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Antioxidant Activities of Steamed Extract from Squid (*Todarodes pacificus*) Muscle

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Abstract

The purpose of this study was to purify antioxidant substances from steamed squid extract (SSE). The yield of SSE was 8% by dry weight. The approximate compositions of SSE proteins, lipids, moisture, carbohydrate and ash were 64.95%, 1.69%, 7.23%, 4.44% and 21.69%, respectively. The major amino acids in SSE were taurine (29.17%), glycine (20.33%), alanine (12.51%), and glutamic acid (9.83%). Antioxidant activities were evaluated using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, which was measured as 24.7% at 1.0 mg/mL. Four SSE fractions were isolated by Sephadex G-25 gel chromatography; the F2 fraction showed the highest DPPH radical scavenging activity. The F2 fraction was separated by reverse-phase high performance liquid chromatography (HPLC) using an octadecylsilane (ODS) column, yielding a purified antioxidant substance with a DPPH radical scavenging activity of 64.41% at 1.0 mg/mL, representing a 2.64-fold increase in the scavenging activity of SSE purified by the 3-step procedure. The amino acid compositions showed that purified SSE was rich in taurine, glycine, glutamic acid and alanine. The purified SSE significantly elevated 2′,7′-dichlorodihydrofluororescein diacetate (DCFH-DA) fluorescence probe, which confirms its effective radical scavenging potential in cellular ROS. In addition, the SSE significantly inhibited oxidative damage of purified genomic DNA. These results suggest that a purified antioxidant substance from SSE can be used as a potential natural compound-based antioxidant in the functional food and pharmaceutical industries.

Key words: antioxidant, taurine, squid steamed extract, DPPH radical scavenging, DCFH-DA

INTRODUCTION

The effects of oxidative stress and the alleviation of oxidative stress by antioxidants are topics currently being addressed by a wide variety of research endeavors. Oxidative stress can cause damage to all major components of cells, and the generation of excess reactive oxygen species (ROS) in tissues can lead to cell death. Moreover, ROS can directly and indirectly cause oxidation of biomolecules involved in many health disorders such as hypertension, inflammation, diabetes, cancer, and aging (1,2).

Antioxidative enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and some food-derived nutritional antioxidants, can protect tissues from free radical-mediated oxidative damage (3). In foods, free radical-mediated oxidation of fatty acids and lipids results in the development of rancid flavors and undesirable chemical compounds. Furthermore, the oxidation of food lipids leads to the deterioration of food quality and shortens shelf life (3). While synthetic antioxidants are commonly used in the food industry to overcome these problems, they are under strict regulation because of the potential health hazards associated with their use. Because some artificial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *t*-butylhydroquinone (TBHQ) pose potential health hazards, many studies have been initiated to identify and develop novel, safe and natural antioxidant compounds (4). For example, natural antioxidants such as vitamin C, α-tocopherol, and phenolic compounds in vegetables, fruits, and seeds can reduce oxidative damage associated with many diseases (4). In addition, there is a growing interest to identify antioxidative properties in dietary protein compounds, including fish products.

Annually, over 100 million metric tons of fish are harvested worldwide (5). Approximately 50% of the total catch is discarded as processing waste or as by-products of the waste stream, while the rapid growth of aquaculture industries leads to large volumes of waste containing high quality components that might be useful for human consumption (5). Despite international attempts to decrease waste through various kinds of waste treat-

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ment systems, the quantity of waste produced has been increasing annually (6). However, these waste by-products are important sources of biomaterials and minerals (7), and several recent studies have reported the recovery of valuable commodities from fish by-products (8,9).

Squid is the most representative fish species in the East Sea. In Korea, squid is a traditional food that is rich in calcium, essential amino acids, n-3 polyunsaturated fatty acids, and vitamins. In addition, squid tissue extracts have been extensively studied as a potential source of natural antioxidants, for example, extracts of squid pen (10), skin gelatin (11), and muscle (12).

In this study, we investigated the antioxidative activity of steamed squid extract (SSE) from squid muscle. Antioxidative substances were isolated by gel filtration and high performance liquid chromatography (HPLC), and antioxidative activities were determined. In addition, the protective effects of the antioxidants purified from SSE were investigated in the context of hydroxyl-induced DNA damage and the inhibitory effect on oxidative stress at the cellular level using 2′,7′-dichloro-dihydrofluororescein diacetate (DCFH-DA) was measured.

