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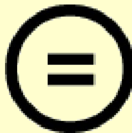

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Thesis for the Degree of Master of Science

Sea squirt (*Halocynthia roretzi*) hydrolysates: Preparation, antioxidant, and anti-cancer effects

by

Sam Sun Kim

Department of Marine-Bio Convergence Science

Specialized Graduate School Science & Technology Convergence

Pukyong National University

February 2018

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멍게(*Halocynthia roretzi*) 가수분해물의 항산화 및 항암 효과

Advisor: Prof. Jae-Young Je

by

Sam Sun Kim

A thesis submitted in partial fulfillment of the requirements

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Specialized Graduate School Science & Technology Convergence

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Sea squirt (*Halocynthia roretzi*) hydrolysates

: Preparation, antioxidant, and anti-cancer effects

A dissertation

by

Sam Sun Kim

Approved by:

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(Chairman) Prof. Won-Kyo Jung

______________________________ ______________________________

(Member) Prof. Yunok Oh (Member) Prof. Jae-Young Je

February 23, 2018

PUKYONG NATIONAL UNIVERSITY
부경대학교

Sea squirt (*Halocynthia roretzi*) hydrolysates
: Preparation, antioxidant, and anti-cancer effects

Sam Sun Kim

Department of Marine-Bio Convergence Science

Specialized Graduate School Science & Technology Convergence

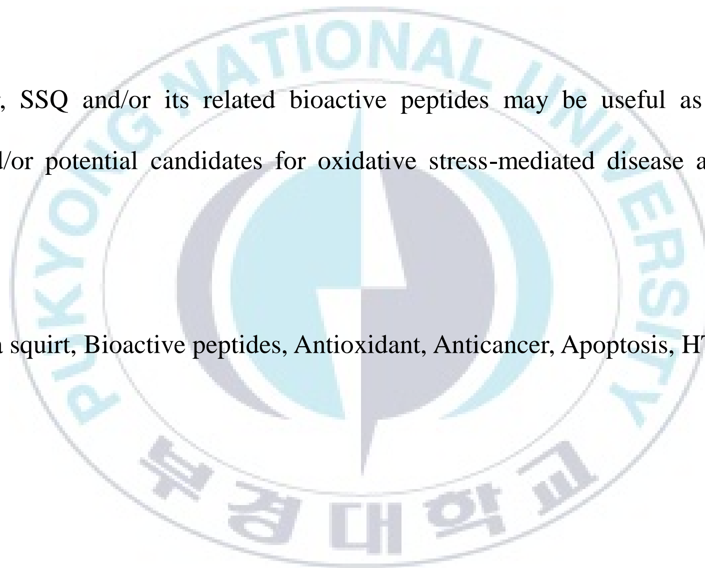
Pukyong National University

Abstract

Sea squirt is widely used as a food material in Korea. The flesh tissue of sea squirt contains about 65 % protein, and it could be a good candidate for antioxidant and anticancer materials. In this study, sea squirt protein hydrolysates were prepared by pepsin hydrolysis at pH 2 and 37°C. Sea squirt protein hydrolysates were separated to purify antioxidant peptides using different chromato - graphic techniques, including gelfiltration chromatography and reverse phase-high performance liquid chromatography. Three antioxidant peptide of Met-Thr-Thr-Leu (P1, 464.58 Da), Leu-Glu-Trp (P2, 446.50 Da) and Tyr-Tyr-Pro-Tyr-Gln-Leu (P3, 845.95 Da) were identifed and the antioxidant activities of three antioxidant peptides were evaluated by DPPH radical scavenging, $ABTS^+$ radical scavenging, Fe^{2+} chelating activity, ORAC and reducing power. All antioxidant peptides exhibited comparable ORAC values than that of glutathione as a positive control and

showed significantly higher Fe^{2+} chelating activity than glutathione. Among three peptides, P2 exhibited higher antioxidant acitivies than P1 and P3. Additionally, anticancer effect of peptic hydrolysates of sea squirts (SSQ) was evaluated on HT-29 human colon cancer cell line. SSQ inhibited cell viability and induced apoptosis in HT-29 ells via intracellular reactive oxygen species (ROS) production. In response to oxidative DNA damage, flow cytometry analysis was carried out for analysis of cell cycle phase transtions by SSQ and the result showed that treatment with SSQ induced G2/M phase arrest in HT-29 cells in a dose-dependent manner. Western blot and real-time PCR analysis showed that treatment with SSQ increased apoptotic caspase-2 expression in HT-29 cells.

Taken together, SSQ and/or its related bioactive peptides may be useful as functional food ingredients and/or potential candidates for oxidative stress-mediated disease and colon cancer treatment.



Keywords: Sea squirt, Bioactive peptides, Antioxidant, Anticancer, Apoptosis, HT-29

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List of abbreviations

CAT (catalase)

CRC (colorectal cancer)

dSSQ (peptic hydrolysate of defatted sea squirts)

DMEM (Dulbecco's modified eagle's medium)

DPPH (2, 2-diphenyl-1-picrylhydrazyl)

GSHPx (glutathione peroxidase)

HT-29 (human colon cancer cell line)

MTT (3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenil tetrazolium bromide)

ROS (reactive oxygen species)

RNS (reactive nitrogen species)

SOD (superoxide dismutase)

SSQ (peptic hydrolysate of sea squirts)

Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid)

Ⅰ. Introduction

Marine organisms have been captivated more and more scientific attention due to various biological activities. Considering their diversity, study of marine organism-derived bioactive compounds is almost unlimited. They live photic or non-photic zones under a wide pressure range from 1 atm to 1,000 atm, and they are exposed to high salinity as well [1, 2]. Even though some marine organisms live in a high oxygen area that lead to generating free radicals, they do not suffer from any serious problems in vivo. The harsh circumstances render them special [3, 4]. They synthesize the ingredients beneficial for human health, which cannot be found in terrestrial organisms. More than 5,000 novel compounds have been detected from marine organisms. For example, trabectidin has been isolated from the Caribbean marine tunicate, *Ecteinascidia turbinate*. It has anticancer effects in breast and prostate and has been approved for use as an anticancer agent in Europe [1].

More than 2,000 species of sea squirts have been reported all over the world [5, 6]. *Halocynthia roretzi*, *Halocynthia aurantium* and *Pyura vittata* are sea squirts inhabiting in the eastern and southern coasts of Korea [7]. Interestingly, protein content is 10 % (w/b) or more not only in cultured sea squirts but also in wild sea squirts on a wet basis. It varies with the season. It is maximum from June until October, while it is minimum between

December and February. In addition, the moisture content of sea squirts is the reverse of protein content of them. Moreover, sea squirts have various and considerable amount of amino acids, especially aspartic acid (Asp), glutamic acid (Glu), and lysine (Lys) [8]. Sea squirts have many components improving human health like other marine organisms: Antioxidant activity in methanol extract of the sea squirt, *Halocynthia roretzi*, has been reported [5] and the powder of edible part of the sea squirt, *Halocynthia roretzi* and *Halocynthia aurantium*, also has been reported as antioxidant [6]; anti-inflammatory activity of polysaccharide derived from the sea squirt, *Ascidiella aspersa*, has been indicated in vivo and in vitro [9]; immune - activity of the sea squirt, *Halocynthia roretzi*, tunic extracts has been well known [10, 11]; and anti-lipase activity has been reported in extracts of the sea squirt, *Halocynthia roretzi*, using ethanol [5]. However, there are no experimental study of antioxidant activity using sea squirts-derived peptic hydrolysate to the best of my knowledge.

Antioxidant effect is one of the most globally interested issues. Over the last decades, there were many studies of antioxidant and reactive species in the cosmetic business, medical research, and foods industry. Reactive species is a molecule with high reactivity, which contributes to the harmful oxidative stress. It consists of ROS (reactive oxygen species) and RNS (reactive nitrogen species) and includes radicals and non-radicals. A free radical has an unpaired electron, which makes it react with another molecule to stabilize [12-14]. In fact, cells produce ROS or RNS in physiological conditions, and

natural antioxidants convert these species into stable molecules by the biological defense system in human body [15, 16]. For instance, NADPH (nicotinamide adenine dinucleotide phosphate's reduced form) is oxidized by oxidase creating $O_2^{\cdot -}$ to produce energy in cells. SOD (superoxide dismutase) changes $O_2^{\cdot -}$ into H_2O_2 that is less damaging, but is still one of ROS, and CAT (catalase) and GSHPx convert H_2O_2 into the water and something safe [14, 17]. Generation of radicals is counterbalanced by eliminating radicals naturally, however, when the ratio of production of radicals to removal of radicals increase significantly, this causes damage to proteins, lipids, and DNA [3, 13, 14] resulting in diverse diseases (e.g. cancer, diabetes, liver diseases and cardiovascular diseases) [13, 17-19]. The rapid increase of radicals may be caused by exogenous factors such as: toxin exposure like heavy metals, lead, and cadmium; life style-related factor like alcohol, strenuous work, cigarette smoke, and excessive exercise; trauma; radiation; stress, cold; allergen; and various disease states, [13, 14]. Antioxidants can be classified according to their activity, solubility, and size, and natural antioxidants such as SOD, CAT, or GSHPx (glutathione peroxidase) are enzymatic large-molecule antioxidants and they modulate the volume of free radicals physiologically [14, 17]. Antioxidants can remove free radicals by transferring hydrogen atom or electron. Antioxidants can also inhibit formation of free radicals by chelating metal ions and retard lipid peroxidation by disturbing radical chain reactions [14, 20, 21]. Lipid peroxidation, oxidative degradation of lipid, causes the loss of the structure and function of cell membranes by removing

electrons of lipid in the cell membranes [12, 22]. It is important to prevent cells particularly polyunsaturated fatty acids (PUFA) in cell membranes from being attacked by free radicals because the damage of PUFA trigger a self-perpetuating chain reaction [17]. It has been reported that antioxidants protect cells from a number of diseases such as cancer, aging, stroke, allergies, asthma, diabetes, arthritis, eczema, cataract, or genetic disorders [14, 23]. Interestingly, the raised production of the radicals is caused by the high concentration of piperine (1-piperoylpiperidine), a well-known natural antioxidant. Whereas, antioxidant effect is shown in low concentration of it [17]. Some phaeophyta-derived crude extracts have not only strong antioxidant activity, but also antitumoral activity [24].

Another area is anticancer peptides. Anticancer has become an important issue in the world. According to the WHO, cancer was globally a leading cause of death [25] accounting for 8.8 million deaths in 2015. Colorectal cancer (CRC) occupied almost 10 % of all tumors. It was the most common cancer besides lung and prostate cancers in men in developed countries [26]. Almost 1.2 million new cases of CRC were reported, responsible for 600,000 deaths in a year [27]. CRC showed 55 % of 5-year survival. However, it can be possible to increase 5-year survival by early detection [25, 28]. Western countries such as Europe or North America are areas with a high incidence rate of cancer, on the other hand, the countries of Asia or Africa are areas with a low incidence rate of cancer. Although the rate is commonly lower in developing countries than that in developed countries,

there's a drift towards increasing rate in developing countries [27]. Unlike lung cancer has a single risk factor, CRC is caused by complex factors. Many studies reported the risk factors are not only non-modifiable factors (e.g. aging, male sex, family history, genetic mutation, inflammatory bowel, and acromegaly) but also modifiable factors (e.g. obesity, smoking, diet, physical activity and high consumption of alcohol and meat) [27-29]. There are already many available anticancer agents using apoptosis-inducers that control pro- and anti-apoptosis proteins and regulate apoptosis-related gene expression. However, the current agents preventing cancer may kill both normal and cancer cells and show adverse effects like anemias, infections, and nausea [30] developing drug resistance [31]. Amusingly, an anticancer effect is closely related to some antioxidants that balance cell proliferation and apoptosis in colorectal cells. It has been reported that vitamin D reduces the incidence rate of colorectal cancer by 26 % [32]. Another study has also shown that flavonoids, in particular resveratrol exhibit the chemo-preventive role in colorectal cancer, although further studies are needed to confirm the optimal dosage [15]. Many studies have reported anticancer effects by substance derived from natural products including *Tualang* honey, maslinic acid, saponins, and chrysin rich *Scutellaria discolor* Colebr [26, 30, 31, 33, 34] It has been reported that halocynthiaxanthin and fucoxanthinol, which are carotenoids, isolated from the sea squirt, *Halocynthia roretzi*, exhibit the ability to induce apoptosis on HL-60, MCF-7 and Caco-2 cells [35]. However, there are few studies of

anticancer effect by sea squirts-derived peptic hydrolysate in HT-29 colon cancer cell line to the best of my knowledge.

Therefore, the purpose of this study is to inform the antioxidant properties of sea squirts-derived peptic hydrolysate by investigating radical scavenging effect after purification and identification. This study also aims at examining the anticancer effect in HT-29 colon cancer cell line by peptic hydrolysate of sea squirts.

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Ⅱ. Materials and Methods

1. Materials

Sea squirts were kindly supplied by National Fisheries Research and Development Institute (Tongyeong, Korea). DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum) and Penicillin streptomycin were purchased from Gibco BRL Co. (Grand Island, NY, USA). MTT (3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenil tetrazolium bromide), DPPH (2, 2-diphenyl-1-picrylhydrazyl), Potassium persulfate, Fluorescein, Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid), ABTS (2, 2'- azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) diammonium salt), TFA (trifluoroacetic acid), AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride), Hoechst 33342, PI (Propidium Iodide) and DCFH-DA (2'7'-dichlorofluorescin diacetate) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). PBS (Phosphate–Buffered Saline) and HBSS (Dulbecco's Phosphate- Hank's Balanced Salts) were purchased from Hyclone (UT, USA). Ethanol and DMSO (dimethyl sulfoxide) were purchased from Junsei Chemical Co. (Tokyo, Japan). Reduced glutathione (GSH) and total antibodies (caspase-3 and β-actin) were purchased from Tokyo Chemical Industry. Co. (Tokyo, Japan) and Santa Cruz Biotechnology (Texas, TX, USA), respectively. The complementary DNA synthesis kit

(ET21025) and QuantiSpeed SYBR No-Rox kit (QS105-05) were obtained from PhileKorea (Seoul, Korea). Pierce® BCA protein assay kit, Trypan Blue Stain 0.4 % and TRIzol reagent were obtained from Thermo scientific Co. (Waltham, MA, USA). All commercial chemicals were used without further purification.

