A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Improvement of Metabolic Syndrome of a Novel Peptide Purified from *Styela clava*

Seok-Chun Ko

Department of Marine Life Sciences

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

20012. 02

Improvement of Metabolic Syndrome of a Novel Peptide Purified from *Styela clava*

Seok-Chun Ko
(Supervised by professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of DOCTOR OF PHILOSOPHY

This thesis has been examined and approved by

Thesis director, Moon-Soo Heo, Professor of Marine Life Sciences

Hee-Guk Byun, Professor of Marine Bioscience and Technology, Gangneung-Wonju National University

Dae-Ho Lee, Professor of Medicine

Seungheon Lee, Professor of Marine Life Sciences

You-Jin Jeon, Professor of Marine Life Sciences

2012.02

Date

Department of Marine Life Sciences

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

국문초록

현대사회의 생활환경(흡연, 음주, 스트레스, 운동부족), 인스턴트 식품의 와 노동력 위주의 경제활동에서 정보통신의 발달로 인한 사회적 업무환경으로의 전환은 과다한 에너지의 저장으로 인하여 발생하는 대사증후군의 발병을 필연적으로 증가시키고 있다. 대사증후군은 만성적인 대사 장애로 인하여 내당능 장애, 고혈압, 인슐린 저항성, 비만, 동맥경화, 고지혈증 등과 관련한 심혈 관계 질환이 한 개인에서 복합적으로 나타나는 것을 대사증후군이라 한다. 또한 National Cholesterol Education Program (NCEP)-Adult Treatment panel III (ATP III) 에서 정의한 자료를 바탕으로 복부비만, HDL cholesterol, 혈압, 중성지방, 공복혈당의 5가지 위험요소 중 제시한 결정수치 범위를 벗어난 것이 3가지 이상 해당되게 되면 대사 증후군 환자라고 할 수 있다. 2008년도 국민영양조사 자료를 바탕 우리나라 20세 이상 성인 인구를 대상으로 대사증후군의 유병율을 살펴보면 1998년도에 24.9%에서 2007년도에 31.3%로 10년 동안 6%이상 증가하였다. 이는 곧 우리나라 성인인구의 3명 중 1명은 대사증후군 환자라고 할 수 있다. 또한, 2010년 통계청 자료의 사망원인 통계를 보면 각종 암의 뒤를 이은 사망원인이 전부 대사증후군과 관련되는 사망원인으로 대사증후군의 위험성을 더해 주고 있다. 현재 시판 되어 지고 있는 대사증후군 약제의 경우 약물에 따라 다양하고 심각한 부작용이 나타날 수 있으며, 복용기간이 지속되면 약물의 효능이 감소될 수 있어 시간이 지남에 따라

약물의 종류를 바꾸거나 복용량을 변화시키는 등 많은 문제점을 지니고 있다.

따라서 부작용이 없고 우수한 효과를 나타내는 대사증후군 개선 물질로서 천연생물자원 유래 소재의 발굴에 대한 연구가 절실하다. 천연생물자원 유래 소재 중 Bioactive peptide 는 그 구조와 생리활성도의 특이성 면에서 질병 예방, 치료제 및 개선제 개발을 위한 강력한 신물질의 후보로 떠오르고 있다. 이러한 Bioactive peptide 들은 항산화, 항고혈압, 항비만, 면역조절 등의 다양한 생리활성을 지니고 있는 것으로 보고 되어 지고 있으며, Bioactive peptide 에 관한 연구는 급속도로 증가하고 있다. 천연생물자원 중 해양생물자원의 하나인 미더덕은 우리나라 에서 양식이 이루어지고 있으며, 식용으로 사용하고 있다. 미더덕은 음식 재료의 용도로만 사용 되어져 이용용도의 한계와 경제성이 낮은 자원으로 분류 되어지고 있다. 최근 미더덕의 소비촉진 및 미더덕의 우수성을 알리기 위하여 다양한 생리활성에 대한 연구결과가 발표 되어지고 있다. 그러나 이들은 거의 대부분이 단순 수용성 추출물이거나 유기용매 추출물에 의한 생리활성 효과로 미더덕이 어떠한 성분을 통해 생리활성을 나타내는지에 대한 연구는 미비하다. 또한 미더덕의 부위 중 육 부위는 65%에 이르는 단백질 함량을 가지고 있음에도 불구하고 이를 이용한 연구가 미비한 실정이다. 따라서 이 연구에서는 양식 미더덕을 대상으로 대사증후군 개선 펩타이드를 분리하고 기능성 식품소재로서의 개발 가능성을 확인하였다.

미더덕을 9종의 단백질 가수분해 효소를 이용하여 가수분해물을 제조하여 안지오텐신 I-전환효소 저해 효과를 스크리닝 한 결과 Protamex 가수분해 효소를 이용하여 획득한 가수분해물이 다른 가수분해물들에 비해 우수한 효과를 나타내는 것을 확인하였으며, 미더덕 부위별 Protamex 가수분해물 제조하여 안지오텐신 I-전환효소 저해 효과를 확인한 결과 껍질보다 육 부위 가수분해물이 더욱 우수한 효과를 나타내어 미더덕의 육부위로부터 Protamex 가수분해물을 획득하여, 안지오텐신 I-전환효소 저해 및 혈관확장 효과를 확인하면서 항고혈압 펩타이드를 분리하였다. 순차적인 분리 과정을 거쳐 획득한 항고혈압 펩타이드의 구조는 Ala-His-Ile-Ile-Ile 이며, 분자량은 565.3 Da 임을 확인하였다. 또한 선천성 고혈압쥐 동물모델에 경구투여 한 후 24시간 이내의 혈압변화를 관찰한 결과 생리식염수 투여군에 비해 현저히 높은 혈압강하 효과를 나타내었다.

획득한 항고혈압 펩타이드를 이용하여 대사증후군 질환의 하나인 항당뇨 효과를 확인하기 위하여 근육세포에서 포도당 흡수 촉진효과를 확인 하였으며, 그 작용기전을 구명하였다. 그 결과 인슐린 보도 처리 농도는 높지만 인슐린과 유사한 포도당 흡수 촉진 효과를 나타냈다. 세포 내 포도당 흡수는 insulin dependent pathway 와 insulin independent pathway 로 이루어 진다. insulin dependent pathway 에 있어서 대표적인 단백질인 Akt 는 insulin 에 의해 활성화 되며, insulin independent pathway 에 있어서 핵심 단백질이 AMPK 로 알려져 있다. 활성화 되어진 Akt

와 AMPK 는 포도당수송운반체인 GLUT4를 세포막으로 translocation 시켜 이를 통해 세포 내로 포도당이 흡수 된다. 항고혈압 펩타이드에 의한 포도당 흡수 촉진 효과가 어떠한 pathway 로 이루어지는 지 Western blot assay 를 통해 확인한 결과 AMPK 를 활성화 시켰으며, 이에 따라 GLUT4가 세포막으로 translocation 되어 포도당 흡수 효과가 나타나는 것을 구명하였다.

대사증후군의 가장 큰 원인으로 꼽히고 있는 비만은 전 세계 국가들이 겪고 있는 심각한 건강문제로 비만인구는 2025년에 이르면 3억 명 이상이 될 것으로 추측 하고 있다. 세계보건기에서 발표한 보고서에 따르면 비만은 각종 질병, 장애 및 사망의 핵심적인 원인이며, 심지어 21세기의 흑사병 이라고도 경고한바 있다. 이에 따라 다양한 작용기전 별 비만 치료제들이 개발 되어 지고 있다. 현재까지의 비만 치료제들의 대부분은 화학적으로 제조된 약품이지만 최근 우리나라에서 천연물로부터 비만 치료제를 개발하려는 연구가 활발히 진행되고 있어 관심을 끌고 있다. 이 연구에서는 작용기전 중 전지방 세포가 지방세포로 분화하는 과정을 저해하는 기전을 통하여 항고혈압 펩타이드의 항비만 효과를 확인하고, 지방세포로 분화화는 과정에 있어서 핵심적으로 작용하는 전사인자들의 발현 감소를 확인 하였다. 그 결과 농도 의존적으로 지방세포로의 분화를 저해하는 것을 Oil Red O staining 을 통하여 확인 하였으며, 핵심적인 전사인자인 SREBP-1, PPARγ, C/EBPα, aP2의 발현

역시 농도의존적으로 억제 시키는 것을 확인하였다. 또한 고지방 식이를 통해 비만을 유도한 쥐에서 항고혈압 펩타이드 투여군이 고지방 식이만을 유도한 쥐와 비교하여 체중 증가가 현저히 감소하는 것을 확인하였다.

이 모든 결과들을 종합하여 볼 때, 미더덕으로부터 획득한 항고혈압 펩타이드는 다양하고 우수한 대사증후군 개선효과를 가지고 있음을 확인하였으며, 그에 따라 미더덕으로부터 획득한 항고혈압 펩타이드는 대사증후군 개선물질로서 잠재적인 기능성 식품 소재로서 충분한 가능성이 있으리라 판단된다.

LIST OF FIGURES

conducted to compare the endothelia (-). *$p < 0.05$.

Fig. 1-5. Sephadex G-25 gel filtration chromatogram of PHFT-I (MW <5 kDa). (A) Separation was performed at 2 ml/min and collected at a fraction volume of 10 ml. Gel columns were separated into four fractions (A~D). (B) Concentration-dependent vasorelaxation of gel filtrated fractions of PHFT-I (MW <5 kDa) in aortic segments with endothelium and without endothelium. The data are expressed as the mean±S.E. Statistical evaluation was conducted to compare the endothelium (-). *$p < 0.05$.

Fig. 1-6. RP-HPLC chromatogram of the fraction A isolated from Sephadex G-25 in aortic segments with endothelium. (A) Separation into sub-fractions (A-I and A-II) was carried out with a linear gradient of acetonitrile from 0% to 15% at a flow rate of 1 ml/min. (B) Vasorelaxation effect of sub-fraction of the fraction A in aortic segments with and without endothelium. The data are expressed as the mean±S.E. Statistical evaluation was carried out to compare the A-I and A-II. *$p < 0.05$.

Fig. 1-7. RP-HPLC and MS profile of the purified antihypertensive peptide.

Fig. 1-8. The effect of the antihypertensive peptide on NO production and eNOS

phosphorylation in human endothelial cells. The eNOS phosphorylation level was determined via Western blot analysis in the endothelial cells treated with one dose of the antihypertensive peptide (0.1 mM).

Fig. 1-9. Change of systolic blood pressure (SBP) of SHRs after the oral administration of test group. (◆) negative control (Saline); (■) positive control (Amlodipine, 30 mg/kg body weight); (•) the antihypertensive peptide (100 mg/kg body weight). The data are expressed as the means±S.E. Statistical evaluation was carried out and compared with the control group. *$p < 0.01$.

Fig. 2-1. Cytotoxicity of antihypertensive peptide. Cytotoxicity of antihypertensive peptide was determined using the MTT assay. Each value is expressed as mean±S.E. in triplicate experiments.

Fig. 2-2. Antihypertensive peptide dose-dependently stimulates glucose uptake in L6 skeletal muscle cells. (A) Cells were starved in serum free (SF) media for 4 h, and incubated for 1 h with increasing of antihypertensive peptide and insulin. Values are expressed as means ± S.E. in triplicate experiments. $^{a-d}$Values with different

alphabets are significantly different at $P<0.05$ as analyzed via Duncan's multiple range test.

Fig. 2-3. Antihypertensive peptide-induced increase of glucose uptake was reduced by wortmannin and compound C. After 4 h starvation, L6 skeletal muscle cells were pretreated with or without 100 nM wortmannin (phosphatidylinositol (PI) 3-kinase inhibitor) and 10 μM compound C, (AMPK inhibitor) for 30 min, and then treated with 100 μg/ml antihypertensive peptide for 1 h. Each value is expressed as mean ± S.E. in triplicate experiments. $^{*}P<0.05$ vs. control or between two groups as indicated.

Fig. 2-4. Effect of antihypertensive peptide on AMPK signaling pathway. Cells were pretreated with or without 10 μM compound C for 30 min, and then treated with the indicated concentrations of antihypertensive peptide and insulin for 1 h and 10 min, respectively. The cell lysates were analyzed via Western blotting using anti-phosphoAMPK (Thr 172) and anti-AMPK. Figures are representative of three independent experiments.

Fig. 2-5. Effect of OPA on GLUT4 translocation to the plasma membrane. Cells were

pretreated with or without 10 μM compound C for 30 min, and then treated with the indicated concentrations of antihypertensive peptide and insulin for 1 h and 10 min, respectively. The cell lysates were analyzed via Western blotting using anti-GLUT4. Figures are representative of three independent experiments.

Fig. 3-1. Effect of antihypertensive peptide on the viability of 3T3-L1 preadipocytes treated for 48 h. Viability was determined via MTT assay. Each value is expressed as mean±S.E. in triplicate experiments.

Fig.3-2. Antihypertensive peptide inhibits lipid accumulation during the differentiation of 3T3-L1 preadipocytes. (A) O Red O staining at day 8 with antihypertensive peptie at 0, 75, 150, 300 μg/ml. (B) The lipid accumulation determined by absorbance at 520 nm. $^{a-d}$Values with different alphabets are significantly different at $P<0.05$ as analyzed via Duncan's multiple range test.

Fig. 3-3. Antihypertensive peptide suppressed the SREBP-1c, C/EBPα, PPARγ, SREBP-1c and aP2 protein expression in the differentiation of 3T3-L1 preadipocyte in to adipocyte. Cells were cultured for 8 days with 75, 150, 300 μg/ml

antihypertensive peptide or without antihypertensive peptide.

Fig. 3-4. Effect of supplementing antihypertensive peptide on body weight gain profile in HFD-induced experimental group for 6 weeks. The values were expressed as mean±S.E. (n=10). Mean separation was performed by Duncan's multiple rang test. Different letters indicate significant differences (p<0.1).

LIST OF TABLES

INTRODUCTION

Recently, metabolic syndrome has become a major problem, and has come to pose a major worldwide threat to human health. It is a clustering of metabolic abnormalities that has been associated with cardiovascular disease (CVD) risk factors of diabetes, abdominal obesity, high blood pressure and high cholesterol (Alberti et al., 2005; Alberti et al., 2006). The Adult Treatment Panel III (ATP III) of the National Cholesterol Education Program (NCEP) published new standard for identifying subjects with metabolic syndrome. Metabolic syndrome was determined using the NCEP-ATP-III guidelines (NIH, NCEP-ATP III panel, 2002), assessing the presence of three or more of the following criteria: abdominal obesity (waist circumference ≥102/88 cm in males/females), dyslipidemia (fasting HDL cholesterol <40/50 mg/dL in males/females), hypertriglyceridemia (triglyceride concentration ≥150 mg/dL under fasting conditions or fibrate or nicotinic acid therapy), hypertension (systolic/diastolic blood pressure ≥130/85 mmHg or antihypertensive treatment), and hyperglycemia (fasting glucose level ≥110 mg/dL or glucose-lowering treatment or previously diagnosed diabetes mellitus) (**Table I**).

Hypertension is one of the major risk factors relevant to the development of cardiovascular diseases including arteriosclerosis, stroke, and myocardial infarction (Lee et al., 2010;

Table I. Clinical identification of the Metabolic Syndrome*

Risk factor	**Defining level**
Abdominal obesity (Waist circumference)	Male: >102 cm (>40 inch) Female: >88 cm (>35 inch)
Triglycerides	≥150 mg/dL
High-density lipoprotein (HDL) cholesterol	Male: <40 mg/dL Female: <50 mg/dL
Blood pressure	≥130/≥85 mmHg
Fasting glucose level	≥110 mg/dL

* Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III), 2001.

Je et al., 2005a). It is triggered by environmental influences including salt intake, obesity, insulin resistance, stress, smoking and lack of exercise (Bohr et al., 1991). Among process related to hypertension, angiotensin I-converting enzyme (ACE, EC 3.4.15.1) participates in regulating blood pressure in the kallikrein kinnin system (KKS) and rennin-angiotensin system (RAS). ACE is a zinc-containing exopeptidase enzyme discovered in vascular, heart, lung and brain tissue, and cleaves dipeptides at the C-terminus of oligopeptides (Je et al., 2005b). ACE converts an inactive form of decapeptide, angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe- His-Leu), to octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), a potent vasoconstrictor, and inactivates bradykinin, which exerts a depressor effect (Je et al., 2005a) (**Fig. I**). ACE inhibition has been used extensively in therapeutic strategies for the prevention and treatment of hypertension, and the literature regarding ACE inhibitory compounds is also rather extensive. Since the discovery of an ACE inhibitor in snake venom, several synthetic ACE inhibitors have been developed, including alacepril, captopril, benazepril, enalapril, fosinopril, ramipril, and zofenopril, all of which are currently extensively used in the treatment of essential hypertension and heart failure in humans (Ondetti, 1977; Je et al., 2005b). However, these synthetic ACE inhibitors are believed to exert certain side effects, including cough, taste disturbances, and skin rashes (Kato and

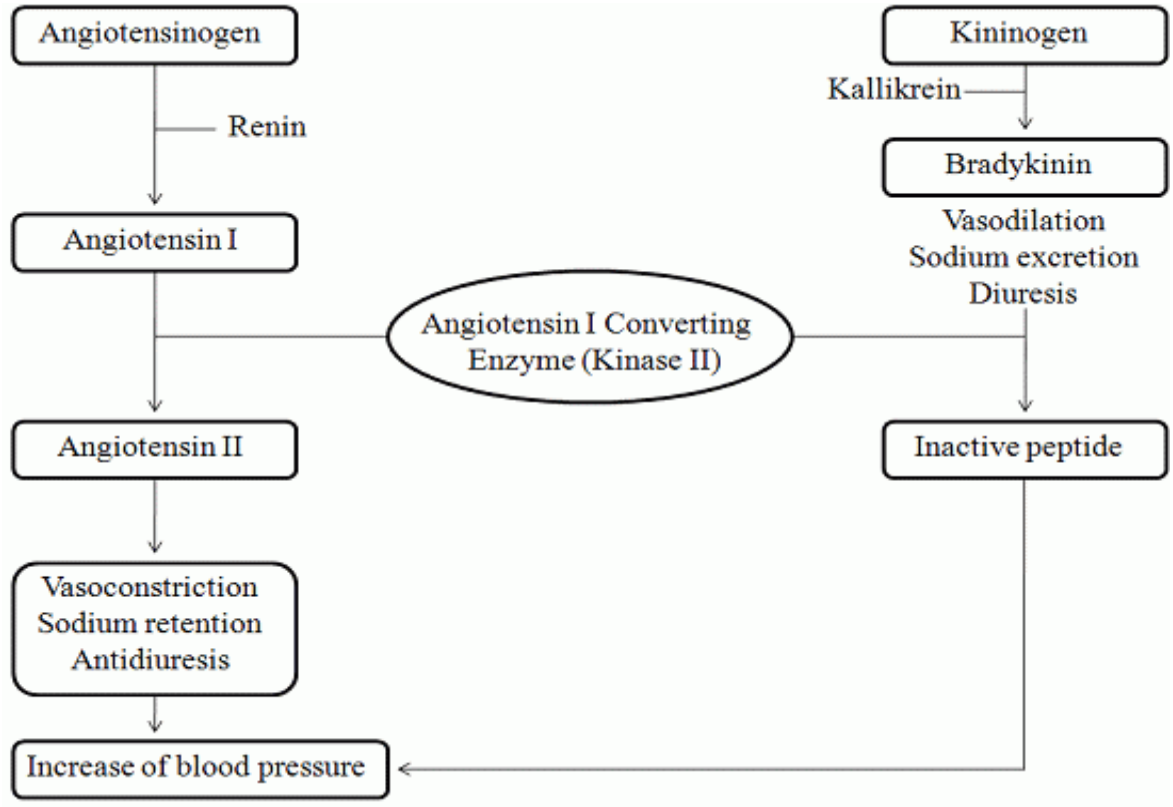

Fig. I. Proposed reaction mechanism catalyzed by angiotensin I converting enzyme (Kininase II).

