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Biological activities of 6-formyl umbelliferone and shrimp by-products on oxidative stress related diseases

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6-포르밀 움벨리페론과 새우 부산물의 산화적 스트레스 관련 질병에서의 생리활성 연구

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List of Abbreviations

(HTN)	Hypertension
(AD)	Alzheimer's disease
(ROS)	Reactive oxygen species
(RNS)	Reactive nitrogen species
(NADPH)	Nicotinamide adenine dinucleotide phosphate
(SOD)	Superoxide dismutase
(NF-κB)	Nuclear factor kappa B
(COX-2)	Cyclooxygenase-2
(iNOS)	Inducible nitric oxide synthase
(NO)	Nitric oxide
(IL)	Interleukin
(γ-IFN)	γ-interferon
(TNF-α)	Tumor necrosis factor-α
(LPS)	Lipopolysaccharide
(Aβ)	Amyloid beta
(APP)	Amyloid precursor protein
(6FU)	6-formyl umbelliferone
(MAPKs)	Mitogen-activated protein kinases
(ERK)	Extracellular signal-regulated kinases
(JNK/SAPK)	c-Jun N-terminal kinase/stress activated proteinkinases

(AA)	Arachidonic acid
(PGE2)	Prostaglandins E2
(EtOAc)	Ethyl acetate
(IF)	Immunofluorescence
(RT-PCR)	Reverse transcription polymerase chain reaction
(DPPH)	2,2-diphenyl-1-picrylhydrazyl
($ABTS^+$)	2,2´-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]
(XO)	Xanthine oxidase
(ACh)	Acetylcholine
(AChE)	Acetylcholinesterase
(PHA)	*P. hypsinotus* acetone extract
(PHC)	*P. hypsinotus* CH_2Cl_2 extract
(PBA)	*P. borealis* acetone extract
(PBC)	*P. borealis* CH_2Cl_2 extract
(PJA)	*P. japonica* acetone extract
(PJC)	*P. japonica* CH_2Cl_2 extract
(PBB)	*P. borealis* shrimp by-product
(IC_{50})	50 % inhibition value
(EC_{50})	0.5 of absorbance
(EDTA)	Ethylenediaminetretraceticacid
(ACE)	Angiotensin I-converting enzyme
(DH)	Degree of hydrolysis

Biological activities of 6-formyl umbelliferone and shrimp by-products on oxidative stress related diseases

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Abstract

The balance of reducing and oxidizing state is essential for cell viability, activation, proliferation, and organ function. Furthermore, disorder of the normal redox state result in toxic effects through the production of free radicals and peroxides. The excess reactive oxygen species (ROS) or dysfunction of the antioxidant system lead to oxidative stress which influenced a number of diseases such as inflammation, hypertension, Alzheimer's disease and could be neutralized by antioxidants for help in preventing diseases.

6-formyl umbelliferone (6FU) derived from *Angelica decursiva* showed the attenuation of the nitric oxide level and down-regulation of over-activated inflammation. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were decreased by 6FU treatment without cell cytotoxicity in lipopolysaccharide (LPS) stimulated RAW264.7 cells. Moreover, translocation activities of ERK1/2 and NF-κB from the cytoplasm to the nucleus were suppressed, which can inhibit translations of pro-inflammatory proteins such as iNOS and COX-2.

It is important to reduce biological wastes and study for sustainable resources. Inedible parts of shrimp were engaged to prevent for hypertension and Alzheimer's diseases. Around the eastern coast of Korea, the shrimp of the family Pandalidae such as *Pandalus borealis*, *Pandalus hypsinotus*, and *Pandalopsis japonica*are known for abundant in nutrients containing protein, calcium, vitamins. The shrimp by-products including the head, shell, and tail were extracted by acetone and dichloromethane (CH_2Cl_2). The acetone extract of *P. hypsinotus* exhibited more effective in antioxidant activity with high total carotenoid contents and inhibition of cholinesterase which is known for a good strategy against Alzheimer's disease.

P. borealis by-products were hydrolyzed by enzymes such as alcalase, protamex, flavourzyme, papain, and trypsin for determining the antioxidant and angiotensin Ⅰ-converting enzyme (ACE) inhibitory activity which is associated with hypertension. The protamex hydrolysate showed potent antioxidant and ACE inhibitory activities. The anti-oxidative effect of protamex hydrolysate fractions (< 3 kDa, 3-10 kDa, and > 10 kDa) showed more potent in < 10 kDa and ACE inhibitory activity showed in > 10 kDa, respectively.

The 6FU single compound is recommended for the treatment of over-expressed inflammation with antioxidant effect and shrimp by-products are valuable to preventive medicine in oxidative stress, hypertension, and Alzheimer’s disease.

Chapter Ⅰ

Overview of oxidative stress and diseases

1. Introduction

In recent years, persuasive evidence has revealed the causal interconnection between inflammation, hypertension (HTN), and Alzheimer's diseases (AD) in oxidative stress and this theory is based on the following observation that oxidative stress is associated with elevated inflammation, HTN, AD [1,2,3].

Oxidative stress reflects the constant manufacture of reactive oxygen species (ROS), and the regulation of reducing and oxidizing state is critical for cell viability, activation, proliferation, and organ function. Disorder in the normal redox state of cells can cause toxic effects through the production of free radicals and peroxides [4].

ROS are produced as a result of normal cellular metabolism and environmental factors such as cigarette smoke, air pollutants or ultraviolet radiation in living organisms. Cellular metabolism is the set of chemical reactions that essential to maintain life and involves complex sequences of controlled biochemical reactions. However, ROS in cellular metabolism are highly reactive molecules that can

damage cell structures including carbohydrates, nucleic acids, lipids, and proteins and modify their functions [5,6].

Considerable quantity of ROS including hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$)are produced by mitochondrial electron transport system and a lot of oxidase such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cycloxygenase, xanthine oxidase and uncoupled nitric oxide synthases in the process of normal aerobic metabolism [7,8].

The aerobic organisms are able to gain significant energy production efficiency compared with anaerobic organisms with the assistance of the mitochondrial respiratory chain. However, the advantage of aerobic respiration accompanies continuous electron leakage to O_2 during mitochondrial ATP synthesis [9].

The antioxidant systems include enzymatic and non-enzymatic antioxidants that are effective against negative influence of ROS such as superoxide dismutase (SOD), catalase, glutathione peroxidase, vitamins, and flavonoids [10]. However, the antioxidant systems can be overwhelmed by oxidative stress that contributes to various pathological conditions and diseases such as cancer, neurological disorders, hypertension, atherosclerosis, diabetes, acute respiratory distress syndrome, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and asthma [11-15].

2. Oxidative stress associated with inflammation, hypertension, and Alzheimer's disease

Oxidative stress is related to a wide spectrum of diseases, including chronic inflammation, hypertension, and Alzheimer's disease. Among these diseases, the sources of inflammation are widespread such as allergens, radiation, autoimmune, obesity, viral and microbial infections [16,17].

During an inflammatory reaction, leukocytes and mast cells are engaged to the site of damage, which leads to the accumulation of ROS as a result of increased uptake of oxygen [18.19]. Inflammatory cells also can produce soluble mediators including cytokines which act by more recruiting inflammatory cells to the site of damage and accumulating more reactive species. These primary mediators can activate not only signal transduction cascades but also the translocation of transcription factors to the nucleus such as nuclear factor kappa B (NF-κB) [20]. Induction of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1 (IL-1), IL-6 and IL-8 have also been reported to play a key role in oxidative stress induced inflammation [21]. NO, synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), is a short-lived free radical that is effective against pathogens and usually synthesized after counter by immunological or inflammatory stimuli [22,23].

The expressed iNOS produces much larger amounts of NO and is induced by cytokines including γ-interferon (γ-IFN), Tumor necrosis factor-α (TNF-α), IL-1, and lipopolysaccharide (LPS) which lead to the translocation of NF-κB from the cytoplasm to the nucleus [24]. The vicious circle result from continuous inflammatory and oxidative environments can damage healthy neighboring epithelial cells and may lead to carcinogenesis over a long period of time [25].

The control vessel tone and neuro humoral transmitter in hypertension have led to the growth of modern antihypertensive drugs including beta-blockers, AT-1 receptor blockers, calcium channel blockers, or angiotensin-converting enzyme inhibitors [26,27]. The pathophysiology of hypertension is highly complex but numbers of experimental studies suggest that this situation is associated with an increased formation of ROS from all layers of the vascular wall. The interrelationship between vascular oxidative stress and raised blood pressure is supported by the control of elevated blood pressure using antihypertensive drugs with the reduction of vascular oxidative stress [28-30]. In addition, decreasing vascular oxidative stress by antioxidants or moderation of ROS-producing enzymes has been shown to reduce blood pressure in animal models [31,32]. Oxidative stress may directly change vascular tone and function by modification of proteins or nucleic acids. The key mechanism of the oxidative stress on vascular tone is the reduction of NO bioavailability through the endothelial dysfunction and may also develop extracellular matrix alterations, vascular cell proliferation, and migration

as well as apoptosis. All of these processes contribute to the promotion of hypertension [33].

A wide range of studies for pathogenesis and progression of AD has reported that oxidative stress which is a serious imbalance between the production of ROS, reactive nitrogen species (RNS) and antioxidant defenses may make a contribution to AD development [34-36]. Sustained production of ROS and a defect in the antioxidant systems lead to neuronal loss in the majority of the neurodegenerative diseases. The brain of suffering AD patients has a high level of oxidative damage associated with the abnormal accumulation of amyloid beta (Aβ) and the deposition of neurofibrillary tangles. Aβ and its amyloid precursor protein (APP) have high-affinity binding sites for bio-metals such as iron, zinc, and copper on the N-terminal metal-binding domains which is a potent mediator of the highly reactive hydroxyl radical (OH•), and consequently supports the increase of oxidative stress [37-41]. In concordance with those findings, the production of hydrogen peroxide and other ROS seems to be related to the length of Aβ fragments. In addition, the binding of zinc which is associated with cognitive regions and memory of the brain such as the amygdala, neocortex, and the hippocampus is leading to the generation of toxic, fibrillary, Aβ aggregates. Consequently, the typical inflammatory response to non-soluble Aβ plaques involves the disruption of uncontrolled cerebral zinc release [42-45].

Mitochondria play crucial roles in cell respiratory processes, energy production, metabolism, intracellular signaling, apoptosis, and free radical generation but the mitochondrial dysfunction in the neurodegenerative diseases results in compromised energy production, impaired calcium buffering, activation of proteases and phospholipases, and increased oxidative stress [46,47].

3. Purpose of this study

Oxidation is a normal and necessary process that takes place in the body and it is the loss of electrons during a reaction by a molecule, atom, or ion. Free radical which is unstable atoms react promptly with other substances in an effort to make up the number of electrons in their outer shell that can damage the body and result in diseases and aging. On the other hand, it can help fight off pathogens when functioning properly [48]. It's impossible to completely evade free radical exposure and oxidative stress. However, the objective of this research is an effort to minimize the effects of oxidative stress and increase the levels of antioxidants for preventing various diseases caused by oxidative stress such as inflammation, Alzheimer's disease, and hypertension using environmental materials.

Chapter Ⅱ is focused on the anti-inflammatory effects of 6-formyl umbelliferone at the condition of increased nitric oxide levels.

Chapter Ⅲ describes on antioxidant and cholinesterase inhibitory activities which is one of the strategies preventing Alzheimer's disease using by-products of three pandalid shrimps.

Chapter Ⅳ represents on antioxidant and angiotensin Ⅰ converting enzyme inhibitory activities for preventing hypertension by enzymatically hydrolyzed northern shrimp by-products.