MATERIALS AND METHODS

Materials

The squid, *Todarodes pacificus* was purchased from Jumunjin Fish Market, Gangwon-do, Korea, and was immediately frozen and stored at -80°C. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Wako Chemical Co., Osaka, Japan. Acetonitrile (HPLC grade) was purchased from Fisher Chemical Co. (Rockford, IL, USA). All reagents used in this study were reagent grade compounds.

Preparation of SSE from squid muscle

Squid were washed twice with distilled water, viscera were removed, and the squid muscle was steam cooked (90°C) for 6 hr. Then, the SSE was centrifuged at $6,000\times g$ for 15 min at 4°C. The squid pellet was discarded, and the supernatant was collected and lyophilized. The yield was measured as the mass ratio of SSE to squid muscle of dry weight.

Analysis of approximate compositions

Approximate compositions of SSE were based on the method published in the 16th Edition of the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) (13). Moisture was determined by oven drying at 105±1°C. Crude lipid was measured in a Soxhlet system by extraction with diethyl ether solvent. Total nitrogen content was analyzed by the Kjeldahl procedure (Kjeltec 1030 Auto Analyzer, Tecator, Hoganas, Sweden). Crude protein content was calculated using a nitrogen : protein conversion factor of 1:6.25. Ash content was determined by incineration of samples at 600°C in a muffle furnace (F6000, Barnstead Thermolyne Co., Dubuque, IA, USA).

Analysis of amino acid compositions

For analysis of total amino acids, SSE was hydrolyzed in 6 N HCl for 24 hr at 110°C. Amino acids were analyzed using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) after pre-derivatization with *o*-phthaldialdehyde and β-mercaptoethanol. Separations were performed with a C_{18} column (5 μm, 4.6×250 mm, Waters Corporation, Milford, MA, USA). The amino acid concentrations of samples were calculated from calibration curves based on amino acid standard solutions (Sigma-Aldrich Co., St. Louis, MO, USA).

Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was estimated using the method of Yen and Hsieh (14) with slight modifications. The sample (40 μL) was mixed with 120 μL of methanol and then added to 40 μL of 0.15 mM DPPH in methanol. The mixture was allowed to stand at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using a spectrophotometer (JASCO, Tokyo, Japan). The control was analyzed in the same manner using distilled water instead of sample. DPPH radical scavenging activity was calculated as follows: $RSA\ (\%) = (A_{control} - A_{sample}) / A_{control} \times 100$, where RSA is the radical scavenging activity, A_{sample} is the absorbance of the sample, and $A_{control}$ is the absorbance of the control.

Purification of antoxidative substance from SSE

The potent fraction of SSE, as determined from the antioxidant activity assay, was further purified by size exclusion chromatography on a Sephadex G-25 gel filtration column (ø 25×750 mm; Sigma-Aldrich Co.) equilibrated with distilled water. Separated fractions were monitored at 215 nm, collected at a volume of 7.5 mL, and measured for antioxidant activity. The highest antioxidative fraction was then injected into a preparative reverse phase HPLC column (ø 10.0×250 mm, 5 μm; ODS-120A, YMC Co., Ltd., Kyoto, Japan) and separated using a linear gradient of acetonitrile (15～20% v/v) containing 0.1% trifluoroacetic acid on an HPLC system (Agilent 1100, Agilent Technologies).

Cell culture

Mouse macrophage cell lines (RAW 264.7) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO/Invitrogen, Grand Island, NY, USA) supplemented with 100 μg/mL penicillin-streptomycin, and

10% fetal bovine serum. The mouse macrophage cell line was cultivated at 37°C in a humidified incubator under 5% CO_2. Confluent cultures were washed twice with phosphate buffered saline (PBS) and then collected with a scraper. Collected cells were resuspended in medium and seeded onto cell culture well plates.

Cell viability assay

Cytotoxicity effects in RAW 264.7 cell lines were measured using the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method as described by Hansen et al. (15) with slight modifications. RAW 264.7 cells were cultured in 96-well plates at a density of 1×10^5 cells/well. After 24 hr, cells were washed with fresh medium and treated with various concentrations (125, 250, 500, 1000, 2000 μg/mL) of sample. After incubation for 24 hr, cells were washed 2 times with PBS, and subsequently 100 μL of MTT solution (1 mg/mL) was added to each well for 3 hr. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formed formazan salt. The amount of formazan salt was determined by measuring the optical density (OD) at 540 nm using a UV microplate reader (Tecan Austria GmbH, Grödig/Salzburg, Austria). Relative cell viability (%) was calculated compared to the non-treated cell samples: $100\times$(OD of non-treated samples−OD of treated samples)/ OD of non-treated samples. The data were expressed as means $\pm$SD of at least 3 independent experiments.