Suzuki, 1972; Thurman and Schrier, 2003). Therefore, the development of ACE inhibitors from natural products has become a major area of research. Also, Vasorelaxation effects were identified as a major mechanism of anti-hypertensive effects. The mechanisms of vasorelaxators may be characterized as endothelium-dependent or endothelium-independent (Kamiya et al., 1998). Endothelium-dependent vasorelaxation induced by increased blood flow and receptor-specific agonists such as bradykinin, adenosine, triphosphate and acetylcholine, are reduced in the presence of classical vascular risk factors, including hypertension (Asselbergs et al., 2005). The endothelium controls a variety of important functions, including the maintenance of blood circulation and fluidity as well as the regulation of vascular tone, coagulation, and inflammatory responses (Furchgott and Zawadzki, 1980). Endothelium was synthesized and releases a broad spectrum of vasoactive substances, including nitric oxide (NO), prostacyclin and endothelin-1 (Bannister, 1995). In particular, NO is believed to be the most important endothelium-derived relaxing factor, and is generated from the amino acid L-arginine by the calcium/calmodulin-dependent enzyme, NO synthase (NOS), in the site of the vascular endothelium (Asselbergs et al., 2005). Recently, many studies have found that NO is capable of an endothelium-dependent vasorelaxant effect in hypertension (Kim et al., 2011; Ushida et al., 2008).

Insulin resistance appears when tissues in the body (skeletal muscle, adipose/fat and liver) become less sensitive and ultimately resistant to insulin, the hormone which is produced by the β-cells in the pancreas (Carrand and Utzschneider, 2004; Hu et al., 2004). Also, insulin resistance is a major defect underlying the development of type II diabetes and is a central factor of the metabolic syndrome, a constellation of abnormalities including obesity and hypertension (Hotamisligil, 2006; Moller and Flier, 1992). Among the tissue in the body, skeletal muscle tissue is responsible for the majority, accounting 75-80% of insulin-stimulated glucose uptake in the post-prandal state and plays a pivotal role in maintain glucose homestasis (Breen et al., 2008; Zygmunt et al., 2010). In skeletal muscle insulin stimulates glucose uptake primarily by increasing translocation of the glucose transporter-4 (GLUT4) from internal membrane to the plasma membrane (Stephens and Pilch, 1995; Zaid et al., 2008) (**Fig. II**). The signaling mechanism by which insulin stimulates muscle glucose uptake is relatively well known and involves binding of insulin to its receptor, phosphorylation of downstream insulin receptor substrates (IRS) and activation of phosphatidylinositol-3 kinase (PI3-K) and protein kinase B (Akt) which promotes GLUT4 glucose transporter translocation from a cellular pool the plasma membrane (Breen et al.,

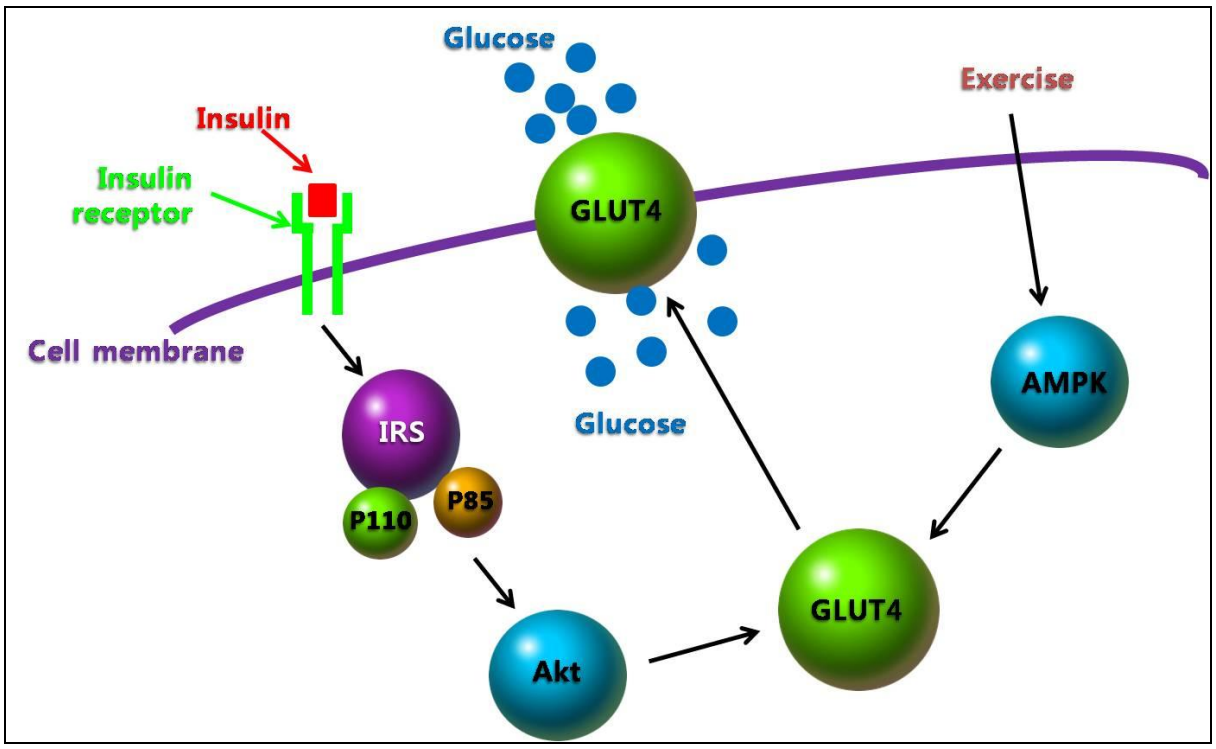

Fig. II. Outline of pathways regulating glucose transport GLUT4 translocation to the plasma membrane in skeletal muscle.

2008; Dugani et al., 2008; Taniguchi et al., 2006). AMP-activated protein kinase (AMPK) complex is αβγ heterorimer, which functions as a cellular energy sensor have been positively correlated with increases in muscle glucose uptake (Kim et al., 2010; Rogers et al., 2009) and recent years has become an attractive pharmacological for the treatment of insulin resistance and type II diabetes (Breen et al., 2008). In skeletal muscle, AMPK is activated by exercise/constraction and numerous compounds including thiazolidineones (Konrad et al., 2005), metformin (Zou et al., 2004), resveratrol (Breen et al., 2008) resulting in stimulation of glucose uptake. The study of new compounds that activate AMPK and stimulate skeletal muscle glucose uptake is pivotal role as the knowledge obtained from such studies could be used towards the development of treatment of insulin resistance and Type II diabetes (Zygmunt et al., 2010). Hypertension is associated with insulin resistance and glucose intolerance. Increased skeletal muscle blood flow and resulting improvements in insulin delivery are pivotal mechanisms by which attenuation of the rennin-angiotensin system (RAS) improves glucose uptake (Stump et al., 2006). One potential mechanism is through the inhibition of angiotensin I-converting enzyme (ACE) inhibitors on kinase II, thereby increasing bradykinin and enhancement of nitric oxide (NO) generation (Henriksen and Jacob,

2003; Stump et al., 2006). Current therapeutics for diabetes are often associated with undesirable side effects and in many cases the precise mechanism of action remains to be completely clarified (Campbell, 2009). Therapeutic approaches with natural products investigate for searching safe, effective and relatively inexpensive new remedies for diabetes mellitus and associated metabolic disorders (Moller, 2001; Tan et al., 2008). Additionally, the use of natural products for the treatment of metabolic diseases has not been explored in depth despite the fact that a number of modern oral hypoglycemic agents such as metformin are derivatives of natural products.

Obesity is associated with insulin resistance and the metabolic syndrome. Obesity contributes to hypertension, high serum cholesterol, low HDL cholesterol and hyperglycaemia, and is independently associated with higher cardiovalscular diseases (CVD) risk (Hu et al., 2004; Zimmet et al., 2001). The risk of serious health consequences in the form of type II diabetes, coronary heart disease (CHD) and a range of other conditions, including some forms of cancer, has been shown to rise with an increase in body mass index (BMI) (Lee et al., 1993), but it is an excess of body fat in the abdomen, measured simply by waist circumference, that is more indicative of the metabolic syndrome profile than BMI (Ohlson et al., 1985; Pouliot et al., 1994). Obesity is characterized by excessive lipid

deposition associated with morphological and functional changes in adipocyte (Kang et al., 2010). Adipocytes are derived from mesenchymal stem cells, which can differentiate into osteoblasts, myoblasts, chondroblasts, or adipocytes (Kim et al., 2010). Adipocytes are critically associated with the regulation of adipose mass and obesity, whereas 3T3-L1 cells are a well established *in vitro* model for assessing adipocyte differentiation and the several stages related to obesity (Cho et al., 2008; Tang et al., 2003). The adipocyte life cycle includes changes in cell shape and terminal differentiation, clonal expansion and complex gene expression cascades which lead to the storage of lipids and finally apoptosis (Gregoire 2001; Kim et al., 2010). The function of adipocytes is to store energy in the form of fatty acids, and these cells play a central role in lipid and glucose metabolism; adipocytes also produce several hormones and cytokines (adipokine) (Rasouli and Kern, 2008). The differentiation of preadipocytes into adipocytes is accompanied by many changes in gene expression, e.g., a dramatic increase in the expression of CCAAR/enhancer binding proteins β (C/EBPβ) followed by the expression of CCAAR/enhancer binding proteins α (C/EBPα) and peroxisome proliferator-activated receptors γ (PPARγ) (Rosen et al. 2002) (**Fig. III**). AMPK acts as a fuel sensor and regulates glucose and lipid homeostasis in adipocytes (Unger, 2004). AMPK activation leads to numerous metabolic changes that would be attractive

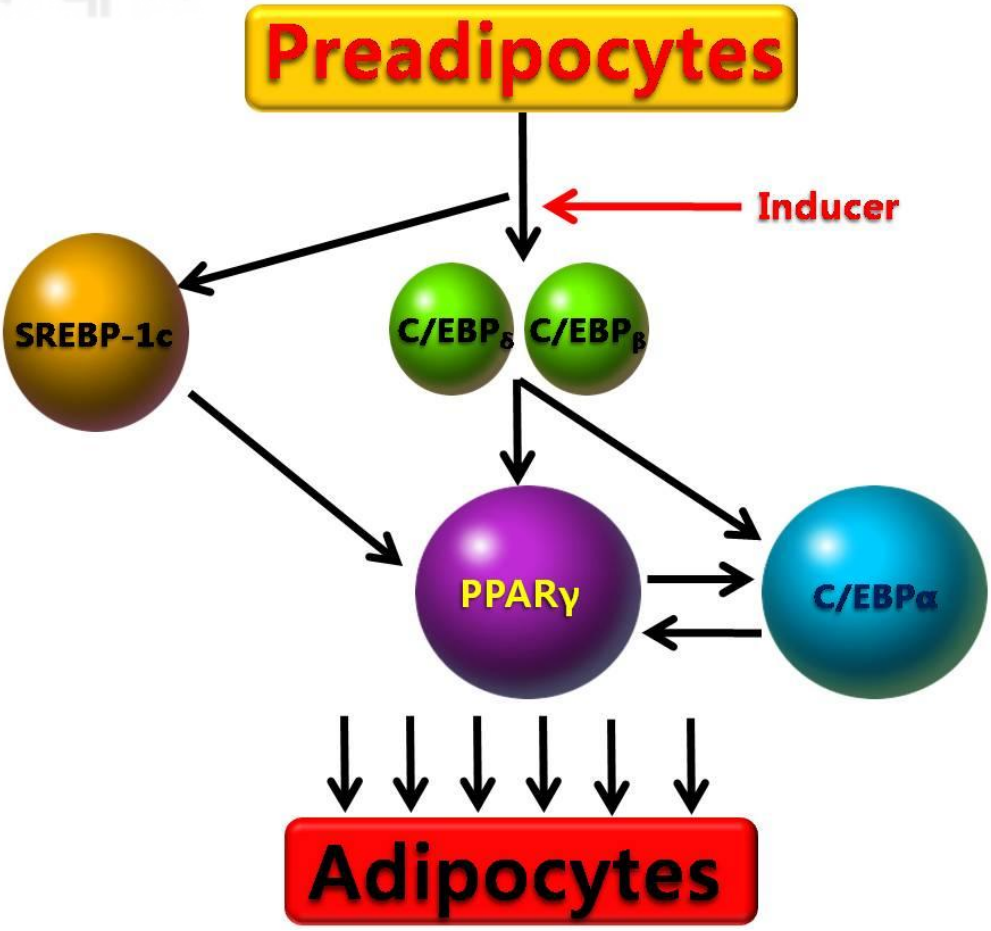

Fig. III. Progression of 3T3-L1 preadipocyte differentiation.

targets in treatment of metabolic disorders such as obesity, type II diabetes and metabolic syndrome (Kong et al., 2009).

Potent bioactive peptides have been produced via the enzymatic hydrolysis of food proteins (Fahmi et al., 2004; Lahl and Braun, 1994). While these peptides are inactive within the sequence of the parent protein, bioactive peptides can be released via enzymatic hydrolysis during the food manufacturing process (Lee et al., 2011). Additionally, enzymatic hydrolysates are also a source of bioactive peptides, which are short peptides released from proteins by hydrolysis that have been shown to exert biological effects such as antihypertensive (Jung et al., 2006), antioxidative (Bougatef et al., 2010), and antimicrobial (Kim et al., 2001) effects.

The *Styela clava* used in this study, a valuable marine resource, is found in Korea and Far East Asia. It is a traditional remedy for healing various internal conditions, and is believed to have profound curative properties. Additionally, in our previous study, *S. clava* was shown to reduce blood pressure in spontaneously hypertensive rats (SHRs). Several studies of the bioactivities of *S. clava*, such as its ACE inhibitory activity (Lee et al., 2010), as well as its antioxidant and anticancer activities (Kim et al. 2006; Lee et al., 2010) have been carried out previously. However, the above studies employed solvent extraction methods, and

there remains a lack of data regarding the biological activities of *S. clava* protein by enzymatic hydrolysis.

The principal objective of this study was to purify and identify an antihypertensive peptide derived from *S. clava*, under *in vitro* and *in vivo* conditions, and investigated its effects on glucose uptake in skeletal muscle cells and adipogenesis suppress in 3T3-L1 preadipocytes.

Part I.

Purification and identification of antihypertensive peptide from enzymatic hydrolysates of *Styela clava* and its antihypertensive effect in spontaneously hypertensive rats

Part I.

Purification and identification of antihypertensive peptide from enzymatic hydrolysates of *Styela clava* and its antihypertensive effect in spontaneously hypertensive rats

1. ABSTRACT

The principal objective of the present study is to isolate antihypertensive peptides derived from *Styela clava* flesh tissue and to characterize the isolated peptide with regard to angiotensin I-converting enzyme (ACE) inhibition and vasorelaxation. Nine proteases were used, and their respective enzymatic hydrolysates were screened to evaluate their potential ACE inhibitory activity. The Protamex hydrolysate possessed the highest ACE inhibitory activity, and the Protamex hydrolysate of flesh tissue showed relatively higher ACE inhibitory activity compared with the Protamex hydrolysate of tunic tissue. The induction of vasorelaxation of Protamex hydrolysate was endothelium-dependent and could be markedly blocked by pretreatment with the nitric oxide synthase (NOS) inhibitor, N^{G}-nitro-$_{L}$-arginine methyl ester ($_{L}$-NAME). During consecutive purification, a potent antihypertensive peptide from *S. clava* flesh tissue, which was composed of five amino acids, Ala-His-Ile-Ile-Ile (MW: 565.3 Da), and enhanced ACE inhibition and vasorelaxation. In human endothelial cells, NO

synthesis was found to be increased and eNOS phosphorylation was upregulated when the cells were cultured with the purified peptide. These results demonstrate that the purified peptide could mediate NO production, thereby exerting an endothelium-dependent vasorelaxation activity. Furthermore, systolic blood pressure (SBP) was reduced following administration of the antihypertensive peptide found in spontaneously hypertensive rats (SHRs).

2. MATERIALS AND METHODS

2.1. Materials

Styela clava (**Fig. 1-1**) used was kindly donated by Miduduk Corporated Association (Masan, Korea) and stored at -70℃ until use. Commercial food grade proteases including Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG and Alcalase 2.4L FG were purchased from Novo Co. (Novozyme Nordisk, Bagasvaerd, Denmark). Other proteases containing pepsin, trypsin, α-chymotrypsin and papain, as well as angiotensin I converting enzyme (from rabbit lung) and N-Hippuryl-His-Leu tetrahydrate (HHL) were purchased from Sigma chemical Co. (St. Louis, Mo, USA). The other chemicals and reagents used were of analytical grade.

2.2. Preparation of enzymatic hydrolysates of *S. clava*

To obtain antihypertensive peptide from *S. clava*, enzymatic hydrolysis was performed using various commercial proteases (Protamex, Kojizyme, Neutrase, Flavourzyme, Alcalase, pepsin, trypsin, a-chymotrypsin, and papain) under optimal conditions for 24 h. One gram of the dried ground *S. clava* powder was homogenized with buffer (100 ml), and then 10 mg or 10 μl enzyme was added. The pHs of the homogenates were adjusted to their respective optimal pH values before the enzymatic hydrolyzes (**Table 1-1**). The enzymatic reactions

Fig. 1-1. The photography of the *Stylea clava*.

were performed for 24 h to achieve optimum degree of the enzymatic hydrolysates. As soon as the enzymatic reaction complete, the hydrolysates were boiled for 10 min at 100℃ to inactivate the enzyme. Each enzymatic hydrolysate was clarified by centrifugation (3500 rpm, for 20 min at 4℃) to remove the residue. The yields of enzymatic hydrolysates were determined by subtracting the dried weight of the residue from one gram of hydrolysates dried and were expressed as a percentage. In all the tested enzymatic hydrolysates for antihypertensive effects of enzymatic hydrolysates, one enzymatic hydrolysate from *S. clava* subjected to molecular weight fractionation to obtain the peptides with molecular weight of <5 kDa (5 kDa or smaller), 5–10 kDa (between 5 and 10 kDa), and >10 kDa (10 kDa or larger). Enzymatic hydrolysate was passed through ultra-filtration membranes (molecular weight cut-off of 5 and 10 kDa) using Millipore's Labscale TFF system (Millipore Corporation, Bedford, Massachusetts, USA) at 4℃. The resultant fractions collected according to molecular weights (>10, 5–10, and <5 kDa) were lyophilized and stored at -20℃ for use in further experiments (Fig. 1-1). All the hydrolysates and molecular weight fractions were kept -20℃ for the further experiments.

Table 1-1. Optimum conditions of enzymatic hydrolysis for various enzymes.