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Chapter Ⅱ

The inhibition of oxidative stress and anti-inflammatory effects of 6-formyl umbelliferone via the NF-κB and ERK/MAPK pathway on LPS-stimulated RAW 264.7 cells

1. Abstract

Inhibition of over activated inflammation has been reported as one of the most efficient strategies for treating inflammatory diseases. In this study, 6-formyl umbelliferone (6FU) was used to evaluate its anti-inflammatory effects on LPS-stimulated Raw 264.7 macrophages. 6FU inhibited chronic inflammatory processes including an increasing nitric oxide levels, expressions of pro-inflammatory genes and producing cytokines. Nitric oxide and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were decreased by 6FU treatment without cell cytotoxicity in LPS stimulated RAW264.7 cells. The expression levels of phosphorylated ERK1/2 and NF-κB were down-regulated in 6FU treated cells. Moreover, translocation activities of ERK1/2 and NF-κB from cytoplasm to nucleus were suppressed, which can inhibit translations of several proteins related to pro-inflammation such as iNOS and COX-2. Therefore, based on these results, it is suggested that 6FU could be a potential candidate for development of agents against chronic inflammation.

2. Introduction

Inflammation, an innate response in immune system, is triggered through the release of specific cytokines, noxious stimuli and tissue injury [1]. Inflammatory response can occur due to damages in tissue or organ and diseases such as cancer, cardiovascular disease, diabetes, obesity, rheumatoid arthritis, depression and Parkinson's disease. Therefore, the inhibition of over-activated inflammation is considered as one of the most efficient strategies for treating the inflammatory diseases [2,3].

In inflammation, primary mediators, such as NO and chemotactic cytokines including TNF-α, IL-1β, and IL-6 were stimulated by LPS which is a component of the gram-negative bacteria cell wall [4]. These mediators react as a toxic agent against infectious organisms and relate to modulation of cellular functions and homeostasis in innate immunity response. However, the over-production of NO lead to producing several proteins, such as mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which are implicated in chronic inflammatory reactions [4,5].

MAPKs consist of three major components including extracellular signal-regulated kinases (ERK),c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) and p38. These proteins are closely associated with a wide range of signaling cascades and play a key role in control of synthesis of inflammation mediators at both transcriptional and translational levels. Therefore, regulation of

MAPKs activity is considered as a target for anti-inflammatory therapeutics [6.7]. NF-κB, one of the downstream components in the MAPK signaling pathway, is translocated to the nucleus by its upstream stimuli. It promotes the transcription of pro-inflammatory genes producing the pro-inflammatory enzymes such as COX-2 and iNOS [8,9]. COX-2 is one of the pro-inflammatory enzymes, which converts arachidonic acid (AA) to prostaglandins E2 (PGE2) and contributes to the progress of chronic inflammatory diseases. In addition, the expression of COX-2 is implicated in the generation of reactive oxygen species in response to LPS stimulation [10,11].

Angelica decursiva has been used for a traditional medicinal plant in Korea, it shows curative effects for cough, thick phlegm and asthma [12]. 6FU was isolated from *A. decursiva* which is one of the uncommon coumarin derivatives in nature. It has been reported that coumarin and its derivatives have numerous pharmacological activities, including anticoagulant, vasodilator, anthelmintic, antimicrobial and antifungal capacity, however, the biological activities of 6FU were poorly studied up to date [12].

The aim of this study was to investigate the therapeutic potential of 6FU in inflammation. LPS-stimulated RAW 264.7 murine macrophages used to monitor anti-inflammatory activity through regulating the production of inflammatory mediators by suppressing expression of MAPKs and NF-κB signaling pathways.

3. Materials and Methods

3.1. Isolation of 6FU from *A.decursiva*

Isolated 6FU was provided by department of food and life science in Pukyung national university, and the isolation process was performed [12]. Briefly, whole *A. decursiva* powder was refluxed in methanol for 3 h and filtered. Then, filtrate was dried in vacuo at 40℃ for concentrating, followed by suspended in distilled water. This extract was partitioned by ethyl acetate (EtOAc), and then EtOAc fraction was served to silica gel chromatography using dichloromethane (CH_2Cl_2)-MeOH (10:1→0:1, gradient). After chromatography, we obtained 20 subfractions (F-1 to F-20), F-6 was partitioned with a silica gel chromatography using CH_2Cl_2-MeOH column (20:1→0:1, gradient). Following these processes, we yielded 6-formyl umbelliferone. Presence of single compound that is 6FU were confirmed by NMR studies.

3.2. Cell culture

Murine RAW264.7 macrophage cell line were obtained from American Type Culture Collection (Rockville, MD, USA) and incubated with DMEM containing 10 % FBS and 1 % penicillin/streptomycin solution at 37℃ with 5 % CO_2 and humidified atmosphere.

3.3. Cell viability

The cell viability in the presence of 6FU was measured by using WST-1 assay [13-15]. 1×10^4 of RAW 264.7 cell was seeded in each well of 96-well cell culture plates. RAW 264.7cells was cultured for 24 h with or without 1 μg/ml LPS and 50 or 100 μM of 6FU. After incubation, 10 μl of EZ-cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Gyeonggi, Korea) was added to each well and reacted for 3 h. Then, the absorbance was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, US) at 460 nm.

3.4. Nitrite assay

The nitrite concentration in the medium was measured with the Griess reaction [13-15]. RAW 264.7 cells were seeded in 24-well cell culture plates (5×10^4 cells/well) and pre-treated with 10, 25 and 50 μM of 6FU for 2 h and further incubated with 1 μg/ml LPS for 24 h. The supernatant of each wells (100 μl) was transferred to 96-well plates and Griess reagent was added in dark condition. Absorbance was measured at 540 nm and the calculated nitrate concentration was considered as an indicator of NO production.

3.5. Western blot analysis

To perform Western blot analysis, RAW264.7 cells were pre-treated with 6FU for 2 h and stimulated with or without LPS (1 μg/ml) for 6 h and 24 h. Whole cells

were harvested and lysed by the cell lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate and 0.1% SDS) (Intron Biotechnology, Gyenggi, Korea), then lysates were centrifuged at 14,000 rpm for 20 min. Separate nuclear and cytoplasmic proteins were obtained using NE-PER® nuclear and cytoplasmic extraction reagents (Life technology, Carlsbad, CA, USA) according to manufacturer's protocol. The protein concentration in the cell lysates were measured by Bradford reagent (Biosesang, Seongnam, Korea). Prepared proteins were separated by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the nitrocellulose membrane (Pall Life Sciences, Ann Harbor, MI, USA). After blocking with 1× PBST buffer containing 5 % skim milk for 2 h, the membranes were incubated overnight with primary rabbit antibodies at 4℃. The membranes were washed three times with PBST, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. The membranes were developed on X-ray film for visualization using an enhanced chemiluminescent (ECL®) detection solution (Pierce, Rockford, IL, USA).

3.6. Immunofluorescence (IF) staining

1×10^5 cells were plated at cover-glass bottom dishes (SPL Lifesciences, Pocheon, Korea) and pre-treated with 25 μM of 6FU for 30 min [16]. To investigate the nuclear translocation activity of p-ERK1/2 or NF-κB, LPS was treated for 6 h or 24

h respectively. Then, cells were stained with 1 μg/ml of DAPI and incubated for 15 min at 37℃, followed by washing with PBS buffer and fixed with 4 % formaldehyde for 15 min at room temperature. After incubation, these cells were blocked with 5 % rabbit normal serum containing 0.3 % Triton X-100 in 1× PBS for 1 h in dark condition and then cells were incubated with the anti-ERK1/2 (Thr202/Tyr204) or NF-κB p65 primary antibody at 4℃ for overnight. Following the reaction, the cells were washed with 1× PBS and then incubated for 50 min with anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor®488 conjugate) as secondary antibodies at room temperature in dark. After staining processes, cells were mounted using ProLong® Gold Anti-fade Reagent. Stained cells were observed by using Carl Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

3.7. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

For total RNA extraction, RAW264.7 cells were pre-treated with 6FU (25 and 50 μM) before the stimulated with LPS (1 μg/ml), then RNA was extracted by 2-mercaptoethanol and RNeasy plus mini kit according to manufacturer's protocol (Qiagen, Venlo, KJ, Netherlands). The concentration of total RNA was measured by nanodrop (MECASYS, Daejeon, Korea) and 2 μg of RNA was synthesized to cDNA using cDNA kit (Genetbio, Daejeon, Korea). The cDNA was amplified by

PCR using specific primers shown in Table 1 and amplified PCR products were observed on 2 % agarose gel.

3.8. Statistical analysis

One-way analysis of variance (ANOVA) with post-hoc tests and Dunnett's multiple comparison tests were used for determining the statistical significance of differences between experimental and control groups. Results were expressed as means ± standard deviation (SD) and all experiments were performed in triplicates independently. p-Values of 0.05 was considered as statistical significance.

4. Results and Discussion

4.1. Structure of 6FU

6-Formyl umbelliferone is an uncommon coumarin derivative in nature and has been isolated from *A. decursiva* which a perennial herb distributed on hillsides, grasslands, and sparse forests within China, Japan, and Korea. According to Ali et al. [12], the structure of the compounds was certificated by NMR spectroscopy and the chemical structure of umbelliferone and 6FU has been revealed as shown in Fig 2-1 [17-20].

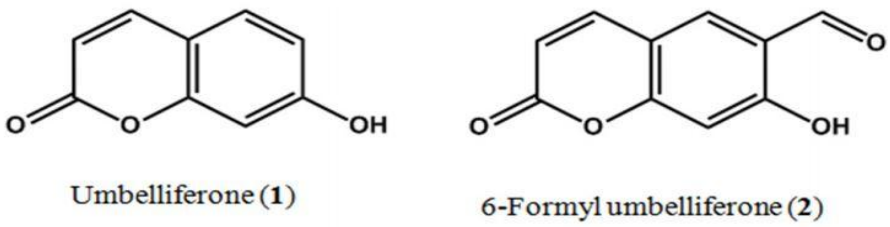

Figure 2-1. Structure of umbelliferone (1) and 6FU (2).

4.2. Cell viability in RAW 264.7

The cell viability of RAW 264.7 was measured by a WST-1 assay. RAW 264.7 cells were treated with or without 6FU (50 and 100 μM) and LPS (1 μg/ml) for 24h. As presented in Fig 2-2, 6FU, and LPS did not demonstrate any cytotoxicity on RAW 264.7 cells. Therefore, <100 μM 6FU was used for investigating its anti-inflammatory capacity in the absence of cytotoxicity.

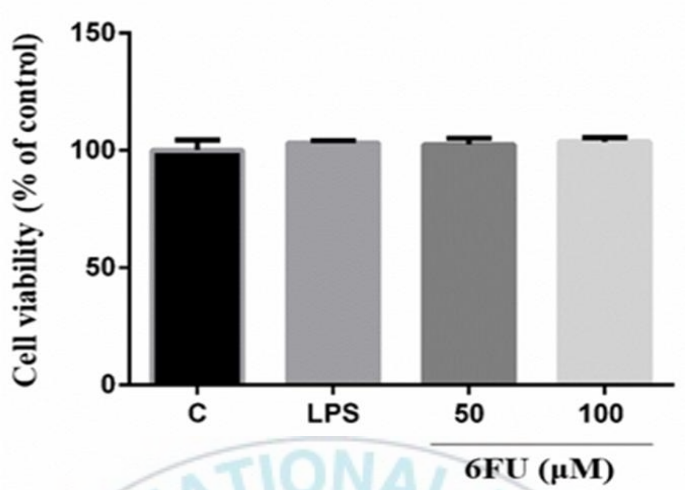

Figure 2-2. Effects of 6FU and LPS on cell viability in RAW 264.7 cells. Cell viability was determined by a WST-1 assay; cells were treated with 50 and 100 μM 6FU and 1 μg/ml LPS for 24 h.

4.3. Characterization of nitric oxide and specific cytokines

RAW 264.7 cells were pretreated with or without 6FU for 2h and subsequently stimulated with LPS for 24 h in order to evaluate the NO production level. The level of NO secretion was significantly increased in LPS-stimulated cells compared with non-stimulated cells (Fig 2-3A, $P<0.01$). However, the expression level of NO was decreased by treatment with 6FU in a dose-dependent manner. Western blot analysis was performed to investigate whether 6FU has an ability to modulate the expression of pro-inflammatory enzymes, including iNOS and COX-2. The results demonstrated that 6FU down-regulated the expression of iNOS and COX-2 in contrast with LPS only-treated cells (Fig 2-3B). 6FU significantly suppressed the mRNA expression of pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β, compared with LPS-stimulated RAW264.7 cells (Fig 2-4, $P<0.01$).