Measurement of intracellular ROS-scavenging activities using DCFH-DA labeling

Intracellular formation of ROS was assessed according to a method described by employing oxidation sensitive dye DCFH-DA, as the substrate. RAW 264.7 cells were grown in 96-well black plates and were labeled with 20 μM DCFH-DA in Hanks balanced salt solution (HBSS) and kept for 20 min in the dark. The non-fluorescent DCFH-DA dye which easily penetrates into cells was then hydrolyzed by intracellular esterase to 2′,7′-dichlorodihydrofluorescein (DCFH), and trapped inside the cells. Cells were then treated with different concentrations (125, 250, 500, 1000, 2000 μg/mL) of test samples and incubated for 1 hr. After washing cells three times with PBS, 500 μM H_2O_2 (in HBSS) was added. The formation of fluorescent dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of several ROS was read after every 30 min at the excitation wavelength of 485 nm and the emission wavelength of 528 nm using a GENios® fluorescence microplate reader (Tecan Austria GmbH). Dose-dependent and time-dependent effects of treatments were plotted and compared with fluorescence intensity of the control group in which samples were not treated.

Protective effects of induced-DNA damage by hydroxyl radical

To evaluate the protective effects of purified antioxidant substance against DNA damage caused by hydroxyl radicals (16), a reaction was induced by placing the following reagents in an Eppendorf tube: 5 μL genomic DNA (obtained from a RAW 264.7 cell line), 2.0 mM $FeSO_4$, and various concentrations (125, 250, 500, 1000, 2000 μg/mL) of purified antioxidant substance. The mixture was incubated at 37°C for 30 min, followed by the addition of 4.0 μL of 10 mM H_2O_2. Next, the mixture was subjected to 1.0% agarose gel electrophoresis, after which the DNA bands were stained with ethidium bromide.

RESULTS AND DISCUSSION

Approximate compositions of SSE

Approximate compositions of SSE are presented in Table 1. The crude protein content was 64.95%, while lipid, moisture, carbohydrate and ash contents were 1.69%, 7.23%, 4.44% and 21.69%, respectively. SSE contains relatively high levels of protein, although not as high as most teleost fishes (17). The amino acid compositions of SSE were as follows: taurine (29.17%), glutamic acid (9.83%), aspartic acid (6.03%), glycine (20.33 %), and alanine (12.51%) (Table 2). Hossain et al. (17) reported similar amino acid compositions for Japanese flying squid and swordtip squid: glutamic acid, glycine, alanine, leucine, and proline. However, taurine is present in the highest concentrations in mollusks, and is soluble in water (18,19). There are many reports on the antioxidant effects of taurine. Taurine is reported to protect cells and tissues from oxidant-induced damage in a variety of models involving inflammation as a pathogenic feature (20). In addition, taurine functions to lower cholesterol levels, enhance immune system function and detoxify tissues (21). In this study, the taurine content of SSE was higher than other amino acids. Therefore, we believe that the higher taurine content (29.17%) of SSE will affect the antioxidant activity.

Table 1. Proximate composition of SSE

Compositions	Contents (%)
Ash	21.69
Protein	64.95
Lipid	1.69
Moisture	7.23
Carbohydrate	4.44
Total	100.00

Table 2. Amino acid compositions of SSE

Amino acids	Contents (%)
Tau	29.17
Glu	9.83
Gly	20.33
Ala	12.61
Cys	0.12
Val	1.70
Met	1.36
Ile	1.30
Leu	3.66
Tyr	0.95
Phe	1.30
Lys	3.69
His	2.47
Arg	6.03
Pro	5.48

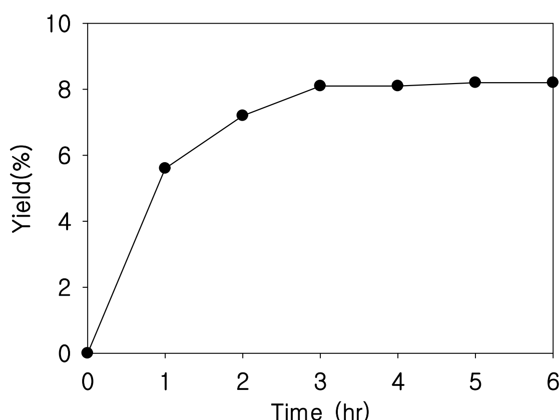

Fig. 1. The yield of squid steamed extract (SSE) from squid muscle during boiling. The boiling was conducted at 90°C for 6 hr.