Enayme	Optimum conditions		
	pH	Temp. (℃)	Buffer
Protamex	6.0	40	50 mM sodium phosphate
Kojizyme	6.0	40	50 mM sodium phosphate
Neutrase	6.0	50	50 mM sodium phosphate
Flavourzyme	7.0	50	50 mM sodium phosphate
Alcalase	8.0	50	50 mM sodium phosphate
α-chymotrypsin	8.0	37	50 mM sodium phosphate
Trypsin	8.0	37	50 mM sodium phosphate
Papain	6.0	37	50 mM sodium phosphate
Pepsin	2.0	37	20 mM glycine-HCl

2.3. Purification and identification of antihypertensive peptide

The strongest ACE inhibition and vasorelaxation fraction was applied to a Sephadex G-25 gel filtration column (2.5 × 75 cm), equilibrated with distilled water. The column was eluted with distilled water at a flow rate of 2 ml/min. The active fraction obtained was then applied to reverse-phase high performance liquid chromatography (RP-HPLC) on a YMC-Pack ODS-A (5 μm, 4.6 × 250 mm, YMC Co., Kyoto, Japan) analytical column with 15% acetonitrile containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. The finally purified peptide was analyzed for the amino acid sequence. Molecular mass and amino acid sequence of the purified peptide was determined by Waters Synapt high definition mass spectrometer (HDMS) coupled with electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). The instrument was operated in positive-ion mode with a capillary voltage of 2.8 kV unless stated otherwise. The cone voltage was maintained at 30 V for intact mass analysis. Following molecular mass determination, peptide was automatically selected for fragmentation and sequence information was obtained tandem MS analysis.

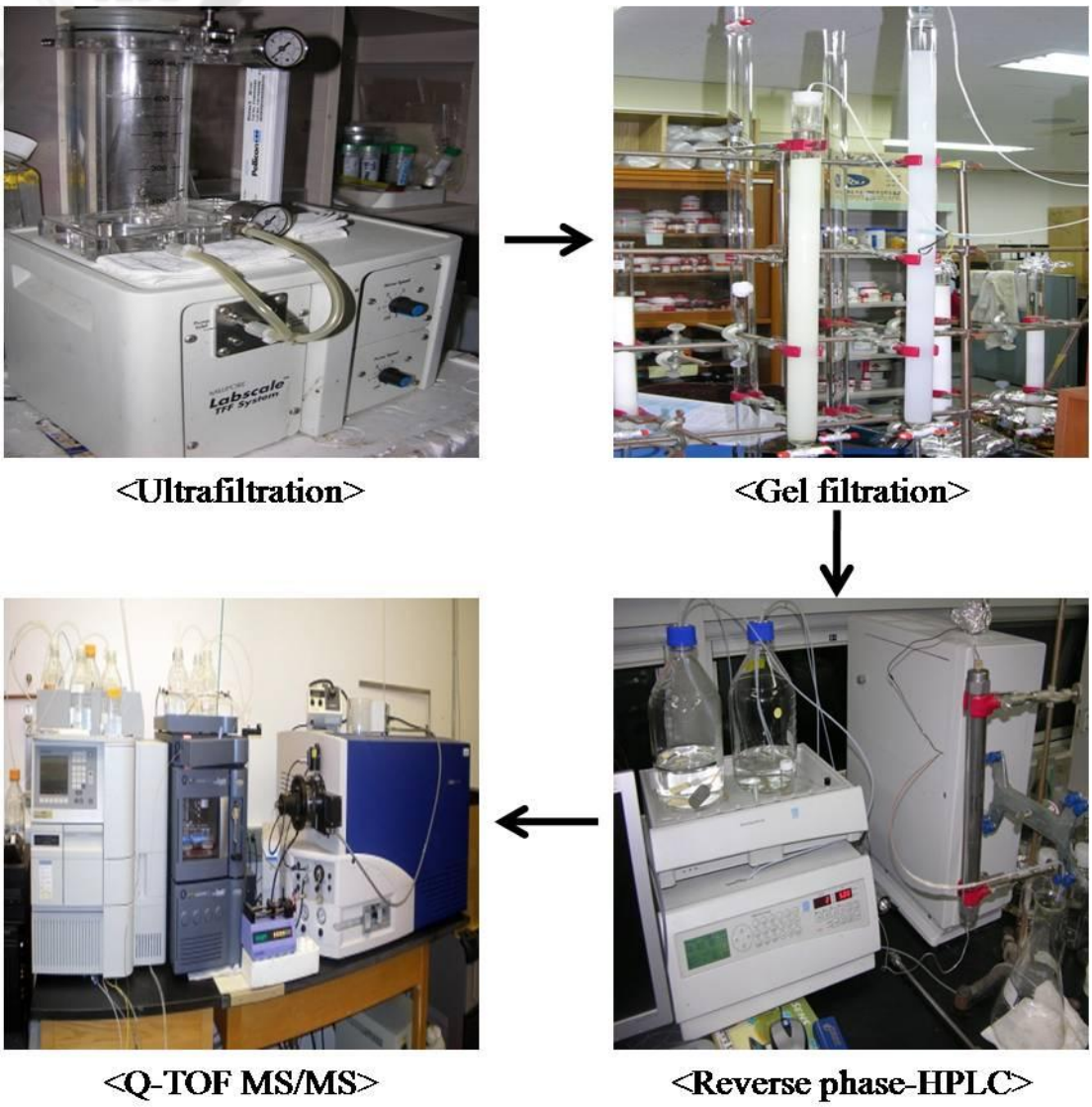

Fig. 1-2. Purification and Identification scheme of antihypertensive peptide from *S. clava.*

2.4. ACE inhibitory activity

The ACE inhibitory activity was assayed by measuring the concentration of hippuric acid liberated from HHL by the method of Cushman and Cheung (1970). For each assay, a 50 μl of the sample solution with 50 μl of ACE solution (25 mU/ml) was pre-incubated at 37℃ for 10 min, and then incubated with 100 μl of substrate (25 mM HHL in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) at 37℃ for 60 min. The reaction stopped by adding 250 μl of 1 N HCl. Hippuric acid was extracted with 500 μl of ethyl acetate. Then a 200 μl aliquot of the extract was removed by evaporation in a dry-oven at 80℃. The residue was dissolved in 1 ml distilled water and its UV spectra absorbance was measured at 228 nm. The IC_{50} value, defined as the concentration required for 50 % inhibition of ACE activity, was determined.

The ACE inhibitory activity was calculated as follows:

Inhibition %=(Ac-As)/(Ac-Ab)

Ac=Absorbance of control sample

As=Absorbance of sample solution

Ab=Absorbance of blank solution

2.5. Animals

All animal procedures were carried out in accordance with the National Institute of *Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Utilization Committee for Veterinary Medicine of Jeju National University. Male Sprague-Dawely (SD) rats with 11-week old and spontaneously hypertensive rats (SHRs) were purchased from Japan SLC. Inc. (Shizuoka, Japan). All rats were maintained at 24±1℃ with 12 h light/dark cycle and were given standard rat chow and water *ad libitum*.

2.6. Determination of vasorelaxation

The descending thoracic aortas obtained from the experimental groups of rats were dissected and placed in an ice-cold and oxygenated Krebs solution containing 120 mM NaCl, 4.75 mM KCl, 6.4 mM glucose, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$ and 1.7 mM $CaCl_2$. Rings of thoracic aorta (4 mm in length) were carefully excised and submerged in organ baths containing 10 ml of Krebs solutions of bathing medium at 37℃ and continuously gas with a carbogen mixture, 95% O_2 and 5% CO_2. The rings were mounted by means of two parallel triangle-shaped stainless-steel holder served as a anchor, while the other was connected to a force-displacement transducer (FT03, Grass, USA) to measure isometric

contractile force recorded by physiograph recorder (PowerLab/800, USA). A basal tension of 1 g was applied. Each preparation was allowed to equilibrate for 60~90 min in Krebs solution prior to the initiation of the experimental procedures, and during this period, the incubation medium was changed every 15 min. After equilibration time, the aortic rings were exposed to norepinephrine (10^{-7} M). When the concentration had stabilized, acetylcholine (10^{-5} M) was added to teat for the presence of the endotheilum. After equilibration time, the aortic rings were contracted by norepineohrine (10^{-7} M), and when the contractile response was stabilized (Steady-state phase, 12~15 min), relaxation was evaluated by cumulative addition of sample. Cumulative concentration-response curves were also performed with in the presence and absence of N^{G}-nitro-$_{L}$-arginine methyl ester ($_{L}$-NAME; 100 μM/L) or endothelium-denuded artery segments. $_{L}$-NAME was pre-incubated for 30 min before experiments. The degrees of pre-contraction of artery segments with all of the treatments were similar to the control. Relaxation responses to samples were plotted as a percentage of relaxation from the maximum contraction (**Fig. 1-3**).

2.7. Cell culture

Human endothelial cell line EA. hy 926 cells were used for NO production and eNOS

Fig. 1-3. Determination of vasorelaxation effect of *S. clava.*

expression by peptides from *S. clava* flesh tissue *in vitro*. Cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (100 mg/ml), and penicillin (100 unit/ml). Cultures were maintained at 37℃ in 5% CO_2 incubator.

2.8. Measurement of nitric oxide (NO) production

After pre-incubation of EA. hy 926 cells (1×10^5 cells/ml) with the antihypertensive peptide for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenthylenediamine dihydrocholoride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment.

2.9. Western blot analysis

Cells (2×10^5 cells/ml) were treated with the purified peptide and harvested. The cell lysates were prepared with lysis buffer [50 mM/L Tris-HCl (pH 7.4), 150 mM/L NaCl, 1% Triton X-100, 0.1% sodium docecyl sulfate (SDS), and 1 mM/L ethylenediamide tetraacetic acid

(EDTA)]. Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit. The lysate containing 40 μg of protein were subjected electrophoresis on 8% SDS-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against p-eNOS and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) in TTBS (25 mM/L Tris-HCl, 137 mM/L NaCl, 0.1% Tween 20, pH 7.4) containing 1% BSA at 1 h. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an enhanced chemiluminescence (ECL) Western blotting detection kit and exposed to X-ray films.

2.10. Anti-hypertensive effect in spontaneously hypertensive rats (SHRs)

The products were orally administered by metal gastric zoned in SHR. Saline solution served as the as negative control, and amlodipine (30 mg/kg body weight), a known calcium channel blocker, served as the positive control. The antihypertensive peptide was dissolved in saline solution at a dose of 100 mg/kg body weight. Before the measurement, the rats were kept at 37℃ for 10 min. The systolic blood pressure (SBP) of the rats was measured by the tail-cuff method, before administration and also 1, 3, 6, 12 and 24 h post-administration using

physiograph recorder (PowerLab 2/25, AD instruments, Colorado springs, USA).

2.11. Statistical analysis

All data were represented as the mean±S.E.M. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncan's multiple-range test using SPSS (11.5) software. Statistical significance was considered at $p<0.05$.

3. RESULTS AND DISCUSSION

Catalytically bioactive peptides can be obtained from a variety of food proteins (Byun & Kim, 2001). Improved nutritional and potent bioactive peptides have been shown to be generated by the enzymatic hydrolysis of proteins (Ondetti, 1997). Bioactive peptides may function as potential physiological modulators in the process of metabolism during the intestinal digestion of the diet (Kato & Suzuki, 1975). Bioactive peptides are liberated depending on their structure, composition and amino acid sequence (Lee et al., 2009). These peptides evidenced a variety of bioactivities, including antioxidative (Liu et al., 2010) and antihypertensive effects (Vercruysse et al., 2008). This study were to isolated antihypertensive peptide from *S. clava* flesh tissue and to investigate the ACE inhibitory and vasorelaxation effects, *in vitro* and *in vivo* spontaneously hypertensive rat (SHR) model.

The approximate chemical composition of *S. clava* is shown in **Table 1-2**. The major chemical component of the tested *S. clava* whole tissue was found to be carbohydrate; carbohydrate contents accounted for more than 40% of the total dry weight. The moisture, ash, protein, and lipid contents of *S. clava* whole tissue were 9.34, 10.77, 33.12, and 4.25%. Additionally, the carbohydrate content (60.38%) of *S. clava* tunic tissue was the highest among all components.

Table 1-2. Chemical compositions of *S. clava.*

Composition	Content (%)		
	Whole	Flesh	Tunic
Moisture	9.34±0.21	1.84±0.18	1.78±0.37
Ash	10.77±0.33	7.05±0.32	3.57±0.25
Protein	33.12±0.29	67.80±0.22	31.51±0.21
Carbohydrate	42.52±0.41	16.77±0.07	60.38±0.21
Lipid	4.25±0.43	6.54±0.21	2.76±0.11

However, *S. clava* flesh tissue evidenced a higher protein content (67.80%) than was observed in the other samples.

To produce antihypertensive peptide, *S. clava* was separated and hydrolyzed using a variety of commercial digestive enzymes. We screened the ACE inhibitory activity of the enzymatic hydrolysates from *S. clava*. The ACE inhibitory activities of the different enzymatic hydrolysates are provided in **Table 1-3**. Among all of the hydrolysates, the Protamex hydrolysate, in particular, evidenced the highest level of activity relative to the other hydrolysates. In terms of the activation of the ACE inhibitory effect, the highest IC_{50} value was observed with the Protamex hydrolysate at a concentration of 1.023 mg/ml. Thus, Protamex was selected for the effective hydrolysis of *S. clava*. We evaluated the effects of the ACE inhibitory activity of Protamex hydrolysate from the flesh and tunic tissues of *S. clava.* The Protamex hydrolysate of flesh tissues (PHFT) evidenced relatively higher levels of ACE inhibitory activity (**Table 1-4**; IC_{50} flesh tissue of 0.455±0.011 mg/ml compared to IC_{50} tunic tissue of 2.060±0.007 mg/ml). Compared to what has been observed in previous reports, the ACE inhibitory activities of enzymatic hydrolysates were more effective than those of the organic solvents and aqueous extracts from *S. clava* (Lee et al., 2010). Many previous reports have demonstrated that enzymes are capable of producing bioactive properties when they are

Table 1-3. ACE inhibitory activity of enzymatic hydrolysates from *S. clava.*

Enzyme	IC_{50} value (mg/ml)[a]
Kojizyme	2.481±0.032
Flavourzyme	2.343±0.022
Neutrase	2.234±0.019
Alcalase	1.781±0.018
Protamex	1.023±0.047
Pepsin	2.147±0.051
Trypsin	2.427±0.033
α-chymotrypsin	2.263±0.028
Papain	2.282±0.042

[a]The concentration of an inhibitor required to inhibit 50% of the ACE activity.

The values of IC_{50} were determined by at triplicate individual experiments.

Table 1-4. ACE inhibitoty activity of Protamex hydrolysates from flesh and tunic tissue of *S. clava*

Tissue	IC_{50} value (mg/ml) [a]
Whole	1.023±0.003
Flesh	0.455±0.011
Tunic	2.060±0.007

[a]The concentration of an inhibitor required to inhibit 50% of the ACE activity.

The values of IC_{50} were determined by at triplicate individual experiments.

incorporated to hydrolyze natural resources (Bougatef et al., 2010; Lee et al., 2009; Li et al., 2006).

The effect of PHFT on vasorelaxation was characterized using rat thoracic aorta prepared with or without endothelium to assess any improvements in endothelium-dependent vasorelaxation. The vasorelaxation effect of PHFT is shown in **Fig. 1-4(A)**. The vasorelaxation of PHFT was increased in a concentration-dependent manner (0.187 to 3 mg/ml) with an EC_{50} value of 2.81 mg/ml on an intact endothelium. However, endothelial denudation abolished vasorelaxation completely. The endothelium, a single layer of the vascular wall, regulates vascular tone via the production of vasoactive factors (Asselbergs et al., 2005; Kim et al., 2011). The endothelium generates potent vasodilators such as endothelium-derived hyperpolarizing factor (EDHF) (Beny and Brunet, 1988; Feletou and Vanhoutte, 2007) and endothelium-derived relaxing factor (EDRF) (Moncada and Vane, 1978). The EDRF has been identified as NO (Kim et al., 2011). NO is produced continuously by eNOS in the healthy endothelium in certain amounts in response to shear and pulsatile stretch of the vascular wall (Asselbergs et al., 2005). To further evaluate the mechanism of the vasorelaxation response in PHFT in endothelium-intact aorta rings, they are pre-incubated with $_L$-NAME (100 μM), a nitric oxide synthesis inhibitor. The pretreatment of aorta rings

with L-NAME markedly attenuated the vasorelaxation effect of PHFT (**Fig. 1-4(A)**). These results demonstrated that PHFT could mediate nitric oxide, thereby exerting an endothelium-dependent vasorelaxation activity. Endothelium-dependent vasorelaxation has been shown to be reflective of endothelial functioning, on the basis of the assumption that impaired endothelium-dependent vasomotion reflects impaired NO production (Asselbergs et al., 2005). Endothelial dysfunction, which manifests as reduced bioactive NO levels, is one of most common pathologic changes occurring in a variety of cardiovascular diseases (Christopher et al., 2008).

Fractionation with different molecular weights of the enzymatic hydrolysates was conducted using ultra-filtration (UF) membranes of different pore sizes (Byun and Kim, 2001). The principal advantage of the UF system is that the molecular weight distribution of the desired digests can be controlled by adopting an appropriate UF membrane (Jeon et al., 2000). The PHFT was further separated into three MW groups, PHFT-I (MW <5 kDa), PHFT-II (MW = 5~10 kDa) and PHFT-III (MW >10 kDa), using UF membrane (MW cut-off of 5 and 10 kDa). The three groups were investigated by vasorelaxation. As shown in **Fig. 1-4(B)~(D)**, most of the MW groups evidenced endothelium-dependent vasorelaxation. Among all of the MW groups, PHFT-I evidenced the strongest vasorelaxation and had an EC_{50} value of 1.58 mg/ml

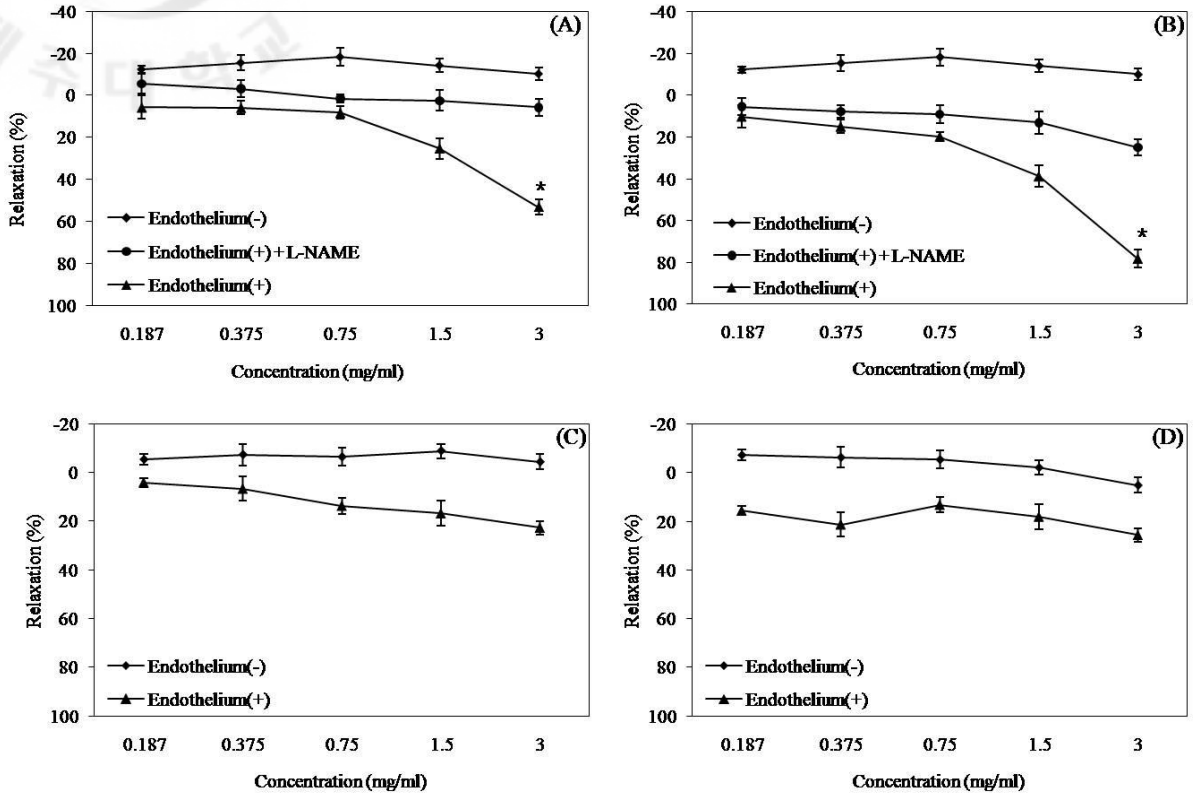

Fig. 1-4. Concentration-dependent vasorelaxation of PHFT and molecular weight fractions of PHFT in aortic segments with and without endothelium. (A) PHFT; (B) PHFT-I (MW < 5 kDa); (C) PHFT-II (MW = 5~10 kDa); (D) PHFT-III (MW >10 kDa). The data are expressed as the means±S.E. Statistical evaluations were conducted to compare the endothelia (-). *$p < 0.05$.