2. Preparation of peptic hydrolysates from sea squirts

The preparation of peptic hydrolysates was conducted according to the method described by Silva *et al*., (2017) after slight modifications [36]. The edible portion of sea quirts was washed with tap water and freeze-dried. The lyophilized sea squirt was defatted with hexane in a ratio of 1:5 (w/v). stirring for 2 h at room temperature. For protein extraction, the defatted sea squirt was suspended in distilled water in a ratio of 1:10 (w/v) followed by adjusting pH 9 with 1 N NaOH. After 2 h stirring at room temperature, the soluble proteins were recovered by centrifugation at 4,000 rpm for 30 min (1248R, Labogene, Seoul, Korea). The supernatant was transferred to other tubes followed by precipitation using 6 N HCl. After centrifugation at 10,000 rpm for 30 min, the precipitates were freeze-dried and suspended in distilled water for 10% (w/v) and pepsin hydrolysis was performed at enzyme to substrate ratio of 1:100 at pH 2 and 37 ℃ for 2 h. Thereafter, pepsin was inactivated by boiling the reaction mixture for 10 min. The supernatants were

recovered by centrifugation at 10,000 rpm for 30 min, lyophilized, and stored at -20 °C until use. This study abbreviates peptic hydrolysate of defatted sea squirts to dSSQ.

To generate peptic hydrolysates of sea squirts the edible portions of sea quirts were prepared as mentioned above. The powder of sea squirts was hydrolyzed with pepsin according to the same condition of preparing dSSQ. SSQ means peptic hydrolysate of sea squirts without the process of defatting for further experimentation with anticancer effect.

3. Purification and identification of antioxidant peptides

Sea squirt protein hydrolysates was fractionated by gelfiltration chromatography (BioBasic SEC-60, 300 × 7.8 mm, 5 µm, Thermo Scientific, PA, USA) equilibrated with distilled water containing 0.05 % Trifluoroacetic acid at a flow rate of 1.5 ml/min for 15 min, and eluate was read at 280 nm. The fraction with the highest antioxidant activity was selected and further separated by RP-HPLC (reverse phase-high performance liquid chromatography) equipped with C18 column (Hypersil Gold, 250 × 4.6 mm, 5 µm, Thermo Scientific) with a linear gradient of acetonitrile (0-80 % in 17 min, 100 % in 17-20 min, and 0 % in 20-25 min) containing 0.05 % TFA at a flow rate of 1.2 ml/min. The elution peaks were detected at 215 nm. To further purification, the preferred fraction was separated using GPC (gel permeation chromatography) on a Superdex™ peptide 10/300 GL high performance column (300 mm × 10 mm, 13 µm, GE Healthcare, Buckinghamshire, UK)

with distilled water containing 0.05 % TFA for 60 min. Flow rate was 0.7 ml/min and eluate was monitored at 215 nm. Finally, the Hypersil Gold C_{18} column was used for divide the fraction had higher antioxidant effect and more much proteins. Flow rate was fixed at 1.2 ml/ml while peaks were monitored at 215 nm, and elution was performed in optimal conditions (0-12 min: 0-60 % acetonitrile, 12-14 min: 100 % acetonitrile, and 14-19 min: 0 % acetonitrile).

An ultra high resolution Q-TOF (quadrupole time-of-flight) LC-MS/MS (liquid chromatography-tandem mass spectrometry) coupled with ESI (electrospray ionization) source (maXis-HD^{TM}, Bruker Daltonics, Bremen, Germany) was used for identification of antioxidant peptides. A 10 µl of peptide was injected and eluted onto a a Acclaim RSLC 120 C_{18} column (2.1 × 100 mm, 2.2 µm, Dionex) at a flow rate of 200 µl/min. Mobile phase A and B consisted of 0.2 % formic acid in water and 0.2 % formic acid in acetonitrile. Gradient elution was performed as: 0-5 min, 5 % B; 5-7 min, 5-10 % B; 7-48 min, 10-30 % B; 48-55 min, 30-50 % B; 55-65 min, 95 % B; 65-66 min, 95-5 % B; 66-71 min, 5 % B. The sequences of antioxidant peptides were evaluated by *De novo* peptide sequencing by MS/MS spectrometry and the Biotools 3.2 software (Bruker Dantonics, Germany).

Three identified antioxidant peptides were synthesized by Fmoc solid-phase peptide synthesis with a PSI 300 synthesizer (NY, USA) from BioStem (Ansan, Korea), and MS of the antioxidant peptides were verified by LC/MS (Shimadzu LC-MS2020, Kyoto, Japan). The purities were over 95%.The synthetic peptides were stored at -20 °C until use.

4. Determinatio of antioxidant activities

4-1. DPPH radical scavenging assay

Scavenging activity of the hydrolysates or peptides was evaluated according to the method of Umayaparvathi et al. (2014) with some modifications [18]. Briefly, a 70 μl of DPPH (0.15 mM) dissolved in methanol was added to sample or distilled water (as blank) in the ratio of 1:1 (v/v) and incubated for 30 min at room temperature in the dark. The absorbance at 540 nm of the mixture was measured by GENios® microplate reader (Tecan Austria GmbH, Austria), and the scavenging effect was calculated using the equation:

$$\% \text{ Scavenging effect} = (1 - A_{sample}/A_{blank}) \times 100$$

Where, A_{blank} is the absorbance of the blank (without dSSQ).

4-2. Oxygen Radical Absorbance Capacity (ORAC)

ORAC of the hydrolysates or peptides was measured according to the published report with a slight modification [37]. The 50 μl of sample (50 μM) was mixed with 50 μl of fluorescein (78 nM, pH 7.0) followed by incubation at 37°C for 15 min and then a 25 μl of AAPH (221 mM, pH 7.0) was added into the reaction mixture as a free radical generator just before measuring the oxidative degradation of the fluorescein. Fluorescence was measured every 5 min for 60 min (Ex 485 nm and Em 582 nm). ORAC was expressed

as μM TE/mg sample or mM peptide. AUC values could be calculated by the following formula:

$$AUC = 0.5 + (F_5/F_0) + (F_{10}/F_0) + \cdots + 0.5 \times (F_{60}/F_0)$$

Net AUC was determined by subtracting the AUC for no compound addition from the other AUC values.

$$\text{Net AUC} = \text{AUC of sample} - \text{AUC of blank}$$

where, all chemical agents were dissolved in sodium phosphate buffer (75 mM, pH 7.0), AUC means an area under the curve, F_0 is an initial fluorescence reading at 0 min, F_n is fluorescence reading at time, and ORAC value was expressed as μM trolox equivalent (TE)/mg sample or mM peptide using standard curve of trolox (1-100 μM).

4-3. $ABTS^+$ radical scavenging assay

Scavenging activity of the hydrolysates or peptides was evaluated according to the published report [38]. ABTS stock solution (7 mM) stayed overnight was mixed with potassium persulfate (2.4 mM) in the ratio of 1:1 (v/v), and the absorbance at 410 nm of ABTS working solution was adjusted to 1.5±0.05 just prior to use. The 150 μl of working solution as $ABTS^+$ radical was added into 50 μl of sample (50 μM), and then the absorbance of the mixture was measured at 410 nm after incubating for 10 min at room temperature. $ABTS^+$ radical scavenging activity was expressed as μM TE/50 μM sample or 50 μM peptide.

4-4. Reducing power assay

The method by Umayaparvathi *et al*. (2014) was used with some modifications to assess reducing power of antioxidant peptides [18]. A 50 μl of peptide (10 mM) was mixed with 50 μl of sodium phosphate buffer (0.2 M, pH 6.6) and 50 μl of 1 % (w/v) potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min followed by addition of 50 μl of 10 % trichloroacetic acid. The 200 μl of the reactant was mixed with 200 μl of distilled water and 40 μl of 0.1 % (w/v) ferric chloride ($FeCl_3$), vortexed gently, and incubated for 10 min at room temperature. The absorbance was obtained at 750 nm.

4-5. Fe^{2+} chelating activity

According to the report described by Aktumsek *et al*. (2013), the chelating activity of peptides was assessed with slight modifications [39]. Briefly, 100 μl of antioxidant peptide (1 mM) was mixed with 20 μl of $FeCl_2$ (2 mM) and added to 40 μl of ferrozine (5 mM). The Absorbance of mixture was monitored at 570 nm after incubation for 10 min at room temperature in the dark. Chelating activity was calculated using the equation:

$$\%\ \text{Metal chelating activity} = (1 - A_{sample}/A_{blank}) \times 100$$

Where, A_{blank} is the absorbance of control reaction (without dSSQ), and A_{sample} is the absorbance in the presence of dSSQ.

5. Cell culture

The human colon cancer cell line HT-29 (KCLB No. 30038) was obtained from Korea Cell Line Bank (Seoul, Korea). The HT-29 cells were cultured in DMEM (Dulbecco's modified eagle's medium) containing 10 % FBS (fetal bovine serum) and 1 % penicillin–streptomycin at 37 °C in a 5 % CO_2 humidified incubator. Cell growth medium was replaced every two days. When the cells reached about 80 % confluence, they were subcultured with a 0.025 % trypsin-EDTA solution.

6. Cell viability analysis

Cell viability was examined by MTT assay [21, 40] and trypan blue dye exclusion assay [41]. For MTT assay, HT-29 cells were seeded at a density of 3×10^4 cells/well into a 96-well cell culture plate. After stabilizing, the cells were then treated with SSQ at different concentrations (0, 0.5, 1 or 2 mg/ml) for 24 h or 48 h. Following incubation, medium was removed, and then 1 mg/ml of MTT solution was added 100 μl/well followed by incubating at 37 °C for 4 h. The MTT solution was removed carefully to dissolve formazan crystals with 100 μl of DMSO, and then the absorbance was measured at a wavelength of 540 nm using a GENIio® microplate reader (GENios, TECAN, Männedorf, Switzerland). Cell viability was calculated by using the equation:

$$\% \text{ Cell viability} = A_{sample}/A_{blank} \times 100$$

Where, A_{blank} is the absorbance of control reaction (without dSSQ), and A_{sample} is the absorbance in the presence of dSSQ.

Trypan blue dye is able to penetrate the lack of intact dead cell membranes, while viable cells exclude dye due to their intact cell membranes. Therefore, the dead cells can be visually examined blue color staining under light microscope. To evaluate live/dead cell, HT-29 cells were seeded at a density of 1×10^5 cells/well into a 12-well plate and incubated for 24 h. The seeded cells were then treated with different concentrations of SSQ (0, 0.5, 1 or 2 mg/ml) for 24 h. The cells were washed with HBSS, and then they treated with pre-warmed 0.5 ml of 0.4 % trypan blue in HBSS for 20 min. The cells were washed with HBSS three times, and then cell morphology was monitored under light microscope (Leica DMI 6000 B, Leica Microsystems GmbH, Wetzlar, Germany).

7. Nuclear condensation

In order to analyze nuclear morphology of apoptotic HT-29 cells, Hoechst 33342 staining was conducted. Nuclear condensation is one of the characteristic features of apoptotic pathway [42]. Hoechst 33342 nuclear staining dye used to distinguish apoptotic cells with changes in nuclear chromatin condensation, and then cell morphology was visualized using fluorescence microscope. The HT-29 cells were seeded at a density of 1

$\times 10^5$ cells/well into a 12 well plate. After 24 h incubation, the cells were further incubated with 0, 0.5, 1 or 2 mg/ml of SSQ for 24 h. After the cells were fixed with ice-cold ethanol (75 %) for 20 min, they were stained by Hoechst 33342 nuclear staining dye (blue) for 20 min at room temperature. The cells were washed with PBS, and then cell morphology was monitored using fluorescence microscope (Leica DMI 6000 B, Leica Microsystems GmbH, Wetzlar, Germany).

8. Detection of intracellular ROS generation

Intracellular ROS levels on the HT-29 cells were evaluated by DCFH-DA assay [43, 44]. Non-fluorescent DCFH-DA (2', 7'-dichlorofluorescein-diacetate, 25 μM in HBSS) can be converted to fluorescent DCF by the action of peroxide in cells. Therefore, DCF green fluorescence reflects ROS generating levels [45], resulted in enhanced oxidative stress. HT-29 cells were seeded at a density of 1×10^5 cells/well into a 12 well plate and incubated for 24 h. After treatment with 0, 0.5, 1 or 2 mg/ml of SSQ on HT-29 cells for 24 h, the cells were incubated in a final concentration of 20 μM DCFH-DA in HBSS for 20 min at 37 °C. Next, the cells were washed with PBS, and then the ROS generation was monitored under fluorescence microscopy and ROS generating levels were measured by microplate reader and flow cytometry analysis according to the manufacture's manual.

9. Cell cycle analysis

To analyze cell cycle arrest in response to DNA damage, the method described by Laishram *et al*. (2015) was operated with slight modifications [21]. HT-29 cells were seeded at a density of 1×10^6 cells/60 mm dish and incubated for 24 h. The seeded cells were then further incubated with SSQ at different concentrations (0, 0.5, 1 or 2 mg/ml) for 24 h. The treated-cells were harvested by trypsinization and washed with PBS (Phosphate buffered saline). The collected cells were fixed with ice-cold ethanol (75 %) and stored at 4 °C at least 3 h. Alcohol-fixed cells are stable for several weeks at 4 ºC. Before analysis, alcohol-fixed cells were washed with cold PBS, resuspended cells with 180 µl of PBS, and incubated with a 20 µl of a 1 mg/ml RNase was added to the and incubated at room temperature for 30 min. This will ensure only DNA, not RNA, is stained. Subsequently, 200 µl of propidium iodide (50 µg/mL was appended to the cells prior to being incubated for 30 min at room temperature in the dark. The cell cycles were performed under flow cytometry and the data of cell proportion were analyzed using CELLQuest software and FlowJo software 7.6.1.

10. Western blot analysis

Western blot analysis was used to examine the expression of caspase-3. HT-29 cells seeded at a density of 1×10^6 cells/60 mm dish and were treated with SSQ at different concentrations (0, 0.5, 1 or 2 mg/ml) for 24 h. The cells were washed twice with PBS, resuspended in RIPA buffer (Sigma Chemical Co.) containing protease and phosphatase inhibitor (Roche Applied Science, IN, USA), and incubated on ice for 10 min before being centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatant was carefully collected, and then the the protein concentration was investigated using a Pierce® BCA protein assay kit (Thermo scientific Co., Waltham, MA, USA). The protein samples (20 μg protein/lane) were loaded and separated onto 15 % SDS–polyacrylamide gel by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes at 0.8 mA of constant current/cm^2 for 1 h. The membranes were blocked by incubation in 5 % dry milk dissolved in TBS (tris-buffered saline) buffer containing 0.1 % Tween 20 (TBS-T) for 1 h at room temperature, washed three times with TBS-T, and blotted overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology Inc.) such as caspase-3 or β-actin. After washing, the membranes were placed in secondary antibodies at room temperature. Finally, all blots were developed by ECL Western Blotting Detection Kit Reagent and imaged on Davinch-Chemi™ imaging system (Core Bio, Seoul, Korea).