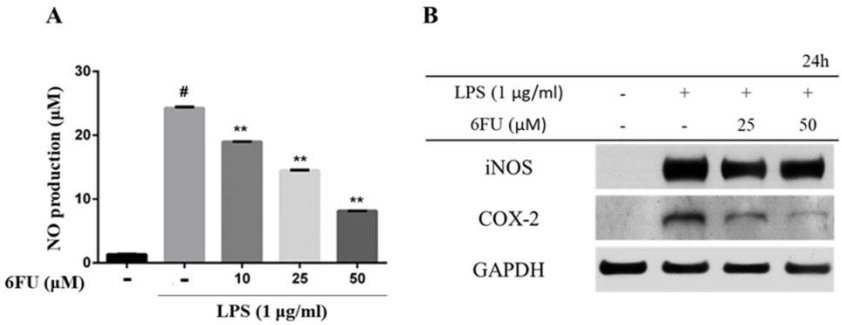

Figure 2-3. Effects of 6FU on NO secretion, and the expression level of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. (A) Production of NO was measured using Griess reagent. (B) Expression levels of iNOS and COX-2 were determined by western blotting in whole cell lysates. Data are presented as the mean ± standard deviation. n=3. #P<0.01 vs. non-treated group; **P<0.01 vs. LPS-stimulated cells.

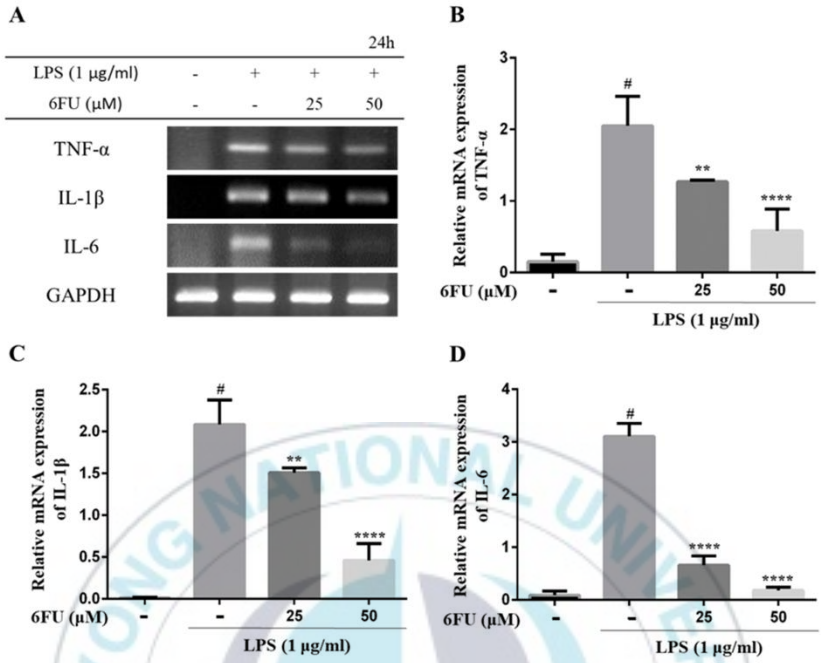

Figure 2-4. Regulation of the mRNA expression on pro-inflammatory cytokines. (A) The expression levels of mRNA were detected by RT-PCR. Relative mRNA expressions of (B) TNF-α, (C) IL-1β (D) and IL-6. Data are presented as the mean ± standard deviation. n=3. #P<0.01 vs. non-treated group; **P<0.01, ****P<0.00001 vs. LPS-stimulated cells.

4.4. Cell signaling pathway of MAPKs and NF- κB against inflammation

The LPS-induced phosphorylation level of MAPKs, including p-ERK, p38 and JNK were measured by western blot analysis. In LPS only-treated RAW 264.7 cells, the phosphorylation level of ERK, p38 and JNK were increased. However, only the expression of p-ERK1/2 was markedly decreased in the 6FU-treated LPS-stimulated RAW264.7 cells in a dose dependent manner compared with the phosphorylation level of p38 and JNK (Fig. 4A). In addition, the translocation of phosphorylated ERK1/2 to the nucleus was inhibited following pretreatment with 6FU in LPS-stimulated RAW 264.7 cells by western blotting and IF staining (Fig 2-5).

Investigate the activity of 6FU on nuclear translocation of NF-κB, western blot analysis and IF staining were performed, demonstrated that 6FU decreased the concentration of NF-κB in the nucleus in LPS-stimulated RAW 264.7 macrophages (Fig 2-6A). In contrast, the expression level of NF-κB in the cytoplasm was up-regulated by 6FU. Furthermore, 6FU inhibited nuclear translocation activity of NF-κB in LPS-treated cells (Fig2-6B). Therefore, 6FU decreased the expression and nuclear translocation of NF-κB in LPS-stimulated macrophages.

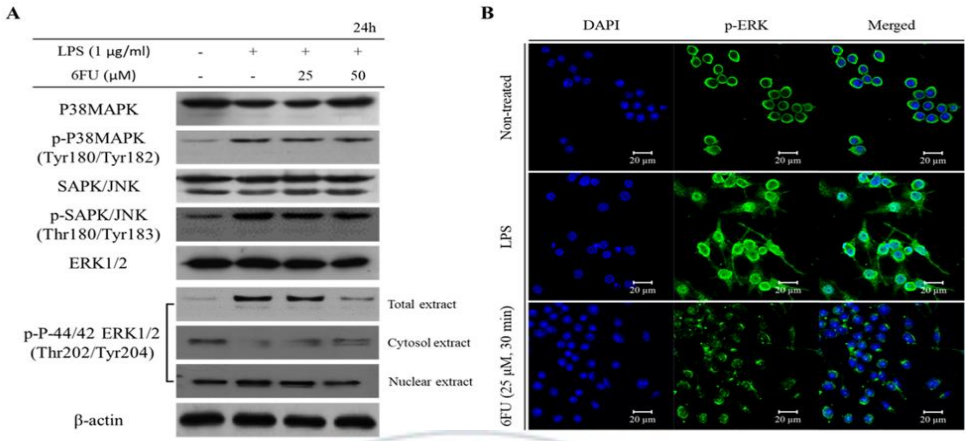

Figure 2-5. Detection of MAPKs expression and phosphorylation in LPS-activated RAW 264.7 macrophages. (A) Cells were pre-treated with 25 μM 6FU for 30 min prior to stimulation with 1 μg/ml LPS for 6 h. MAPK proteins and its phosphorylated forms were detected using western blotting in whole cell lysates. Only p-ERK1/2 was investigated for expression in cytoplasmic and nuclear levels. β-actin was used as a loading control. (B) Effect of 6FU on nuclear translocation of p-ERK1/2, detected by immunofluorescence staining. Blue indicates DAPI and green indicates p-ERK1/2.

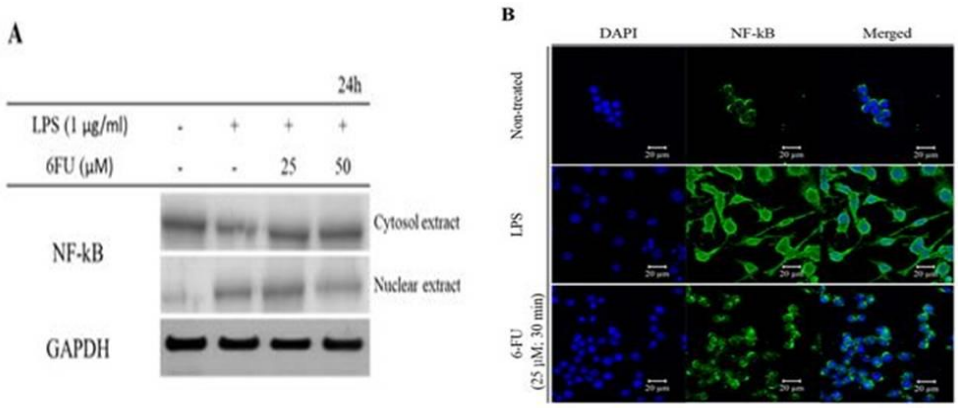

Figure 2-6. Translocation of NF-κB to the nucleus in LPS-stimulated RAW 264.7 cells. Effect of 6FU on nuclear translocation of NF-κB in LPS-stimulated macrophages. Cells were pretreated with 25 μM 6FU for 30 min and subsequently stimulated with LPS for 24 h. (A) Expression levels of cytoplasmic and nuclear NF-κB were investigated by western blot analysis and GAPDH was used as the loading control. (B) Nuclear translocation activity of NF-κB was detected using immunofluorescence staining. Blue indicates DAPI and green indicates NF-κB.

The aim of the present study was to investigate the anti-inflammatory properties of 6FU. The cytotoxicity of 6FU on RAW 264.7 and 293 cell lines was determined. The results demonstrated that 6FU did not exhibit any cytotoxicity on RAW 264.7 cells ≤100 μM. In 293 cells, 6FU did not demonstrate any significant cytotoxic effect ≤50 μM. However, 100 μM 6FU in 293 cells resulted in 88.1 % cell viability. These results suggested that ≤50 μM 6FU did not demonstrate any cytotoxicity on the murine and human cell lines, thus, ≤50 μM 6FU was used for further investigation. It was investigated whether 6FU may regulate production of NO in LPS-stimulated RAW 264.7 murine macrophages, as NO is one of the principal contributors to the formation of reactive nitrogen species and mediates the inflammatory response [21-22]. NO production was decreased by 6FU without cytotoxic effects, compared with LPS-only-treated RAW264.7 cells. It has been demonstrated that iNOS catalyzes the formation and release of a large amount of NO, and COX-2 serves an essential role in the inflammatory response as a precursor of various biological active mediators, including PGE_2 [23, 24]. The present results demonstrated that 6FU markedly inhibited the protein expression level of iNOS and COX-2 against a stimulus of inflammation in RAW 264.7 cells. Therefore, it was suggested that 6FU has an ability to suppress production of NO and PGE_2 through down-regulation of iNOS and COX-2 expression. Additionally, endotoxins, including LPS in the present study, stimulate macrophages to express

cytokines, including TNF-α, IL-1β and IL-6, which activate inflammation-associated signaling pathways [25,26]. It was demonstrated that the mRNA expression levels of TNF-α, IL-1β and IL-6 were significantly decreased by 6FU compared with LPS-stimulated cells. These results suggested that 6FU attenuated the inflammatory response by regulating expression of iNOS, COX-2 and numerous pro-inflammatory cytokines. Based on these results, it was hypothesized that 6FU may regulate the cellular signaling pathway, which is associated with the production of NO and pro-inflammatory cytokines in macrophages. To further investigate the mechanisms of NO and cytokine production, the expressions of MAPK signaling proteins were examined, which have been demonstrated to regulate various cellular activities, cell proliferation, differentiation, migration and the inflammatory response [6]. MAPK signaling pathway proteins consist of ERK1/2, JNK/SAPK and p38, which mediate intracellular signaling initiated by extracellular stimuli. Among them, activated ERK1/2 serves an essential role in the regulation of the inflammatory response by promoting phosphorylation of its downstream proteins [27,28]. It was identified that p-ERK1/2 was markedly decreased by treatment with 6FU; however, expression of p-p38 and p-JNK/SAPK did not demonstrate any difference.

Furthermore, the nuclear translocation activity of p-ERK1/2 was inhibited by 6FU in LPS-stimulated RAW 264.7 macrophages. These results suggested that 6FU

inhibits the ERK-mediated inflammatory response by suppressing phosphorylation and translocation of ERK1/2.NF-κB additionally serves as one of the key regulators of the inflammatory gene expression, which induces the synthesis of pro-inflammatory cytokines, including iNOS and COX-2 [8]. It has been investigated that inflammatory stimuli activate NF-κB translocation from the cytoplasm to the nucleus, and its transcriptional activity through degradation of inhibitor of NF-κB by proteasomes [29,30].