(**Fig. 1(B)**). Additionally, the pretreatment of aorta rings with $_{L}$-NAME markedly attenuated the vasorelaxation effect exhibited by PHFT-I. Molecular size of fractions became smaller, and evidenced a low extent as compared with molecular weight size. ACE inhibitory activity was widely observed in all of the fractions, thus suggesting that many ACE inhibitory substances with various molecular weight ranges were contained in the PHFT. However, the most potent ACE inhibition was noted at PHFT-I, and evidenced an IC_{50} value of 0.281 mg/ml (**Table 1-5**). This result was consistent with the previous studies of anti-hypertensive peptides, in which the lower molecular weight fraction had more potent ACE inhibition and vasorelaxation when separated by an UF membrane (Miguel et al., 2007).

To purify the strong antihypertensive peptide, gel filtration chromatography of PHFT-I was conducted using a Sephadex G-25 column, and a total of four fractions were collected (**Fig. 1-5(A)**). Among the fractions, fraction A evidenced the strongest vasorelaxation, with an EC_{50} value of 0.761 mg/ml on an intact endothelium (**Fig. 1-5(B)**). Additionally, the pretreatment of aorta rings with $_{L}$-NAME markedly attenuated the vasorelaxation effect exhibited by fraction A. Also, fraction A evidenced the highest ACE inhibitory activity, with an IC_{50} value of 0.162 mg/ml (**Table 1-6**). Fraction A was concentrated and used for further isolation using reverse phase-HPLC on a YMC-Pack ODS-A column ($C_{18,}$ 5 μm, 4.6 × 250

Table 1-5. ACE inhibitory activity of molecular weight fractions of PHFT.

Fraction	IC_{50} value (mg/ml)[a]
PHFT-I (<5 kDa)	0.281±0.003
PHFT-II (5-10 kDa)	0.552±0.005
PHFT-III (>10 kDa)	0.325±0.008

[a]The concentration of an inhibitor required to inhibit 50% of the ACE activity. The values of IC_{50} were determined by at triplicate individual experiments.

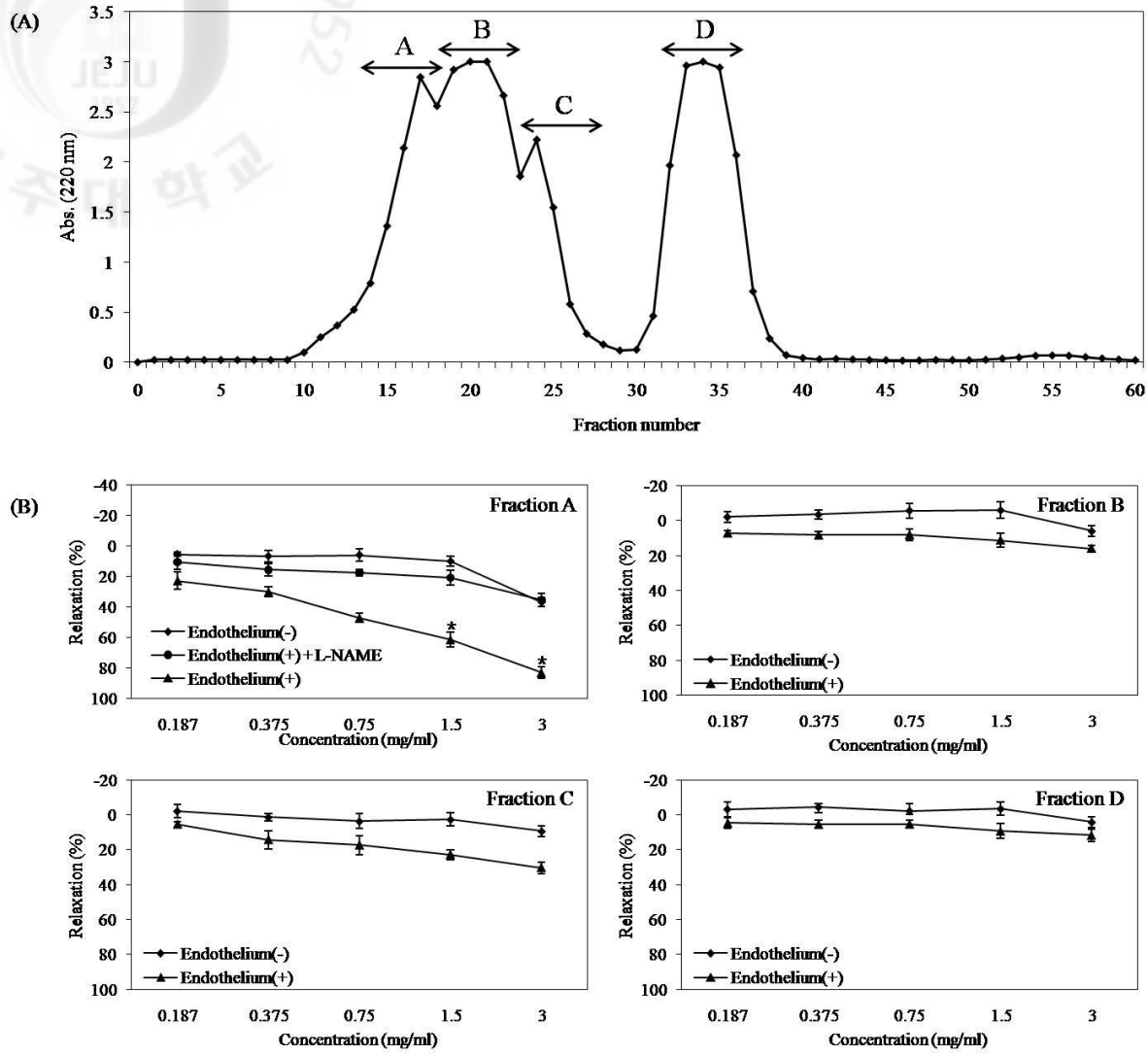

Fig. 1-5. Sephadex G-25 gel filtration chromatogram of PHFT-I (MW <5 kDa). (A) Separation was performed at 2 ml/min and collected at a fraction volume of 10 ml. Gel columns were separated into four fractions (A~D). (B) Concentration-dependent vasorelaxation of gel filtrated fractions of PHFT-I (MW <5 kDa) in aortic segments with endothelium and without endothelium. The data are expressed as the mean±S.E. Statistical evaluation was conducted to compare the endothelium (-). *$p < 0.05$.

Table 1-6. ACE inhibitory activity of gel filtrated fractions of PHFT-I (MW <5 kDa).

Fraction	IC_{50} value (mg/ml)[a]
A	0.162±0.012
B	0.926±0.025
C	0.285±0.009
D	1.140±0.029

[a]The concentration of an inhibitor required to inhibit 50% of the ACE activity.

The values of IC_{50} were determined by at triplicate individual experiments.

mm, YMC, Kyoto, Japan). Of the two fractions collected, fraction A-II evidenced the most profound vasorelaxation (**Fig. 1-6**). Also, fraction A-II exhibited the most potent ACE inhibitory activity, with an IC_{50} value of 0.021 mg/ml (**Table 1-7**). The purity of fraction A-II was confirmed by RP-HPLC analysis (**Fig. 1-**7). The molecular mass of the purified peptide was 565.3 Da, as determined by a Waters Synapt high-definition mass spectrometer (HDMS). The purified peptide was analyzed using a peptide sequencer and was identified as a pentapeptide, Ala-His-Ile-Ile-Ile (**Fig. 1-7**). These findings indicate that the anti-hypertensive effect of Ala-His-Ile-Ile-Ile from PHFT is caused by ACE inhibition and endothelium-dependent vasorelaxation. Anti-hypertensive treatment with ACE-inhibitors, calcium–channel blockers, angiotensin II receptor blockers, and diuretics can improve endothelium-dependent vasorelaxation (Clozel et al., 1990; Hayakawa et al. 1997; Modena et al., 2002; Modena et al., 1994). The most potent improvement of endothelium function might be obtained by ACE inhibitors, and reduce blood pressure by inhibiting not only the production of angiotensin II, but also via the degradation of bradykinin, the vasorelaxing activity of which is caused by NO (Zhao et al., 2008). The direct effects of the antihypertensive peptide on the endothelium were investigated in human endothelial cells. In order to observe the

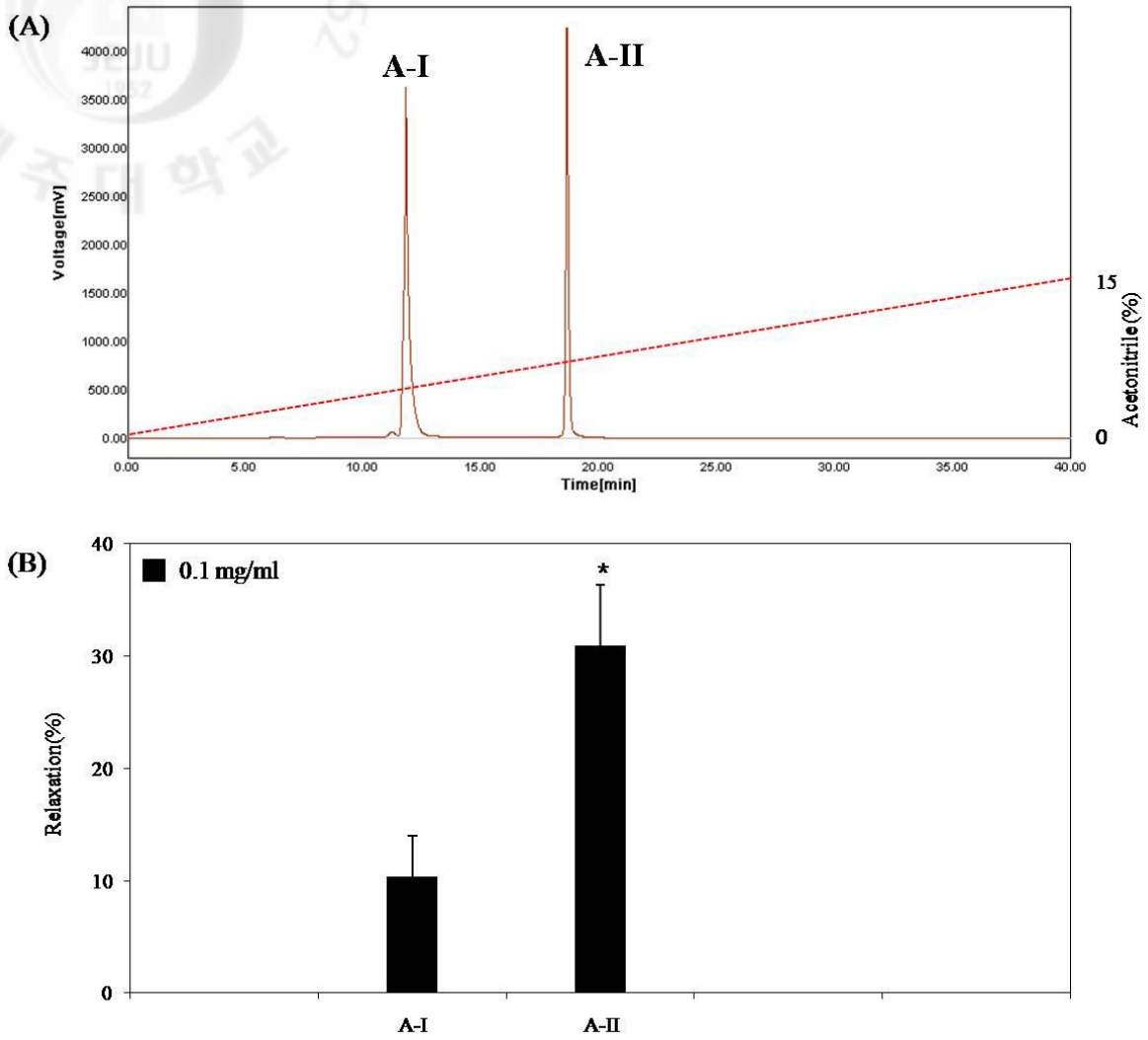

Fig. 1-6. RP-HPLC chromatogram of the fraction A isolated from Sephadex G-25 in aortic segments with endothelium. (A) Separation into sub-fractions (A-I and A-II) was carried out with a linear gradient of acetonitrile from 0% to 15% at a flow rate of 1 ml/min. (B) Vasorelaxation effect of sub-fraction of the fraction A in aortic segments with and without endothelium. The data are expressed as the mean±S.E. Statistical evaluation was carried out to compare the A-I and A-II. *$p < 0.05$.

Table 1-7. ACE inhibitory activity of sub-fraction of the fraction A.

Fraction	IC_{50} value (mg/ml)[a]
A-I	0.132±0.025
A-II	0.021±0.012

[a]The concentration of an inhibitor required to inhibit 50% of the ACE activity. The values of IC_{50} were determined by at triplicate individual experiments.

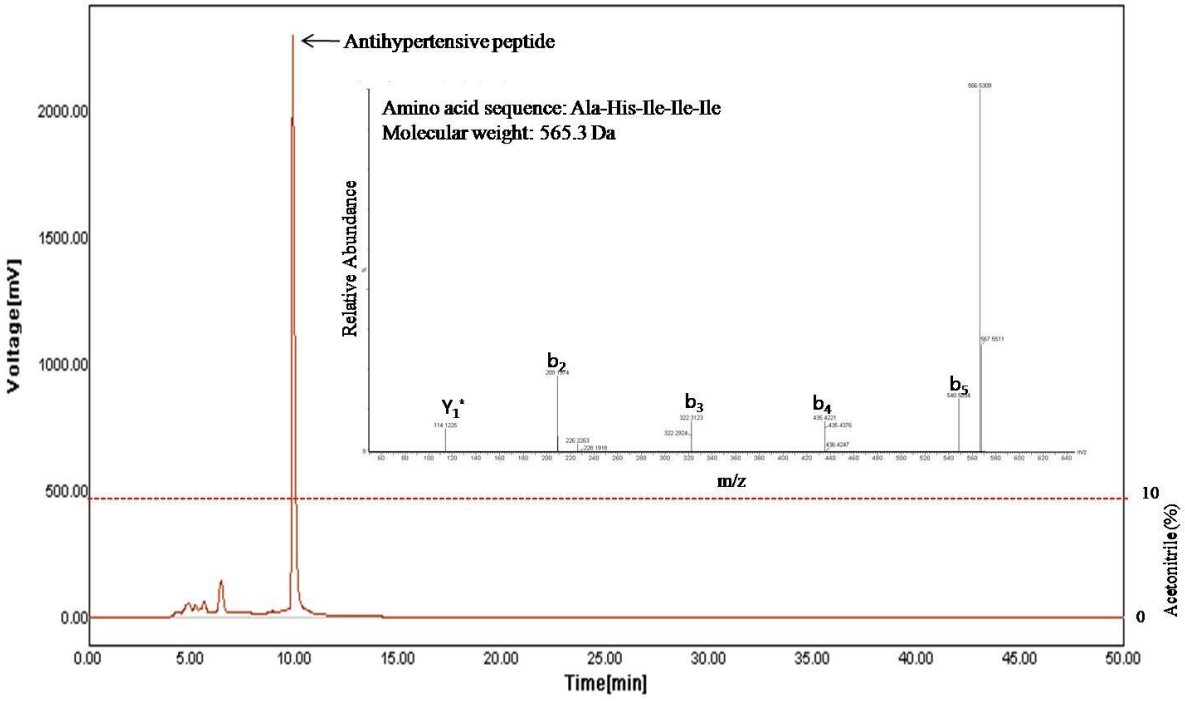

Fig. 1-7. RP-HPLC and MS profile of the purified antihypertensive peptide.

effect of the antihypertensive peptide on NO production, human endothelial cells (EA.hy 926 cells) were incubated in medium and the levels of released NO were measured at varying concentrations of the antihypertensive peptide from PHFT. As demonstrated in **Fig. 1-8(A)**, the level of NO released in the incubation media of endothelial cells were increased by treatment with the antihypertensive peptide at a concentration of 0.1 mM. This result suggests that the antihypertensive peptide directly affects NO production in endothelial cells. Reduced NO activity in the vasculature promotes the progression of cardiovascular disease (Schmitt and Dirsch, 2009). An important characteristic of healthy endothelium is an adequate output of NO, which is generated by endothelial nitric oxide synthase (eNOS) (Alderton et al., 2001; Palmer et al., 1988; Schmitt and Dirsch, 2009). As the antihypertensive peptide enhanced NO production from endothelial cells, this study further investigated the effect of the antihypertensive peptide on NO synthase activation. On the basis of Western blot analysis, the treatments of the antihypertensive peptide markedly enhanced eNOS phosphorylation (ser 1177) in endothelial cells (**Fig. 1-8(B)**). The overall data indicate that NO production via the activation of eNOS is involved in the antihypertensive peptide-induced vasorelaxation. The phosphorylation of eNOS is a major post-translational regulatory mechanism of eNOS

