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Abalone Protein Hydrolysates: Preparation, Angiotensin I Converting Enzyme Inhibition and Cellular Antioxidant Activity

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ABSTRACT: Abalone protein was hydrolyzed by enzymatic hydrolysis and the optimal enzyme/substrate (E/S) ratios were determined. Abalone protein hydrolysates (APH) produced by Protamex at E/S ratio of 1:100 showed angiotensin I converting enzyme inhibitory activity with IC_{50} of 0.46 mg/mL, and APH obtained by Flavourzyme at E/S ratio of 1:100 possessed the oxygen radical absorbance capacity value of 457.6 μM trolox equivalent/mg sample. Flavourzyme abalone protein hydrolysates (FAPH) also exhibited H_2O_2 scavenging activity with IC_{50} of 0.48 mg/mL and Fe^{2+} chelating activity with IC_{50} of 2.26 mg/mL as well as high reducing power. FAPH significantly ($P<0.05$) protected H_2O_2-induced hepatic cell damage in cultured hepatocytes, and the cell viability was restored to 90.27% in the presence of FAPH. FAPH exhibited 46.20% intracellular ROS scavenging activity and 57.89% lipid peroxidation inhibition activity in cultured hepatocytes. Overall, APH may be useful as an ingredient for functional foods.

Keywords: abalone, angiotensin I converting enzyme, antioxidant, enzymatic hydrolysis

INTRODUCTION

Fish and shellfish protein hydrolysates have been extensively studied due to their potential health benefits such as antioxidant (1,2), antihypertensive (3), anti-inflammatory (4), anti-cancer (5), and enzyme inhibition (2). These bioactive protein hydrolysates are specific protein fragments with the ability to impact body functions. Some of these protein hydrolysates may exhibit multifunctional properties, thereby indicating that they may be useful as ingredients for functional foods. Enzymatic hydrolysis is generally considered one of the best techniques to produce bioactive protein hydrolysates without nutrient loss. The bioactivities of produced protein hydrolysates are strongly dependent on amino acid composition, peptide size and sequence as well as proteases for production of protein hydrolysates (1).

One of the most valuable marine gastropods in Korea is abalone (*Haliotis discus hannai*). Abalone has been used in traditional medicine and several researches reported that abalone exhibited anti-fatigue and detoxification properties (6,7). More than 90% of the world abalone production is based on farming, and abalone mariculture has been increasing due to its nutritive and pharmaceutical values (8). In our previous research, we attempted to produce protein hydrolysates with multifunctional bioactivities from abalone viscera, which is normally considered as an inedible part by producers and customers. The produced protein hydrolysates exhibited strong antioxidant and cytoprotective activity in a model cell line (1). However, scanty information for protein hydrolysates with bioactivities from abalone muscle by enzymatic hydrolysis has been reported. The objective of this study is to produce bioactive protein hydrolysates from abalone muscle by enzymatic hydrolysis and to determine multifunctional bioactivities including cellular antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities.

MATERIALS AND METHODS

Materials

Alcalase, Flavourzyme, Neutrase, and Protamex were purchased from Novozyme Nordisk (Bagsvaerd, Denmark). Fluorescein, trolox (6-hydroxy-2,5,7,8-tetrameth-

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ylchroman-2-carboxylic acid), 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and ferrozine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diphenyl-1-pyrenylphosphine (DPPP) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Preparation of abalone protein hydrolysates

Before digestion, the abalone was washed using tap water, and then freeze dried. The abalone protein hydrolysates (APH) were obtained by enzymatic hydrolysis with Alcalase, Flavourzyme, Neutrase, and Protamax at pH 7.0 and 50°C according to the manufacturer's instructions. Briefly, the enzyme was mixed with a 12% substrate solution at a ratio of 1:25 (w/w), and then hydrolysis was performed with simple agitation for 8 h. The mixture was then boiled for 10 min to inactivate the protease. After selecting the optimal enzyme based on bioactivities [ACE inhibitory and oxygen radical absorbance capacity (ORAC) assays], new digestions using enzyme/substrate (E/S) ratios of 1:50 and 1:100 were performed to determine the optimal E/S ratio for this enzyme. Unhydrolyzed protein was removed using a filter cloth, and the supernatant was lyophilized and stored at −20°C until use.

