bioactivity of peptides [17]. In addition, Zhu et al. also reported the antioxidant activity of sulfated polysaccharide conjugates from *H. discus hannai* viscera [18]. Also, it has been studied that abalones can contain large amounts of polyphenols and gamma-aminobutyric acid (GABA) whose amount depends on how much abalone feeds on seaweeds [20]. In this regard, mucus-secreting parts and mucus-based products of abalones in regard to binding their body to shells are suggested to mainly include mucosubstances, sulfated glycans, polyphenols, and GABA too. In this regard, potent antioxidant efficiency of AM can be credited to the presence of these substances as all the aforementioned molecules have been shown to possess antioxidant properties [21, 22].

3.2. Anti-Inflammatory Effect of H. discus hannai. Studies reported that strong antioxidant agents are effective against inflammation referring to the biochemical mechanism relation between two conditions [8]. Therefore, the potential anti-inflammatory activity of AM on LPS-stimulated RAW 264.7 mouse macrophages has been evaluated. When activated with pathogenic substances, macrophages initiate and regulate inflammatory responses through a broad range of inflammatory mediators. Lipopolysaccharides are among the most effective macrophage activators, and it has been known that LPS-stimulated macrophages produce excessive inflammatory mediators that have been in close relation with elevated oxidative stress such as free radicals, NO, and cytokines such as TNF-α, IL-1β, and IL-6 [23]. Following confirming anti-oxidative effect of *H. discus hannai*, anti-inflammatory potential was firstly evaluated by assessing NO production in the presence and absence of AM in LPS-stimulated RAW 264.7 cells (Figure 2). Production of NO was evaluated in two different time periods, 24 h and 48 h. Introduction of LPS activated the mouse macrophages and elevated NO production to 28 μM and to 91 μM from 3.5 μM (blank) after 24 (Figure 2(a)) and 48 h (Figure 2(b)) of incubation, respectively. Results also showed that treatment with AM significantly lowered NO production compared to control cells. Presence of AM decreased the produced NO amount under LPS-stimulation to approximately 3 μM in mouse macrophages incubated 24 h at the concentration of 1 mg/mL. On the other hand, after 48 h incubation AM

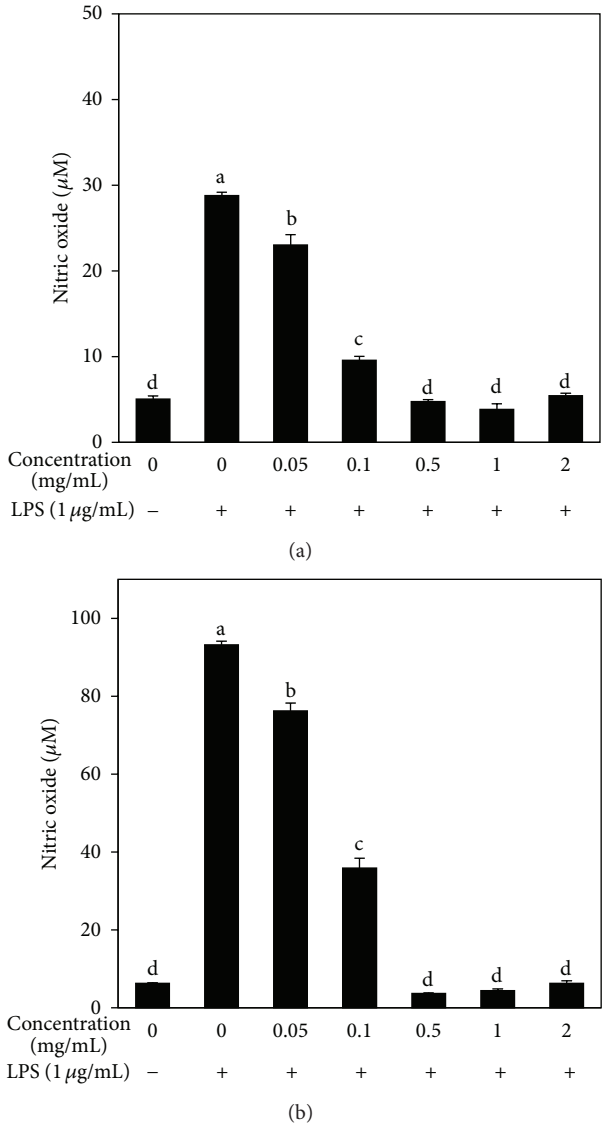

Figure 2: Effect of AM on intracellular NO level in LPS-stimulated RAW 264.7 cells. The cells were pretreated with LPS (1 μg/mL) and followed by treatment with the AM for 24 h (a) and 48 h (b). The nitrite content of culture media was analyzed. $^{a-d}$Mean with the different letters is significantly different ($P < 0.05$) by Duncan's multiple range test.