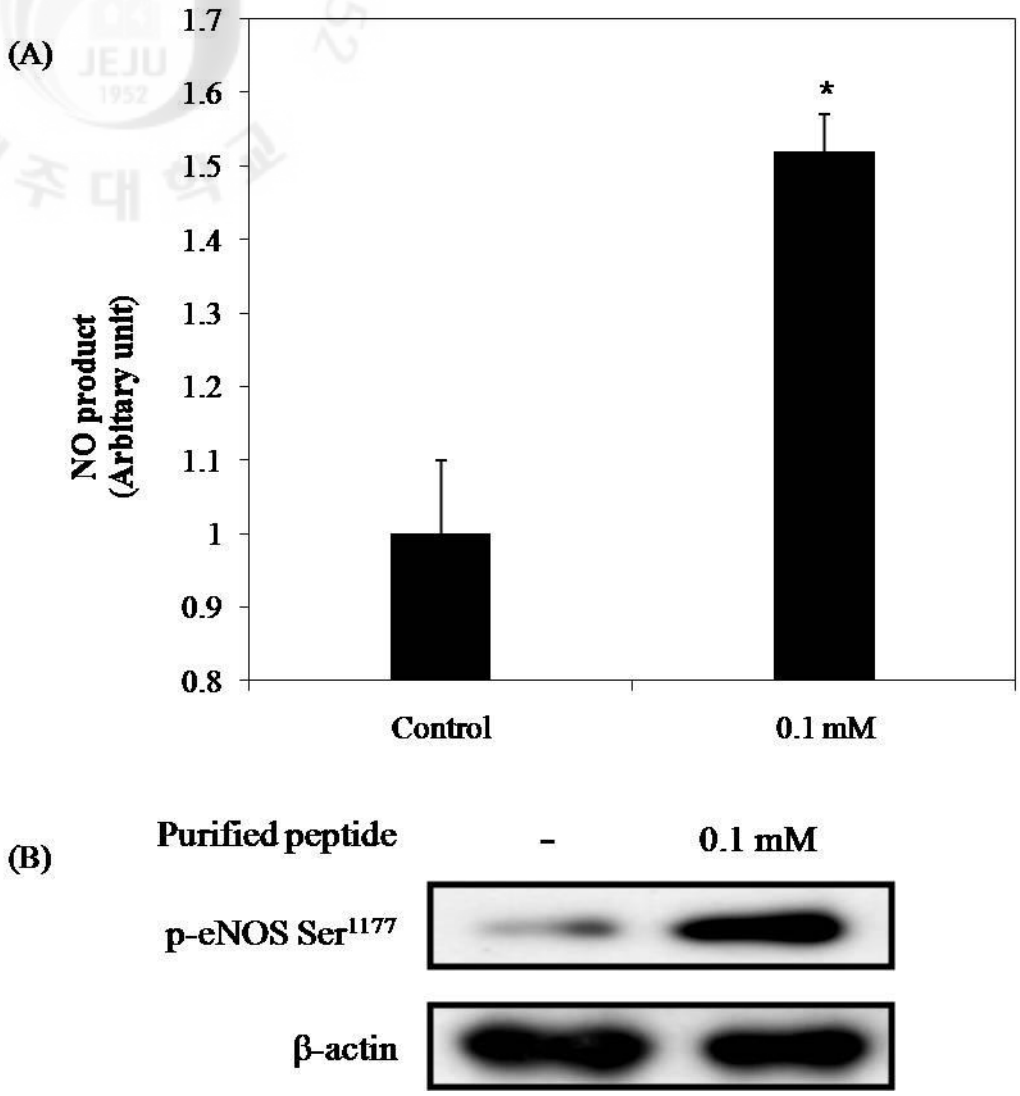

Fig. 1-8. The effect of the antihypertensive peptide on NO production and eNOS phosphorylation in human endothelial cells. The eNOS phosphorylation level was determined via Western blot analysis in the endothelial cells treated with one dose of the antihypertensive peptide (0.1 mM).

activity and involves a number of kinases and phosphatases. The eNOS phosphorylation sites have been identified thus far; the amino acid numbers refer to the human eNOS sequence, unless started otherwise (Schmitt and Dirsch, 2009). Among the phosphorylation sites, Ser 1177 is the most important positive regulatory domain, which is phosphorylated in response to most stimuli promoting eNOS activation. eNOS phosphorylation is catalyzed by protein kinase B (Akt), as well as a number of other kinases (Schmitt and Dirsch, 2009; McCabe et al., 2000; Mount et al., 2007).

The antihypertensive effect of the purified peptide was evaluated by measuring the change in systolic blood pressure (SBP) at 1, 3, 6, 12 and 24 h after the oral administration of 100 mg/kg of body weight. Amlodipine (30 mg/kg bodyweight) was employed as a positive control, and the control group was injected with an identical volume of saline. In our acute oral administration experiments (**Fig. 1-9**), little SBP changes were observed in the SHR model control group. The maximum SBP reduction levels of these treatment groups were, in descending order: amlodipine group (53.67 mmHg); and the purified peptide group (46.83 mmHg). The antihypertensive effects of these treatments were maintained for 12 h. The SBP began to recover 12 h after treatment (the purified peptide) and returned to initial levels 24 h after administration. Overall, our results clearly indicated that the purified peptide could

depress SBP in SHRs. Many antihypertensive peptides have recently been isolated from enzyme-digested food proteins. Small peptides (di- or tripeptides) are readily absorbed in their intact forms in the intestine (Lee et al., 2010). In order to exert an antihypertensive effect after oral ingestion, bioactive peptides must be absorbed from the intestine intact and be resistant to degradation (Zhao et al., 2007). Even through the purified peptide has relativity high MW compared to di- or tripeptides, the results clearly demonstrated that purified peptide exerts a substantial effect on the reduction of SBP in SHRs. If this peptide was not effectively absorbed without digestion, it was quite difficult to observe this type of anti-hypertensive effect on SHRs.

In conclusion, we have purified and identified a novel antihypertensive peptide (Ala-His-Ile-Ile-Ile) from *Styela clava* flesh tissue via ACE inhibitory and NO mediated vasorelaxation effects. Additionally, the anti-hypertensive effect in SHR also showed that the oral administration of purified peptide could reduce SBP significantly. The results of this study indicate that the antihypertensive peptide from *S. clava* flesh tissue could be employed as a functional food ingredient with potential therapeutic benefits in the prevention and treatment of hypertension and other associated diseases.

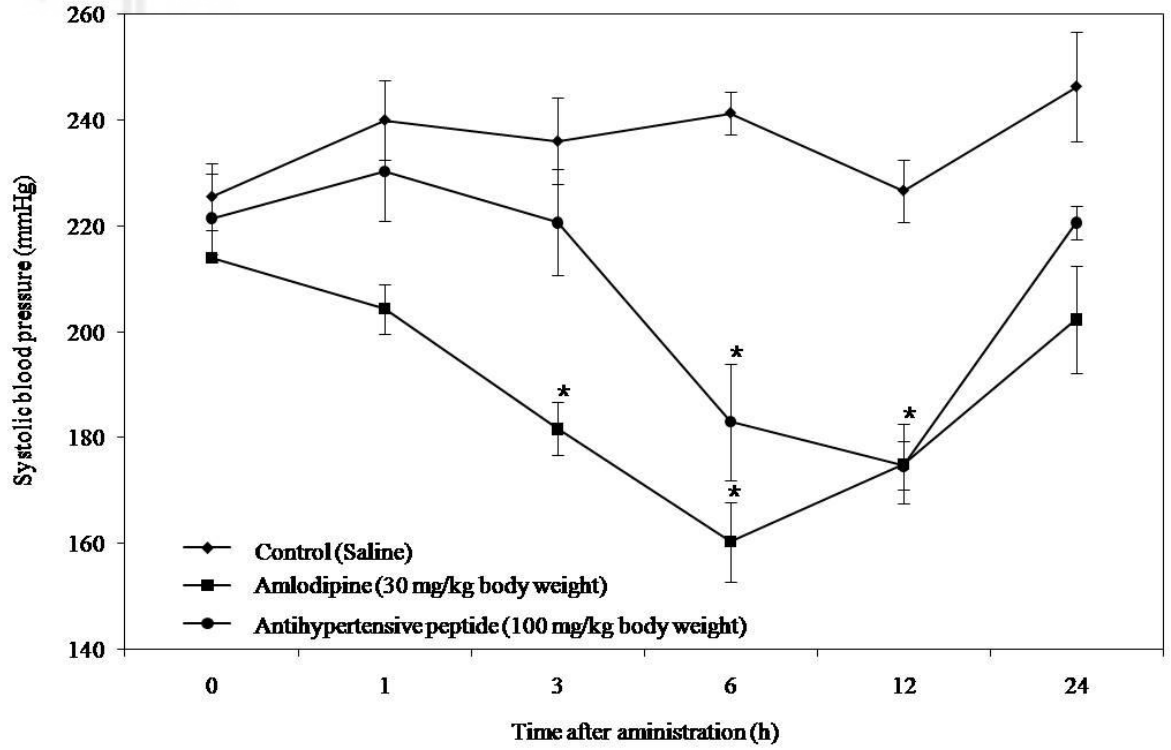

Fig. 1-9. Change of systolic blood pressure (SBP) of SHRs after the oral administration of test group. (◆) negative control (Saline); (■) positive control (Amlodipine, 30 mg/kg body weight); (•) the antihypertensive peptide (100 mg/kg body weight);. The data are expressed as the means±S.E. Statistical evaluation was carried out and compared with the control group. *$p < 0.01$.

Part II.

Antihypertensive peptide purified from *Styela clava* flesh tissue stimulates glucose uptake through AMP-activated protein kinase (AMPK) activation in skeletal muscle cells

Part II.

Antihypertensive peptide purified from *Styela clava* flesh tissue stimulates glucose uptake through AMP-activated protein kinase (AMPK) activation in skeletal muscle cells

1. Abstract

In the present study, we reported the glucose uptake effects of the antihypertensive peptide purified from *Styela clava*, on skeletal muscle cells. The antihypertensive peptide dose-dependently increased glucose uptake in differentiated L6 rat myoblast cells compared to control. Inhibition of AMP-activated protein kinase (AMPK) by compound C exhibited significant inhibitory effect on antihypertensive peptide-stimulated glucose uptake. Western blotting analyses revealed that antihypertensive peptide increased the phosphorylation level of AMPK and such enhancement can be specifically inhibited by compound C. In addition, we demonstrated that glucose transporter GLUT4 translocation to plasma membrane was increased by antihypertensive peptide. In summary, AMPK activation was involved in the effects of antihypertensive peptide on glucose transport activation. The antihypertensive peptide can be further developed as potential compound for the anti-diabetic therapy.

2. MATERIALS AND METHODS

2.1. Materials

The rat myoblast cell line L6 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). DMEM (Dulbecco's modified Eagle's medium), wortmannin, and compound C were purchased from Sigma (St. Louis, MO, USA). Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (Thr 172), and glucose transporter 4 (GLUT4) were from Cell Signaling Technology (Bedford, Massachusetts, USA). Second IgG HRP-linked antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other chemicals and reagents used were of analytical grade.

2.2. Cell culture

Rat myoblast L6 cells were maintained in high glucose-DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37℃ in 5 % CO_2 incubator. For differentiation, the cells were seeded in appropriate culture plates, and after sub-confluence (about 80%), the medium was changed to DMEM containing 2% horse serum for 7 days, with medium changes every day. All experiments were performed in differentiated L6 myotubes after 7 days.

2.3. MTT assay

The cytotoxicity of antihypertensive peptide against the L6 cells was determined by a colorimetric MTT assay. Cells were seeded in a 24-well plate. After 24 h, the cells were treated with various concentrations (9.38, 18.8, 37.5, 75 and 150 μM) of the antihypertensive peptide. The cells were then incubated for an additional 24 h at 37℃. MTT stock solution (100 μl; 2 mg/ml in PBS) was then added to each well. After incubating for 4 h, the plate was centrifuged at 2,000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

2.4. Glucose uptake assay

L6 cells were seeded in a 24-well plate. After differentiation, the cells were starved in serum-free low glucose DMEM for 4 h, and then washed with PBS and incubated with fresh serum-free low glucose DMEM. After that, the cells were treated with insulin (100 nM) for 1 h, or the indicated concentrations (to determine the dose response of L6 myotubes to antihypertensive peptide) of antihypertensive peptide. Glucose uptake was measured by glucose concentration in the media solution using glucose oxidase assay kit (Asan

Pharmaceutical corp., Korea).

In some experiments, 100 nM of Wortmannin (PI3-kinase inhibitor) and 10 μM of Compound C (AMPK inhibitor) were added 30 min before the antihypertensive peptide treatment.

2.5. Western blot analysis

L6 myotubes were grown 100 mm dishes and were starved in serum-free low glucose DMEM for 4 h prior to treatment with the indicated agents. Following treatment the media were aspirated and the cells were washed twice in ice-cold PBS. The cells were lysated in NucBuster^{TM} Protein Extraction Kit (Novagen, San Diego, CA, USA) for 10 min and then centrifuged at 16,000 rpm for 5 min at 4℃. The protein concentrations were determined by using BCA^{TM} protein assay kit. The lysate containing 40 μg of protein were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane. The membrane was blocked in 1% bovine serum albumin (BSA) in TBST (25 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4) for 2 h. The primary antibodies were used at a 1:1000 dilution. Membranes incubated with the primary antibodies at 4℃ for overnight. Then the membranes were washed with TTBT and

then incubated with the secondary antibodies used at 1:3000 dilution. Signals were developed using an ECL western blotting detection kit and exposed to X-ray films.

2.6. Plasma membrane fractionation and immunoblot analysis

L6 myotubes were treated with the indicated agents and harvested. The cell lysates were prepared with lysis buffer [250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCI, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors (1 mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin)] and kept on ice for 10 min. The cell lysate were ultracentrifuged at 22,000 rpm for 1 h at 4℃. The pallet was re-suspended in a lysis buffer and kept on ice for 10 min and then centrifuged at 8,000 rpm for 5 min at 4℃ to obtain plasma membrane fraction from the middle layer of the supernatant. Immunoblot analyses of GLUT4 described in the method to 2.6.

2.7. Statistical analysis

All data were represented as the mean ± S.E.M. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncan's multiple-range test using SPSS (11.5) software. Statistical significance was considered at $p<0.05$.

3. RESULTS AND DISCUSSION

Type II diabetes has come to pose a major worldwide threat to human health and is the most common type of diabetes (Zimmet et al., 2001). Hyperglycemia plays a pivotal role in the development type II diabetes and complications associated with the diseases such as arteriosclerosis, stroke, nephropathy and myocardial infarction (Baron, 1998). Therefore, the effective control of blood glucose level is the key to prevent or reverse diabetic complications and improve the quality of the life in diabetic patients (DeFronzo, 1999). Currently available drugs for type II diabetes have a number of limitations, including adverse effects and high rates of secondary failure. In recent years, natural products have become the focus of a considerable amount of attention from researchers searching for alternative therapies of diabetes (Chang et al., 2006; Jung et al., 2007). This is because natural products are usually considered to be less toxic with fewer side effects than synthetic drugs. Recently, interest has emerged to identify and characterize bioactive peptides from natural products (Sarmadi and Ismail, 2010).

Skeletal muscle has been identified as the major tissue in glucose metabolism, accounting for nearly 75% of whole-body insulin-stimulated glucose uptake (Defronzo et al., 1981). Insulin-stimulated glucose uptake in skeletal muscle is critical for reducing blood glucose

levels. Failure of glucose uptake due to decreased insulin sensitivity leads to the development of type II diabetes. In skeletal muscle, glucose transport can be activated by at least two major mechanisms. In this article, we have studied the effects of antihypertensive peptide purified from *S. clava* on glucose uptake in cell cultures of differentiated L6-myotubes. Furthermore, we analyzed the phosphorylation status of key components of signaling pathways that are involved in the molecular mechanisms regulating glucose uptake.

Cytotoxicity of antihypertensive peptide was evaluated using the MTT assay in various concentrations (9.38, 18.8, 37.5, 75, and 150 μM). The antihypertensive peptide did not affect the cytotoxic of L6 skeletal muscle cells compared control (**Fig 2-1**). Thus, the concentrations were used in subsequent experiments.

In order to determine the role of antihypertensive peptide did not shown cytotoxicity up to 150 μM compared with control in glucose metabolism of muscle cells, the effect of antihypertensive peptide on glucose uptake was investigated in L6 skeletal muscle cells. It was found that antihypertensive peptide dose-dependently stimulated glucose uptake as shown in **Fig. 2-2**, and the effect of antihypertensive peptide (150 μM) was comparable to that of insulin, which indicates that antihypertensive peptide may have metabolic effects in skeletal muscle cells.

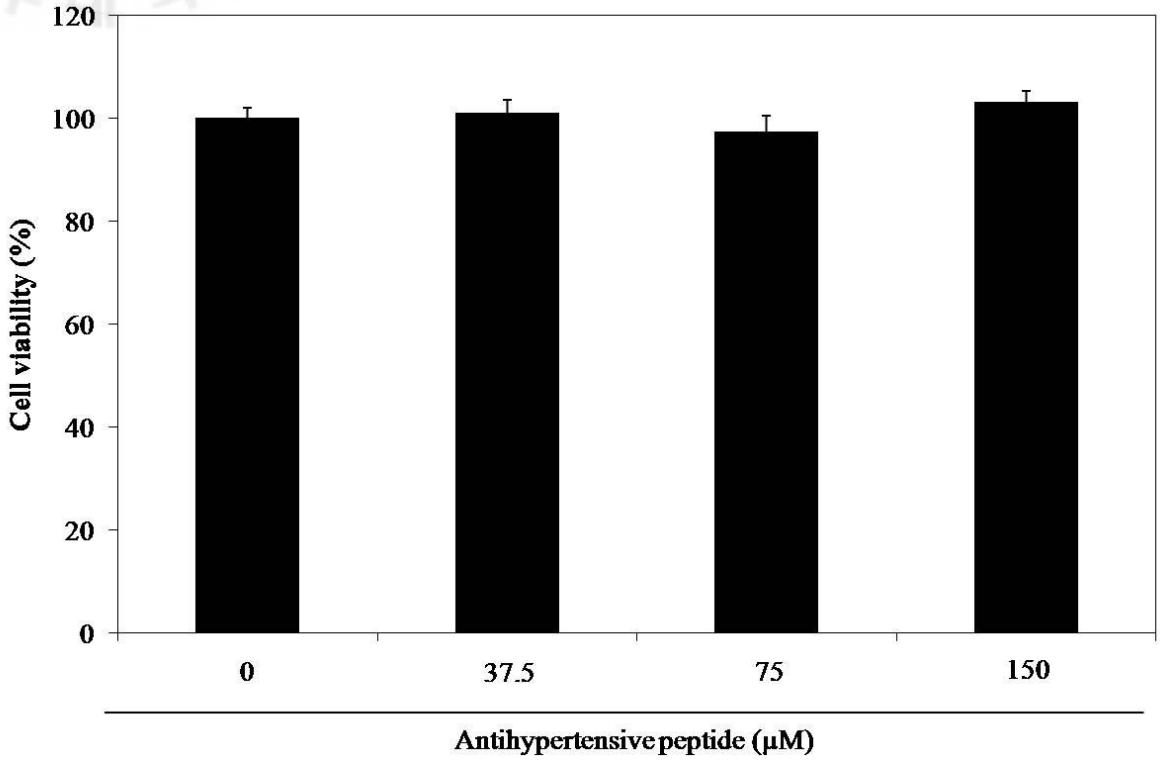

Fig. 2-1. Cytotoxicity of antihypertensive peptide. Cytotoxicity of antihypertensive peptide was determined using the MTT assay. Each value is expressed as mean±S.E. in triplicate experiments.