ACE inhibitory assay

ACE inhibitory activity was measured according to the method of Cushman and Cheung (9) with slight modifications. The mixture of APH solution (50 μL) and 50 μL of ACE (25 mU/mL) was pre-incubated at 37°C for 10 min, after which the mixture was re-incubated with 150 μL of substrate (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl, at pH 8.3) for 30 min at the same temperature. The reaction was terminated by adding 250 μL of 1.0 M HCl. The resulting hippuric acid was extracted with 0.5 mL of ethyl acetate. After centrifugation (800 *g*, 15 min), 0.2 mL of the upper layer was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV-spectrophotometer (SpectraMax® M2/M2e, Molecular Devices, Sunnyvale, CA, USA). The IC_{50} value was defined as the concentration required for inhibiting 50% of ACE.

Determination of antioxidant activity

***ORAC assay*:** The ORAC values of APH were measured using a previously described method with slight modifications (10). Briefly, APH (50 μL) or distilled water (control) was mixed with fluorescein (50 μL, 78 nM) and incubated at 37°C for 15 min, followed by addition of AAPH (25 μL, 221 mM). The fluorescence intensity was measured every 5 min for 60 min at Ex 485 nm/Em 535 nm (SpectraMax® M2/M2e). The ORAC values were expressed as μM trolox equivalent (TE)/mg APH using a trolox standard curve.

***H_2O_2 scavenging assay*:** The H_2O_2 scavenging activity of APH was measured using a previously described method (1). Briefly, APH (100 μL) was mixed with sodium phosphate buffer (100 μL, 0.1 M, pH 5.0) in a 96-well plate followed by addition of H_2O_2 (20 μL, 20 mM), and then the mixture was incubated at 37°C for 5 min. Thereafter, ABTS (30 μL, 1.25 mM) and peroxidase (30 μL, 1 U/mL) were added to the mixture followed by incubation at 37°C for 10 min. The absorbance values of APH or buffer were measured at 405 nm. The IC_{50} value was defined as the concentration required for scavenging 50% of H_2O_2.

***Fe^{2+} chelating assay*:** The Fe^{2+} chelating activity of APH was measured using a previously described method (1). Briefly, APH (100 μL) or distilled water (control) was mixed with $FeCl_2$ (100 μL, 0.1 mM) for 30 s, followed by addition of ferrozine (100 μL, 0.25 mM), and then allowed to equilibrate at room temperature for 10 min. The absorbance was measured at 562 nm. The IC_{50} value was defined as the concentration required for scavenging 50% of Fe^{2+}.

Reducing power assay

The reducing power of APH was measured using a previously described method (11). Briefly, APH (200 μL) was mixed with sodium phosphate buffer (300 μL, 0.1 M, pH 6.6) and potassium ferricyanide (500 μL, 1%) followed by incubation at 50°C for 20 min. Thereafter, TCA (500 μL, 10%) was added to the mixture followed by centrifugation at 1,036 *g* for 10 min. Finally, 100 μL of the supernatant was mixed with 100 μL of distilled water and 20 μL of $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm.

Cellular antioxidant activity

***MTT assay and cytoprotective activity of APH*:** Chang liver hepatocytes were purchased from ATCC (Rockville, MD, USA), maintained in an incubator at 37°C with a humidified atmosphere of 5% CO_2, and cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 unit/mL).