have lowered the NO production as low as 2.5 μM at the most effective concentration (0.5 mg/mL). However, unlike antioxidant effect, AM showed NO efficiency on lowering NO production in a dose-dependent fashion. In case of 24 h incubation NO lowering effect was increased by elevated doses until 1 mg/mL. At the concentration of 2 mg/mL NO production was slightly higher than that of 1 mg/mL. However, this difference was evaluated to be statistically nondifferent. Samples continued to be effective by means of lowering NO production after 48 h of incubation. Presence of AM was able to keep the NO levels as low as they were after 24 h incubation, at the concentrations of 0.5, 1, and 2 mg/mL.

In order to evaluate anti-inflammatory effect of AM in detail, expression of key indicators of inflammatory response was observed by RT-PCR and immunoblotting. Inflammatory response of macrophages is also accompanied by induced inflammatory gene expression. Inducement of inflammatory response is mediated by generation of transcriptional factors by inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) [24]. Various reports have suggested that elevated expressions of COX-2 and inducible NOS (iNOS) are closely related in the pathogenesis of inflammation and several other diseases including cancer and Alzheimer's [25, 26]. In this regard, effect of AM on gene and protein expression of inflammation mediator enzymes iNOS and COX-2 was observed in LPS-stimulated mouse macrophages. As shown in Figure 3, AM were able to suppress the expression of inflammatory mediators in a concentration-dependent fashion. According to results, presence of AM was able to suppress the mRNA expression (Figure 3(a)) and protein levels (Figure 3(b)) of iNOS and COX-2 in a direct dose-dependent manner. Further, effect of samples on gene expression of inflammatory cytokines was also evaluated by means of TNF-α, IL-1β, and IL-6 mRNA levels. Expectedly, AM treatment decreased the expression of inflammatory cytokines, namely, TNF-α, IL-1β, and IL-6 (Figure 4(a)). In order to assess the ability of sample to promote anti-inflammatory response in addition to suppressing inflammatory response, gene expression of HO-1 was observed in the presence of *H. discus hannai*. The ability of HO-1 to upregulate IL-10 and IL-1R makes it a crucial enzyme for development of anti-inflammatory response [27]. Results indicated that presence of sample in a dose-dependent manner strongly elevated the mRNA expression of HO-1 (Figure 4(b)). Treatment with abalone by-products raised HO-1 mRNA expession approximately double of the blank group.

Several other parts of abalone are reported to possess anti-inflammatory effect on *in vitro* models [28]. As mentioned earlier, abalones are considered to contain several bioactive materials, namely, sulfated glycans, polysaccharides, polyphenols, and GABA. On the other hand, reports evidently suggested that mucus-secreting salivary glands of abalone contain several mucosubstances, sulfated glycans, and mucin-like components. Considering earlier reports of antioxidant and anti-inflammatory sulfated compounds from shellfish it can be suggested that mucus-based by-products of abalone production contain mucus- and salivary gland-based materials, especially sulfated mucosubstances as well as polysaccharides. In this case, the presented antioxidant and anti-inflammatory effect of AM may be credited to presence of these compounds.

In order to have a better idea about the antioxidative and anti-inflammatory compounds that were present in the extract, FT-IR analysis was conducted. Results indicated a glycoprotein and polysaccharide-rich constituent with the stretchings between 1700 and 2000 (Figure 5). Also protein peaks between 3700 and 4000 exerted a possible inclusion of bioactive peptides that could be suggested for the aforementioned bioactivities.

Results clearly indicate that AM is effective in relieving inflammation through suppressing pathogen-induced overexpression of inflammatory mediators and cytokines as well