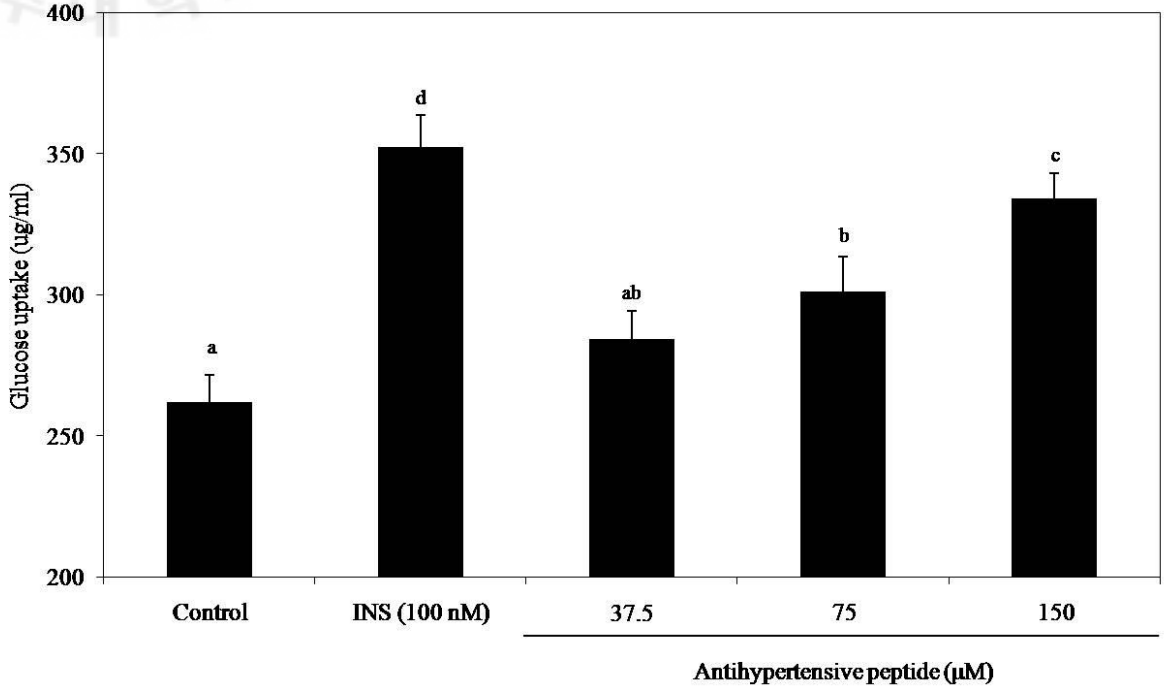

Fig. 2-2. Antihypertensive peptide dose-dependently stimulates glucose uptake in L6 skeletal muscle cells. (A) Cells were starved in serum free (SF) media for 4 h, and incubated for 1 h with increasing of antihypertensive peptide and insulin. Values are expressed as means ± S.E. in triplicate experiments. $^{a-d}$Values with different alphabets are significantly different at $P<0.05$ as analyzed via Duncan's multiple range test.

To look into which pathway may be involved in the effect of antihypertensive peptide on glucose uptake in L6 cells, L6 cells were pretreated with wortmannin, an inhibitor of phosphatidylinositol (PI) 3-kinase and compound C, a selective AMPK inhibitor. As shown in **Fig. 2-3**, compound C exhibited significant inhibition on glucose uptake stimulated by antihypertensive peptide (150 μM). The results indicate that antihypertensive peptide-induced increase in the glucose uptake may involve AMPK activation. To look into the roles of antihypertensive peptide in AMPK signaling pathway, we investigated the effects of antihypertensive peptide on AMPK activation. We found that treatment of antihypertensive peptide induced increase in AMPK phosphorylation in L6 cells (**Fig. 2-4**). However, the increase in phosphorylation of AMPK was inhibited by pretreatment of compound C, a selective AMPK inhibitor for 30 min before treatment of antihypertensive peptide (**Fig. 2-4**). This result, together with above results, strongly indicates that antihypertensive peptide plays a metabolic role in skeletal muscle cells through the AMPK pathway. AMPK is known to play a major role in energy homeostasis in ATP-depleting metabolic states such as ischemia, hypoxia, heart shock, oxidative stress, and especially exercise (Harder et al., 2001; Raj and Dentino, 2002). Once activated under such condition, it accelerates ATP-generating catabolic pathway including glucose uptake and fatty acid oxidation through direct regulation of key

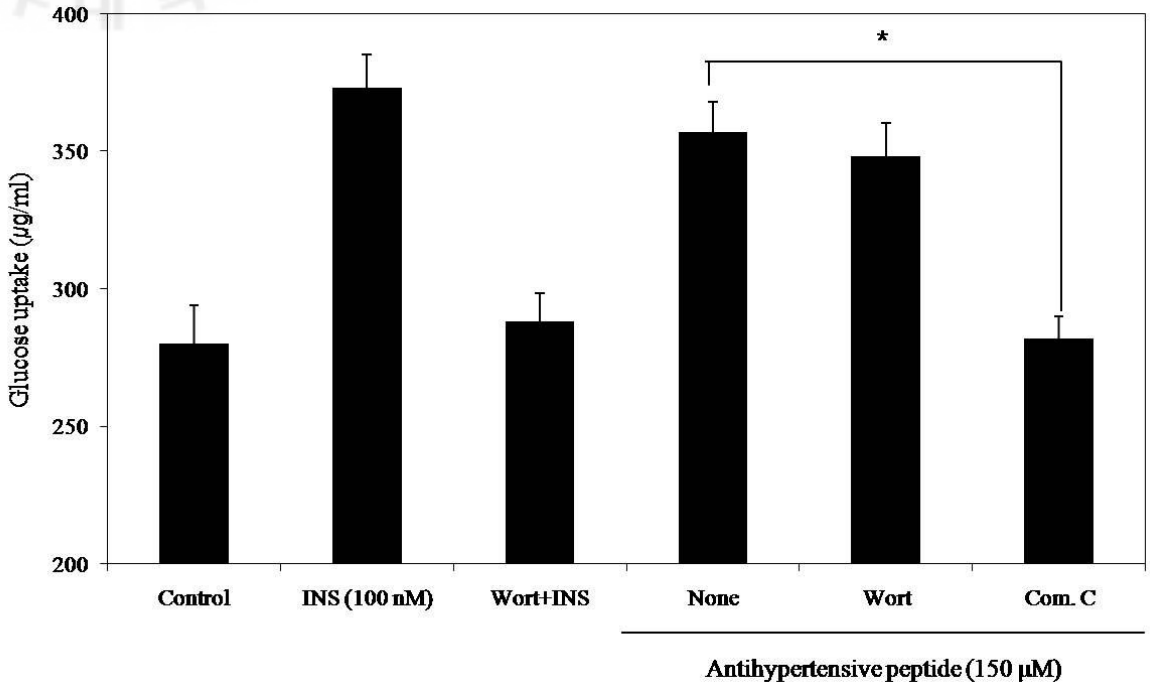

Fig. 2-3. Antihypertensive peptide-induced increase of glucose uptake was reduced by wortmannin and compound C. After 4 h starvation, L6 skeletal muscle cells were pretreated with or without 100 nM wortmannin (phosphatidylinositol (PI) 3-kinase inhibitor) and 10 μM compound C, (AMPK inhibitor) for 30 min, and then treated with 150 μM antihypertensive peptide for 1 h. Each value is expressed as mean ± S.E. in triplicate experiments. $^{*}P<0.05$ vs. control or between two groups as indicated.

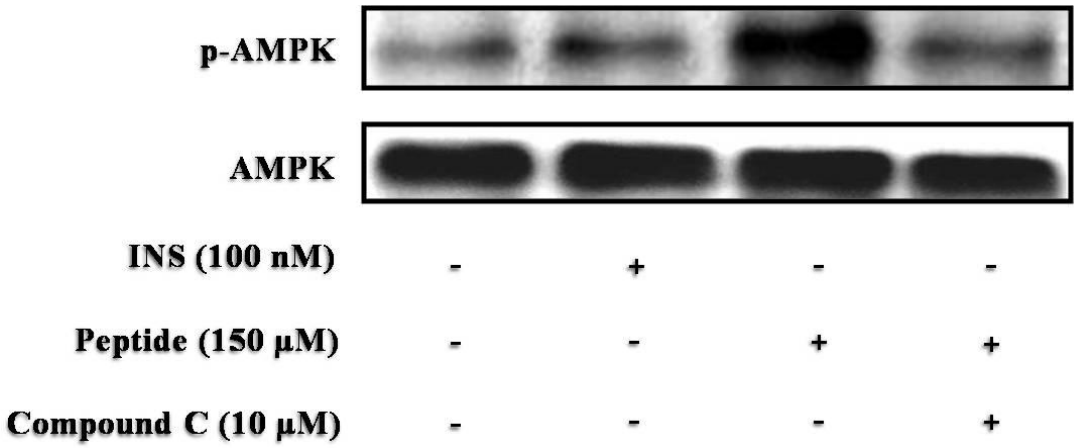

Fig. 2-4. Effect of antihypertensive peptide on AMPK signaling pathway. Cells were pretreated with or without 10 μM compound C for 30 min, and then treated with the indicated concentrations of antihypertensive peptide and insulin for 1 h and 10 min, respectively. The cell lysates were analyzed via Western blotting using anti-phosphoAMPK (Thr 172) and anti-AMPK. Figures are representative of three independent experiments.

metabolic enzymes (Sheetz and King, 2002). In recent papers, it has been reported that AMPK serves as a key metabolic sensor through cellular regulation of insulin-independent glucose uptake and glycogen metabolism as described previously (Ozcan et al., 2004; Hotamisligil, 2006). From this, AMPK is emerging as a potentially interesting target for the treatment of diabetes (Nakatani et al., 2005), especially because it could plays a principal role in exercisedinduced adaptation of skeletal muscle (Ozawa et al., 2005), type II diabetes, obesity and the metabolic syndrome. Present study showed a significant increase in AMPK phosphorylation by antihypertensive peptide. And also, the antihypertensive peptide -mediated activation of AMPK is abolished by pretreatment of compound C, highly-selective AMPK inhibitor. Therefore, these results indicate that AMPK is a principal factor in antihypertensive peptide -stimulated glucose uptake.

We next examined the effect of antihypertensive peptide on the AMPK signaling pathway that leads to the translocation of glucose transport 4 (GLUT4) to the plasma membrane and increases the uptake of glucose. After L6 myotubes cells were treated with antihypertensive peptide for 1 h, the translocation of GLUT4 was determined. As seen in **Fig. 2-5**, GLUT4 translocation to the plasma membrane of L6 myotubes cells were markedly increased by treatment of antihypertensive peptide. However, increased translocation of GLUT4 to the

plasma membrane of antihypertensive peptide-treated L6 myotubes cells were almost completely abolished by compound C pretreatment. These results suggest that antihypertensive peptide stimulated increase in GLUT4 translocation to the plasma membrane possibly via activating AMPK pathway. Hyperglycemic-hyperinsulinemic clamp analyses of human type II diabetic patients show that insulin resistance in muscle is caused by a defect in glucose transport.

In consequence, these results demonstrate that antihypertensive peptide improve glucose uptake via activating AMPK pathway in skeletal muscles. Especially, the skeletal muscle has a major role in the regulation of energy balance (Ozcan et al., 2006) and is the primary tissue for glucose uptake and disposal. Indeed, the glucose uptake, by skeletal muscle, accounts for >70% of the glucose removal from the serum in humans (Cormont et al., 1993). With this, it is considered an important target tissue for type II diabetes (Sheetz and King, 2002).

In conclusion, antihypertensive peptide increases glucose uptake through activating AMPK pathway, a novel target for treatment of type II diabetes and we can find a new possibility of antihypertensive peptide as a anti-diabetic agent.

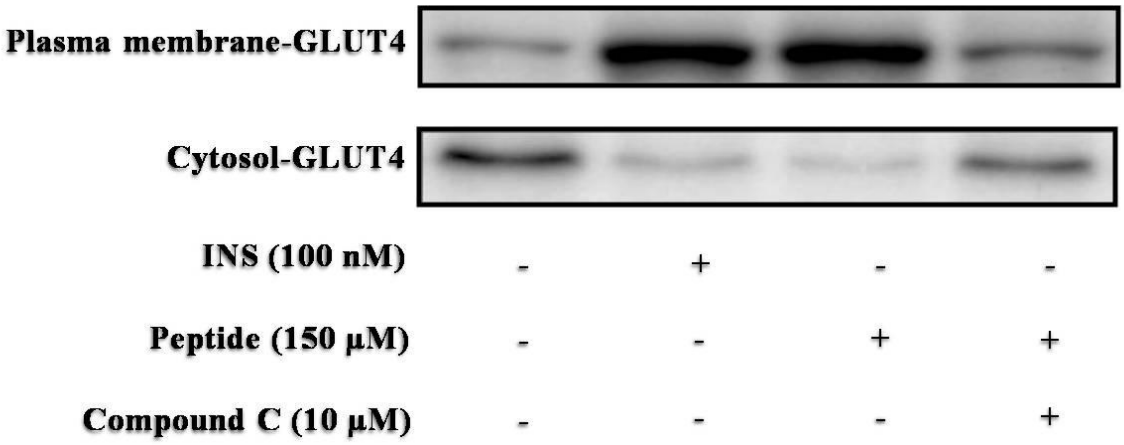

Fig. 2-5. Effect of antihypertensive peptide on GLUT4 translocation to the plasma membrane. Cells were pretreated with or without 10 μM compound C for 30 min, and then treated with the indicated concentrations of antihypertensive peptide and insulin for 1 h and 10 min, respectively. The cell lysates were analyzed via Western blotting using anti-GLUT4. Figures are representative of three independent experiments.

Part III.

Antihypertensive peptide purified from *Styela clava* inhibits the expression of adipogenic regulators 3T3-L1 preadipocytes and reduces weight gain in mice fed a high-fat diet

Part III.

Antihypertensive peptide purified from *Styela clava* inhibits the expression of adipogenic regulators 3T3-L1 preadipocytes and reduces weight gain in mice fed a high-fat diet

1. Abstract

In this study, we investigated the effect of antihypertensive peptide on adipogenesis of 3T3-L1 adipocytes during differentiation of preadipocytes into adipocytes by measuring lipid accumulation and adipogenesis related factors and an animal model of obesity. antihypertensive peptide treatment inhibits adipocyte differentiation, evidenced by decreased lipid accumulation and down regulation of adipocyte markers. antihypertensive peptide then inhibited the expression of both early CCAAT-enhancer-binding proteins α (C/EBPα) and peroxisome proliferator-activated receptors γ (PPARγ), differentiation- dependent factor 1/sterol regulatory element-binding protein (SREBP-1) and late activating protein 2 (aP2) adipogenic transcription factors, which is a crucial role for adipocyte development. Antihypertensive peptide administration significantly reduced the body weight of mice fed a high-fat diet. These results demonstrate an inhibitory effect of antihypertensive peptide on adipogenesis through reduction of an adipogenic factor and reducing body weight gain in a

high-fat diet-induced animal model of obesity.

2. MATERIALS AND METHODS

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum (BS), Phosphate-buffered saline (pH 7.4; PBS) and penicillin–streptomycin (PS) were from Gibco BRL (Grand Island, NY, USA). Antibodies to PPARγ, aP2 and C/EBPα were purchased were from Cell Signaling Technology (Bedford, Massachusetts, USA). Antibody to SREBP-1c was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO, USA). A All other reagents were purchased from Sigma Chemical Co. unless otherwise stated.

2.2. Cell culture and differentiation

3T3-L1 preadipocyte cells obtained from American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing 1% PS and 10% bovine calf serum (Gibco BRL) at 37℃ under a 5% CO_2 atmosphere. To induce differentiation, 2-day post confluent preadipocytes (designated Day 0) were cultured in MDI differentiation medium (DMEM

containing 1% PS, 10% FBS, 0.5 mM IBMX, 0.25 μM dexamethasone and 5 μg/ml insulin) for 2 days. The cells were then cultured for another 2 days in DMEM containing 1% PS, 10% FBS and 5 μg/ml insulin. Thereafter, the cells were maintained in post differentiation medium (DMEM containing 1% PS and 10% FBS), with replacement of the medium every 2 days. To examine the effects of antihypertensive peptide on the differentiation of preadipocytes to adipocytes, the cells were cultured with MDI in the presence of various concentrations of antihypertensive peptide. Differentiation, as measured by the expression of adipogenic markers and the appearance of lipid droplets, was complete on Day 8. The effect of antihypertensive peptide on cell viability and cytotoxicity was determined by the MTT assay. Cells were seeded at a density of 1×10^5 cells/well into a 96-well plate, then treated antihypertensive peptide after 24 h, and then incubated for 48 h. The MTT stock solution (100 μl; 2 mg/ml in PBS) was added to each well, and the plates incubated for 4 h at 37℃. The liquid in the plate was removed, and dimethyl sulfoxide was added to dissolve the MTT–formazan complex. Optical density was measured at 540 nm.

2.3. Determination of lipid accumulation by Oil Red O staining

To induce adipogenesis, 3T3-L1 cells were seeded on 6-well plates and maintained for

2 days after reaching confluence. Then media was exchanged with differentiation medium (DMEM containing 10% FBS, 0.5 mM IBMX, 0.25 μM Dex and 10 μg/ml insulin) and cells were treated with antihypertensive peptide (0, 112.5, 225 or 450 μM). After two days, the differentiation medium was replaced with adipocyte growth medium (DMEM supplemented with 10% FBS and 5 μg/ml insulin), which was refreshed every 2 days. After adipocyte differentiation, the cells were stained with Oil Red O, an indicator of cell lipid content with slight modifications. Briefly, cells were washed with phosphate-buffered saline, fixed with 10% buffered formalin and stained with Oil Red O solution (0.5 g in 100 ml isopropanol) for 10 min. After removing the staining solution, the dye retained in the cells was eluted into isopropanol and Optical density was measured at 520 nm.

2.4. Western blot analysis

Cells were lysated in NucBuster™ Protein Extraction Kit (Novagen, San Diego, CA, USA) for 10 min and then centrifuged at 16,000 rpm for 5 min at 4℃. The protein concentrations were determined by using BCA™ protein assay kit. The lysate containing 40 μg of protein were subjected to electrophoresis on 12 % sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane. The membrane was blocked in 1%

bovine serum albumin (BSA) in TBST (25 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4) for 2 h. The primary antibodies were used at a 1:1000 dilution. Membranes incubated with the primary antibodies at 4℃ for overnight. Then the membranes were washed with TTBT and then incubated with the secondary antibodies used at 1:3000 dilution. Signals were developed using an ECL western blotting detection kit and exposed to X-ray films.

2.5. *In vivo* anti-obesity effect

All animal procedures were carried out in accordance with the National Institute of *Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Utilization Committee for Veterinary Medicine of Jeju National University. After purchase, 40 male 5-week-old C57BL/6N mice (Japan SLC. Inc. Shizuoka, Japan) were adapted for 1 week to specific conditions of temperature (23±2℃), humidity (50±5%) and 12 h light/dark cycle and were given standard mouse chow and water *ad libitum*. After adaptation, the C57BL/6N mice (now 6 weeks old) were randomly divided into a normal diet group (ND; n=10), high-fat diet group (HFD; n=10), high-fat diet + 5 mg/kg BW of AP (HFD + 5 mg/kg BW of AP) and high-fat diet + 10 mg/kg BW of AP (HFD + 10 mg/kg BW of AP). Mice in the HFD group were orally administered saline once a day while mice in the

HFD + AP group were orally administered 5 and 10 mg AP/kg BW in saline. Body weights were recorded weekly for six weeks.