It was observed that 6FU suppressed the translocation activity of NF-κB from the cytoplasm to the nucleus by western blot analysis and IF staining, which demonstrated the anti-inflammatory capacity of 6FU.In conclusion, the present results demonstrated that 6FU down-regulates the production of NO and pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 by inhibition of the inflammation-associated signaling pathways, including ERK1/2 and NF-κB. In the present study, it was identified that 6FU has potential as one of the therapeutic candidates for chronic inflammation.

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ChapterⅢ

Antioxidant and cholinesterase inhibitory activities of shrimp by-products (family Pandalidae)

1. Abstract

The antioxidant and cholinesterase inhibitory activities of the dichloromethane (CH_2Cl_2) and acetone extracts of the by-products (heads, shells, and tails) of family Pandalidae including *Pandalus borealis*, *P. hypsinotus*, and *P. japonica* were investigated and their bioactivities were compared. The antioxidant activity of extracted three shrimp by-products using organic solvent were evaluated followed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^+$) radical scavenging activities, reducing power and xanthine oxidase (XO) inhibitory activity assays and Ellman's colorimetric method was engaged for cholinesterase inhibitory activity. The extracts of *P. hypsinotus* showed the potent antioxidant and cholinesterase inhibitory activities. The acetone extracts exhibited to more effective activities of antioxidant and cholinesterase inhibition than those of the CH_2Cl_2 extracts. In addition, the total carotenoid contents of the acetone extract more potent activity than CH_2Cl_2 extracts and the carotenoid contents may influence antioxidant and cholinesterase inhibition. According to the results, the shrimp by-products are able to role as a nutraceutical agent to prevent oxidative stress and Alzheimer's disease.

2. Introduction

Alzheimer's disease (AD) is a progressive neurologic disorder results in irreversible loss of neurons, especially in the cortex and hippocampus [1]. The clinical features are progressive impediment in memory, judgment decision making, and language. The pathological hallmarks are extracellular senile plagues containing the peptide beta-amyloid, neurofibrillary tangles, and neuronal loss [2]. The reason of AD has not yet been clarified but it is one of the convincing theories that cholinergic hypothesis of deficiency in the brain levels of cerebral neuro-transmitters including acetylcholine (ACh) which is hydrolyzed by acetylcholinesterase (AChE) and butyrylcholine (BCh) [3, 4]. Butyrylcholinesterase (BChE) inhibition is considered a potentially important aspect of treating AD because BChE activity is increased by 40 % – 90 % during the progression of AD and may have a greater role in cholinergic transmission [5, 6]. Furthermore, excessive free radicals observed in AD are associated with the pathological changes produced by various oxidative stress [7]. The fact that age is a crucial risk factor and provides considerable support for the free radical hypothesis in AD because effects of the invasions by free radicals, particularly those produced by Reactive oxygen species (ROS), can accumulate over the years [8]. Accordingly, the activation of cholinergic interaction by decrease of cholinesterase which hydrolyzes the cholinergic neuromediators and reduction of oxidative stress has been used as a key role in the treatment of AD [9, 10].

Around the eastern coast of Korea, the shrimp of the family Pandalidae such as *P. borealis*, *P. hypsinotus*, and *P. japonica* are distributed in the deep sea at depths of 40-1,300 m widely [11]. They are the most popular shrimp species and abundant in nutrients containing protein, calcium, and vitamins. But the inedible parts of the shrimp including the head, shell, and tail can occur environmental pollution with high volume of these by-products [12]. However, the shrimp by-products consist of various bioactive materials including pigments, chitin, chitosan, carotenoprotein and have a high economic value as a helpful raw material [13, 14]. The use of their valuable constituents such as β-carotene, α-carotene, lycopene, and lutein are effective in treating cancer, cardiovascular disease, osteoporosis, and hypertension [15, 16]. However, there are few studies on the potential of shrimp by-products of the family Pandalidae and their carotenoid contents against AD.

The aim of this study is determining that effects on the antioxidant and ChEs activities by extracting the by-products of three shrimp species using acetone and CH_2Cl_2 and the correlation between the carotenoid contents and their bioactivities.

3. Materials and methods

3.1. Preparation of samples

The *P. hypsinotus, P. borealis and P. japonica* were purchased from the market of Donghae-si, Gangwondo, Korea (Fig3-1). The lyophilized shrimp by-products (1 kg) were stored at -20℃ until use. The 20 g of lyophilized samples were extracted with 50 mL acetone or CH_2Cl_2, and then used in the analysis of various lipophilic materials such as carotenoids (Fig 3-2) [17, 18]. The yields of the extracts were shown by following *P. hypsinotus* acetone extract (PHA, 2.72 g), *P. hypsinotus* CH_2Cl_2 extract (PHC, 2.49 g), *P. borealis* acetone extract (PBA, 2.97 g), *P. borealis* CH_2Cl_2 extract (PBC, 2.52 g), *P. japonica* acetone extract (PJA, 2.62 g), and *P. japonica* CH_2Cl_2 extract (PJC, 2.18 g).

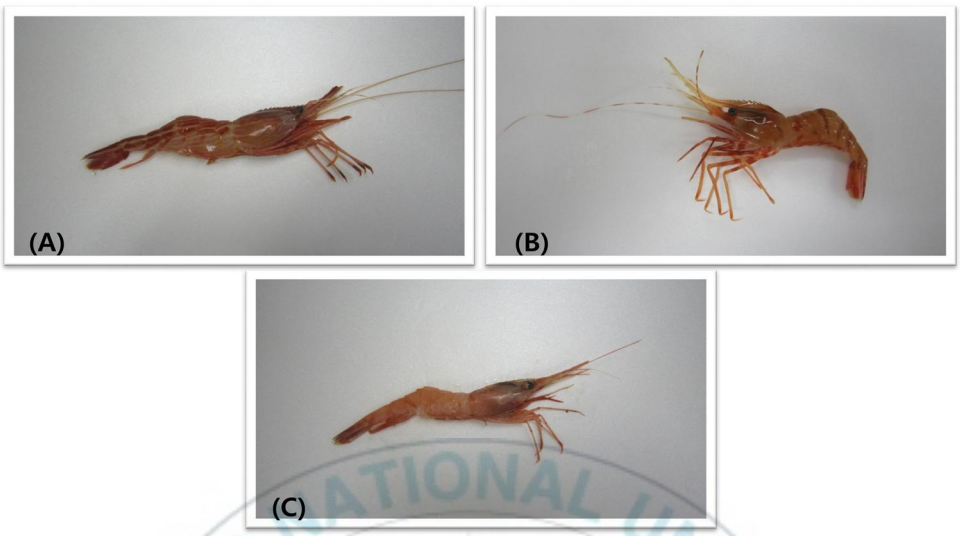

Figure 3-1. The family Pandalidae. (A), *P. japonica*. (B), *P. hypsinotus*. (C) *P. borealis*. They were lyophilized and stored at -20℃ until use.

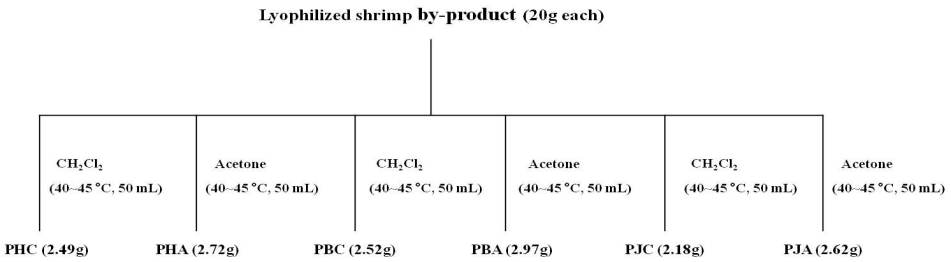

Figure 3-2. Preparation of samples. The 20 g of lyophilized shrimp by-products were extracted with 50 mL acetone or CH_2Cl_2.

3.2. DPPH radical scavenging activity

The method of Blois was modified for the determination of DPPH radical-scavenging activity [19]. 6.5 mM of DPPH solution was added to samples before incubation in the dark for 10 min at room temperature. The absorbance was measured at 540 nm by using a spectrophotometer (BIOTEK US/MQX 200, USA). The DPPH radical scavenging activity of each sample was expressed as the 50 % inhibition value (IC_{50}) and L-ascorbic acid was used as a positive control.

3.3. $ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activity was measured using the method of Re et al. [20]. $ABTS^+$ solution was diluted to the absorbance of 0.75 ± 0.03 at 734 nm with water. $ABTS^+$ solution was added to samples of different concentrations. The samples were measured at 734 nm after incubation in the dark for 10 min. The $ABTS^+$ radical scavenging activity of each sample was expressed as an IC_{50} value and Trolox was used as a positive control.

3.4. Reducing power

The reducing power of the extracts was examined using a method of Jayaprakasha et al. [21]. The extracts in 10 % dimethylsulfoxide (DMSO) were mixed with the 10 mg/mL of potassium ferricyanide ($K_3Fe(CN)_6$) and 0.2 M sodium phosphate buffer (pH 6.6). The mixtures were incubated for 20 min at 50℃. Then, 100

mg/mL of TCA were added before the mixture centrifugation at 2,000 × g for 10 min. After centrifugation, 100 μL of supernatant was mixed with 1 mg/mL of iron (III) chloride. L-ascorbic acid was used as a positive control and the sample concentration resulting in 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm.

3.5. XOinhibitory activity

The method of Nongonierma and FitzGerald was modified for the investigation of XO inhibition assays [22]. The sample were treated in a 96-well plate containing 12.5 μM of ethylenediaminetretraceticacid (EDTA), hydroxylamine phosphate (25 μM), and xanthine (0.125 mM). Then, 50 μL XO (0.1 U/mL) was treated and incubated at 37°C for 30 min. The absorbance of the uric acid formed was measured at 290 nm. The XO inhibitory activity of each sample was expressed as an IC_{50} value and BHA was used as a positive control.

3.6. ChEs inhibitory activity

The inhibitory activity of ChEs was performed using the spectrophotometric method of Ellman [23]. The reaction mixture including 140 μL of 100 mM sodium phosphate buffer (pH 8.0), 20 μL of the sample, and 20 μL of either AChE (0.36 U/mL) or BChE (0.36 U/mL). The solution was measured in a 96-well plate and mixed. After incubation at room temperature for 15 min, 10 μL of 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB) solution and 10 μL acetylthiocholine iodide or

butylthiocholine iodide were added. The absorbance of Ball reactions was measured at 412 nm and eserine was used as a positive control. The ChEs inhibitory activity was expressed as an IC_{50} value.

3.7. Total carotenoid contents

The mehod of Tolasa was used to evaluate the total carotenoid contents with a modification [24]. The astaxanthin standard (3.0 mg) and 3,5′-di-tert-butylhydroxytoluene (BHT) (100 mg) were dissolved in 10 mL of CH_2Cl_2. Then, 1 mL of this stock solution was diluted to 10 mL with n-hexane before the measuring absorbance in a UV-visible spectrophotometer at a wavelength between 350 and 600 nm. The maximum absorbance was observed at 472 nm and the concentration of astaxanthin was measured and corrected according to the following formula:

$$C_{astaxanthin}(\mu g/mL) = A \times 10{,}000/E,$$

where $C_{astaxanthin}$ is the total carotenoid contents, A is the absorbance 472 nm, E is the extinction coefficient, and 10,000 is the scale factor. 0.1, 0.25, 0.50, 0.75, 1.0, 1.25, and 1.5 mL of stock solutions were placed in separate 10 mL flasks and made up to the appropriate volume with n-hexane to provide data to plot the standard curve.