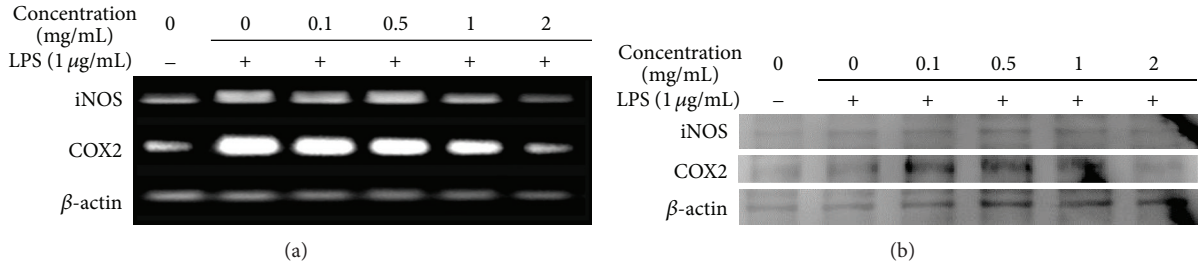

Figure 3: Effect of AM on the iNOS and COX-2 gene expressions (a) and protein levels (b) in LPS-stimulated RAW 264.7 cells shown by RT-PCR and Western blotting, respectively. The cells were treated with various concentrations of AM for 1 h prior to the addition of LPS and the cells were further incubated for 24 h.

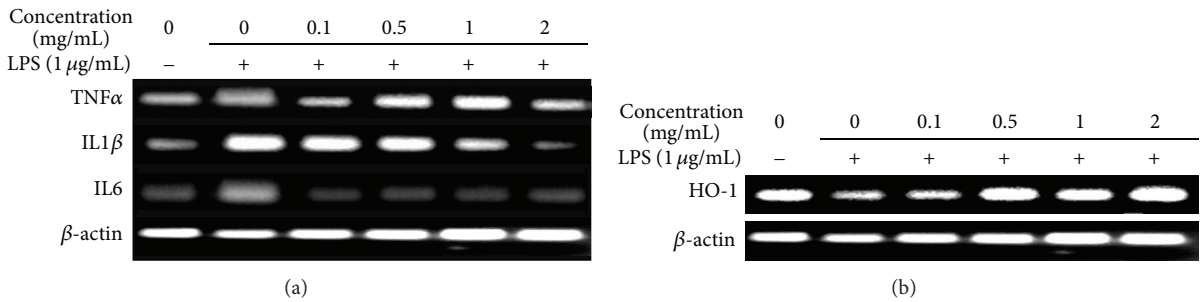

Figure 4: Effect of AM on expressions of inflammatory cytokines and HO-1 gene in LPS-stimulated RAW 264.7 cells shown by RT-PCR. The cells were treated with various concentrations of AM for 1 h prior to the addition of LPS and the cells were further incubated for 24 h.

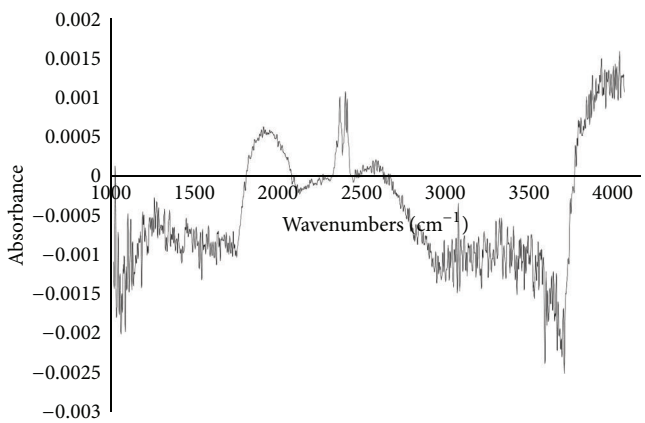

Figure 5: FT-IR spectrum of *H. discus hannai* extract.

as regulating the expression of anti-inflammatory response by related cytokines. Inhibition of NO generation by AM can also be related to suppression of these mediators. Numerous studies report that therapeutic agents mostly contain antioxidant and anti-inflammatory activities together due to a similar action mechanism for both bioactivities [8]. In addition, the ability to scavenge radicals in a structure-related activity is also considered to indicate a structure that can act as anti-inflammatory agent to suppress inflammatory response by downgraded expression of cytokines. In this regard, AM was able to decrease the mRNA expression of cytokines TNF-α, IL-1β, and IL-6 and inflammatory mediator enzymes iNOS and COX-2 and increase the expression of anti-inflammatory mediator enzyme HO-1. Also, AM was shown to be efficient in inhibiting NO generation. Taken together, AM could be suggested as a significant source of therapeutic agents. By-products of abalone shucking are currently regarded as waste and discarded. However, to the best of our knowledge, there is no published research addressing the antioxidant and anti-inflammatory effect of AM. Therefore, waste of bioactive material-rich AM left on shell after shucking was evidently exhibited to possess anti-inflammatory activity and great potential for utilization as functional food ingredient.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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