11.RT-qPCR (quantitative real time polymerase chain reaction)

In order to compare mRNA expression of caspase-3, RT-qPCR was performed. HT-29 cells were seeded at a density of 1×10^6 cells/60 mm dish, the seeded cells then treated with 0, 0.5, 1 or 2 mg/ml of SSQ for 24 h. Total RNA was isolated using TRIzol reagent (Thermo scientific Co., Waltham, MA, USA) according to manufacturer's instructions. RNA purity was calculated by the ratio of $absorbance_{260}/absorbance_{280}$ and the concentration of total RNA was determined using microplate reader at 260 nm. The complementary DNA was synthesized by reverse transcription of 2 μg RNA in 20 μl of total volume using cDNA synthesis kit (ET21025, PhileKorea, Seoul, Korea). Reverse transcription was conducted at 42 ℃ for 30 min and then the synthesized cDNA was incubated at 70 ℃ for 10 min to denature RTase using Magnetic Induction Cycler (BMS, Australia). The primer used in PCR was listed in Table 1. For PCR reaction, 2 μl of the above product was used with QuantiSpeed SYBR No-Rox kit (QS105-05, PhileKorea, Seoul, Korea). The PCR reaction was carried out at 95 ℃ for 2 min to activation of polymerase followed by 40 cycles at 95 ℃ for 5 s and at 60 ℃ for 20 s for denaturation, annealing, and extension. Melting temperature was set from 50 ℃ to 95 ℃ at 0.3 ℃/s. The β-actin was used a housekeeping gene.

$$\text{Relative mRNA expression} = 2^{-(\Delta C,SSQ - \Delta C,blank)}$$

Where, $\Delta C_x = C_{x\ interest\ gene} - C_{x\ housekeeping\ gene}$, x is blank or SSQ.

12. Statistics

All values are presented as mean±S.D. (standard deviation) (n=3) from three independent experiments. Differences between means of each group were analyzed using Student's t-test. The statistical significances were achieved as a P-value < 0.05.

Table 1. The sequences of primers used in RT-qPCR.

Gene		Primer (5’-3’)	Reference
caspase-3	Forward	TTTGTTTGTGTGCTTCTGAGCC	[46]
	Reverse	ATTCTGTTGCCACCTTTCGG	
β-Actin	Forward	CTGTCTGGCGGCACCACCAT	
	Reverse	GCAACTAAGTCATAGTCCGC	

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Ⅲ. Results and Discussion

1. Antioxidant effects of the defatted peptic hydrolysate

Many studies have concentrated on the marine organisms which have biological activities. Like other marine organisms, sea squirts were also studied in the field of antioxidant, anti-inflammatory, anti-lipase, anticancer, and immune effects using its edible parts or tunic [9-11, 47]. However, no experimental study of antioxidant effect of peptic hydrolysate derived from sea squirts has been reported to the best of my knowledge. Therefore, radical scavenging activities of the sea squirts peptic hydrolysates have been investigated by determining DPPH radical and $ABTS^+$ radical scavenging activity as well as ORAC value (Table 2). At a concentration of 1.0 mg/ml, dSSQ showed DPPH radical scavenging activity of 30.59 ± 0.90 %, $ABTS^+$ radical scavenging activity of 37.73 ± 0.67 μM TE/mg dSSQ, and ORAC of 576.0 ± 0.50 μM TE/mg dSSQ. The DPPH and $ABTS^+$ radical scavenging activities were lower than that of GSH (glutathione), as a positive control. However, dSSQ showed a significant ORAC value that was higher than the ORAC value of GSH (314.2 μM TE/mg GSH). It has been reported that the IC_{50} of ethanol extract, methanol extract, water extract, and control (added nothing) from *Halocynthia roretzi* were 1515.85, 1176.99, 2242.08, and 10.00 μg/ml in DPPH radical scavenging assay,

respectively [5]. The antioxidant activities of *Halocynthia roretzi* and *Halocynthia aurantium* have been shown by Ji-Eun Jo *et al*. (2010). They reported that extracts of *Halocynthia roretzi* and *Halocynthia aurantium* had 42.9 % and 3.2 % of DPPH radical scavenging effects at 50 mg/ml. The extracts had 56.1 % and 30.1 % of $ABTS^+$ radical scavenging effects at the same concentration as well [6]. Antioxidant activities of natural products differ from each other reports due to the different extracting methods including sample concentration, extraction time, extraction temperature or solvent [3]. Some antioxidants behave different against different radical sources depending on their solubility or mechanism of action. Measuring ORAC value can provide better data than using $ABTS^+$ or DPPH radicals. This is because unlike $ABTS^+$ or DPPH radical scavenging assay, ORAC assay has an obvious starting point and an obvious endpoint and measure the ability of hydrogen donating by quenching free radicals. This represents a “physiological” radical source [48]. Therefore, the results reveal that dSSQ transfers electrons or hydrogen effectively, it has antioxidant effects as well.

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Table 2. Radical scavenging effects of dSSQ. ABTS$^+$ radical, DPPH radical scavenging assay and ORAC assay. The values were described as mean±S.D. (n=3).

ABTS, 410 nm	dSSQ			GSH
Concentration (μg/ml)	100	1000	2000	20
Scavenging effect	21.77	37.73	46.05	69.02
±S.D. (μM TE/sample)	±0.43	±0.67	±0.71	±0.33

DPPH, 540 nm	dSSQ	GSH
Final concentration (μg/ml)	1000	20
Scavenging effect	30.59	58.17
±S.D. (%)	±0.90	±4.67

ORAC Ex. 485 nm, Em. 535 nm	dSSQ	GSH
Concentration (μg/ml)	100	100
Scavenging effect	57.60	37.42
±S.D. (μM TE/sample)	±0.05	±4.04

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2. Purification and identification of antioxidant peptides

In order to purify antioxidant peptides, chromatographic techniques were used consecutively. Six separated fractions were obtained using a size-exclusion chromatography at 280 nm (Figure 1). The DPPH radical scavenging activities of the fractions were detailed in Figure 2. Fraction F exhibited the highest radical scavenging activity at a final concentration of 1 mg/ml (65.08 %), so it was selected for further study. Fraction A was excluded from selection because it precipitated. Fraction F was loaded onto a Hypersil Gold C_{18} column with a linear gradient of acetonitrile at 215 nm and fractionated into eight sub-fractions (Figure 3). At a concentration of 500 μg/ml (Figure 4), fraction 2 had the highest DPPH radical scavenging, however, its volume was not enough to purify. Fraction 1 had a little amount of eluate and Fraction 3 precipitated. Given that the result of DPPH assay and the volumes of fractions, peak 6 was chosen for the next step. The peak 6 was divided into eight sub-fractions (Figure 5) through a Superdex™ peptide 10/300 GL high performance column at 215 nm. The sub-fraction h indicated 62.77 % of the highest DPPH radical scavenging activities at 150 μg/ml of final concentration (Figure 6). The Hypersil Gold C_{18} column with a linear gradient of acetonitrile was used for eluting the sub-fraction h (Figure 7). Finally, sub-fraction 2 was selected as the purified antioxidant peptide for further study because the sub-fraction 1 and 3 were small in volume.

The selected fraction was identified using Q-TOF LC-MS/MS system and the amino acid sequence was determined by de novo sequencing program. Their MS/MS spectra were illustrated in Figure 8. The antioxidant peptides derived from sea squirts by peptic hydrolysis were identified to be Met-Thr-Thr-Leu (P1, MTTL, 464.58 Da), Leu-Glu-Trp (P2, LEW, 446.50 Da), and Tyr-Tyr-Pro-Tyr-Gln-Leu (P3, YYPYQL, 845.95 Da). To be specific, peptides are broken at different sites because of the addition of a proton which causes different fragments. The fragment ions were categorized into two parts. One is the N-terminal charged fragment ions such as a, b, or c. The other is the C-terminal charged fragment ions such as x, y, or z. The sum of residue masses is calculated by the formula:

Mass of b-ions = $\sum$ (residue masses) + 1 (H^+)

Mass of y-ions = $\sum$ (residue masses) + 19 (H_2O+H^+)

For example, leucine of P1 (MTTL) sequence (in Figure 8) was determined by above formula. The 132.081 of y1-ion mass minus 19 is 113.08 which is completely the same as 113.08 Da of leucine (L) and isoleucine (I) mass. Considering leucine (L) is more bountiful than isoleucine (I) in sea squirts, leucine (L) is suitable for the component of the antioxidant peptides [8]. The sequences of antioxidant peptides have been reported in the past decades. Three antioxidant peptides from peptic hydrolysate of prawn were purified and identified as Ile-Lys-Lys (388 Da), Phe-Lys-Lys (422 Da), and Phe-Ile-Lys-Lys (535 Da) [49]. Trp-Pro-Pro (398.44 Da) was derived from blood clam muscle as an antioxidant amino acid [50]. Tyrosine (Tyr), Tryptophan (Trp), and Phenylalanine (Phe) are typical

examples of antioxidant amino acids. They have aromatic residues which can donate protons to unstable radicals while maintaining their own stability [51]. Methionine (Met), Lysine (Lys), and Proline (Pro) are also examples of amino acids which cause antioxidant activity [52, 53]. In this study, purified and identified antioxidant peptides have those amino acids. P1 and P2 have one antioxidant amino acid such as Met or Trp, respectively. Tyr and Pro which are antioxidant amino acids account for 67 % of P3’s sequence. Identified antioxidant peptides were synthesized for evaluating their antioxidant activities.

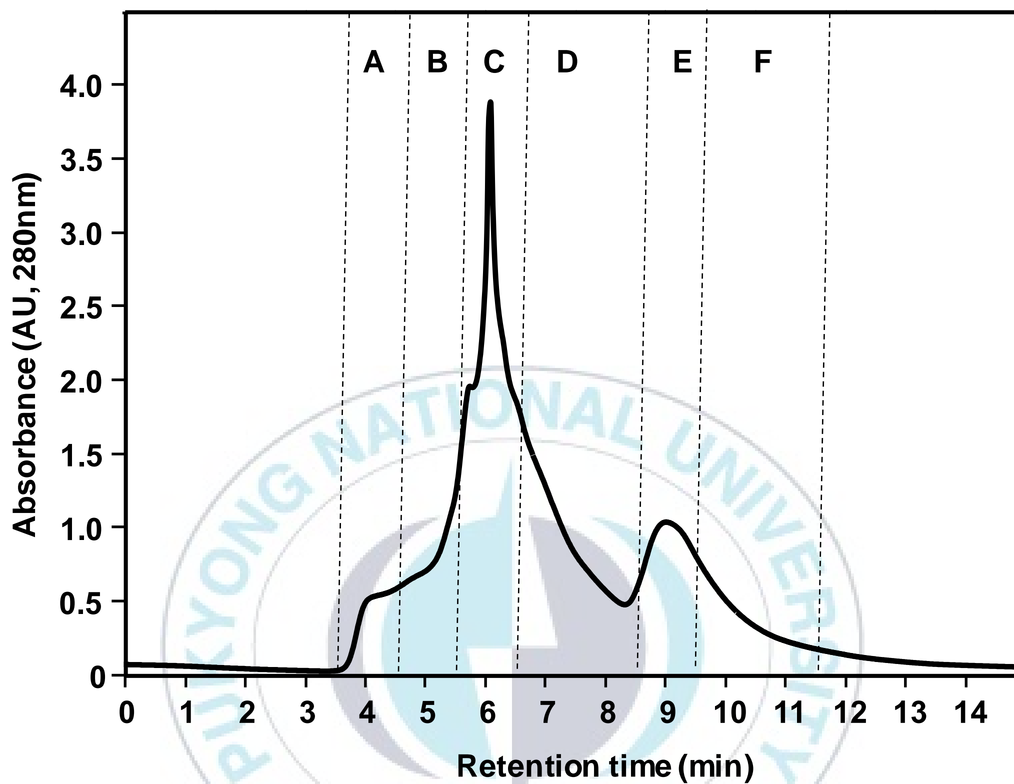


Figure 1. Chromatogram of peptic hydrolysate of sea squirts performed by means of size-exclusion chromatography (300 mm×7.8 mm, 5 μm) with distilled water containing 0.05 % Trifluoroacetic acid (TFA) at 1.5 ml/min of flow rate for 15 min. The peaks were read at 280 nm.

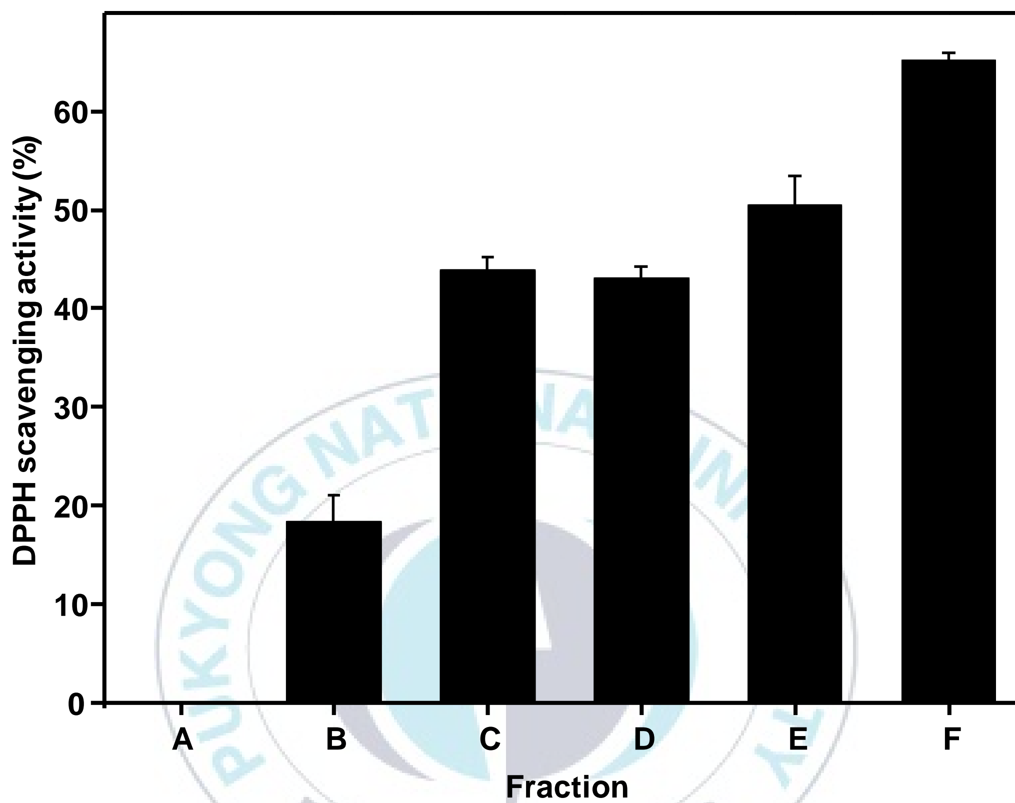


Figure 2. DPPH radical scavenging effect of fractions from first purification. Each values were described as mean±S.D. (n=3).