3.8. Statistical analysis

The data were analyzed using analysis of variance following the general linear model procedure (SAS Institute, Cary, NC, USA). Duncan's multiple range test was applied to confirm the significance of differences between means ($P < 0.05$).

4. Results and Discussion

4.1. Antioxidant activity

ROS such as superoxide (O_2-), hydroxyl radical (HO), and hydrogen peroxide (H_2O_2) are generated by normal metabolism and respiration [25] and generated ROS in the body can occur various diseases including atherosclerosis, cancer, rheumatoid arthritis, and neurodegenerative diseases through cell or nucleic acid damage [26-28]. The by-products of three shrimp species were extracted by acetone and CH_2Cl_2 that were used to compare antioxidant activities with ChEs inhibitory activities based on the DPPH and $ABTS^+$ radical scavenging activities (Table 3-1, Fig3-3), xanthine oxidase inhibitory activity, and reducing power assays (Table 3-2, Fig3-4). The one of the shrimp extracts, PHA was exhibited the most potent DPPH and $ABTS^+$ radical scavenging activities with IC_{50} values of 1.43 ± 0.02 and 1.44 ± 0.02 mg/mL, respectively. But, the activities of all extracts were not exceeded than those of the positive controls, L-ascorbic acid and trolox. The XO inhibitory activities were shown in Table 3-2: PHA (IC_{50} = 0.03 ± 0.00 mg/mL) > PHC (IC_{50} = 0.11 ± 0.00 mg/mL) > PBA (IC_{50} =0.12 ± 0.01 mg/mL) > PBC (IC_{50} = 0.57 ± 0.01 mg/mL) > PJA (IC_{50} = 1.03 ± 0.03 mg/mL) > PJC (IC_{50} = 1.30 ± 0.03 mg/mL).PHA appeared to much stronger than the positive control, BHA (IC_{50} = 0.14 ± 0.00 mg/mL). Moreover, PHC and PBA against the XO showed activity similarly to that of BHA used as a positive control. But PJA

showed no notable activity on XO inhibition. The electron donation capacity of extracts by the acetone and $CH_2Cl_2$is shown in Table 3-2. PHA (EC_{50} = 0.57 ± 0.01 mg/mL) appeared to the greatest reducing power but other extracts showed moderate electron donation capacity. The reducing power of all extracts was lower than that of L-ascorbic acid, used as a positive control. These results suggest that the acetone extracts of the shrimp by-products be concerned more potent antioxidant activity than those of the CH_2Cl_2 extracts including DPPH and $ABTS^+$ radical scavenging activity, as well as XO inhibitory activity and reducing power.

Samples	Radical Scavenging activity (IC_{50}, mg/mL)	
	DPPH	$ABTS^+$
PHA	1.43 ± 0.02[1,f]	1.44 ± 0.02[f]
PHC	3.22 ± 0.03[b]	3.34 ± 0.00[e]
PBA	2.49 ± 0.01[d]	6.76 ± 0.02[c]
PBC	3.45 ± 0.01[a]	6.96 ± 0.03[b]
PJA	2.12 ± 0.01[e]	6.46 ± 0.03[d]
PJC	2.98 ± 0.01[c]	7.94 ± 0.01[a]
L-ascorbic acid	0.02 ± 0.00[g]	-
Trolox	-	0.11 ± 0.00[g]

Table 3-1. DPPH and $ABTS^+$ radical scavenging activities. The IC_{50} values (mg/mL) of DPPH and $ABTS^+$ radical scavenging activities were expressed as the means ± SD. L-ascorbic acid and trolox were used as positive controls.

[1]The different superscripts are significantly distinct by Duncan's multiple range test ($P< 0.05$).

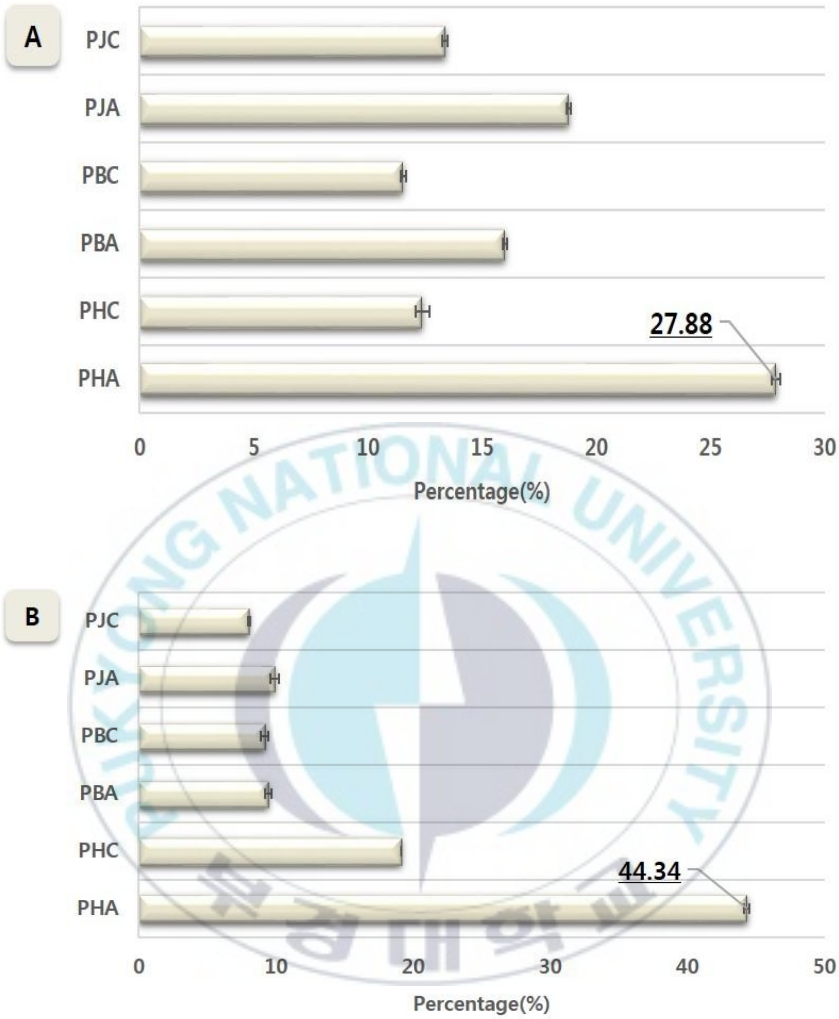

Figure 3-3. DPPH and $ABTS^{+}$ radical scavenging activities. A: DPPH and B: $ABTS^{+}$ radical scavenging activities were described as a percentage.

Samples	Xanthine oxidase (IC50, mg/mL)	Reducing power (EC50, mg/mL)
PHA	0.03 ± 0.00[1,f]	0.57 ± 0.01[e]
PHC	0.11 ± 0.00[e]	1.08 ± 0.27[d]
PBA	0.12 ± 0.01[de]	1.56 ± 0.01[c]
PBC	0.57 ± 0.01[c]	6.02 ± 0.00[a]
PJA	1.03 ± 0.03[b]	1.60 ± 0.00[c]
PJC	1.30 ± 0.03[a]	3.35 ± 0.00[b]
BHA	0.14 ± 0.00[d]	-
L-ascorbic acid	-	0.03 ± 0.00[f]

Table 3-2. XO inhibitory activity and Reducing power. The IC_{50} value of XO inhibitory activity and EC_{50} value of Reducing power were expressed as the means ± SD respectively. BHA and L-ascorbic acid were used as positive controls.

[1]The different superscripts are significantly distinct by Duncan's multiple range test ($P< 0.05$).

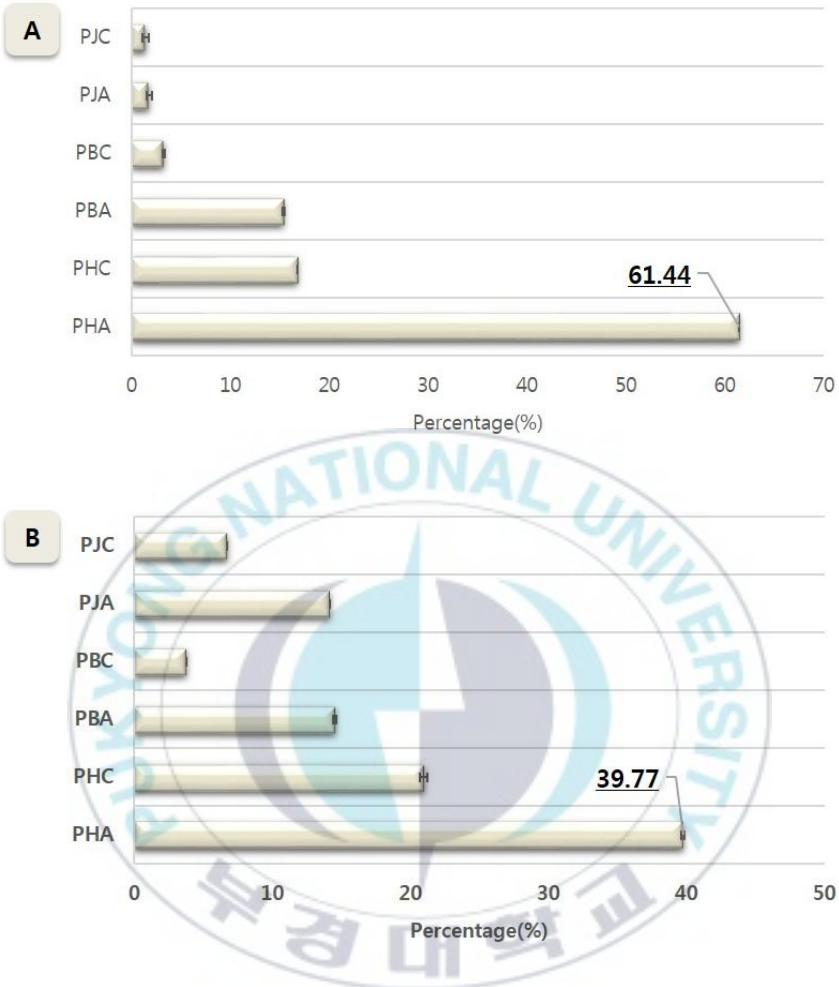

Figure 3-4. XO inhibitory activity and Reducing power. A: XO and B: Reducing power were described as a percentage.

4.2. ChEs inhibitory activity

The ACh and BCh are the member of neurotransmitters and can be hydrolyzed by ChEs [3]. Lack of neurotransmitters at the synaptic site of the brain is associated with memory and cognitive deficits [29]. Therefore, prevention of neurotransmitter deficiency through the inhibition of ChEs activation has been accepted as the most effective treatment in the pathology of AD[30]. The ChEs inhibitory activity of the acetone and CH_2Cl_2 extracts of the shrimp by-products was described in Table 3-3 (Fig 3-5). PHA exhibited to remarkable inhibitory activity against AChE and BChE with IC_{50} values of 0.20 ± 0.01 and 0.67 ± 0.03 mg/mL, respectively. PJA(IC_{50} = 0.30 ± 0.03, 1.16 ±0.02 mg/mL) and PBA (IC_{50}=0.36±0.01,1.18±0.02mg/mL) also exhibited to exceptional ChEs inhibitory activity. However, The CH_2Cl_2 extracts, PHC (0.56 ± 0.02 and 0.76 ± 0.03 mg/mL), PBC (0.64 ± 0.02 and 2.05 ± 0.02 mg/mL), and PJC (0.94 ± 0.03 and 1.76 ±0.04 mg/mL), showed moderate ChEs inhibitory activity. As a result, Acetone extracts of shrimp by-products more suitable than CH_2Cl_2 extracts for preventing ChEs hydrolyzation activity.