2.5. Statistical analysis

All data were represented as the mean±S.E. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncan's multiple-range test using SPSS (11.5) software. Statistical significance was considered at $p<0.05$.

3. RESULTS AND DISCUSSION

Obesity is associated with the development of metabolic diseases such as type II diabetes, hypertension, heart, disease and myocardial infarction, where its prevalence is greatly increasing (Lee et al., 2005). The possible proposed mechanisms for the treatment of obesity are as follows: balance energy intake and expenditure, reduction of preadipocyte differentiation, decrease in lipogenesis, increase in lipolysis and induction of adipocyte apoptosis (Evans et al., 2002). Adipocytes play an important role in lipid homeostasis and energy balance by relating to triglyceride storage and release of free fatty acids. Adipocyte differentiation and the amount of fat accumulation are associated with the occurrence and development of obesity (Jeon et al., 2004). For these reasons, anti-obesity works have been conducted in 3T3-L1 adipocytes. In this study, we investigated anti-obesity effect of antihypertensive peptide in the 3T3-L1 cell model by understanding the molecular mechanisms through AMPK signaling pathway.

To identify a concentration of antihypertensive peptide that did not affect viability or cause cytotoxicity in 3T3-L1 preadipocytes, cell viability and cytotoxicity after 48 h incubation periods in 3T3-L1 preadipocytes were evaluated in MTT assays. At concentrations of 112.5, 225 or 450 μM, antihypertensive peptide did not affect viability or cause cytotoxicity in 3T3-

L1 cells, as determined by MTT assay (**Fig. 3-1**).

Next, we tested whether antihypertensive peptide inhibited MDI-induced differentiation in 3T3-L1 preadipocytes. On day 0, antihypertensive peptide was added to the MDI differentiation medium, containing IBMX, dexamethasone, and insulin; on day 8, the adipocytes were stained using Oil Red O. Oil Red O staining demonstrated that treatment with antihypertensive peptide at a concentration of 112.5, 225 or 450 μM dose-dependently inhibited 3T3-L1 adipocyte differentiation and lipid accumulation (**Fig. 3-2(A) and (B)**).

Adipocyte differentiation causes a series of programmed changes in specific gene expressions. Adipogenesis can be induced through the action of several enzymes such as FAS, ACC, ACS and glycerol-3-phosphate acyltransferase. They are regulated by transcription factors such as PPARγ, C/EBPα and SREBP-1c, which are known to be critical activators for adipogenesis. These transcription factors are regulated in early stage of adipocyte differentiation (Latasa et al., 2000; Luong et al., 2000; Ericsson et al., 1997). Overexpression of these transcription factors can accelerate adipocyte differentiation. PPARγ and C/EBPα are induced prior to the transcriptional activation of most adipocyte specific genes and coordinate expression of genes involved in creating or maintaining the phenotype of adipocytes (Rosen, 2005). SREBP-1c critically crossactivates a ligand binding domain of PPARγ and regulates

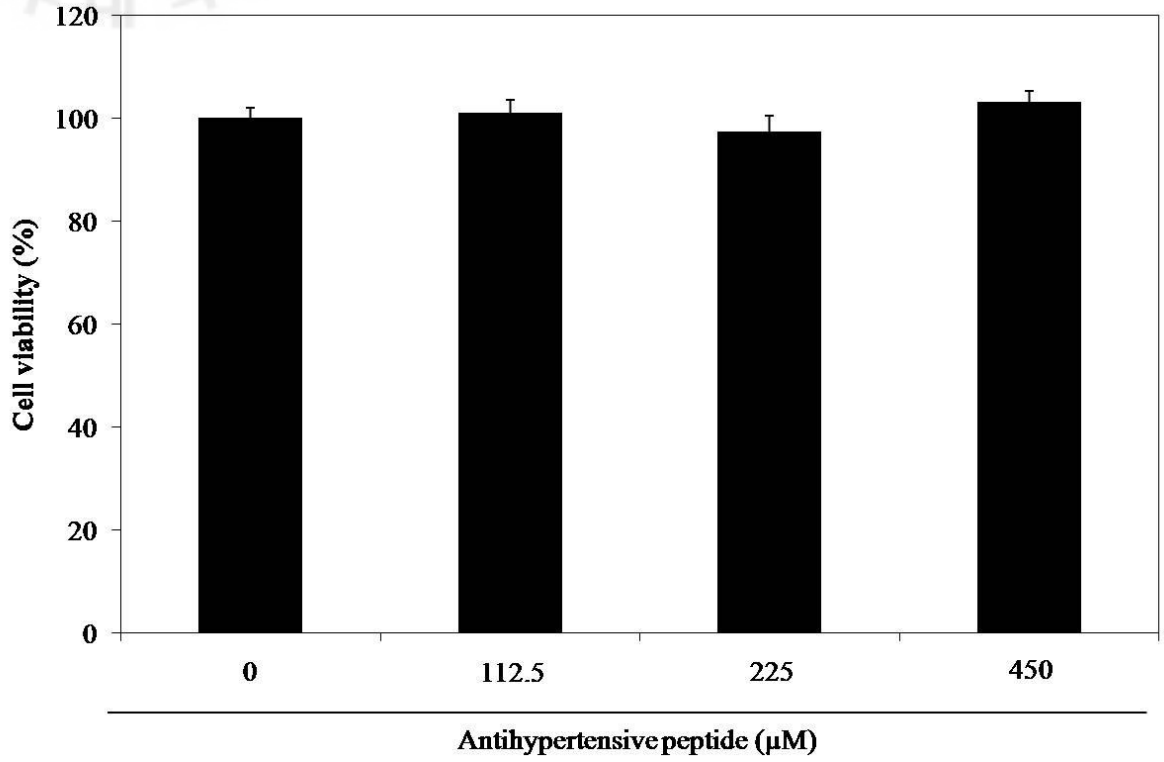

Fig. 3-1. Effect of antihypertensive peptide on the viability of 3T3-L1 preadipocytes treated for 48 h. Viability was determined via MTT assay. Each value is expressed as mean±S.E. in triplicate experiments.

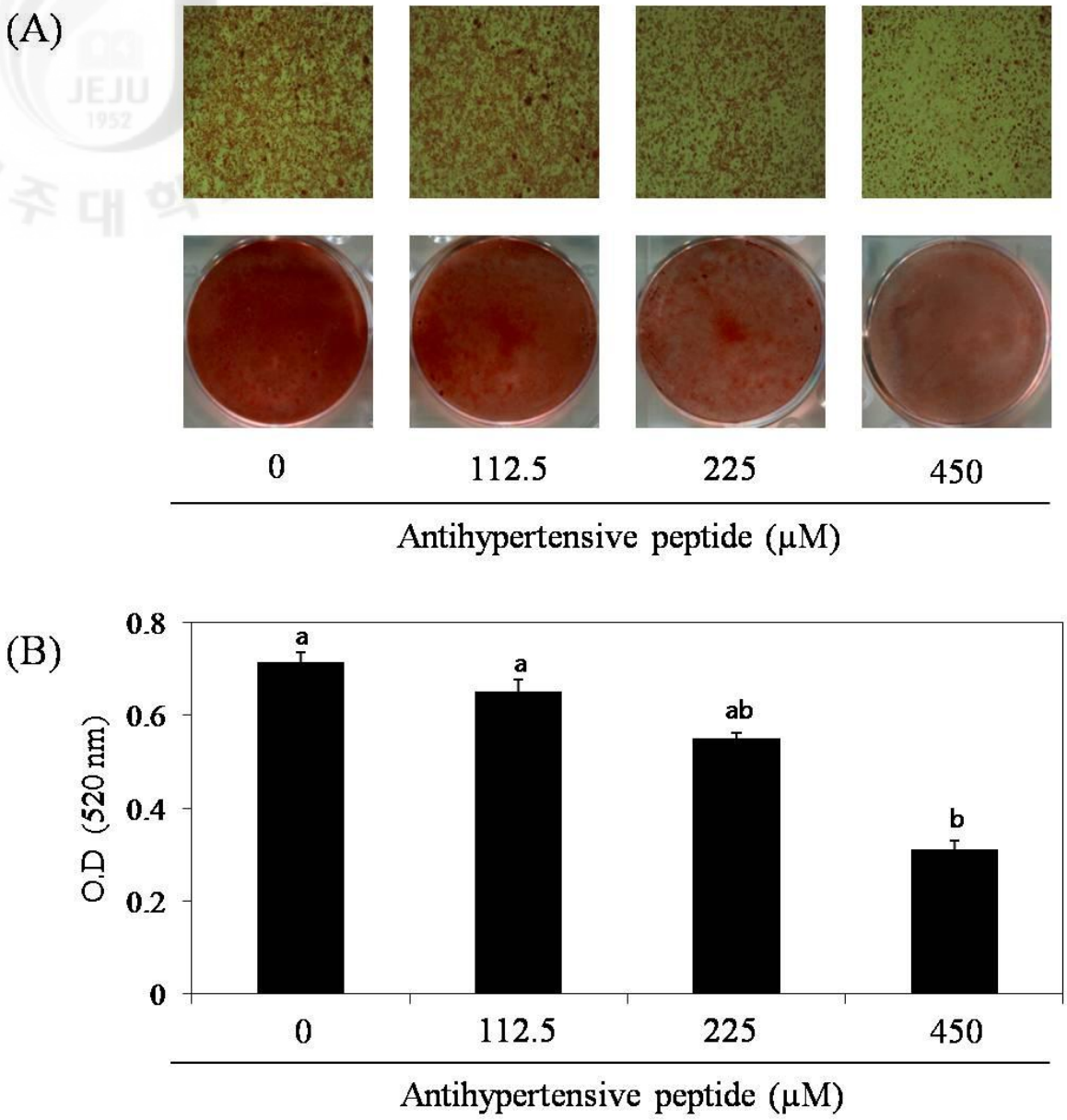

Fig.3-2. Antihypertensive peptide inhibits lipid accumulation during the differentiation of 3T3-L1 preadipocytes. (A) O Red O staining at day 8 with antihypertensive peptie at 0, 112.5, 225, 450 μM. (B) The lipid accumulation determined by absorbance at 520 nm. $^{a-d}$Values with different alphabets are significantly different at $P<0.05$ as analyzed via Duncan's multiple range test.

the expression of the enzymes involved in lipogenesis and fatty acid desaturation (Bruce and Jeffery, 2001; Rosen et al., 2000).

To determine whether antihypertensive peptide affects the expression of transcriptional factors, Western blotting analysis was conducted (**Fig. 3-3**). Treatment with antihypertensive peptide lessened regulation of peroxisome proliferator-activated receptor-γ (PPARγ), differentiation- dependent factor 1/sterol regulatory element-binding protein (SREBP-1c) and CCAAT/enhancer-binding proteins α (C/EBPα), compared to fully differentiated control adipocytes. The inhibitory effects of antihypertensive peptide exhibited dose-dependent pattern.

PPARγ, C/EBPα and SREBP-1c synergistically activate the adipocyte specific gene promoters such as aP2, FAS, LPL, ACS1 and leptin (Gregoire et al., 1998). The aP2 gene is the terminal differentiation marker of adipocytes and plays central roles in the pathway which link obesity to insulin resistance and fatty acid metabolism. Further investigations were carried out to find the effect of antihypertensive peptide on regulation of adipogenic target genes such as adipocyte fatty acid binding protein (aP2) (**Fig. 3-3**). Presence of antihypertensive peptide lessened the expression level of aP2 gene.

To investigate the anti-adipogenic properties of antihypertensive peptide *in vivo*, we

recorded body weights after oral administration of antihypertensive peptide 5 and 10 mg/kg per day to HFD-induced obese mice for six weeks. **Fig. 3-4** shows that the HFD increased body weight significantly compared to the normal diet (ND) over six week period. Moreover, final body weight was significantly lower in the HFD + 10 mg/kg of antihypertensive peptide group compared to the HFD group after six weeks.

In conclusion, we evaluated the anti-obesity effects of antihypertensive peptide on adipocyte differentiation and associated mechanisms in 3T3-L1 cells and confirmed and confirmed our findings in an obese animal model fed HFD. Atihypertensive peptide decrease lipid accumulation in 3T3-L1 adipocytes. Moreover, administration of antihypertensive peptide effectively suppressed body weight gain. These results suggest that the anti-obesity effect of antihypertensive peptide results from a decrease in adipogenesis and the antihypertensive peptide has a beneficial effect, reducing body weight gain in an experimental animal model.

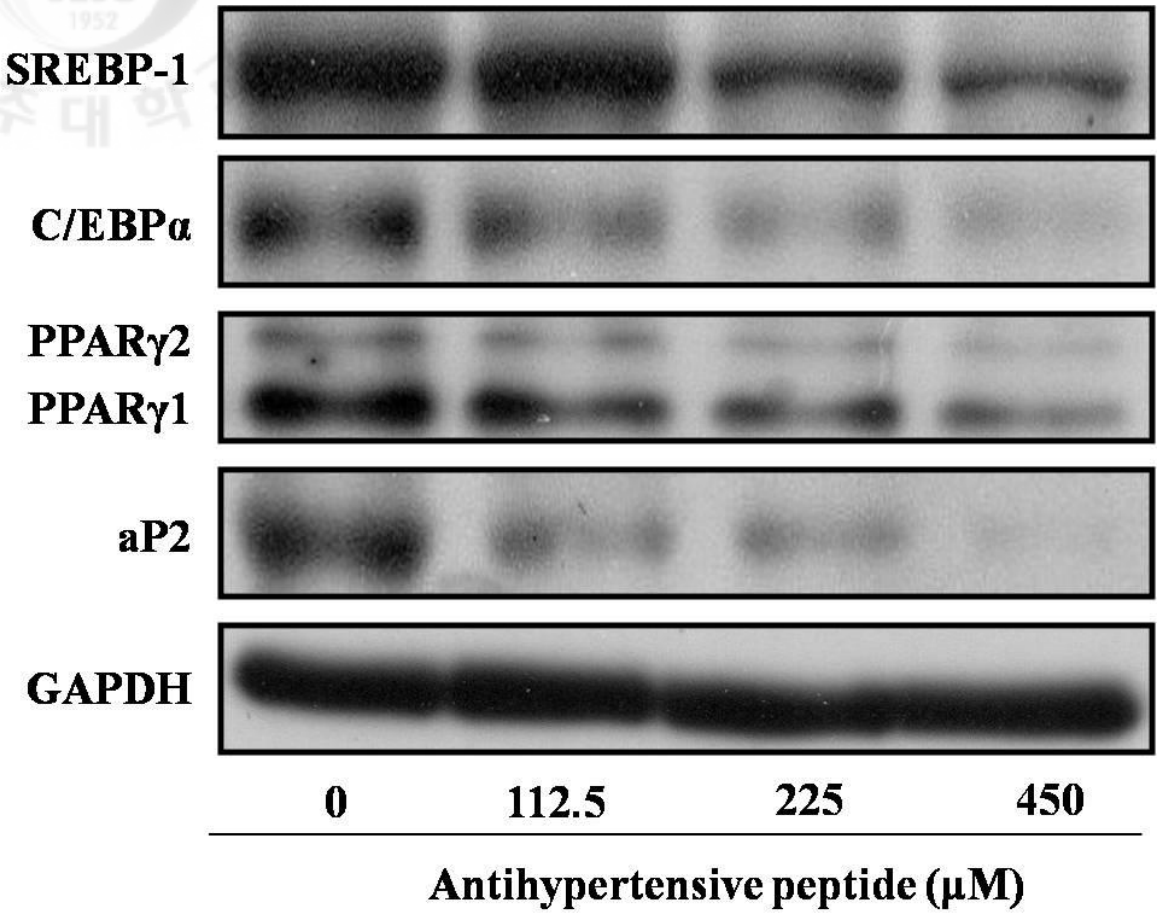

Fig. 3-3. Antihypertensive peptide suppressed the SREBP-1c, C/EBPα, PPARγ and aP2 protein expression in the differentiation of 3T3-L1 preadipocyte in to adipocyte. Cells were cultured for 8 days with 112.5, 225 or 450 μM antihypertensive peptide or without antihypertensive peptide.

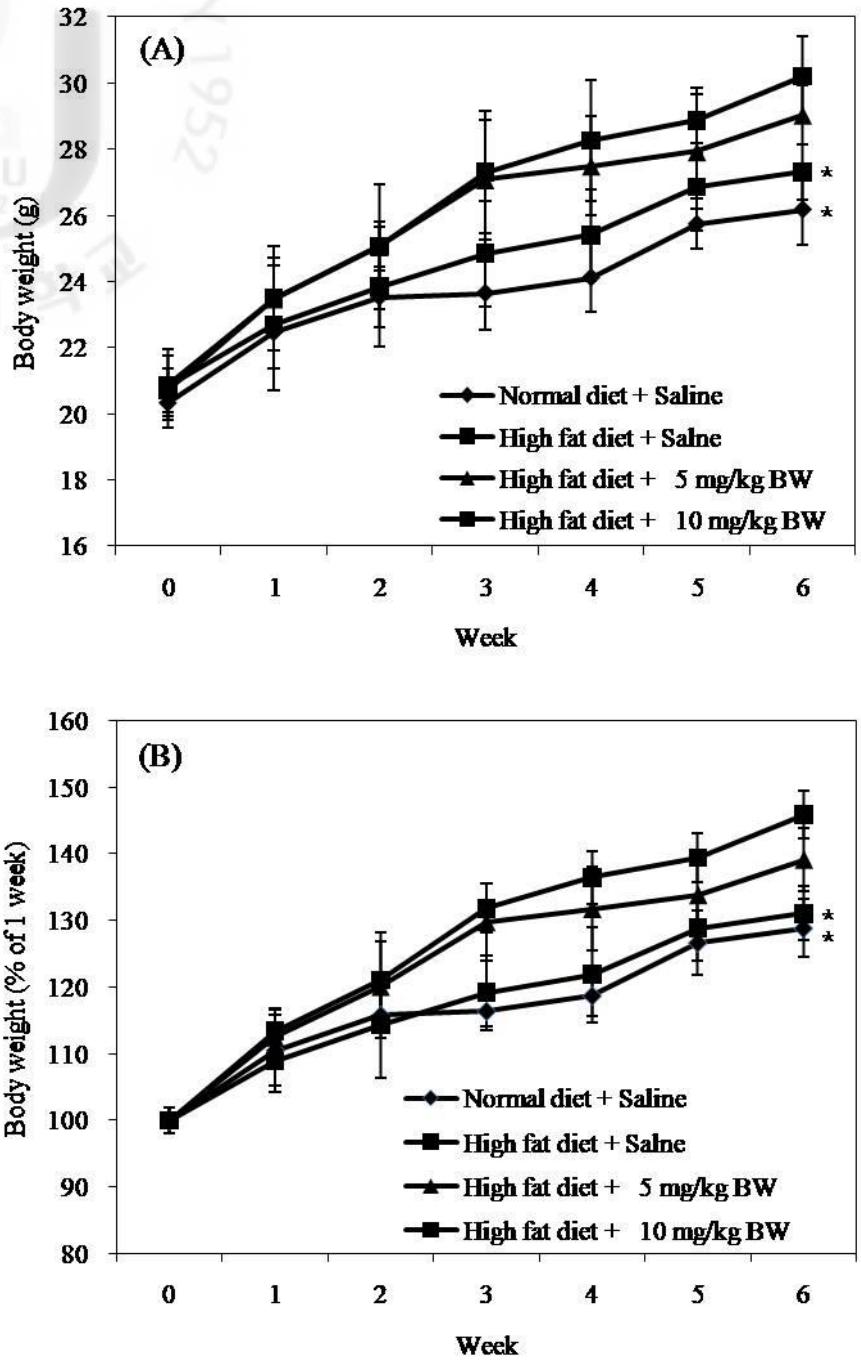

Fig. 3-4. Effect of supplementing antihypertensive peptide on body weight gain profile in HFD-induced experimental group for 6 weeks. The values were expressed as mean±S.E. (n=10). Mean separation was performed by Duncan's multiple rang test. Different letters indicate significant differences (p<0.1).