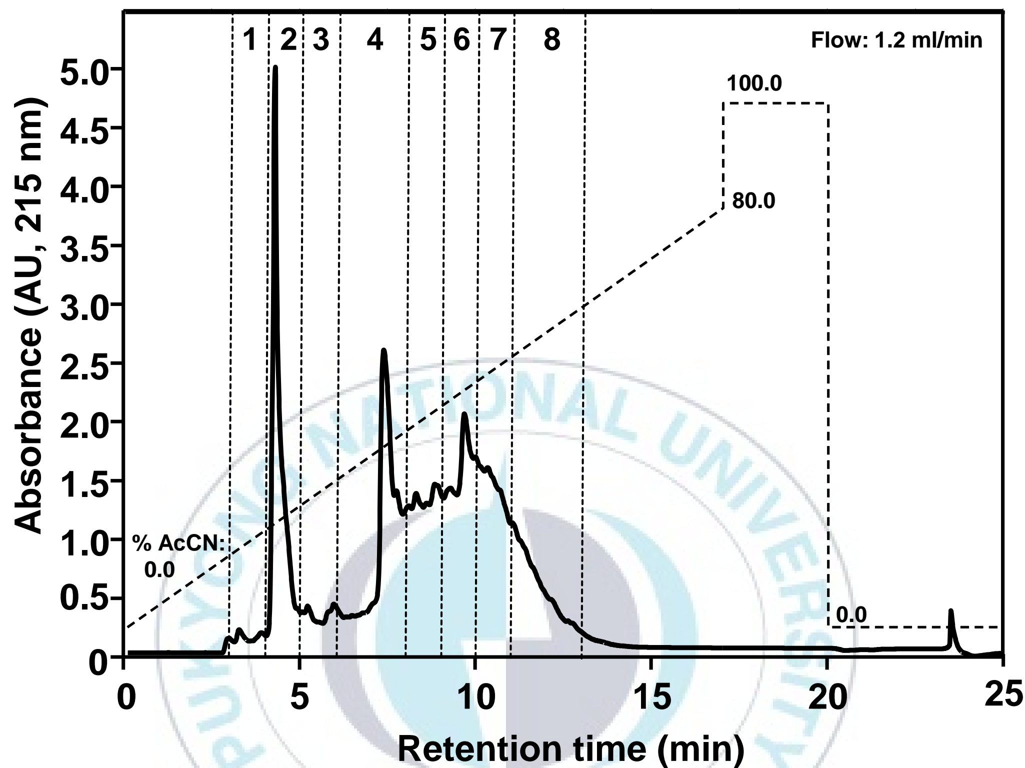


Figure 3. Chromatogram of the fraction F from dSSQ accomplished on a Hypersil Gold C_{18} column (250 mm×4.6 mm, 5 μm) with a linear gradient of acetonitrile (0-80 % in 0-17 min, 100 % in 17-20 min and 0 % in 20-25 min). Flow rate was set at 1.2 ml/min and the elution peaks were detected at 215 nm.

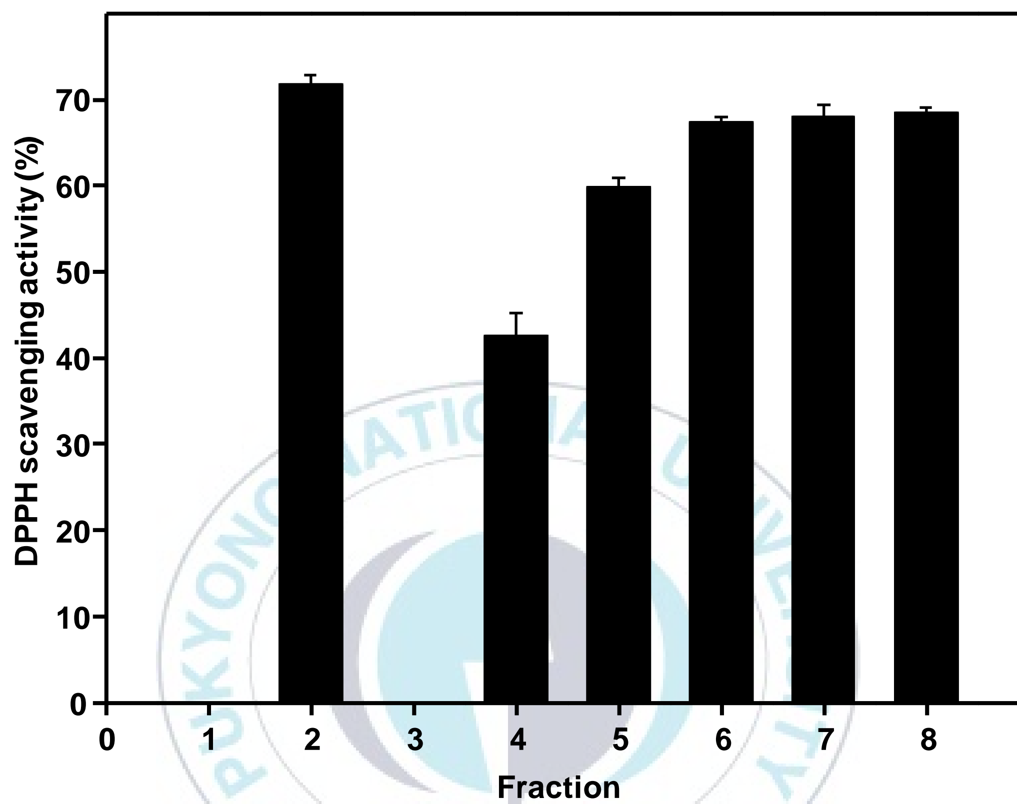


Figure 4. DPPH radical scavenging effect of fractions from second purification. The values were described as mean±S.D. (n=3).

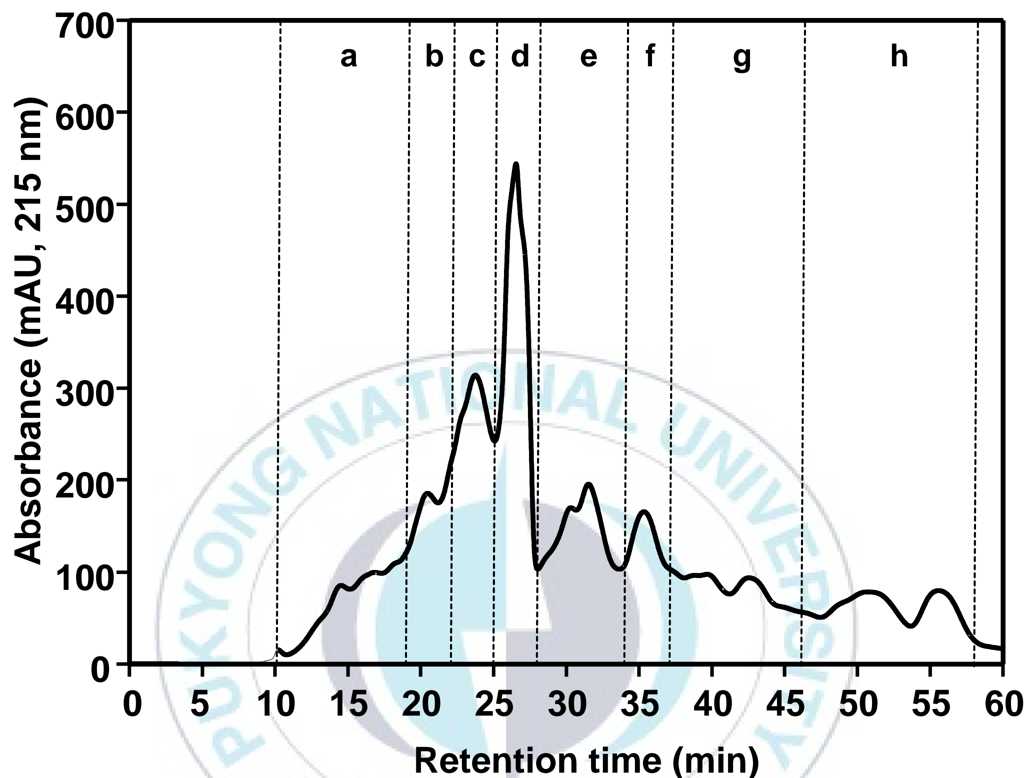


Figure 5. Chromatogram of fraction 6 from fraction F achieved using GPC (gel permeation chromatography) on a Superdex™ peptide 10/300 GL high performance column (300 mm×10 mm, 13 μm) with distilled water containing 0.05 % TFA. Flow rate was 0.7 ml/min and eluate was monitored at 215 nm.

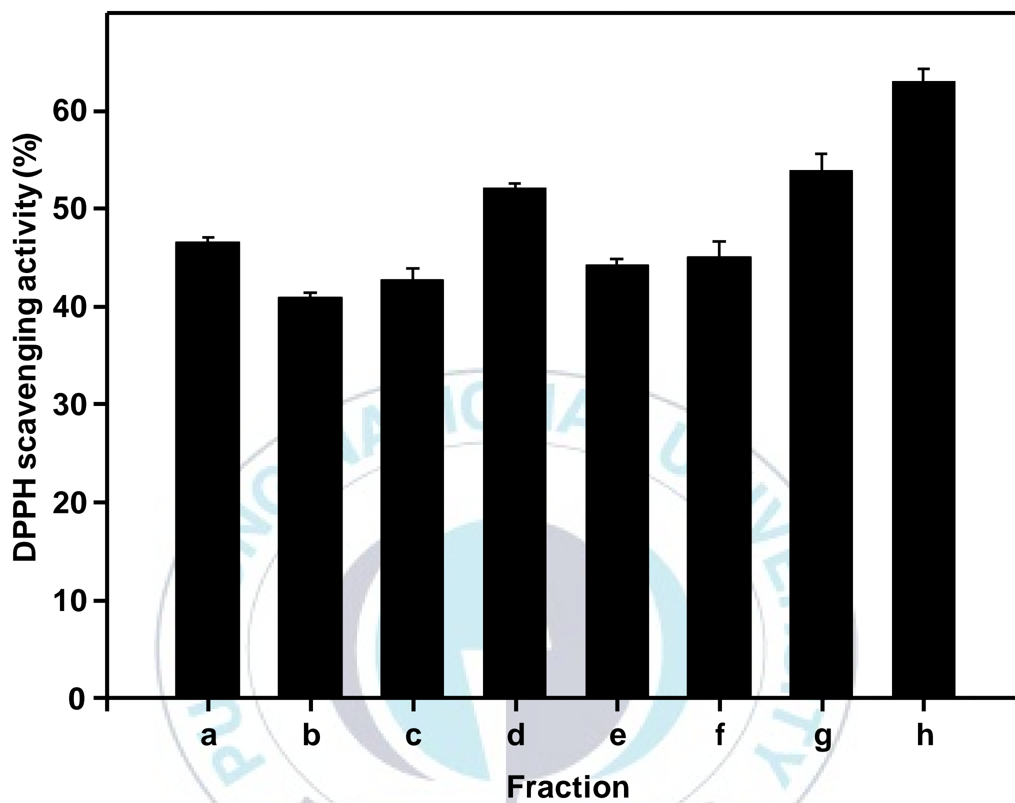


Figure 6. DPPH radical scavenging effect of fractions from third purification. The values were described as mean±S.D. (n=3).

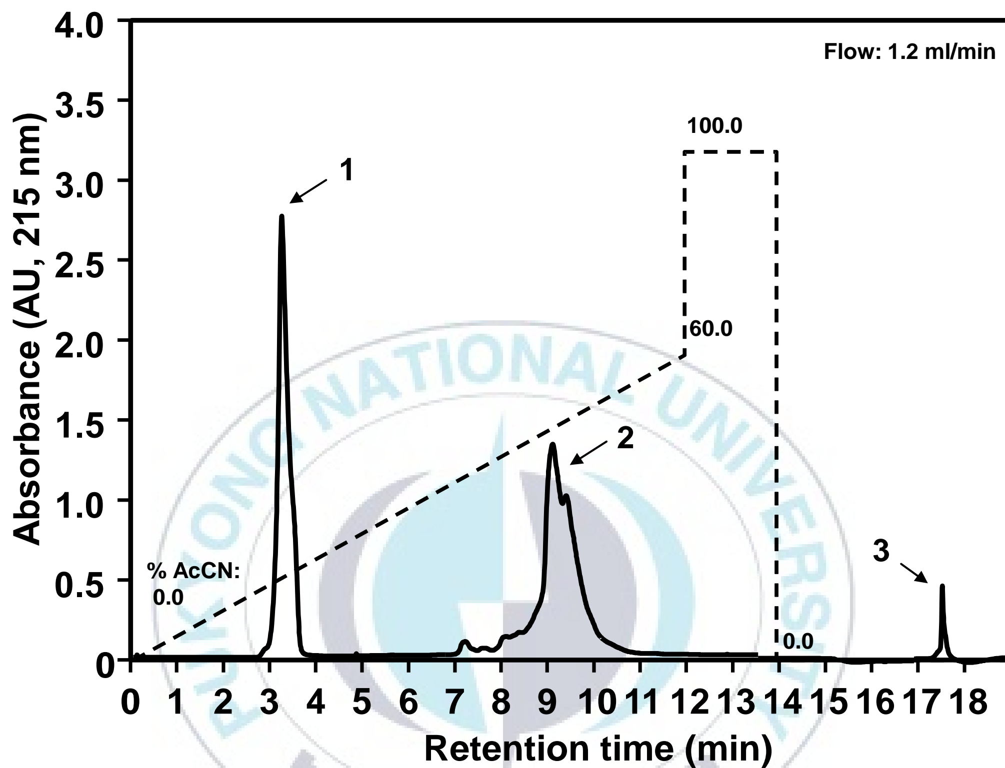


Figure 7. Chromatogram of fraction h from fraction 6 purified on the Hypersil Gold C_{18} column (250 mm×4.6 mm, 5 μm) in optimal conditions (0-12 min: 0-60 % acetonitrile, 12-14 min: 100 % acetonitrile, 14-19 min: 0 % acetonitrile). Flow rate was fixed at 1.2 ml/ml while peaks were monitored at 215 nm.