Samples	Cholinesterase inhibitory activity (IC_{50}, mg/mL)	
	AChE	BChE
PHA	0.20 ± 0.01[1,f]	0.67 ± 0.03[e]
PHC	0.56 ± 0.02[c]	0.76 ± 0.03[d]
PBA	0.36 ± 0.01[d]	1.18 ± 0.02[c]
PBC	0.64 ± 0.02[b]	2.05 ± 0.02[a]
PJA	0.30 ± 0.03[e]	1.16 ± 0.02[c]
PJC	0.94 ± 0.03[a]	1.76 ± 0.04[b]
Eserine (μg/mL)	0.11 ± 0.00[g]	0.13 ± 0.00[f]

Table3-3. Cholinesterase inhibitory activities. The IC_{50} values (mg/mL) of AChE and BChE inhibitory activities were expressed as the means ± SD. Eserine (μg/mL) was used as positive controls.

[1]The different superscripts are significantly distinct by Duncan's multiple range test ($P< 0.05$).

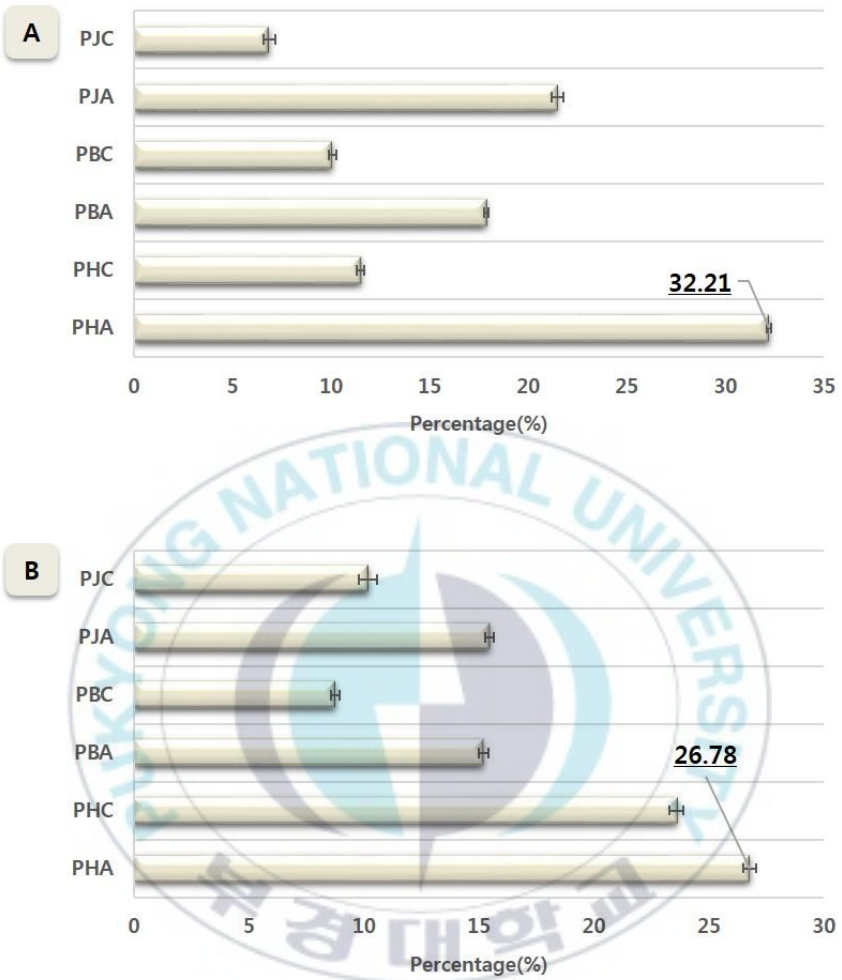

Figure 3-5. Cholinesterase inhibitory activities. A: AChE and B: BChE were described as a percentage.

4.3. Total carotenoid contents

Carotenoids are known for effective natural pigments which exist in terrestrial plants, seaweeds, microorganisms, and crustaceans by preventing that oxidative damage, cardiovascular disease, and cancer [31-34]. Therefore, the evaluation of the total carotenoid contents of the acetone and CH_2Cl_2 extracts are important for the correlation between the carotenoid contents and biological activities. The total carotenoid contents of the acetone and CH_2Cl_2 extracts showed in Table 3-4 (Figure 3-6): PHA (11.12 ± 0.11 mg/g) > PBA (11.06 ± 0.00 mg/g) > PBC (9.51 ± 0.39 mg/g) > PJA (8.93 ± 0.00 mg/g) > PHC (7.76 ± 0.19 mg/g) > PJC (6.79 ± 0.19 mg/g). As a results, the acetone extracts including PHA, PBA, and PJA were exhibited to higher total carotenoid contents compared with the CH_2Cl_2 extracts. In this study, total carotenoid contents seem to be related to antioxidant and ChEs inhibitory activities, and it has been suggested that effect of carotenoids in neurodegenerative diseases, such as AD and oxidative stress [15].

Samples	Total Carotenoid contents (mg/g)
PHA	11.12 ± 0.11[1,a]
PHC	7.76 ± 0.19[d]
PBA	11.06 ± 0.00[a]
PBC	9.51 ± 0.39[b]
PJA	8.93 ± 0.00[c]
PJC	6.79 ± 0.19[e]

Table 3-4. Total carotenoid contents. The method of Tolasa was used to confirming the total carotenoid contents with a modification.

[1]The different superscripts are significantly distinct by Duncan's multiple range test ($P< 0.05$).

Figure3-6. Total carotenoid contents were described as a percentage.

High levels of ROS and low antioxidant capacity occur to damage the human brain and lead to neurodegenerative diseases. Moreover, the considerable decrease of carotenoids level has been reported in Alzheimer's and Parkinson's diseases and vascular dementia patients [35]. Therefore, the effects of antioxidant and ChE inhibitory activities of shrimp by-product extracts are possibly associated with carotenoids contents.

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ChapterⅣ

Antioxidant and angiotensin Ⅰ-converting enzyme inhibitory activities of *Pandalus borealis* by-products hydrolysate

1. Abstract

In this study, the northern shrimp (*Pandalus borealis*) by-products (PBB) was hydrolyzed by enzymes such as alcalase, protamex, flavourzyme, papain, and trypsin for determining the antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities. The antioxidant activity of five enzymatic hydrolysates of PBB was examined through the 2, 2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] ($ABTS^{+}$) radical scavenging, reducing power, superoxide dismutase (SOD)-like activities. Of these five enzymes, the protamex hydrolysate appeared to the significant antioxidant and ACE inhibitory activity. Therefore, the PBB protamex hydrolysate was fractionated using two ultrafiltration membranes 3 and 10 kDa (below 3 kDa, between 3 and 10 kDa, and above 10 kDa). These three fractions were measured that the total amino acids composition, antioxidant, and ACE inhibitory activities.

Among these fractions, the < 3 kDa and 3–10 kDa fractions exhibited to more potent $ABTS^{+}$ radical scavenging activity than that of > 10 kDa fraction, but the >

10 kDa fraction showed the potent reducing power than others. In addition, 3–10 kDa and > 10 kDa fractions appeared to the significant ACE inhibitory activity. These results indicated that the high molecular weight enzymatic hydrolysate derived from PBB more suitable for regulating oxidative stress and prevent hypertension.

2. Introduction

Hypertension is one of the primary reasons to the cardiovascular disease which leads to stroke, coronary artery disease, and sudden cardiac death [1]. The renin-angiotensin system (RAS) is important in adjusting blood volume and hypertension and ACE plays a key role in the functioning of the RAS [2, 3]. Renin changes angiotensinogen to angiotensin I and ACE catalyzes cleavage of angiotensin I into angiotensin II that is the main active element of hypertension [4, 5]. The increased angiotensin II occur vasoconstriction with increased blood volume and water possession [6]. Several ACE inhibitors have been studied to inhibit angiotensin II-mediated hypertension. Most synthetic ACE inhibitors such as enalapril, captopril, ramipril, Lisinopril and benzapril have been reported with their side effects including skin rash, dry cough, and loss of taste [7]. Approximately 50 % of the catch contained the valuable and useful bioactive materials such as pigments, chitin, caroteno-protein and chitosan [8-10] and utilization of protein has been studied [11-13]. Therefore, it is appropriated that investigation of new ACE inhibitors with side effects from natural resources.

A recent research has been reported that oxidative stress is a crucial factor for hypertension. Moreover, over expressed reactive oxygen species affects cellular functions such as decrease of the bioavailability of endothelial nitric oxide and enhance of low-density lipoprotein oxidation in the vascular system. PBB is one of

the most popular shrimp species in Korea and existing widely in the deep sea at depths of 20 – 1330m with the temperature of 2–14℃ [15].

Northern shrimp is abundant in nutrients including minerals, proteins, and vitamins. But, the inedible parts of shrimp by-products such as head, shell, and tail portions possess nutraceuticals and nutritional supplements with high nutrient [16-18]. The aim of this study is determining the inhibitory activities of hypertension and oxidative stress using enzymatic hydrolysates of *P. borealis* by-products and its molecular weight cut-off fractions.

3. Material and methods

3.1. Enzymatic hydrolysis and fractionation of shrimp by-product

The PBB were lyophilized and stored at −20℃ until use. The crude protein portions of PBB was measured by AOAC method as shown in Table 4-1 [19] and enzymatic hydrolysis was performed using five enzymes including alcalase, papain, trypsin, protamex, and flavourzyme, under their optimal conditions (Table 4-2). The 100 g sample on the base of protein weight and 1 % enzyme were mixed and then incubated for 8 h at each optimal temperature with stirring. After incubation, the mixtures were heated at 100℃ in order to inactivate the enzyme. The inactivated mixtures were centrifuged at 2000 × g for 20 min and the supernatant was lyophilized and stored at −20℃ until use. The 38 g of protamex hydrolysate was dissolved in 50 mL deionized water and filtered using two ultrafiltration membranes (Amicon Ultra-filter devices; Millipore, Billerica, MA, USA) with 3 and 10 kDa molecular weight cut-offs (<3 kDa, 3–10 kDa, and >10 kDa). The soluble fractions were obtained by centrifuging at 3000 × g for 20 min and filtering the membrane with the largest molecular weight cut-off membrane cartridge (10 kDa) sequentially.

The retentate and permeate were prepared separately, and the retentate was re-circulated into the feed to gain the maximum permeate yield. Permeate from the 10 kDa membrane was filtered through the 3kDa membrane with recirculation.

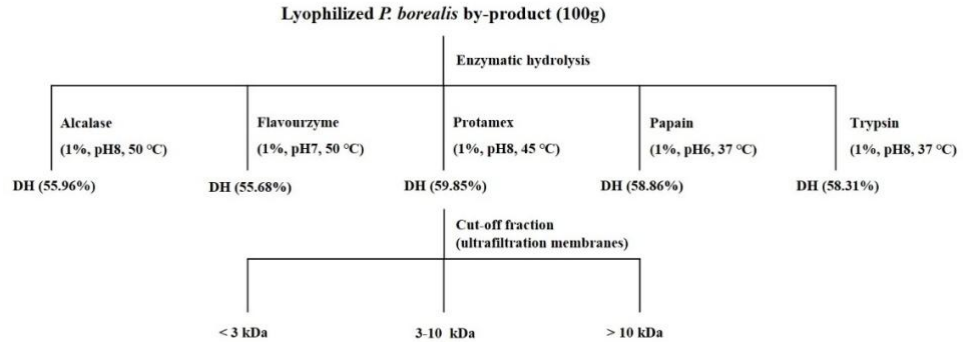

Figure4-1. Preparation of samples. The enzymatic hydrolysis and cut-off fractionation were measured using Lyophilized PBB by-product.

Samples	Moisture (%)	Crude fat (%)	Ash (%)	Crude protein (%)
PBB	21.07 ± 0.22	12.19 ± 0.12	17.18 ± 0.64	44.50 ± 0.35
Protamex	7.05 ± 0.21	1.12 ± 0.15	18.35 ± 0.21	73.92 ± 0.95
< 3K	14.04 ± 0.13	4.66 ± 0.21	15.85 ± 0.18	62.07 ± 3.23
3-10 K	7.26 ± 0.17	2.27 ± 0.25	11.75 ± 0.26	69.31 ± 2.11
> 10K	5.48 ± 0.22	2.30 ± 0.17	12.69 ± 0.31	74.02 ± 2.63

Table 4-1. The proximate compositions.