The cytotoxicity of APH was determined using the MTT assay. Chang liver hepatocytes were seeded in a 96-well plate at a density of 1.0×10^5 cells/well. After 18 h, the hepatocytes were pretreated with various concentrations of APH for 1 h, and then washed with phosphate buffered saline (PBS). The cells were then exposed to 650 μM H_2O_2 to induce oxidative stress, followed by incubation for 24 h at 37°C. After the 24 h incubation,

the MTT assay was performed.

Intracellular ROS scavenging activity: The intracellular ROS scavenging activity of APH was measured using a previously described method (12). Hepatocytes were seeded in a 96-well black plate at a density of 1.0×10^5 cells/well. Hepatocytes were labeled with 20 μM DCFH-DA in Hank's balanced salt solution (HBSS) for 20 min, followed by treatment with APH for 1 h. Then 500 μM H_2O_2 in HBSS was added after washing the cells with PBS. The formation of 2'7'-dichlorofluorescin due to oxidation of DCFH in the presence of ROS was measured every 30 min at Ex 485 nm/Em 528 nm. The percentage of fluorescence intensity (ROS generation) was compared with that of the control cells without APH, which were arbitrarily assigned a value of 100%.

Lipid peroxidation inhibition activity: The lipid peroxidation inhibition activity of APH was measured using a previously described method (13). Hepatocytes were grown in culture dishes and washed with PBS, followed by staining of 13 μM DPPP (in DMSO) for 30 min at 37°C in the dark. The cells were then washed three times with PBS and seeded in a 96-well black plate at a density of 1.0×10^7 cells/mL using serum-free media. Following complete attachment, the cells were treated with the extracts for 1 h and then challenged with 3 mM AAPH in PBS to initiate cell membrane lipid peroxidation. DPPP oxide fluorescence intensity after 6 h was measured at Ex 361 nm/Em 380 nm. The percentage of fluorescence intensity (lipid peroxidation) was compared with that of the control cells without APH, which were arbitrarily assigned a value of 100%.

Statistical analysis

The data are presented as the mean±standard deviation (SD) of at least three independent experiments (n=3). Differences between means of each group were assessed by one-way analysis of variance followed by Duncan's multiple range test using PASW Statistics 19.0 software (SPSS, Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

ACE inhibitory activity of APHs

ACE is a zinc metallopeptidase important for blood pressure control by converting angiotensin I to angiotensin II, which constricts the vessels (14). ACE also inactivates bradykinin, which has a depressor action. Thus, the inhibition of ACE activity is thought to be a major target for antihypertension. Abalone protein was hydrolyzed using various proteases and the yields of unhydrolyzed abalone protein and APHs were 11.84% (unhydrolysate), 62.40% (Alcalase), 47.24% (Flavourzyme), 44.88% (Neutrase), and 56.50% (Protamex), respectively. The ACE inhibitory activity of APHs produced by various proteases showed different inhibitory activities (Fig. 1A). At E/S ratio of 1:25, protamex abalone protein hydrolysates (PAPH) showed the highest ACE inhibitory activity with 92.69±1.08% (at 4 mg/mL) than that of the other protease hydrolysates. Thus, we conducted the enzymatic hydrolysis using protamex with different E/S ratios in order to determine the optimal E/S ratio that exhibited the best ACE inhibitory activity. As depicted in Fig. 1B, ACE inhibitory activities varied with the E/S ratios. At E/S ratio of 1:100, PAPH showed the highest ACE inhibitory activity with 85.93±0.81% (at 2 mg/mL) than that of the other E/S ratios, and the optimal E/S ratio was found to be 1:100. The calculated IC_{50} values of APHs are shown in Table 1, and the IC_{50} value of PAPH against ACE was found to be 0.46±0.01 mg/mL, which was lower than the 2.45 mg/mL of oyster sauce (15), 1.17 mg/mL of Atlantic salmon skin collagen peptide (16), and 0.79 mg/mL of Atlantic salmon hydrolysate (17), but much higher than the 0.27 mg/mL of sea cucumber digest (18), and 0.014