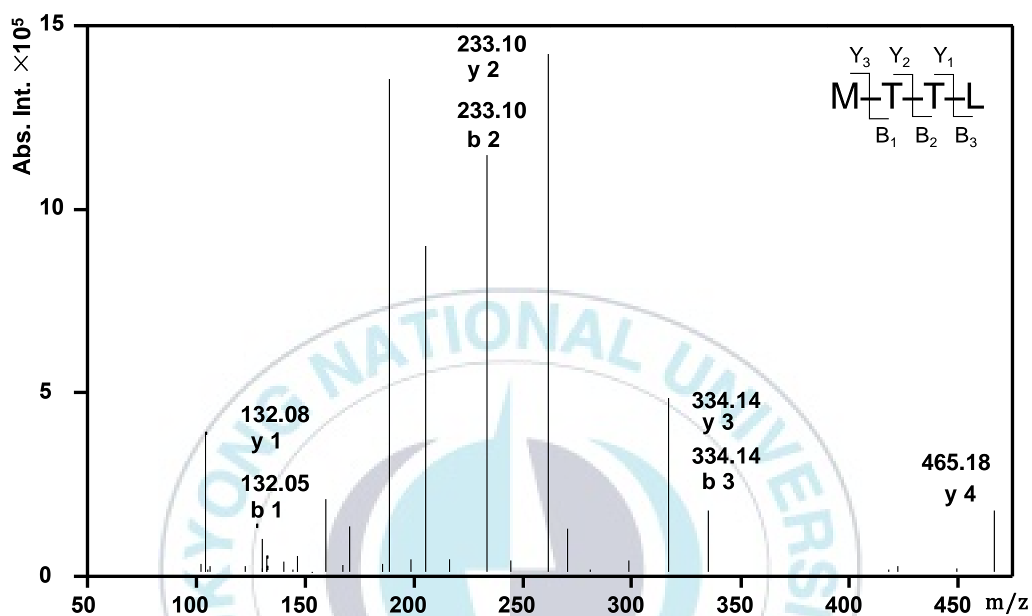
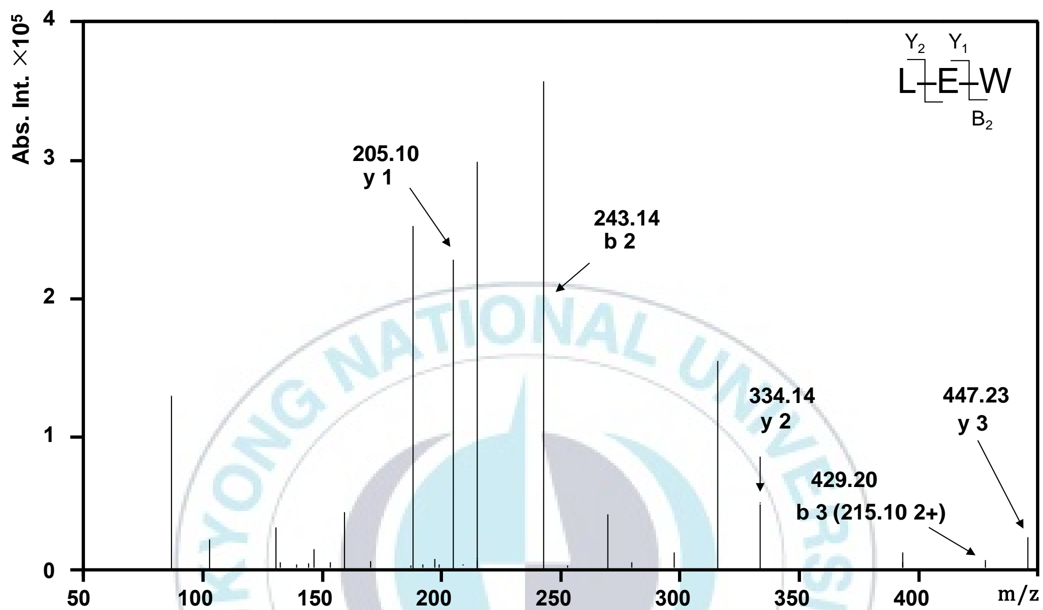


Figure 8. LC-MS/MS spectra and sequences of antioxidant peptides. The sequences of Peptide P1, P2 and P3 are MTTL (464.58 Da), LEW (446.50 Da) and YYPYQL (845.95 Da), respectively.



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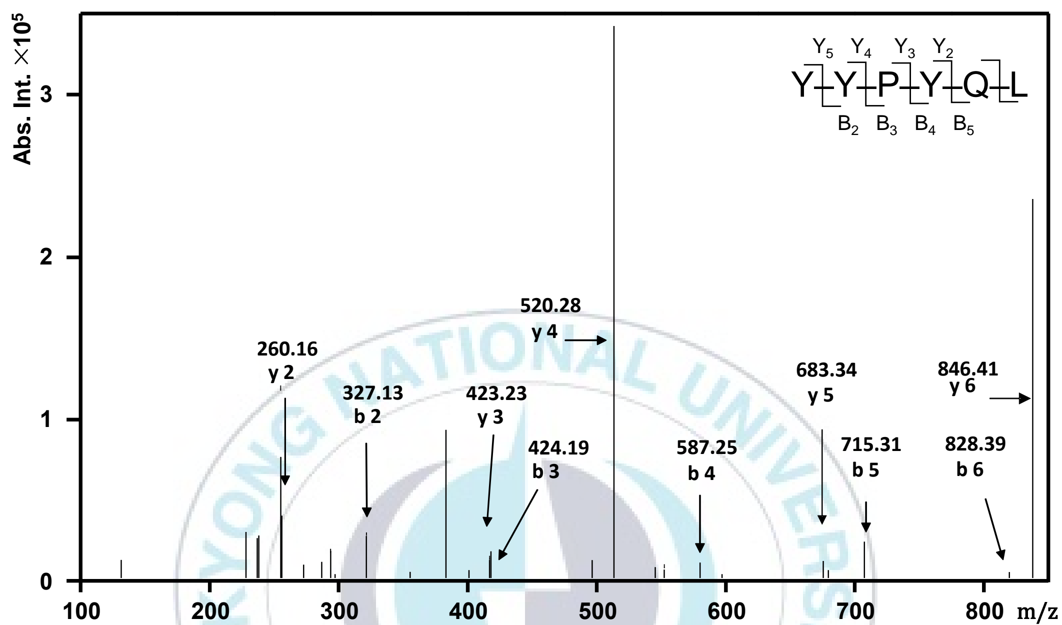


Figure 8. LC-MS/MS spectra and sequences of antioxidant peptides. The sequences of Peptide P1, P2 and P3 are MTTL (464.58 Da), LEW (446.50 Da) and YYPYQL (845.95 Da), respectively.

3. Antioxidant effects of the synthesized antioxidant peptides

To evaluate antioxidant abilities, DPPH radical scavenging, $ABTS^{+}$ radical scavenging, ORAC, Fe^{2+} chelating activity, and reducing power were measured.

DPPH radical scavenging assay bases on SET (single electron transfer) whose reaction is slow and long time required to reach a fixed endpoint. DPPH radical is one of the stable nitrogen radicals and is so prominent that it is used as an indicator of radical reaction. It changes color from deep purple to yellow exchanging electrons and this property permit monitoring of the reaction visually [48]. DPPH radical scavenging activity curves of P1, P2, and P3 were shown as Figure 9 at a dose-dependent manner. DPPH radical scavenging activity of P2 was stronger than those of P1 and P3. IC_{50} value of P2 was 2 mM which was higher than that of LPHPSF (0.17 mM) from tryptic hydrolysate of Styela plicata [54], however, IC_{50} value of P2 was lower than that of WPP (3.48 mM) [50], WDR (7.63 mM) [55], YPPAK (4.56 mM) [56], and PSYV (36.62 mM) [57] from protein hydrolysate of blood clam muscle, ethanol soluble protein hydrolysate of *Sphyrna lewini* muscle, blue mussel protein hydrolysate, and loach protein hydrolysate, respectively. This result reveals that P2 is in possession of an electron donating capability.

Peroxyl radicals or other oxidants oxidize ABTS, and antioxidants neutralize $ABTS^{+}$ radicals via electron transfers directly [48]. Figure 10 shows that $ABTS^{+}$ radical

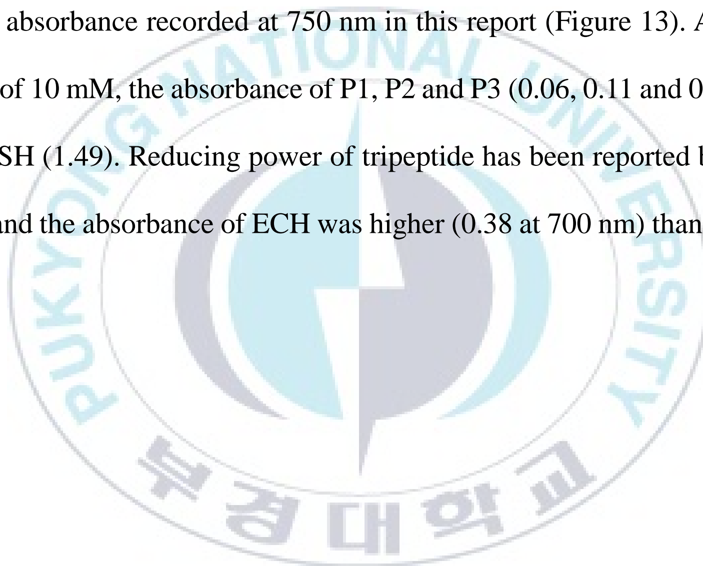
scavenging activities of peptides. P1 showed no $ABTS^+$ scavenging activity and the activities of P2 and P3 were 13.41 and 11.87 μM TE/50 μM peptide, respectively. These activities were lower than that of GSH (78.89 μM TE/50 μM GSH). Longjian Gu *et al*. (2012) have reported $ABTS^+$ radical scavenging capacities of two novel peptides, 2.43 mM TE/mM for ECH and 8.88 mM TE/mM for YECG [58]. These capacities were higher than $ABTS^+$ radical scavenging activities of P1, P2 and P3.

The ORAC values of synthesized peptides were higher than that of GSH (18.47 μM TE/50 μM GSH), especially P2 (93.69 μM TE/50 μM P2) and P3 (95.71 μM TE/50 μM P3) (Figure 11). Chamila Nimalaratne *et al*. (2015) have reported some ORAC values of 1.53 μmol TE/μmol for EERYP, 3.32 μmol TE/μmol for AEERYP, and 0.44 μmol TE/μmol for LPDEVSG [59]. ORAC assay uses oxygen radicals and measures the capacity of hydrogen donating by quenching free radicals. This differs from $ABTS^+$ radicals scavenging assay via electron transfers directly [48]. As a result, ORAC values used physiological radicals are more dependable to analyze antioxidant activity on human body than $ABTS^+$ scavenging activities [60]. Hence, ORAC values show P2 and P3 have significant capability of transferring hydrogen.

As shown in Figure 12, the peptides showed stronger Fe^{2+} chelating activities than GSH and the Fe^{2+} chelating levels among the antioxidant peptides were in the order of P2 (12.49 %) > P3 (10.91 %) > P1 (9.20 %) > GSH (0.60 %). Antioxidant peptides form a complex with ferrous ion (Fe^{2+}) resulting in inhibiting the formation of a ferrozine-Fe^{2+}

complex, whose color is purple. The more the purple color dims, the more active the chelating effect reactions [20, 61]. This result indicates that the three antioxidant peptides are efficient hydroxyl radical scavenger and behave efficient in Fe^{2+} chelating.

Reducing power assay base on single-electron-transfer reaction which is similar to $ABTS^{+}$ scavenging assay [58]. Antioxidants reduce potassium ferricyanide to potassium ferricyanide which reacts on ferric chloride. This reaction result in the formation of ferric-ferrocyanide which is insoluble bluish green pigment [62]. Reducing power is higher as increasing the absorbance recorded at 750 nm in this report (Figure 13). At the treatment concentration of 10 mM, the absorbance of P1, P2 and P3 (0.06, 0.11 and 0.09) were lower than that of GSH (1.49). Reducing power of tripeptide has been reported by Longjian Gu *et al*. (2012), and the absorbance of ECH was higher (0.38 at 700 nm) than those of P1, P2 and P3 [58].



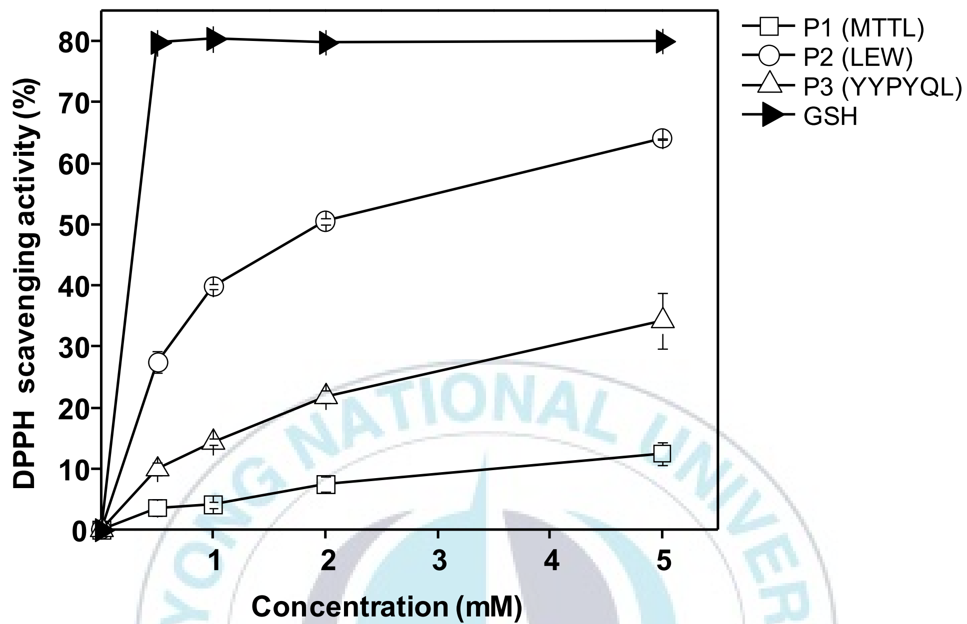


Figure 9. DPPH radical scavenging activities of P1, P2, and P3 at 540 nm in a dose-dependent manner. Data expressed as mean±S.D. (n=3).