The yield and antioxidant activity of SSE

SSE extracts were produced by steam cooking for 6 hr. The yield was highest after three hours of steam treatment (Fig. 1), with a yield of 8%, and the extraction was not influenced by antioxidant activity at or beyond this time point. Therefore, the optimum extraction time was three hours. SSE antioxidant activity was evaluated using a DPPH radical, which is a potent free radical-scavenging molecule. The DPPH radical is one of the few stable radical sources and is widely used to test the free radical-scavenging ability of various samples (22, 23). The highest antioxidative activity (24.7% at 1.0 mg/mL) was observed three hours after introducing DPPH to the sample (Fig. 2). The results of these investigations suggest that the antioxidative activity of SSE was not influenced after this period of time. Previous reports have demonstrated antioxidant activities from squid products. Nam et al. (24) found radical scavenging ability in squid collagen. Similarly, Lin and Li (25) reported that hydrolysates from jumbo flying squid skin gelatin were able to scavenge hydroxyl and superoxide radicals. The antioxidant activity of squid gelatin extract is 49.3% (11) and the initial DPPH scavenging activity of tuna cooking juice is approximately 18% (26). The antioxidant activity of SSE appears to be greater than tuna cooking juice, but less than squid gelatin extract, suggesting that SSE contains some antioxidative substance.

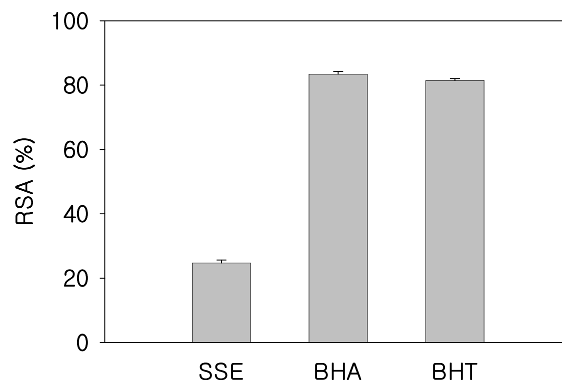

Fig. 2. DPPH radical scavenging activity of SSE. Radical scavenging activity was measured at a concentration of 1.0 mg/mL. BHA and BHT were measured at concentration of 0.1 mg/mL.

Purification of antioxidative substance from SSE

The antioxidative effect of SSE, determined by DPPH radical scavenging activity, was determined using purified samples. Initially, the SSE was dissolved in distilled water and fractionated using a Sephadex G-25 open column chromatograph. Sephadex G-25 gel chromatography allows the separation of substances with different molecular weights, and has been used for separating low-molecular-weight substances. Pihlanto-Leppala (27) reported that molecular sizes tend to be smaller in bioactive compounds. Purified antioxidant extract was fractionated according to molecular weights, where F1 and F2 represent larger molecular weight compounds and fractions F3 and F4 represent smaller molecular weight compounds. Our results indicated that DPPH radical scavenging activity was highest for fraction F2 compounds (Fig. 3). Fraction F2 obtained from the Sephadex G-25 column was separated by reverse-phase HPLC using an ODS column and fractionated into 3 parts (F2-1, F2-2, and F2-3 (Fig. 4); the purified F2-3 fraction possessed the highest antioxidant activity, with a DPPH radical scavenging activity of 64.41% at 1.0 mg/mL (Fig. 5). Thus, the antioxidant activity of SSE purified by a 3-step purification procedure was increased 2.64-fold relative to unpurified SSE.