Enzymes	Temperature (℃)	pH	Crude protein (%)	DH (%)
Alcalase	50	8.0	70.57 ± 2.05	55.96 ± 0.04^{1,d}
Protamex	45	8.0	73.92 ± 0.95	59.85 ± 0.09^{a}
Flavourzyme	50	7.0	67.40 ± 0.16	55.68 ± 0.08^{e}
Papain	37	6.0	69.45 ± 1.33	55.86 ± 0.08^{b}
Trypsin	37	8.0	66.62 ± 0.66	58.31 ± 0.08^{c}

Table 4-2. The condition of enzymatic hydrolysis, Crude protein (%) and DH (%). They were measured using AOAC and Nielsen methods with modification respectively.

3.2. Degree of hydrolysis

The Degree of hydrolysis (DH) of enzymatic hydrolysates of PBB was calculated to determining free amino groups with o-phthaldialdehyde (OPA) [20].

$$DH \text{ ¼ } h = htot\ 100$$

Where h is the number of hydrolyzed bonds and htot is the total number of peptide bonds per protein equivalent. The factor htot is dependent on the amino acids composition of the raw material [21].

3.3. Total amino acids contents

The total amino acids composition was measured using an amino acid analyzer (S43000; Sykam, Eresing, Germany). The hydrolyzed samples obtained by the 6 N hydrochloric acid in vacuum-sealed tubes at 110℃ for 24 h and tryptophan was measured after alkaline hydrolysis [22]. The 10 mg of each Sample were dissolved in 10 mL of 4.2 N sodium hydroxide and hydrolysed at 110℃ for 20 h and then hydrolysates were filtered (ADVANTEC No. 5B) and mass up 50 mL by 0.2 N sodium citrate buffer (pH 4.2). The 5 mL of solution was dried in a waterbath at 70℃ and the pH of the hydrolysates was adjusted to 4.2 and mess up 25 mL. The solutions were filtered using 0.2 μm membrane filter and analyzed by amino acid analyzer (Sykam 4300, Sykam, Germany).

3.4. $ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activity was measured followed by the method of Roberta [23]. The ABTS solution was diluted to achieve an absorbance of 0.75 ± 0.03 at 734 nm and 180 μL of ABTS solution was added to 20 μL of different concentrations of samples. The incubated samples in the dark for 10 min were measured the absorbance by spectrophotometer (BIO-TEK US/MQX 200, USA) at 734 nm. The $ABTS^+$ radical scavenging activity of each sample was expressed as IC_{50} value and L-ascorbic acid was used as a positive control.

3.5. Superoxide dismutase like activity

The SOD-like activity of the sample was evaluated according to the method of Marklund and Marklund with a slight modification [24]. The sample solutions were mixed with 100 μL of pyrogallol (7.2 mM) and 100 μL of 50 mM Tris-HCl buffer containing 0.2 mM EDTA (pH8.5). After 10 min, 50 μL of 1 N HCl was added to stop the reaction and measured at 420 nm and L-ascorbic acid was used as a positive control.

3.6. Reducing power

The reducing power of PBB extracts were determined by the method of Oyaizu [25]. 10 % DMSO were mixed with 50 μL of 0.2 M sodium phosphate buffer (pH 6.6), 50 μL of potassium ferricyanide (10 mg/mL) in different concentrations of

samples. After incubation at 50℃ for 20 min mixtures was added into 50 μL of TCA (100 mg/mL) and centrifuged at 2000 × g for 10 min. Then, 100 μL of the supernatant was mixed with 20 μL of iron (III) chloride (1 mg/mL) and measured at 700 nm. L-ascorbic acid was used as a positive control and reducing power was expressed as the 0.5 of absorbance (EC_{50}).

3.7. ACE inhibitory activity

The inhibitory activity of ACE was measured followed by the procedure of Li et al. [26]. The Mixtures with 20 μL of sample, 50 μL of 5 mM HHL, and 100 mM of sodium borate buffer (pH 8.3) containing 300 mM NaCl were pre-incubated at 37℃ for 5 min. The 10 μL of ACE solution (100 mU/mL) initiated reaction and the mixture was incubated at 37℃ for 30 min. The reaction was terminated by adding 100 μL of 1 M HCl and then sodium borate buffer (320 μL), pyridine (600 μL), and BSC (200 μL) were added to the reaction mixture. The absorbance of reaction mixture was measured at 492 nm after incubation at room temperature for 30 min and the captopril was used as a positive control.

3.8. Statistical analysis

The data were analyzed through the general linear model procedure (SAS Institute, Cary, NC, USA) and Duncan's multiple range test was applied to verify the significance of the differences between means ($P<0.05$).

4. Results and Discussion

4.1. Enzymatic hydrolysis and fractionation

Recently, the enzymatic hydrolysates have been investigated and used as nutraceutical resources. In particular, various biological activities derived from fish and shrimp processing by-products were performed such as antioxidant, anti-obesity, antibacterial and antihypertensive activities [27, 28, 29]. In this study, PBB was hydrolyzed by five enzymes such asalcalase, papain, trypsin, protamex and flavourzyme for 8 h respectively and then DH values of their hydrolysates were appeared in Table 4-2.

Decomposition of protein tertiary structure and reduction of the molecular weight of proteins were influenced by the cleavage of peptide bonds by protease [21]. In addition, this reaction also up regulated the concentration of free amino and carboxyl groups and its functional properties of proteins [30]. The DH values of PBB hydrolysates were showed followed byprotamex (59.85 ± 0.09 %) > papain (58.86 ± 0.08 %) > trypsin (58.31 ± 0.08 %) >alcalase (55.96 ± 0.04 %) >flavourzyme (55.68 ± 0.08 %).These enzymatic hydrolysates, protamex hydrolysate of PBB exhibited to the high DH value.

4.2. Antioxidant activity

ROS containing superoxide (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH−), and singlet oxygen (O·2), can trigger the oxidative damage to the essential components including protein, lipid, nucleic acids and associated with the outbreak of hypertension [31,32]. The five PBB enzymatic hydrolysates were determined by the scavenging activity on $ABTS^+$ radicals, SOD-like activity, and reducing power (Table 4-4, Figure 4-2). The alcalase and protamex hydrolysates of PBB appeared exceptional $ABTS^+$ radical scavenging activity with IC_{50} value of 0.16 ± 0.02 and 0.17 ± 0.00 mg/mL, respectively. But all hydrolysates were exhibited to lower ABTS+ radical scavenging activity than that of L-ascorbic acid. SOD, an excellent antioxidant defense enzyme, catalyzes the dismutation of the hydrogen peroxide (H_2O_2) and superoxide anion (O^{-2}) into oxygen (O_2). The SOD-like activity is used to measure the inhibition of pyrogallol autoxidation widely. The protamex hydrolysate exhibited to the noticeable SOD-like activity (2.04± 0.15 mg/mL) among hydrolysates, while the alcalase hydrolysate has moderate activity as shown in Table 4-4 (Fig 4-2). The reducing power is the ability to donate an electron or hydrogen [33]. The electron donation capacity of the PBB enzymatic hydrolysates were measured and shown in Table 4-4 (Fig 4-2). All the PBB hydrolysates showed moderate reducing power. The antioxidant activities of the three MWCO fractions (protamex hydrolysate) were evaluated as shown in Table 2-5. The < 3 kDa and 3–10 kDa fractions exhibited to potent $ABTS^+$ radical scavenging activity

(IC_{50} = 0.22 ± 0.01 mg/mL and 0.22 ± 0.00 mg/mL) and the > 10 kDa fraction was appeared to the strong reducing power with EC_{50} value of 7.04 ± 0.83 mg/mL than those of the < 3 kDa and 3–10 kDa fractions.

4.3. Total amino acids contents

Total amino acid composition of the MWCO fractions (protamex hydrolysate) were presented in Table 2-3. As a result, the total amino acid contents of MWCO fractions were followed by 47.44 g/100 g (<3 kDa), 48.17 g/100 g (3–10 kDa), and 47.48 g/100 g (>10 kDa), respectively. The total amino acid compositions were shown similarly to each other and all MWCO fractions abundant to Glu, Gly, Asp, Ala, Leu, and Lys, while these fractions contained low levels of Cys and Trp.

Amino acids	< 3kDa	3-10 kDa	> 10 kDa
Asp	3.66	4.77	3.65
Thr	1.96	2.21	1.94
Ser	1.91	2.18	1.10
Glu	6.14	7.16	6.11
Pro	2.95	0.34	3.22
Gly	5.06	4.72	5.15
Ala	4.44	4.10	4.43
Cys	0.08	0.18	0.17
Val	2.53	2.62	2.11
Met	1.44	1.24	1.74
Ile	2.21	2.54	1.88
Leu	3.80	3.68	3.73
Tyr	1.51	1.43	1.50
Phe	2.24	2.27	2.25
His	1.29	1.73	1.24
Lys	3.06	3.49	3.89
Arg	2.94	3.36	3.07
Trp	0.22	0.15	0.30
Total	47.44	48.17	47.48

Table 4-3. Total amino acids compostion of the molecular weight cut-off fractions of protamex hydrolysate (g/100g).

4.4. ACE inhibitory activity

The inhibition of ACE is crucial role regulating the blood pressure and recognized as the potent effective therapy for the treatment of hypertension. However, many synthetic ACE inhibitors including captopril, enalapril, alacepril, fosinopril, and lisinopril can induce the side effects such as taste alterations, cough, skin rashes, and angioneurotic edema [34-36]. Therefore, it is necessary to investigate effective ACE inhibitors from natural products and the ACE inhibitory activity of the enzymatic hydrolysates of PBB was presented in Table 2-4 (Figure 2-3). According to the results, the protamex hydrolysate exhibited to the most potent ACE inhibitory activity with IC_{50} value of 0.08 ± 0.00 mg/mL and flavourzyme (IC_{50} = 0.11 ± 0.00 mg/mL) > alcalase (IC_{50} = 0.11 ± 0.01 mg/mL) > papain (IC_{50}=0.11±0.00 mg/mL) > trypsin (IC_{50}=0.13±0.00 mg/mL) sequentially. The ACE inhibitory activity of the three MWCO fractions of protamex hydrolysate was shown in Table 4-5. Among the MWCO fractions, the >3 kDa fraction including 3–10 kDa and >10 kDa fractions exhibited to potent ACE inhibitory activity with IC_{50} value of 0.03±0.00 mg/mL. This result also suggested that high molecular weight fraction, 3–10 kDa and >10 kDa, exhibited to significant ACE inhibitory activity compared with protamex enzymatic hydrolysate.

Samples	ABTS^{+} IC_{50} (mg/mL)[1]	SOD-like activity IC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL) [2]	ACE inhibition IC_{50} (mg/mL)
Alcalase	0.16 ± 0.02[a,c]	2.82 ± 0.72[a]	9.42 ± 0.82[a]	0.11 ± 0.01[b]
Protamex	0.17 ± 0.00[b,c]	2.04 ± 0.15[a]	6.75 ± 0.94[b]	0.08 ± 0.00[c]
Flavourzyme	0.20 ± 0.00[a,b]	3.13 ± 0.51[a]	4.63 ± 0.15[c]	0.11 ± 0.00[b]
Papain	0.21 ± 0.03[a]	2.59 ± 0.48[a]	4.46 ± 0.31[c]	0.11 ± 0.01[b]
Trypsin	0.22 ± 0.02[a]	2.44 ± 0.13[a]	6.62 ± 0.06[b]	0.13 ± 0.00[a]
L-Ascorbic acid	0.004 ± 0.000[d]	0.02 ± 0.00[b]	0.04 ± 0.00[d]	-
Captopril	-	-	-	0.00002 ± 0.00000[d]

Table 4-4. The antioxidant and ACE inhibitory activity of enzymatic hydrolysates. ABTS^{+}, SOD-like activity and Reducing power were engaged to determine on antioxidant activity. ACE inhibitory activity was performed using Li's method with a slight modification.