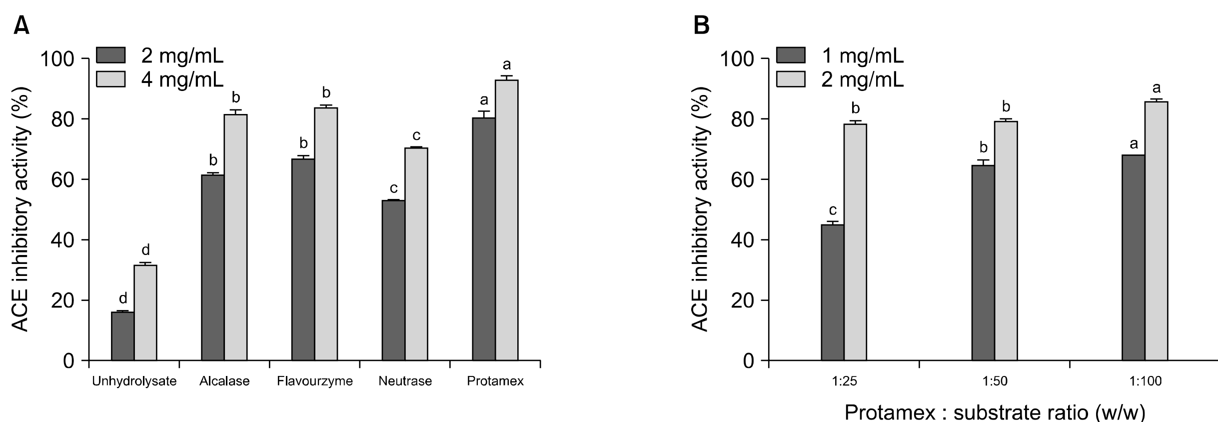

Fig. 1. (A) Angiotensin I converting enzyme (ACE) inhibitory activities of abalone protein hydrolysates (APHs) and (B) determination of optimal E/S ratio for production of ACE inhibitory APH by Protamex. Bars with different letters (a-d) indicate significant differences at the same concentration ($P<0.05$). Values are expressed as means±SD (n=3).

Table 1. ACE inhibitory activities of abalone protein hydrolysates by various proteases (unit: mg/mL)

	IC_{50} values
Unhydrolysate	>5.0
Alcalase	0.86±0.04
Flavourzyme	0.77±0.03
Neutrase	1.64±0.05
Protamex	0.46±0.01

mg/mL of pentapeptide purified from a sea squirt (19). It is well known that bioactivities of protein hydrolysates are strongly dependent on the amino acid composition of the parent protein, the type of hydrolyzing enzymes, and the distribution of molecular weights of hydrolysates. However, the IC_{50} comparison indicates that PAPH may have potential ACE inhibitory peptides, thus purification of ACE inhibitory peptides is in progress.

Antioxidant activity of APHs

Antioxidant activity of APHs was determined by measuring the ORAC value and is depicted in Fig. 2. At E/S ratio of 1:25, all APHs exhibited better ORAC values than the unhydrolyzed abalone, and Flavourzyme abalone protein hydrolysates (FAPH) showed the highest ORAC value of 441.8±10.2 μM TE/mg FAPH. Thus, enzymatic hydrolysis with different E/S ratios was conducted to obtain FAPH with the highest ORAC value. As shown in Fig. 2B, the ORAC values varied with different E/S ratios, and FAPH exhibited the ORAC value of 457.6±7.8 μM TE/mg FAPH at E/S ratio of 1:100. The ORAC value observed in this study was higher than that of other reported ORAC values including abalone viscera protein hydrolysates (415.8 μM) (1), sea cucumber digests (4.4 μM) (18), catfish hydrolysates (3.5 μM) (20), and pacific hake hydrolysates (225 μM) (21). The ORAC assay evaluates the ability to scavenge peroxyl radicals, a common radical in human biology, and thus it is considered the most reliable method for evaluating the antioxidant capacity of food-derived antioxidants (22). In this study, we showed that FAPH possessed high ORAC value, indicating FH might be useful as an ingredient for dietary antioxidants.