Amino acid composition of purified antioxidant substance

Amino acid sequencing of purified antioxidant sub-

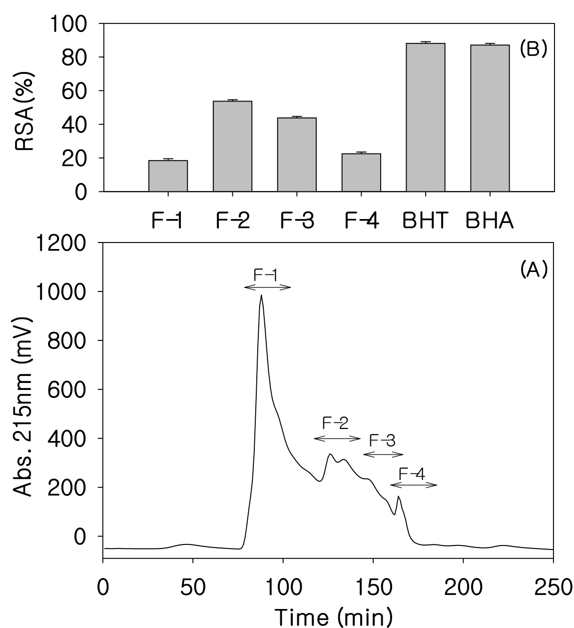

Fig. 3. Sephadex G-25 Gel filtration chromatogram of the squid steamed extract. Separation was performed with 1.5 mL/min and collected at a fraction volume of 7.5 mL (A). The fractions isolated by Sephadex G-25 Gel column were separated (F1～F4) and antioxidant activity determined as upper panel (B).

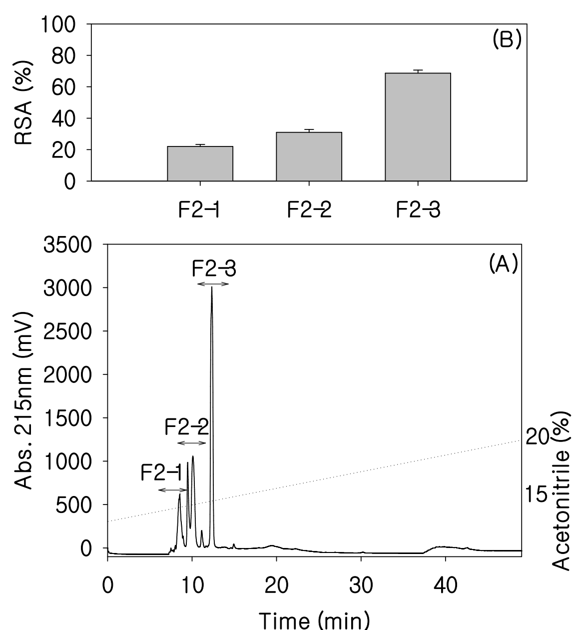

Fig. 4. HPLC chromatogram of potent antioxidant activity F2 isolated from Sephadex G-25. Separation was performed with linear gradient of acetonitrile from 15% to 20% in 50 min at a flow rate of 1.5 mL/min and YMC-Pack ODS-A 120A column (5 μm, 10×250 mm) (A). Elution was monitored at 215 nm. The fractions showing antioxidant activity were designated F2-1～F2-3 and antioxidant activity determined as upper panel (B).

stance using quantitative time-of-flight mass spectrometry (Q-TOF MS) analysis did not reveal information

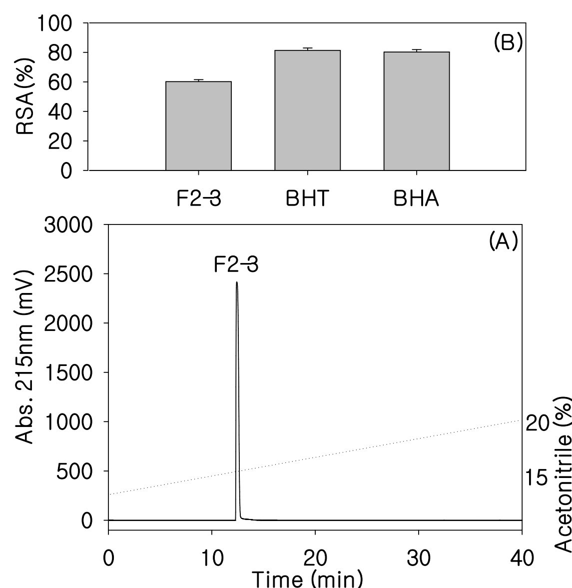

Fig. 5. HPLC chromatogram of potent antioxidant activity F2-3 isolated from Fig. 4. Separation was performed with linear gradient of acetonitrile from 15% to 20% in 50 min at a flow rate of 1.5 mL/min and YMC-Pack ODS-120A column (5 μm, 10×250 mm) (A). Elution was monitored at 215 nm. The fractions showing antioxidant activity were designated F2-3 and antioxidant activity determined as upper panel (B).