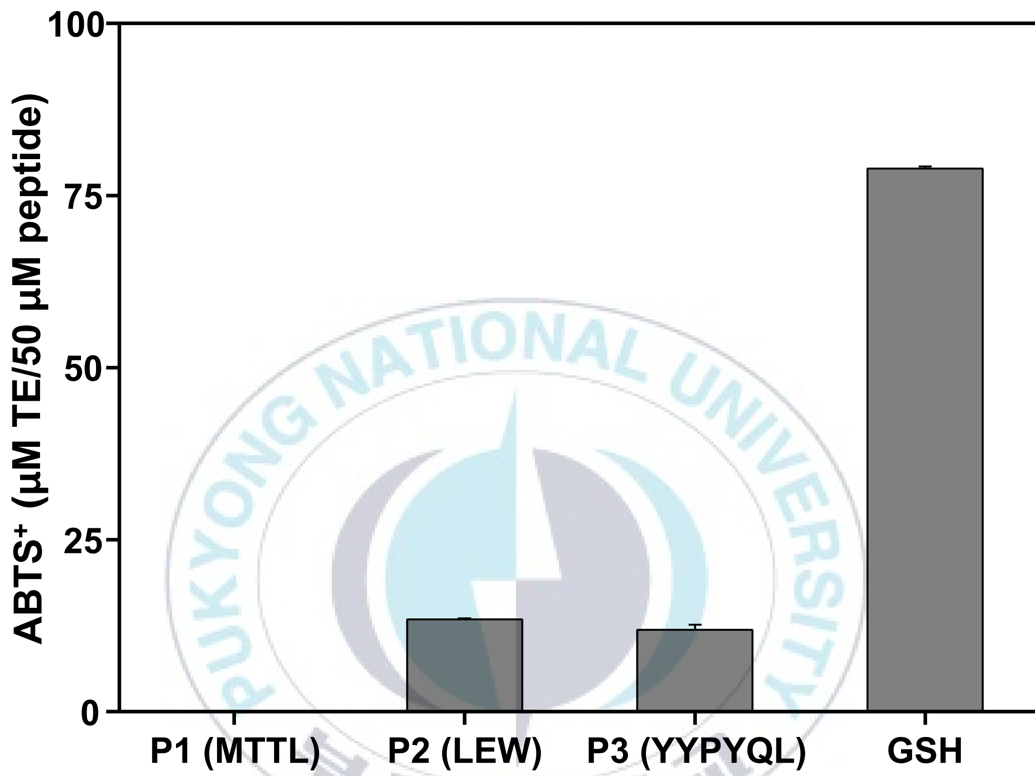


Figure 10. ABTS^{+} radical scavenging effects of P1, P2, and P3 measured at 410 nm. The values were described as mean±S.D. (n=3).

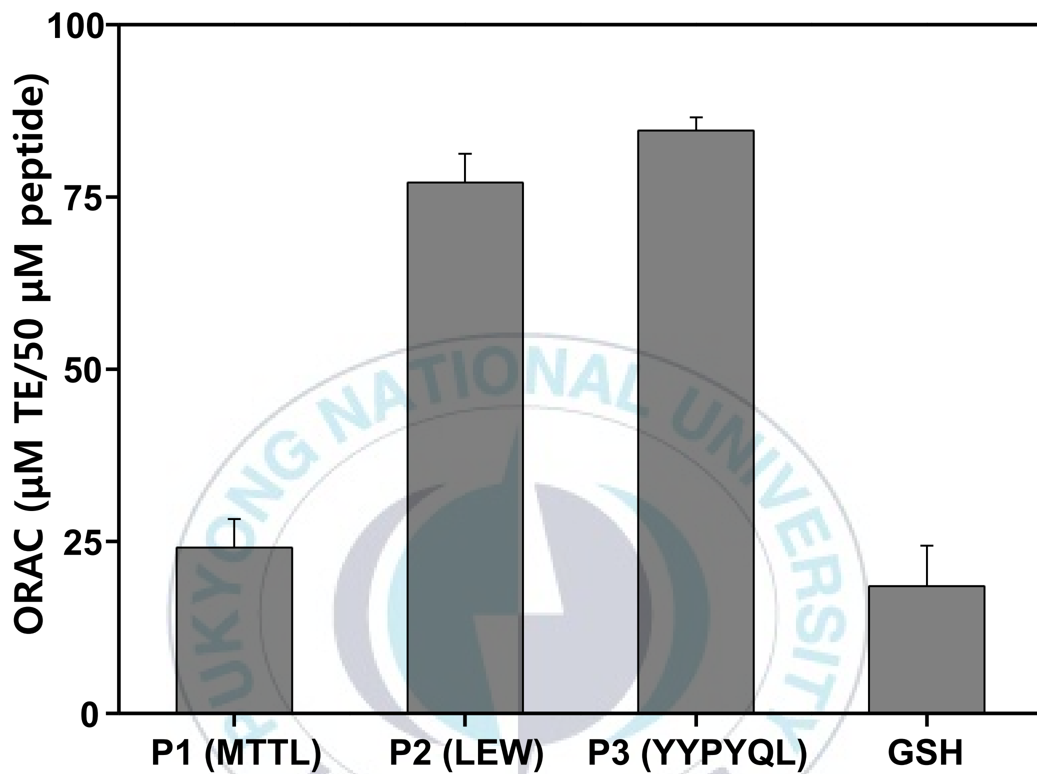


Figure 11. ORAC values of P1, P2, and P3 measured every 5 min for 60 min (Ex. 485 nm, Em. 535 nm). The values were described as mean±S.D. (n=3).

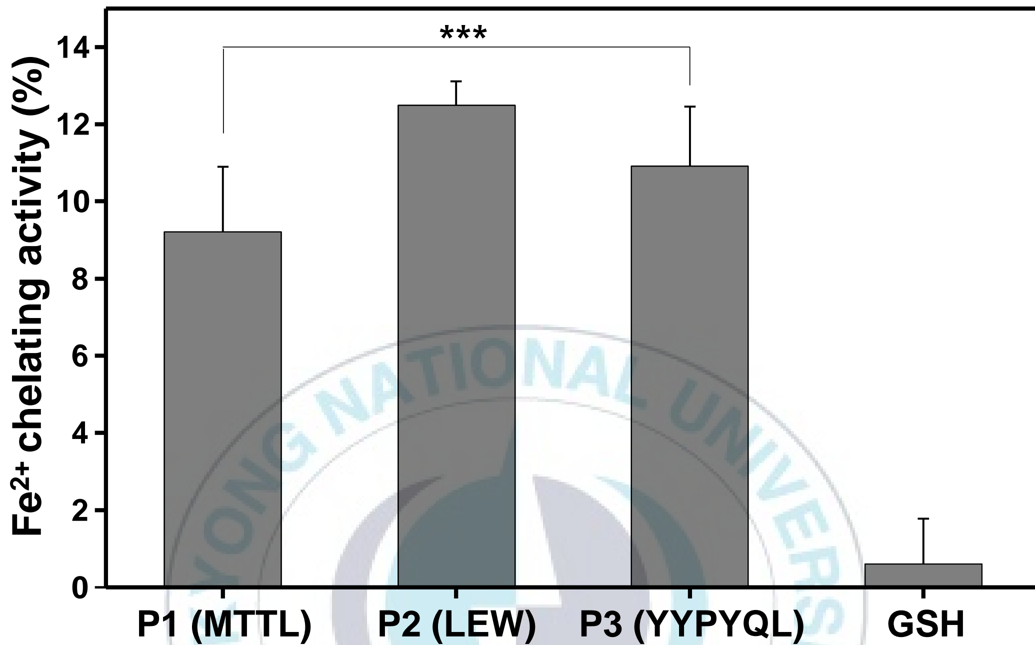


Figure 12. Fe^{2+} chelating activity of P1, P2, and P3 recorded at 570 nm. Each bar shows the mean±S.D. (n=3). The mark, * represents P<0.001 compared with positive control, GSH, as analyzed by t-test.**

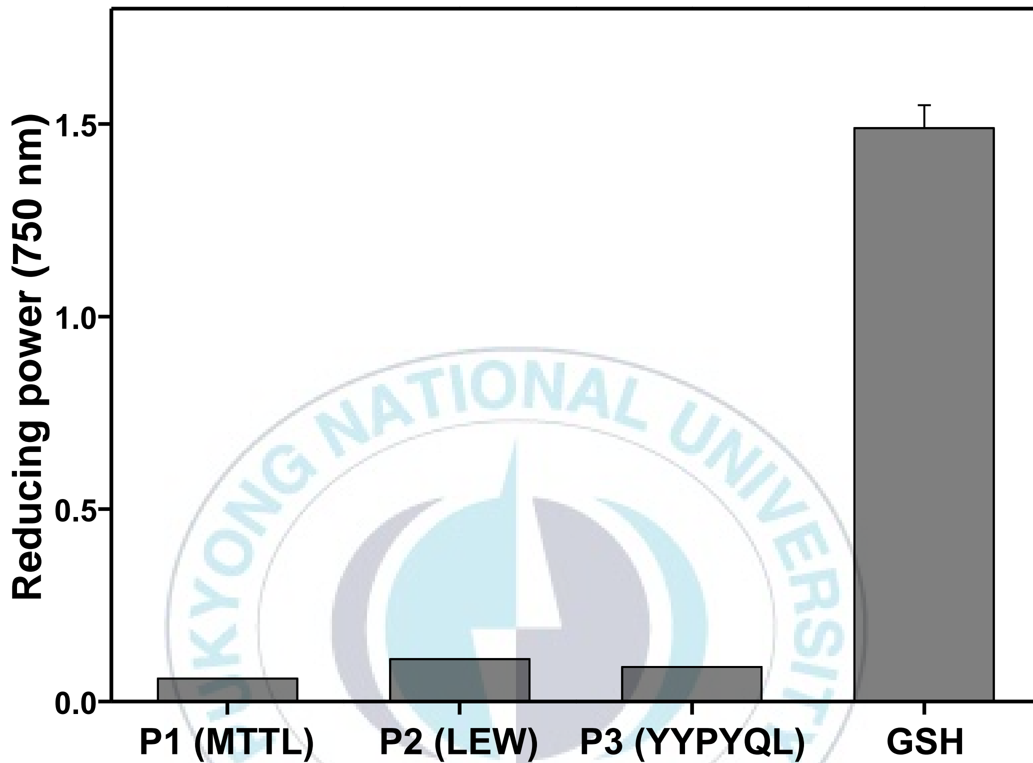


Figure 13. Reducing power of P1, P2, and P3 were reckoned at 3.33 mM of the final concentration of those peptides. The values were described as mean±S.D. (n=3).

4. Inhibition of cell proliferation

In order to assess the inhibition of proliferation of SSQ in HT-29 colon cancer cell line, the cell viability was analyzed using MTT assay and trypan blue staining in sequence. The water-soluble tetrazolium (MTT) convert to the DMSO-soluble purple formazan by Living cells but not dead cells. Trypan blue stain dead cells but not living cells. These methods can quantify living cells. HT-29 cells grew in DMEM with different concentrations of SSQ or distilled water as a blank for 24 h or 48 h. Then MTT assay was conducted Figure 14. The cells treated with SSQ for 24 h shows that cell viabilities were 82.17, 70.88 and 60.85 % in concentrations of 0.5, 1 and 2 mg/ml compared with blank (only treated with distilled water), respectively. The cell viabilities of the cells stained by trypan blue were observed using bright field microscopy (Figure 15). The result showed a number of dead bodies compared with blank in a dose-dependent manner. The cells treated with SSQ for 48 h showed lower cell viabilities (66.72, 62.13 and 51.19 % at 0.5, 1 and 2 mg/ml of SSQ) than cell viabilities of the cell treated with SSQ for 24 h but not significant difference. On the other hand, Derek Thomson *et al*. (2016) have shown that polysaccharides from the sea squirt, Ascidiella aspersa, have no cytotoxicity in Hela cells [9]. Si-Hyang Park *et al*. (2013) have also demonstrated that *Halocynthia roretzi* tunic extracts using alcalase or hot water have no inhibition of cell viability in AGS human stomach cancer cells or HT-29 cells [10]. The anti-proliferative property of extracts from

natural products vary with cell lines [19, 24], extraction processes, (by ultrasonification and/or by high pressure) [63] or the extracting conditions such as temperature, time or enzyme [10]. The results obtained from MTT assay and trypan blue staining assay reveal that SSQ prohibits cell proliferation in HT-29. The results indicate that SSQ inhibited the proliferation of HT-29 cells in a dose-dependent manner.

5. Apoptotic cell morphological change and DNA damage

To analyze morphological changes accompanied with anti-proliferation on HT-29 treated with SSQ, Hoechst 33342 staining was carried out and the results are depicted in Figure 16. HT-29 cells were treated with 0, 0.5, 1 and 2 mg/ml of SSQ for 24 h, then cells were stained by Hoechst 33342 nuclear staining dye (blue) for 20 min. Apoptotic nuclei were monitored by fluorescence microscope (scale bar, 100 mm). As shown in Figure 16, Hoechst 33342 staining displayed many bright apoptotic nuclei in HT-29 cells treated with SSQ compared with blank. This means that DNA fragmentation was induced by SSQ in HT-29 cells because Hoechst 33342 stains nuclei containing damaged DNA [64] and morphological changes, nuclear condensation and DNA fragmentation are characteristics of apoptosis [26, 65]. DNA is a major target for cancer cell killing [18]. Therefore, Hoechst 33342 staining by fluorescence microscope shows that SSQ induces apoptosis in HT-29 cells, as apparent from nuclear fragmentation.

6. ROS generation

In order to determine intracellular ROS generation by SSQ in HT-29 cells, the intracellular oxidation of $DCFH_2$ were observed under fluorescence microscope (scale bar, 100 μm) (Figure 17). ROS was considered as a great marker of the oxidative stress that led to cell cycle arrest or cell death in living cells [16, 66]. In this study, intracellular ROS in HT-29 cells treated with SSQ for 24 h increased proportionally to the concentration of SSQ. ROS acts as a key to determine initiating cell cycle arrest and/or apoptosis [67]. Although a modest increase in ROS advance cell proliferation, significant amounts of ROS can trigger oxidative damage to cells, inducing cytotoxicity to cancer cells [67, 68]. Additionally, Inordinate amounts of ROS can be a major cause of cancer [13, 27]. Therefore, the results indicate that SSQ induces ROS-related cell death in HT-29 cells.