[1]IC_{50} (50% inhibitory concentration) values of ABTS^{+} scavenging, SOD-like, and ACE inhibitory activities were expressed as a mean ± SD.

[2]The reducing power was expressed as an EC_{50} (concentration of the 0.5 absorbance) value.

[3]Means within the same row with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

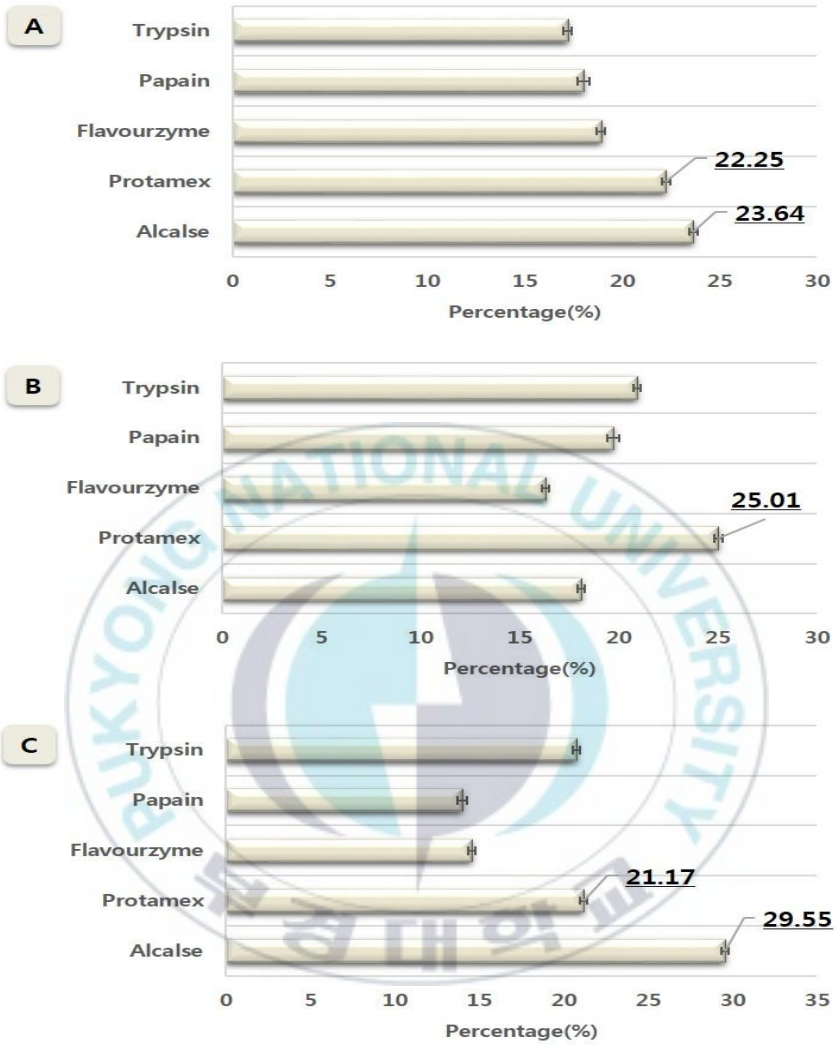

Figure 4-2. The antioxidant activities. A: $ABTS^{+}$, B: SOD-like activity and C: Reducing power were described as a percentage.

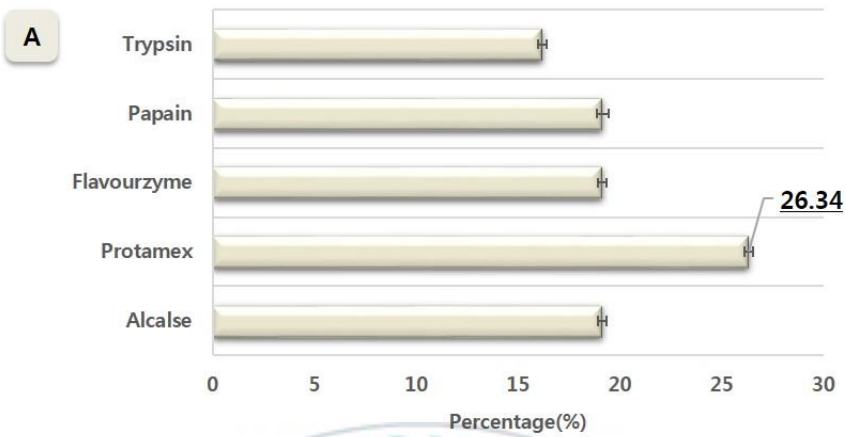

Figure 4-3. The ACE inhibitory activity was described as a percentage.

Samples	ABTS^{+} IC_{50} (mg/mL)[1]	SOD-like activity IC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL) [2]	ACE inhibition IC_{50} (mg/mL)
< 3 K	0.22 ± 0.01[a,b]	> 10	20.14 ± 0.39[a]	0.06 ± 0.00[a]
3-10 K	0.22 ± 0.00[b]	> 10	13.84 ± 0.16[b]	0.03 ± 0.00[b]
> 10 K	0.24 ± 0.01[a]	> 10	7.04 ± 0.83[c]	0.03 ± 0.00[b]
L-Ascorbic acid	0.005 ± 0.000[c]	0.07 ± 0.00[d]	0.04 ± 0.00[d]	-
Captopril	-	-	-	0.00001 ± 0.00000[c]

Table 4-5. The antioxidant and ACE inhibitory activity of fractionated protamex hydrolysate.

[1]IC_{50} (50% inhibitory concentration) values of $ABTS^{+}$ scavenging, SOD-like, and ACE inhibitory activities were expressed as a mean ± SD.

[2]The reducing power was expressed as an EC_{50} (concentration of the 0.5 absorbance) value.

[3]Means within the same row with different superscripts are significantly different by Duncan's multiple range test ($P < 0.05$).

Recently, several studies have been reported that various bioactive peptides derived from fisheries processing by-products including Pacific cod skin [31, 32], crab shell [37], squid skin and muscle [38, 39] and tuna [40-42]. According to the results, PBB protamex hydrolysate exhibited to remarkable $ABTS^{+}$ radical scavenging and ACE inhibitory activities. In addition, its MWCO fractions showed potent ACE inhibitory activities.

In this study, the five enzymatic hydrolysates of PBB were investigated to the antioxidant and ACE inhibitory activities. The PBB Protamex hydrolysate showed the most significant ACE inhibitory activity than other fractions and it is fractionated as the below 3 kDa, between 3 and 10 kDa, and above 10 kDa fractions to isolate the active materials. But these fractions exhibited to lower antioxidant activities than those of enzymatic hydrolysate but it is showed the significant ACE inhibitory activity. In addition, 3-10 kDa and >10 kDa fractions appeared the better ACE inhibitory activity than <3 kDa fraction. These results indicated that the high molecular weight enzymatic hydrolysate derived from PBB more valuable for prevent hypertension.

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6-포르밀 움벨리페론과 새우 부산물의 산화적 스트레스 관련 질병에서의 생리활성 연구

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국문요약

산화는 몸에서 일어나는 정상적이고 필요한 과정이며 분자, 원자 또는 이온에 의한 반응 중에 전자가 손실 되는 것이다. 안정한 원자인 활성산소는 외부껍질 전자의 수를 보충 하기 위해 다른 물질과 즉시 반응하며 병원균을 퇴치하는데 도움을 줄 수 있지만, 몸을 손상시키고 질병과 노화를 초래할 수 있다. 또한, 활성산소의 노출과 지속적인 발생은 산화적 스트레스를 유발시키며, 염증, 고혈압, 치매 등 각종 질병을 발생시키는 원인이 될 수 있다. 본 연구는 산화적 스트레스와 관련된 질병을 대상으로 하여 이를 예방하고 치료에 도움을 줄 수 있는 천연물 유래 기능성 물질의 효과를 확인하였다.

바디나물 추출물에서 분리된 6-포르밀 움벨리페론 (6FU)은 지질다당류 (LPS)로 인하여 심화된 염증환경에서 질소산화물(NO) 발생을 완화하였다. 질소산화물의 역기능으로 염증을 심화시키는 산화적 스트레스로 알려져 있으며, 질소 산화물의 발생감소는 세포질에서 핵으로 이동하는 ERK1/2 와 NF-κB 의 활동이 억제되어 질소산화물의 발생을 유도하는 iNOS 효소 생성의 감소가 이루어졌고, 염증 상태를 더욱 심화시키는 COX-2 의 발현 감소로 효과적으로 염증을 억제하는 경로를 확인하였다. 뿐만 아니라 평상시 면역반응을 촉진하여 몸을 방어하는 기작에 사용되지만, 염증반응을 촉진 시킬 수 있는 종양괴사인자(TNF-α), 인터루킨(IL)-1β, IL-6 등의 사이토 카인의 발생을 감소시켜 염증을 억제 시킬 수 있는 효과를 증명하였다.

지속 가능한 자원의 개발은 인류의 미래를 위한 중요한 가치를 지니고 있다. 특히, 갑각류의 부산물은 폐기물로 버려지고 있어, 이를 활용하여 산화적 스트레스와 관련된 질병의 예방에 도움을 줄 수 있는 기능성 물질로 연구 하고자 하였다. 동해안을 중심으로 북쪽분홍새우, 도화새우, 물렁가시 붉은새우 등 도화새우과의

새우는 단백질, 칼슘, 비타민 등이 함유되어 영양소가 풍부하다고 알려져 있다. 머리, 껍질, 꼬리를 포함한 새우 부산물을 아세톤과 디클로로메탄 유기용매를 이용하여 추출하고, 치매를 예방하는데 도움을 줄 수 있는 효과를 확인하였다. 치매환자에서 발견되는 뇌세포의 병변현상은 다양하고 이를 타깃으로 치매를 억제하는 기능성 연구는 계속해서 보고 되고 있다. 그 중 뇌세포간의 신경전달물질로 알려진 아세틸콜린을 콜린과 아세테이트로 분리하여 신경전달물질의 이동을 차단하는 효소인 아세틸콜린에스테라제의 활성을 억제하는 것은 치매예방의 좋은 전략 중 하나로 알려져 있다. 실험에 이용된 새우 부산물 중에서도 도화새우의 아세톤 추출물은 항산화 실험과 환원력 측정에서 다른 새우 추출물에 비해 높게 나타났으며, 콜린에스테라제 억제효과 및 총 카로티노이드함량에서도 높게 측정되어 활용 가능성이 높을 것으로 예상된다.

다음은 새우 부산물에 가수분해효소를 이용하여 펩타이드를 분리하고 산화적 스트레스 관련 고혈압의 억제효과를 확인하였다. 우리 몸의 혈압은 레닌-엔지오텐신-알도스테롤시스템 (RAAS)에 의하여 조절되며, 혈압을 상승시킬때 작용하는 엔지오텐신변환효소 (ACE)의 활성을 억제하여 고혈압 예방에 도움을 줄 수 있는 실험을 진행하였다. 북쪽 분홍새우 부산물은 알칼라아제, 프로타멕스, 플라보자임, 파파인, 트립신 5 종의 효소에 의해 가수분해되었다. 그 중 프로타멕스효소에 의해 분리된 물질에서 항산화 및 엔지오텐신변환효소 활성억제가 가장 높게 나타났다. 프로타멕스 가수분해물을 질량별로 3 그룹으로 분리하여 (<3 kDa, 3-10 kDa, >10 kDa)활성을 비교하였고, 항산화 효과는 <10 kDa 에서 ACE 억제활성도는 10 kDa > 에서 더 강력하였다.

본 연구 에서는 산화적 스트레스와 관련된 질병을 예방하고 치료하는데 도움을 줄 수 있는 기능성 물질에 대해서 그 효과를 확인하였다. 6-포르밀 움벨리페론의 항염증효과는 2019 년 영국분자의학저널(Int. J. Mol. Med)에, 새우 부산물을 이용한 치매 및 고혈압 예방 실험은 각각 2014 년, 2016 년 수산 과학회지에 개제되었다.

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