about amino acid contents. However, proximate amino acid compositions of purified and unpurified SSE were compared in Fig. 6. Taurine content increased 1.5 times in purified antioxidant substance and accounted for 57% of the total amino acid composition. Other dominant amino acids present in the purified antioxidant substance were glutamic acid, glycine, and alanine, all of which are standard amino acids. Generally, taurine acts as an antioxidant and protects against toxicity of various substances, such as lead and cadmium (28,29). Taurine supplements have also been shown to prevent oxidative stress induced by exercise (30). Therefore, the high taurine content of purified antioxidant substance is consid-

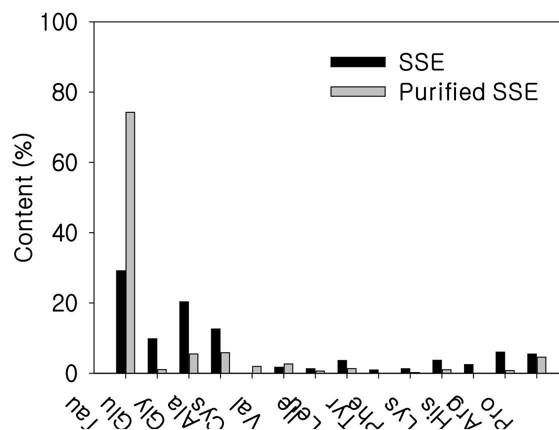

Fig. 6. Amino acid compositions of SSE and purified SSE.

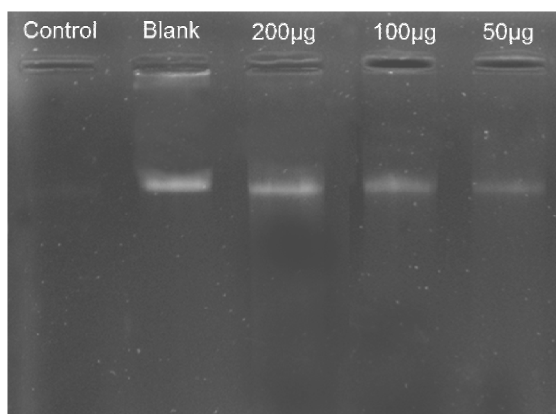

Fig. 7. Protective effect of purified SSE on hydroxyl radicals induced oxidation of genomic DNA. Blank: untreated sample and H_2O_2, Control: distilled water instead of sample.

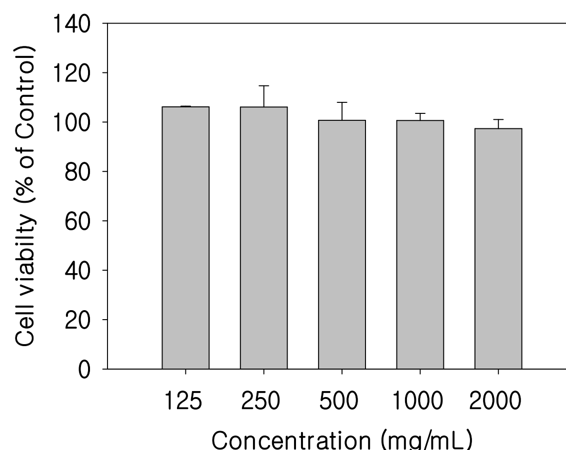

Fig. 8. Cytotoxic effect of purified antioxidant substrate from SSE on cell viability in RAW 246.7 macrophage.

ered to contribute to the high radical scavenging activity of SSE antioxidants.

Prevention of the purified antioxidant substance on oxidation-induced DNA damage

We evaluated the protective activity of purified antioxidant substance against hydroxyl radical-induced DNA damage in *in vitro* studies using RAW 264.7 cell line. Fig. 7 shows that increasing the purified antioxidant substance concentration from 50 to 200 μM decreased hydroxyl radical-induced DNA damage. In our experiments, DNA was exposed to OH radical generated by catalysis of H_2O_2 by Fe^{2+}. Catalysis of H_2O_2 by Fe^{2+} is an important pathway for OH radical production in physical systems. OH radical is highly reactive with all components of the DNA molecule, for example, it causes damage to purine and pyrimidine bases and creates lesions in the deoxyribose backbone. DNA is a sensitive bio-target for ROS-mediated oxidative damage (31). DNA damage by ROS is known to initiate carcinogenesis and contribute to pathogenesis of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. Among ROS, the hydroxyl radical is recognized as a significant physiological DNA-damaging agent (32).