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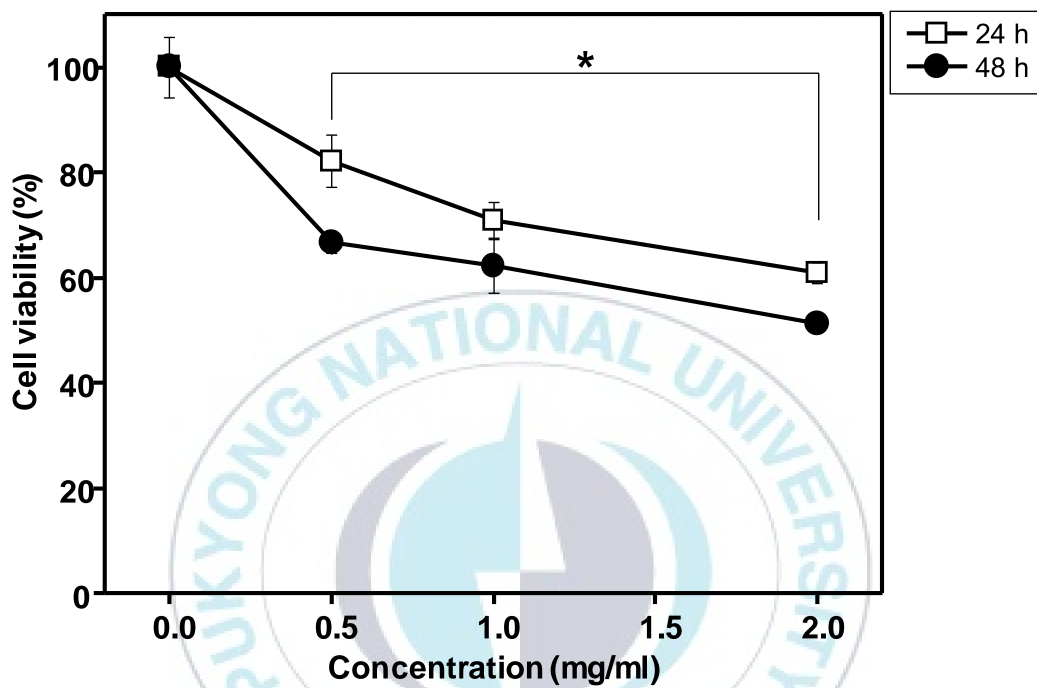


Figure 14. Cell viability of HT-29 treated with SSQ for 24 h or 48 h Cell viabilities were analyzed using MTT assay. Each value was expressed as the mean±S.D. (n=3). The mark, *, means $p < 0.05$ compared with blank, as analyzed by t-test.

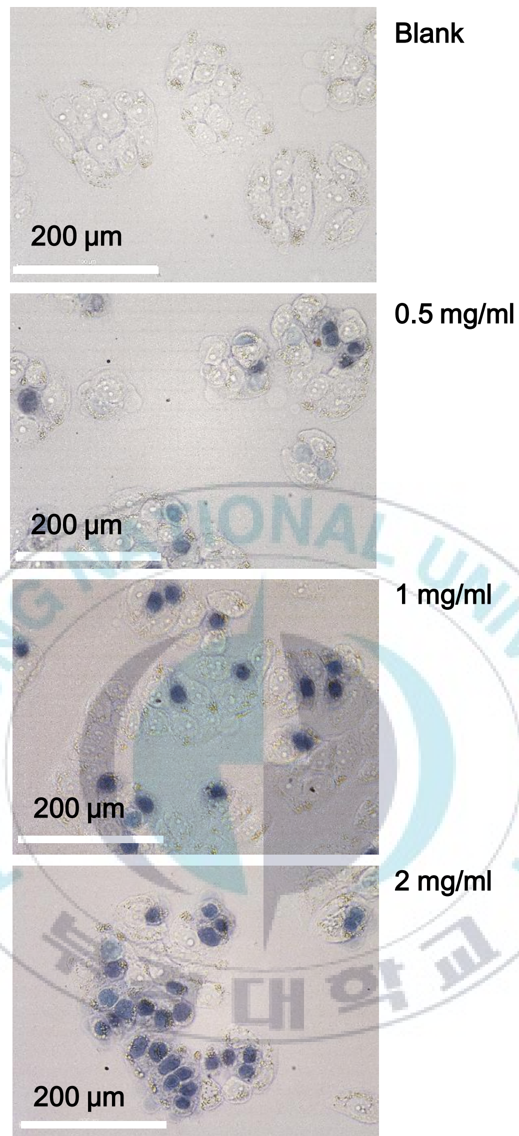


Figure 15. Cell viability of HT-29 treated with SSQ for 24 h by trypan blue staining observed using bright field microscopy (scale bar, 200 µm).

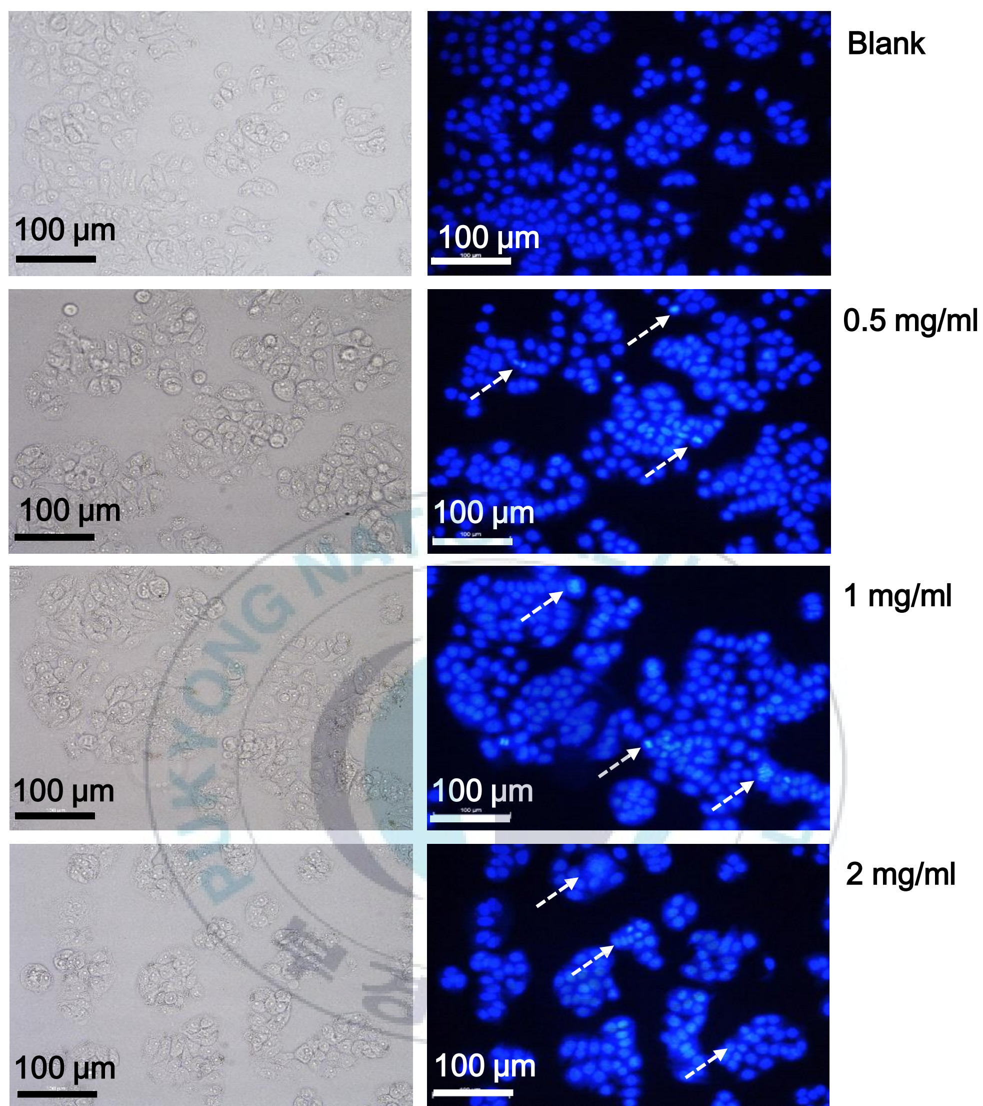


Figure 16. Apoptotic nuclear staining by Hoechst 33342 staining dye (blue) in HT-29 cells treated with SSQ for 24 h (scale bar, 100 μm).

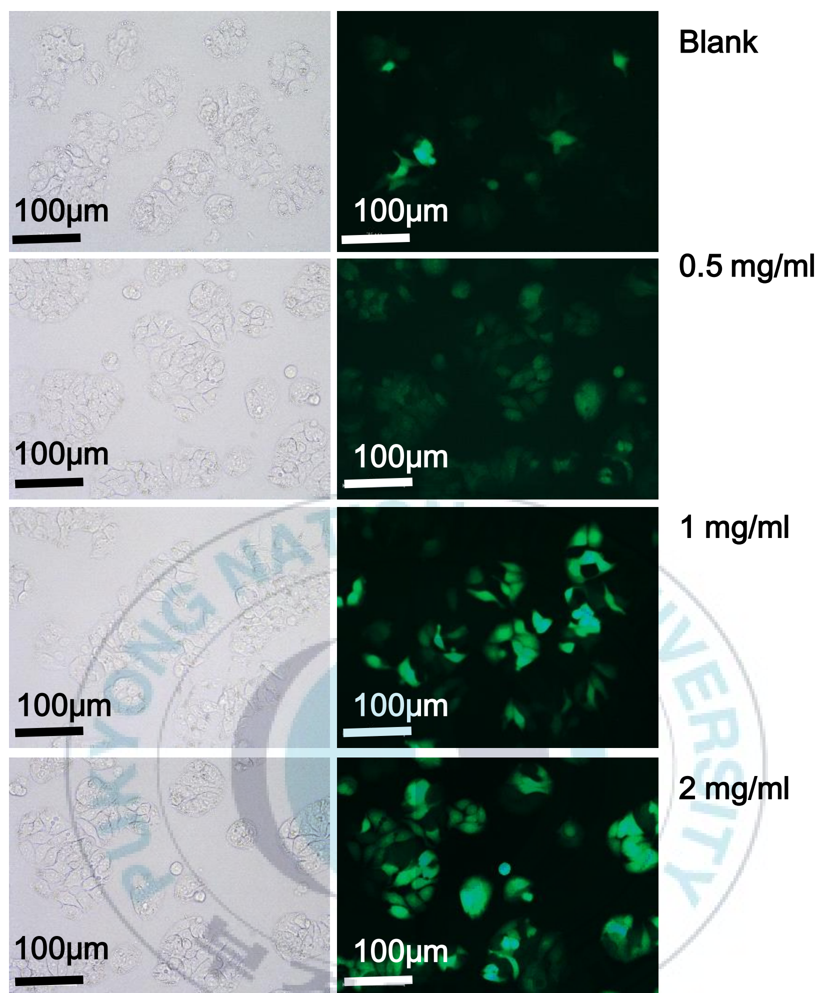


Figure 17. ROS generation in HT-29 cells treated with SSQ for 24 h. Intracellular ROS generation in HT-29 cells stained by DCFH was observed by DCF green fluorescence under fluorescence microscope (scale bar, 100 μm).

7. Cell cycle arrest

Cell cycle distribution was analyzed by flow cytometry to evaluate cell cycle arrest in HT-29 cells exposed to SSQ for 24 h (Figure 18). The G_2/M phase populations increased by significantly 5.9 % at blank, 30.8 % at 0.5 mg/ml, 35.7 % at 1 mg/ml and 41.7 % at 2 mg/ml compared with blank (vehicle-treated cells). On the contrary, SSQ diminished the G_0/G_1 phase populations in HT-29 cells by 50.3, 43.8, 38.9 and 34.3 % at 0, 0.5, 1 and 2 mg/ml, respectively. The populations of S phase were decreased by SSQ as well (31.7, 22.6, 21.8 and 20.3 % at 0, 0.5, 1 and 2 mg/ml, respectively). Many studies have reported G_2 phase arrest by nature-derived extracts. Bo Li et al. (2015) has reported G_2 cell cycle arrest chaetoglobosin K (ChK) isolated from the fungus *Diplodia macrospora* at first. They demonstrated that ChK caused a significant up-regulating in the proportion of cancer cells at the G_2 phase, while treatment of ChK resulted in a decrease at the G_1 and S phase [69]. Scutellaria discolor acetone extract showed a significant accumulation of G_2 phase cells in Hela cells [21]. Allicin, which is a compound derived garlic, has an ability of up-regulating at G_2 phase cells in human oesophageal cancer cell line Eca109 and EC9706. This increase accompanied with decrease in cell population at G_0/G_1 and S phase [70]. Another study has shown that the pericarp extract of Baneh, *Pistacia atlantica sub kurdica*, induced S phase delay in HT-29 cells [71]. A number of studies have demonstrated cell cycle arrest at G_0/G_1 phase by natural substances. Sara Jaramillo *et al*. (2016) have reported saponins

from wild asparagus led to G_0/G_1 cell cycle arrest [26]. The blocking cell cycle progression at G_0/G_1 phase has been also shown by Swadesh K. Das *et al*. (2005). They have reported that fucoxanthin, a natural carotenoid, induces increasing population during the G_0/G_1 phase [72]. It has been reported that the damaged cells repair themselves or induce growth arrest to correct damage. If it is impossible to repair the cells, they trigger apoptosis [73, 74]. Phosphorylation, acetylation and ubiquitination of p53 act as a key role to determine beginning cell cycle arrest or apoptosis [73]. Depending on the cells' damage, apoptosis and cell cycle arrest can occur simultaneously [67, 75]. In addition, 14-3-3 proteins control cell cycle. Especially, 14-3-3σ is a crucial p53 response gene and maintain in the G_2/M checkpoint by sequester Cdc25. The Cdc family is composed of three isoform, Cdc25A, Cdc25B and Cdc25C. Cdc25B and Cdc25C govern entry into M phase, while Cdc25A is vital for S phase entry [76]. This result shows that SSQ induces cell cycle arrest at G_2/M phase in HT-29 cells.

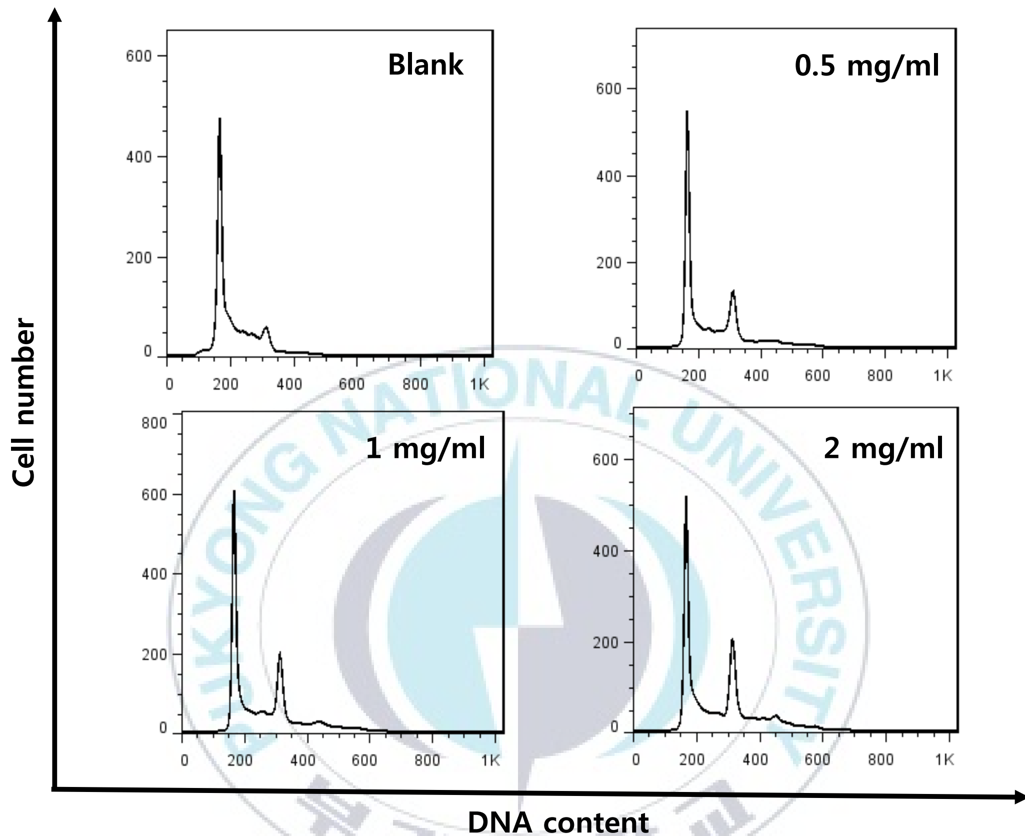


Figure 18. Cell cycle arrest by SSQ in HT-29 cells treated with different concentrations of SSQ for 24 h followed by analyzing of cell arrest using flow cytometry.