We also evaluated H_2O_2 scavenging activity, Fe^{2+} chelating activity, and reducing power of FAPH obtained at E/S ratio of 1:100 (Table 2). The IC_{50} values of FAPH against H_2O_2 scavenging and Fe^{2+} chelating were 0.48±0.02 mg/mL and 2.26±0.03 mg/mL, respectively. The reducing power of FAPH was 0.83±0.01 at 2.0 mg/mL. In the body, highly reactive hydroxyl radicals can be generated by the Fenton reaction in the presence of H_2O_2 and Fe^{2+}, which can damage virtually all types of macromolecules including carbohydrates, nucleic acids, lipids, and proteins (23). The use of antioxidants could prevent the generation of hydroxyl radicals through scavenging and/or chelating of H_2O_2 and Fe^{2+}, thus antioxidants could reduce hydroxyl radical-induced damage in the body. Another method for measuring the antioxidant potential of FAPH is the ferric reducing antioxidant power. This assay is based on the donation of an electron or hydrogen and an increase in absorbance at 700 nm would indicate an increase in the reducing power. Our previous work reported that abalone viscera protein hydrolysates possessed reducing power with an absorbance of 0.98 at 2.0 mg/mL, which is higher than the absorbance value obtained in this work (1). However, the absorbance value of FAPH is higher than in smooth

Table 2. Antioxidant activities of abalone protein hydrolysates by Flavourzyme at E/S ratio of 1:100

	FAPH[2)]
H_2O_2 scavenging (IC_{50}, mg/mL)	0.48±0.02
Fe^{2+} chelating (IC_{50}, mg/mL)	2.26±0.03
Reducing power[1)] (A_{700})	0.83±0.01

[1)]Reducing power was evaluated at 2.0 mg/mL.
[2)]FAPH: Flavourzyme abalone protein hydrolysate.

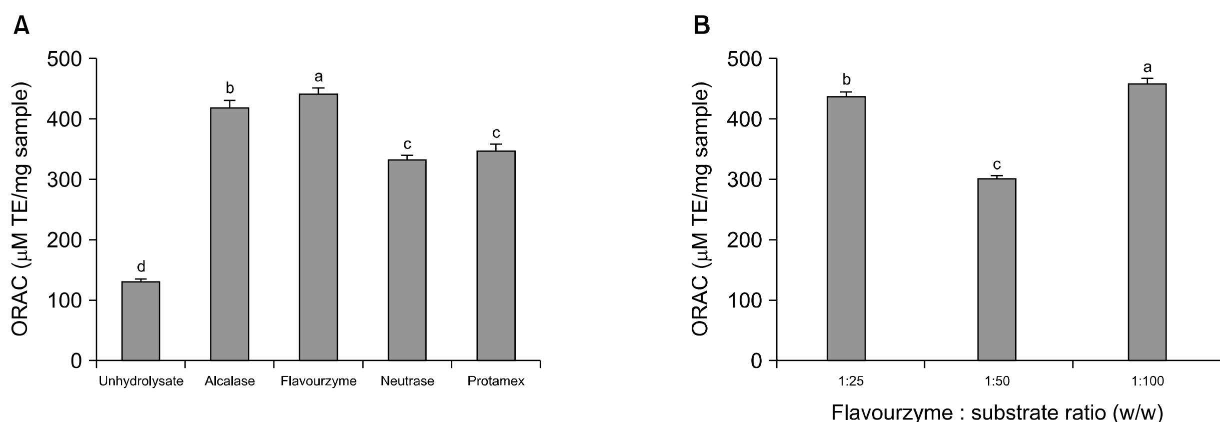

Fig. 2. (A) Oxygen radical absorbance capacity (ORAC) values of abalone protein hydrolysates (APHs) and (B) determination of optimal E/S ratio for production of APH with the highest ORAC value by Flavourzyme. Bars with different letters (a-d) indicate significant differences at the same concentration ($P<0.05$). Values are expressed as means±SD (n=3).

muscle protein hydrolysates (0.60 at 2.0 mg/mL) and yellow stripe trevally protein hydrolysates (0.52 at 3.6 mg/mL), respectively (24,25).