Effect of purified antioxidant substance on the viability of RAW 264.7 cells

One of the aims of this study was to investigate possible cytotoxic effects of the SSE purified antioxidant substance on RAW 264.7 cell lines. Cell viability data confirmed that the purified antioxidant substance was non-cytotoxic to RAW 264.7 cells (Fig. 8). Interestingly, the purified antioxidant substance exerted no cytotoxic effects on normal cells, even at the highest test concentrations (2,000 μg/mL) (Fig. 8). Therefore, concentrations of purified antioxidant substance ranging from 125 μg/mL to 2,000 μg/mL were selected for antioxidant activity assays.

ROS determination of the purified antioxidant substance using DCFH-DA assay

The scavenging effects of purified antioxidant substance on cellular radicals were studied in order to confirm their ability to scavenge free radicals in a cellular environment. RAW 264.7 cells were labeled with DCFH-DA fluorescence probe. DCFH-DA is a specific probe for ROS. The principle of this assay is that DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by esterase to become DCFH, which reacts with ROS to form DCF, a fluorescent product. When cells are labeled with DCFH-DA and incubated with H_2O_2 for 30 min, DCF fluorescence intensity indicates oxidation of DCFH by intracellular radicals (Fig. 9). Monitoring of DCF fluorescence intensity every 30 min for 3 hr indicated that radical-mediated oxidation increased with incubation time. However, pre-treatment of cells with purified SSE antioxidant substance decreased DCF fluorescence in a time-dependent manner. Purified antioxidant substance at varying concentrations (125, 250, 500, 1,000, and 2,000 μg/mL) exhibited free radical scavenging effects after only 30 min of incubation, compared with controls (H_2O_2 treatment without sample) (Fig. 9). Our results showed that antioxidant activity varies with the concentration of purified antioxidant substance, and that the free radical scavenging effect of the purified antioxidant substance at 2,000 μg/mL was higher than other tested concentrations. We observed that RAW 264.7 cells exposed to antioxidant substance resulted in enhanced DCFH oxidation, which is essentially dependent on reactive species (33). Therefore, we confirmed that the purified antioxidant substance scavenged free radicals in a dose- and time-dependent manner in RAW 264.7 cells.

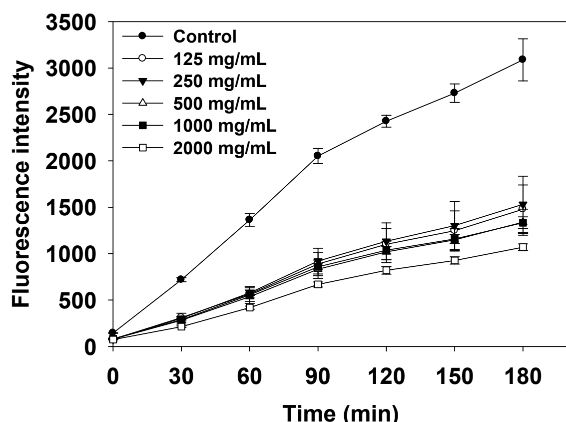

Fig. 9. The intracellular ROS generation level of cells was measured by DCFH-DA to detect produced H_2O_2. RAW 264.7 cells were treated with 20 nM/mL DCFH-DA, and then cells were treated with different concentrations of purified SSE for 1 hr. Results are presented as means ± standard error of three independent experiments.

CONCLUSION

Squid is the most abundant fish species in the East Sea, and waste products from squid processing, particularly waste water, are causing environmental pollution. The bioactivity of substances isolated from these waste products can be applied to high-value industries such as food and nutraceutical applications. In this study, SSE was purified using chromatography, and the purified antioxidant substance exhibited high antioxidant activities in stress-induced cells. We suggest that SSE could be used as a natural antioxidant, enhancing the antioxidant properties of food additives and preventing oxidation reactions in food processing. In addition, it is expected that SSE antioxidant activity will contribute to an interest in basic research in the potential applications of fish processing wastes.

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