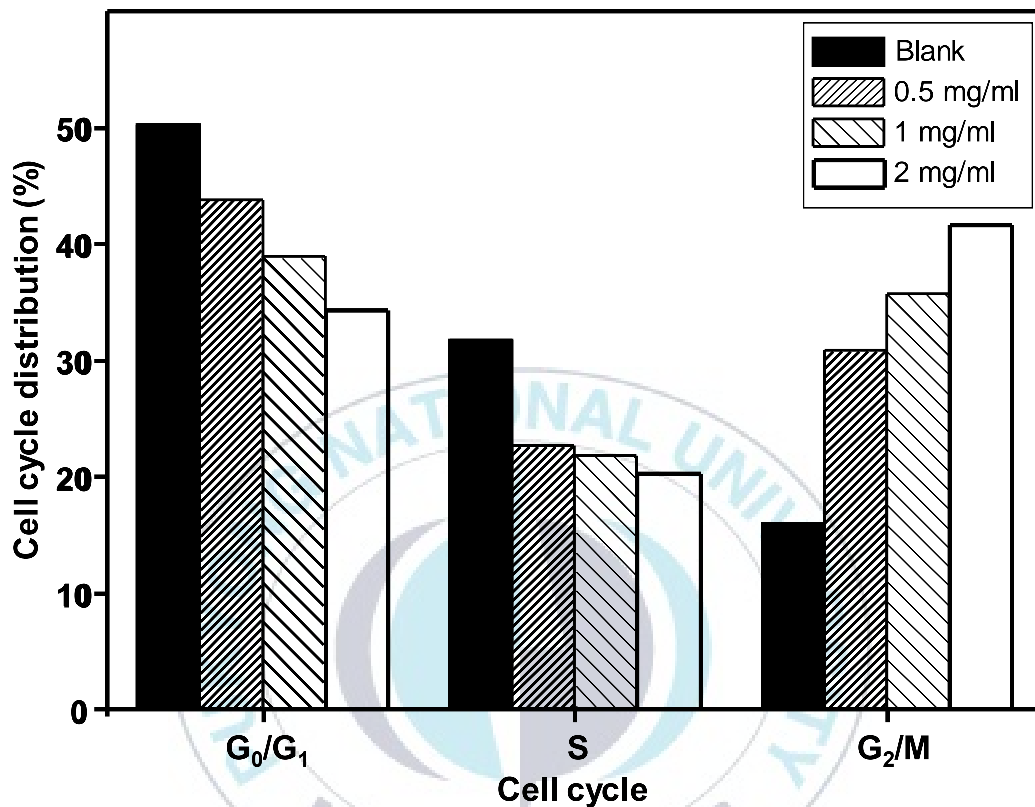


Figure 18. Cell cycle arrest by SSQ in HT-29 cells treated with different concentrations of SSQ for 24 h followed by analyzing of cell arrest using flow cytometry.

8. The apoptotic protein and mRNA expression

To establish whether SSQ induces apoptosis in HT-29 cells or not, the apoptotic protein and mRNA expression were detected by western blot analysis and RT-qPCR, respectively. Treatment with SSQ for 24 h induced the elevation of caspase-3, a pro-apoptotic protein in HT-29 cells (Figure 19). Additionally, the significant up-regulating the gene expression of caspase-3 resulted from SSQ in HT-29 cells. (Figure 20). The protein expression level was higher at 1 mg/ml of SSQ than the other concentrations. However, the gene expression level at 0.5 mg/ml of SSQ was 1.7 times as high as blank. The expression of gene may not be the same protein expression occasionally [77, 78]. Apoptosis pathway is various and complex. Among them, mitochondrial pathway is initiated mitochondrial dysfunction. Bax inhibit bcl-2, anti-apoptosis protein, resulting in releasing cytochrome c from mitochondria. This lead to activate caspase-9, initiator caspase, by forming apoptosome which is made of cytochrome c, caspase-9, and Apaf-1. Activated caspase-9 cleave and activate caspase-3, executioner, which cause PARP cleaving and cell death [34, 79-82]. These results suggest that SSQ causes apoptosis by up-regulating caspase-3 in HT-29 cells.

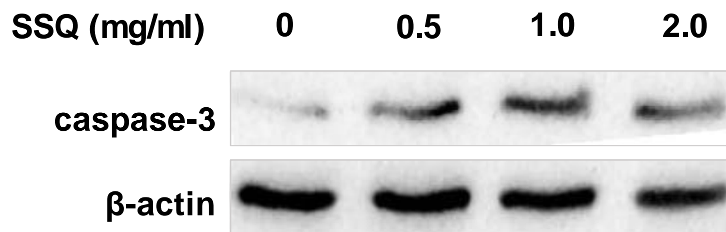
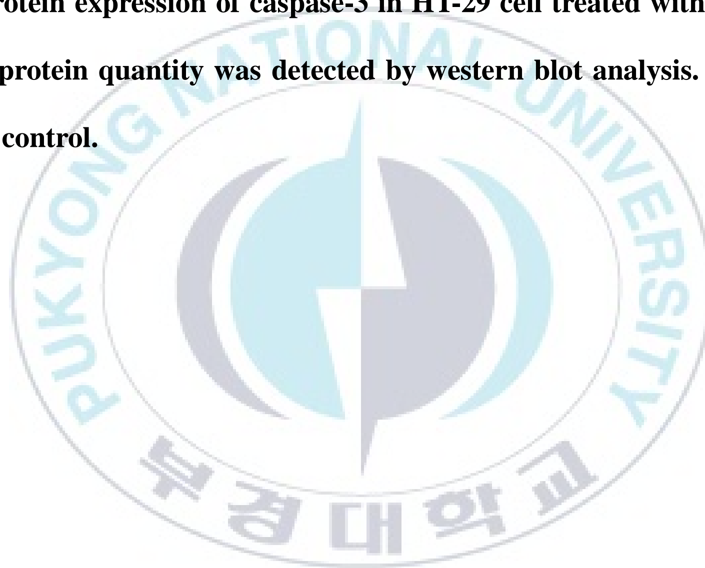


Figure 19. Protein expression of caspase-3 in HT-29 cell treated with SSQ for 24 h. The relative protein quantity was detected by western blot analysis. β-actin served for a loading control.



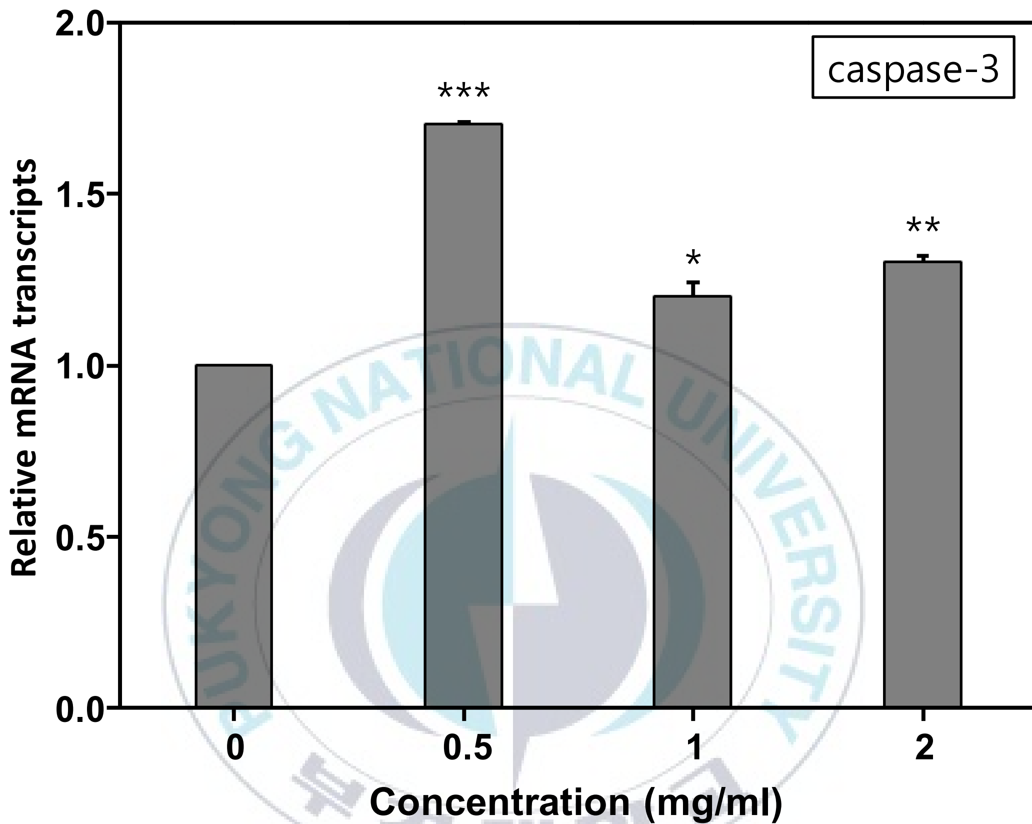


Figure 20. The relative mRNA expression of caspase-3 in HT-29 cell treated with SSQ for 24 h. The cDNA was synthesized for PCR. β-actin was a reference gene. Each value was expressed as the mean±S.D. (n=3). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ compared with untreated RNA sample, as analyzed by t-test.

Ⅳ. Conclusion

In this study, the protein hydrolysates of sea squirts (SSQ) were prepared by enzymatic hydrolysis with pepsin, and their antioxidant and anticancer effects were demonstrated. Antioxidant peptides derived from sea squirts were separated using consecutive chromatographic methods, and the sequences of separated peptides were identified as Met-Thr-Thr-Leu (P1, 464.58 Da), Leu-Glu-Trp (P2, 446.50 Da), and Tyr-Tyr-Pro-Tyr-Gln-Leu (P3, 845.95 Da). Among three peptides, ORAC values of P2 and P3 were comparable to glutathione, and P2 exhibited the highest DPPH radical scavenging activity. All three peptides were not effective as antioxidant agents in $ABTS^{+}$ radical scavenging activity and reducing power. However, they exhibited high levels of Fe^{2+} chelating activity compared to GSH. Furthermore, the anticancer activities of the SSQ were estimated against HT-29 colon cancer cells. SSQ inhibited cell proliferation against HT-29 colon cancer cell line. In the results of fluorescence microscopic analyses, generation of ROS was surged up and nuclear condensation was detected. After SSQ treatment for 24 h, the population of cells in G_2/M phase was up-regulated, which was accompanied by a decrease in G_0/G_1 and S phase cells. Western blotting and mRNA expression by RT-qPCR revealed caspase-3 activation for the detection of apoptotic pathway. These results confirm SSQ induces apoptosis in HT-29 cells in response to the

oxdative DNA damage. In summary, the results show that the peptic hydrolysate of sea squirts possesses not only strong antioxidant activity, but also higher anticancer activity in HT-29 colon cancer cell line. On the whole, the bioactive peptic hydrolysate of sea squirts could be a promising antioxidant and anticancer nourishment in new functional foods.

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멍게 (*Halocynthia roretzi*) 가수분해물의 항산화 및 항암 효과

김 삼 선

부 경 대 학 교

과 학 기 술 융 합 전 문 대 학 원

해 양 바 이 오 융 합 과 학 전 공

요 약

멍게의 펩신 가수 분해물에서 항산화 펩타이드를 얻기 위해 역상 고성능 액체 크로마토그래피 (reverse phase-high performance liquid chromatography), 겔 투과 크로마토그래피 (gel permeation chromatography) 등의 연속적인 분리 · 정제 과정을 거친 후, 크로마토그래피 질량 분광계 (liquid chromatography tandem mass spectrometry)를 이용하여 MTTL (464.58 Da, P1), LEW (446.50 Da, P2), YYPYQL (845.95 Da, P3)의 세 가지 펩타이드 서열을 확인하였다. 펩타이드의 항산화능을 분석하기 위해 DPPH 라디칼 소거능, $ABTS^{+}$ 라디칼 소거능, Fe^{2+} 켈라이팅 활성, ORAC, 환원력 등을 측정하였다. 세 가지 펩타이드 중 P2 의 DPPH 라디칼 활성이 가장 높게 나타났으며, P2 와 P3 의 ORAC 은 glutathione (GSH)에 상당하는 값을 보였다. Fe^{2+} 켈라이팅 활성은 세 가지

펩타이드 모두 GSH 보다 높게 나타났으나, ABTS+ 라디칼 소거 활성과 환원력은 GSH 의 값이 펩타이드들보다 높게 나타났다. 펩신 가수분해물을 HT-29 대장암 세포에 처리한 결과, 세포 생존율 저해, 세포 내 활성 산소종 (reactive oxygen species, ROS)의 증가, 손상된 DNA 를 확인하였다. 또한, 펩신 가수분해물의 농도에 따라 G_0/G_1 기와 S 기는 감소, G_2/M 기는 증가하여 G_2/M 기에서 세포 주기가 정지하는 것을 확인하였다. Western blot 분석과 RT-qPCR (quantitative real time polymerase chain reaction)을 통해 caspase-3 단백질과 mRNA 의 발현이 증가하는 것을 확인하였다. 그러므로 멍게의 펩신 가수 분해물은 HT-29 대장암 세포에 대해 활성 산소종을 증가시켜 세포 주기를 정지시키며 caspase-3 활성에 의한 apoptosis 를 유도한다. 또한, 탈지 과정을 거처 추출된 멍게의 펩신 가수 분해물에서 분리, 합성한 펩타이드들은 일부 라디칼에 대해 높은 항산화 활성을 가진다.

Keywords: Sea squirt, Bioactive peptides, Antioxidant, Anticancer, Apoptosis, HT-29