Cellular antioxidant capacity of FAPH

Since FAPH showed the highest antioxidant activity, we evaluated the cellular antioxidant activity of FAPH in cultured hepatocytes under oxidative stress. Prior to evaluating cellular antioxidant activity, cytotoxicity of FAPH in cultured hepatocytes was determined using the MTT assay. As depicted in Fig. 3A, FAPH did not show any cytotoxic effect against cultured hepatocytes in the tested concentrations. Further, we investigated whether FAPH could protect H_2O_2-induced hepatic damage in cultured hepatocytes. FAPH was added to cultured hepatocytes for 1 h, and washed with PBS, followed by exposure of 650 μM H_2O_2 to induce oxidative stress in cultured hepatocytes. In the absence of FAPH, hepatocytes were significantly ($P<0.05$) damaged by oxidative stress, and the cell viability was 60.16±5.96% compared to the blank group (without FAPH and H_2O_2). However, this decrease was significantly ($P<0.05$) restored by pretreatment with FAPH in a dose-dependent manner (Fig. 3B), and the cell viability was restored to 90.27±1.00% at 250 μg/mL of FAPH. To verify the antioxidant effect of FAPH, cellular ROS scavenging activity was measured in cultured hepatocytes under oxidative stress conditions. As shown in Fig. 3C, FAPH significantly ($P<0.05$) scavenged intracellular ROS in a dose-dependent manner, and intracellular ROS levels were decreased by 53.80±0.04% compared to the blank (without FAPH and H_2O_2). To measure lipid peroxidation inhibition ability of FAPH, AAPH was exposed to cultured hepatocytes in order to cause lipid peroxidation in the presence or absence of FAPH. As depicted in Fig. 3D, lipid peroxidation in cultured hepatocytes was significantly ($P<0.05$) decreased by pretreatment with FAPH in a dose-dependent manner and the inhibition activity was 57.89±0.26% at 250 μg/mL of FAPH.

Oxidative stress is thought to be involved in the development of human diseases such as cancer, atherosclerosis, Alzheimer's, and heart failure (26). Therefore, preventing or delaying the pathogenesis of these chronic diseases is an essential strategy to promote health conditions. In the present study, H_2O_2 was used to induce oxidative cell injury, and the protective ability of FAPH was investigated. Previous reports had demonstrated that treatment with H_2O_2 induced cell death and this damage was suppressed by pretreatment with antioxidants (27-29). Our results also agreed with these obser-

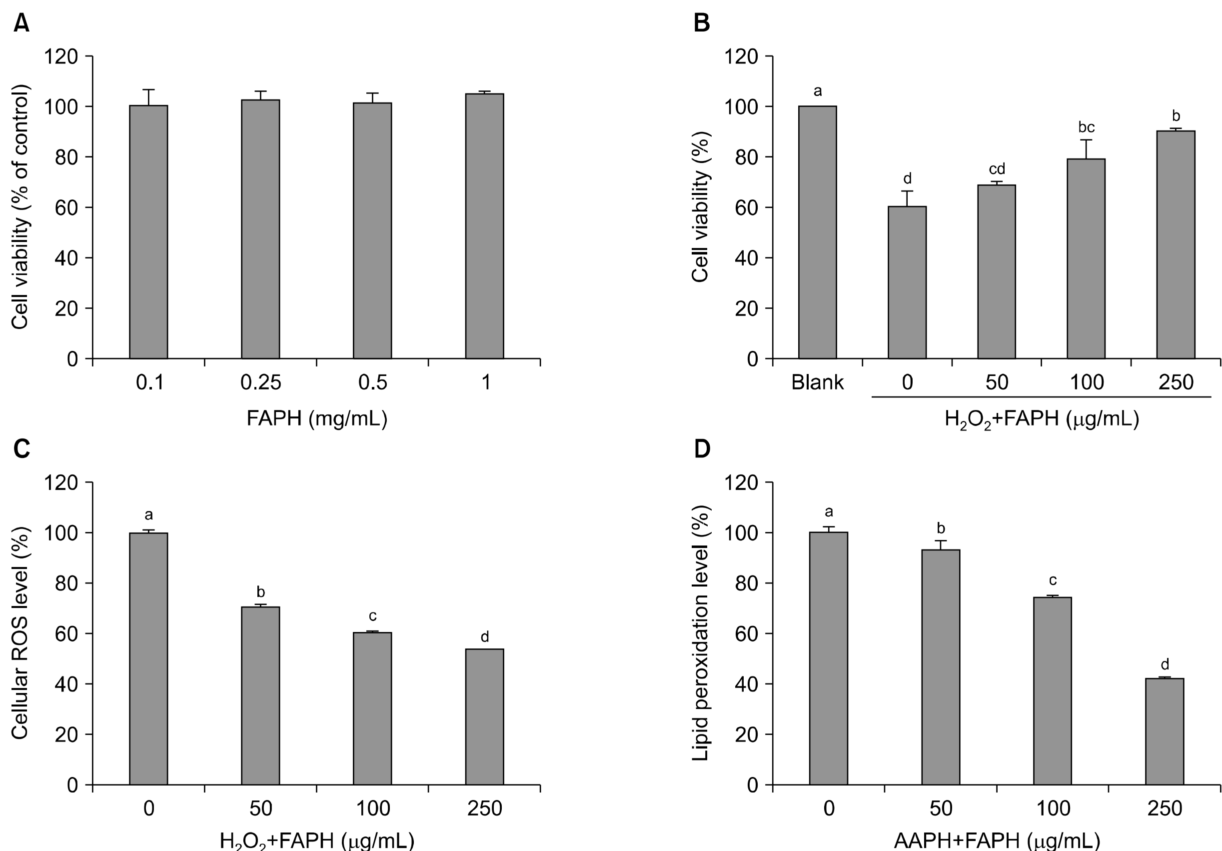

Fig. 3. (A) Cytotoxicity of Flavourzyme abalone protein hydrolysates (FAPH) from abalone protein, (B) protective effect of FAPH against H_2O_2-induced hepatic cell damage in cultured hepatocytes, (C) intracellular ROS scavenging activity of FAPH in cultured hepatocytes, and (D) lipid peroxidation inhibition of FAPH in cultured hepatocytes. Bars with different letters (a-d) indicate significant differences ($P<0.05$). Values are expressed as means±SD (n=3).

vations. In addition, overproduction of ROS is involved in the pathogenesis of hepatic fibrosis through lipid peroxidation in hepatocytes (30). Previous studies revealed that marine-derived hydrolysates scavenged cellular ROS; however, FAPH showed comparable or even higher cellular ROS scavenging activity as well as inhibition of lipid peroxidation (29,31).

CONCLUSIONS

Abalone protein hydrolysates (APH) were produced by enzymatic hydrolysis, and the optimal E/S ratios for ACE inhibitory and cellular antioxidant activities were determined. APH produced by Protamex at E/S ratio of 1:100 showed the best ACE inhibitory activity, and APH produced by Flavourzyme at E/S ratio of 1:100 possessed the highest ORAC value. Furthermore, Flavourzyme abalone protein hydrolysates (FAPH) exhibited protective effects against oxidative stress-induced hepatic cell damage through cellular ROS scavenging and lipid peroxidation inhibition. Collectively, these results suggest that APH can be used as an ingredient for functional food applications.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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