REFERENCES

Afsana, F., Latif, Z.A., Haq, M., Ahmed, T., Habib, S.H., Mahtab, H., 2010. Characteristics of different parameters of metabolic syndrome in subjects undergoing coronary angiogram and their association with peripheral vascular disease.

Alberti, K.G., Zimmet, P., Shaw. J., 2005. IDF Epidemiology Task Force Consensus Group. The metabolic syndrome new worldwide definition. Lancet 366, 1059-1062.

Alberti K.G., Zimmet, P., Shaw, J., 2006. Metabolic syndrome-a new world-wide definition. A Consensus Statement from the International Diabetes Federation. Diabet. Med. 23, 469-480.

Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357, 593–615.

Asselbergs, F.W., Harst, P., Jessurum, G.A.J., Tio, R.A., van Gilst, W.H., 2005. Clinical impact of vasomotor function assessment and the role of ACE-inhibitors and statins. Vasc. Pharmacol. 42, 125-140.

Bannister, L. 1995) Haemolymphoid system - endothelium. In L.W. Peter (Ed.), *Gray's Anatomy* (pp. 1456-1458). New York: Churchill Livingstone.

Baron, A.D., 1998. Postprandial hyperglycemia and α-glucosidase inhibitors. Diabetes Res.

Clin. Pract. 40, S51-S55.

Beny, J.L., and Brunet, P.C., 1988. Electrophysiological and mechanical effects of substance P and acetylcholine on rabbit aorta. J. Physiol. 398,277-289.

Bohr, D.F., Dominiczak, A.F., Webb, R.C., 1991. Pathophysiology of the vasculature in hypertension. Hypertension 18(suppl 111), S69-S75.

Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., Nasri, M. 2010. Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of saldinelle (*Sardinella aurita*) by-products proteins. Food Chem. 118, 559-565.

Byun, H.G., & Kim, S.K., 2001. Purification and characterization of angiotensin I convering enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. Process Biochem. 36, 1155-1162.

Campbell, R.K., 2009. Type 2 diabetes: where we are today: an overview of disease burden, current treatments, and treatment strategies. J. Am. Pharm. Assoc. 49, S3–S9.

Carr, D.B., and Utzschneider, K.M., 2004. Intra-abdominal fat is a major determinant of NCEP ATP III criteria for the metabolic syndrome. Diabetes 53, 2087–2094.

Chang, M.S., Oh, M.S., Kim, D.R., Jung, K.J., Park, S., Choi, S.B., Ko, B.S., Park, S.K..,

2006. Effects of Okchun-San, a herbal formulation, on blood glucose levels and body weight in a model of type 2 diabetes. J. Ethnopharmacol. 103, 491-495.

Cho, E.J., Rahman,A., Kim, S.W., Baek, Y.M., Hwang, H.J., Oh, J.Y., Hwang, H.S., Lee, S.H., Yun, J.W., 2008. Chitosan oligosaccharides inhibit adipogenesis in 3T3-L1 adipocytes. J. Microbiol. Biotechnol. 18, 80–87.

Christopher, T.A., Lopez, B.L., Stillwagon, J.C., Gao, F., Gao, E., Ma, X.L., Ohlstein, E.H., Yue, T.L., 2008. Idoxifene causes endothelium-dependent, nitric oxide-mediated vasorelaxation in male rats. Eur. J. Pharmacol. 446, 139-143.

Clozel, M., Kuhn, H., Hefti, F., 1990. Effects of angiotensin converting enzyme inhibitors and of hydralazine on endothelial function in hypertensive rats. Hypertension, 1990, 16: 532–540.

Cushman, D.W., and Cheung, H.S., 1970. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20, 1637-1648.

Defronzo, R.A., Jacot, E., Jequier, E, Maeder, E., Wahren, J, Felber, J.P., 1981. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 30, 1000-1007.

Dugani, C.B., Randhawa, V.K., Cheng, A.W., Patel, N., Klip, A., 2008. Selective regulation

of the perinuclear distribution of glucose transporter 4 (GLUT4) by insulin signals in muscle cells. Eur. J. Cell Biol. 87, 337-351.

Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. 2001. *Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III)*. JAMA. 285, 2486-2497.

Fahmi, A., Morimura, S, Guo, H.C., Shigematsu, T, Kida, K., Ueyama, Y., 2004. Production of angiotensin I converting enzyme inhibitory peptides from sea bream scales. Process Biochem. 39, 1195-1200.

Feletou, M., and Vanhoutte, P.M., 2007. Endothelium-dependent hyperpolarizations: past beliefs and present facts. Ann. Med. 39, 495-516.

Fryer, L.G., Parbu-Patel, A., Carling, D., 2002. The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J. Biol. Chem. 277, 25226–25232.

Furchgott, R.F., and Zawadzki, J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-376.

Gregoire, F.M., 2001. Adipocyte differentiation: from fibroblast to endocrine cell.

Experimental Biology and Medicine (Maywood) 226, 997–1002

Hayakawa, H., Coffee, K., Raij, L., 1997. Endothelial dysfunction and cardiorenal injury in experimental salt-sensitive hypertension: effects of antihypertensive therapy. Circulation 96, 2407–13.

Hotamisligil, G.S., 2006. Inflammation and metabolic disorders. Nature 444, 860–867.

Hu, G., Qiao, Q., Tuomilehto, J., Eliasson, M., Feskens, E.J., Pyörälä, K., 2004. Plasma insulin and cardiovascular mortality in non-diabetic European men and women: a meta-analysis of data from eleven prospective studies. The DECODE Insulin Study Group. Diabetologia 47, 1245–1256.

Je, J.Y., Park, J.Y., Jung, W.K., Park, P.J., Kim. S.K., 2005a. Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce, *Crassostrea gigas*. Food Chem. 90, 809-814.

Je, J.Y., Park, P.J., Byun HG, Jung, W.K., Kim, S.K., 2005b. Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, *Mytilus edulis*. Bioresource Technol. 96,1624-1629.

Jeon, Y.J., Byun, H.G., Kim, S.K., 2000. Improvement of functional properties of cod frame protein hydrolysate using ultrafiltration membranes. Process Biochem. 35, 471-478.

Jung, U.J., Baek, N.I., Chung, H.G., Bang, M.H., Yoo, J.S., Jeong, T.S., Lee, K.T., Kang, Y.J., Lee, M.K., Yeo, J.Y., Choi, M.S., 2007. The anti-diabetic effects of ethanol extract from two variants of *Artemisia princeps* Pampanini in C57BL/KsJ-*db/db* mice. Food Chem. Toxicol. 45, 2022-2029.

Jung, W.K., Mendis, E., Je, J.Y., Park, P.J., Son, B.W., Kim, H.C., Choi, Y.K., Kim, S.K., 2006. Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 94, 26-32.

Kamiya, A., Ando, J., Shibata, M., Masuda, H., 1998. Roles of fluid shear stress in physiological regulation of vascular structure and function. Biorheology 25, 272-278.

Kang, S.I., Kim M.H., Shin, H.S., Kim, H.M., Hong, YS., Park, J.G., Ko, H.C., Lee, N.H., Chung, W.S., Kim, S.J., 2010. A water-soluble extract of Petalonia binghamiae inhibits the expression of adipogenic regulators in 3T3-L1 preadipocytes and reduces adiposity and weight gain in rats fed a high-fat diet. J. Nutr. Biochem. 21, 1251-1257.

Kato, H., Suzuk,i T., 1972. Bradykinin-potentiating peptides from the venom of Agkistrodon halys blomhoffii: Isolation of five bradykinin potentiators B and C. Toxicon. 10, 313.

Kato, H., & Suzuki, T., 1975. Bradykinin-potentiating peptides from the venom of Agkistrodonhalys blomhoffii: Their amino acid sequence and the inhibitory activity

on angiotensin-I converting enzyme from rabbit lung. Life Sci. 16, 810-811.

Kim, H.Y., Oh, H., Li, X., Cho, K.W., Kang, D.G., Lee, H.S., 2011. Ethanol extract of seeds of *Oenothera odorata* induces vasorelaxation via endotheliump-dependent NO-cGMP signaling through activation of Akt-eNOS-sGC pathway. J. Ethnophamacol, 133, 315-323.

Kim, J.B., Iwamuro, S, Knoop, F.C., Conlon, J.M., 2011. Antimicrobial peptides from the skin of the Japanese mountain brown frog, *Rona ornativentris*. J.Pept.Res.58:349-356.

Kim, J.J., Kim, S.J., Kim, S.H., Park, H.R., Lee, S.C., 2006. Antioxidant and anticancer activities of extracts from *Styela clava* according to the processing methods and solvents. J. Soc. Food Sci. Nutr. 35, 278-283.

Kim, K.J., Lee, O.H., Lee, B.Y., 2010. Fucoidan, a sulfated polysaccharide, inhibits adipogenesis through the mitogen-activated protein kinase pathway in 3T3-L1 preadipocytes. Life Sci. 86, 791-797.

Lahl, W.J., and Braun, S.D., 1994. Enzymatic production of protein hydrolysate for food use. Food Technol. 48, 68-71.

Lee D.W., You, D.H., Yang, E.K., Jang, I.C., Bae, M.S., Jeon, Y.J., Kim, S.J., Lee, S.C., 2010. Antioxidant and ACE inhibitory activities of *Styela clava* according to harvesting time. J. Food Sci. Nutr. 39, 331-336.

Lee, E.S., Uhm, K.O., Lee, Y.M., Han, M.S., Lee,M.S., Park,J.M., Suh, P.G., Park, S.H., Kim, H.S., 2007. CAPE (caffeic acid phenethyl ester) stimulates glucose uptake through AMPK (AMP-activated protein kinase) activation in skeletal muscle cells. Biochem. Bioph. Res. Co. 361, 854-858.

Lee, J.K., Hong, S., Jeon, J.K., Kim, S.K., Byun, H.G., 2009. Purification and characterization of angiotensin I converting enzyme inhibitory peptides from the rotifer, *Brachionus rotundufirmis*. Bioresour. Technol.100, 5255-5259.

Lee, J.K., Jeon, J.K., Byun, H.G., 2011. Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate. Food Chem. 125, 495-499.

Lee, I., Manson, J.E., Hennekens, C.H., Paffenbarger, R.S., 1993. Body weight and mortality. A 27-year follow-up of middle-aged men. JAMA 270, 2823-2828.

Lee, S.H., Qian, Z.J., & Kim, S.K., 2010. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 118, 96-102.

Li, G.H., Wan, J.Z., Le, G.W., Shi, Y.H., 2006. Novel angiotensin I-converting enzyme inhibitory peptides isolated from alcalase hydrolysate of mung bean protein. J. Pept. Sci. 12, 509-514.

Liu, R., Wang, M., Duan, J.A., Guo, J.M., Tang, Y.P., 2010. Purification and identification of three novel antioxidant peptides from *Cornu Bubali* (water buffalo horn). Peptides 31, 786-793.

McCabe, T.J., Fulton, D., Roman, L.J., Sessa, W.C., 2000. Enhanced electron flux and reduced calmodulin dissociation may explain ‘‘calcium-independent” eNOS activation by phosphorylation, J. Biol. Chem. 275, 6123–6128.

Miguel, M., Alonso, M.J., Salaices, M., Aleixandre, A., Lopeze-Fandiño, R., 2007. Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. Food Chem. 104, 163-168.

Modena, M.G., Bonetti, L., Coppi, F., Bursi, F., Rossi, R., 2002. Prognostic role of reversible endothelial dysfunction in hypertensive postmenopausal women. J. Am. Coll. Cardiol. 40, 505– 510.

Mount, P.F., Kemp, B.E., Power, D.A., 2007. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J. Mol. Cell. Cardiol. 42, 271-279.

Moller, D.E., 2001.Newdrug targets for type 2 diabetes and the metabolic syndrome. Nature 414, 821–827.

Moller, D.E., and Flier, J.S., 1992. Insulin resistance: mechanisms, syndromes, and implications. N. Engl. J. Med. 325, 938–942.

Musi, N., and Goodyear, L.J., 2003. AMP-activated protein kinase and muscle glucose uptake. Acta Physiol. Scand. 178, 337–345.

Novosel, D., Lang, M.G., Noll, G., Luscher, T.F., 1994. Endothelial dysfunction in aorta of the spontaneously hypertensive, stroke-prone rat: effects of therapy with verapamil and trandolapril alone and in combination. J. Cardiovasc. Pharmacol. 24, 979–85.

Ohlson, L.O., Larsson, B., Svardsudd, K., Welin, L., Eriksson, H., Wilhelmsen, L., Björntorp, P., Tibblin, G., 1985. The influence of body fat distribution on the incidence of diabetes mellitus: 13.5years of follow-up of the participants in the study of men born in 1913. Diabetes 34, 1055-1058.

Ondetti, M. A., 1977. Design of specific inhibitors of angiotensin converting enzyme: New class of orally active antihypertensive agents. Science 196, 441-444.

Palmer, R.M., Ashton, D.S., Moncada, S., 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333, 664-666.

Petersen, F.K., and Shulman, G.I., 2006. Etiology of insulin resistance. J. Am. Med. 119(Suppl1), S10-S16.

Pouliot, M.C., Després, J.P., Lemieux, S., Moorjani, S., Bouchard, C., Tremblay, A., Nadeau, A., Lupien P.J., 1994. Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. Am. J. Cardiol. 73, 460-468.

Rasouli, N., Kern, P.A. 2008. Adipocytokines and the metabolic complications of obesity. J. Clin. Endocr. Metab. 93, S64–S73.

Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., Spiegelman, B.M. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. 16, 22–26, 2002.

Sarmadi, B.H., and Ismail, A., 2010. Antioxidant peptides from food proteins: A review. Peptides 31, 1949-1956.

Schmitt, C.A., and Dirsch, V.M., 2009. Modulation of endothelial nitric oxide by plant-derived products. Nitric Oxide 21, 77-91.

Stephens, J.M., Pilch, P.F., 1995. The metabolic regulation and vesicular transport of GLUT4, the major insulin-responsive glucose transporter. Endocr. Rev. 16, 529–546.

Tan, M.J., Ye, J.M., Turner, N., Hohnen-Behrens, C., Ke, C.Q., Tang, C.P., Chen, T., Weiss, H.C., Gesing, E.R., Rowland, A., James, D.E., Ye, Y., 2008. Antidiabetic activities of

triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. Chem. Biol. 15, 263–273.

Tang, Q.Q., Otto, T.C., Lane, M.D., 2003. Mitotic clonal expansion: a synchronous process required for adipogenesis, Proc. Natl. Acad. Sci. U.S.A. 100, 44–49.

Taniguchi,C.M., Emanuelli, B., Kahn, C.R., 2006. Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. 7, 85–96.

Ushida, Y., Matsui, T., Tanaka, M., Matsumoto, K., Hosoyama, H., Mitomi, A., Sagesaka, Y., Kakuda, T., 2008. Endothelium-dependent vasorelaxation effect of rutin-free tartary buckwheat extract in isolated rat thoracic aorta. J. Nutr. Biochem. 19, 700-707.

Vercruysse, L., Smagghe, G., Matsui, T., Camp, J.V., 2008. Purification and identification of an angiotensin I converting enzyme (ACE) inhibitory peptide from the gastrointestinal hydrolysate of the cotton leafworm, *Spodoptera littoralis.* Process Biochem. 43, 900-904.

Zaid, H., Antonescu, C.N., Randhawa, V.K., Klip, A., 2008. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem. J. 413, 201–215.

Zhao, H., Usui, H., Ohinata, K., Yoshikawa, M., 2008. Met-Arg-Trp derived from Rubisco lowers blood pressure via prostaglandin D2-dependent vasorelaxation in spontaneously

hypertensive rats. Peptides 29, 345-349.

Zhao, Y., Li, B., Liu, Z., Dong, S., Zhao, X., Zeng, M., 2007. Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. Process Biochem. 42, 1586-1591.

Zhou, G., Myers, R., Li, Y., Chen,Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber,T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., Moller, D.E., 2001. Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest. 108, 1167–1174.

Zimmet, P., Alberti, K., Shaw, J., 2001. Global and societal implications of the diabetes epidemic. Nature 414, 782-787

Acknowledgement

학부 2학년으로 연구실 생활을 시작하여 박사학위논문이라는 내 노력의 열매를 열 때까지 많은 분들의 관심과 조언이 있었기에 이 글을 빌어 감사의 말씀을 올리고자 합니다. 먼저 가야 할 길을 제대로 가지 않고 방황하는 저를 끝까지 포기하지 않으시고 가야 할 방향을 잡아주시고, 학위논문의 귀한 연구를 할 수 있도록 물심양면 지원을 아끼지 않으신 전유진 교수님께 머리 숙여 진심으로 감사 드립니다. 또한 바쁘신 가운데에서도 논문이 좀 더 좋은 방향으로 나아 갈 수 있게 심사해 주시고 관심을 보여주신 허문수 교수님, 이승헌 교수님, 의과대학 이대호 교수님, 강릉원주대 변희국 교수님과 학부시절부터 대학원 기간 동안 늘 많은 관심과 조언을 해주셨던 이기완 교수님, 송춘복 교수님, 최광식 교수님, 이제희 교수님, 여인규 교수님, 이경준 교수님, 정준범 교수님, 김수현 교수님께도 깊은 감사를 드립니다.

다사다난한 연구실에서 동고동락하며 부족한 후배를 잘 이끌어주어 많은 조언을 통해 저의 롤 모델이 되어주신 이승홍 선배님과 연구실의 동기이지만 선배 같은 역할을 해주었던 강성명, 연구실장으로서 역할에 부족함이 없는 이원우 후배님, 부족한 선배 밑에서 혼자 해낼 수 없는 연구에 있어서 묵묵히 일을 도와준

강민철, 고주영, 이지혁, 고창익, 양혜미, 강나래, 김은아, 오재영 후배님 및 한국생활을 잘 적응해 나가는 양수동, 자나카, 칼파 이 모든 해양생물자원이용공학연구실 가족에게 감사의 마음을 전합니다. 또한 저희 연구실 출신으로 참된 연구자로서 항상 모범을 보여주고 계시는 허수진 선배님, 김길남 선배님, 동기인 차선희, 안긴내, 김원석 선배님, 양현필 선배님의 많은 조언과 관심에 깊은 감사를 드립니다.

학부시절과 대학원생활 동안 항상 옆에 있어주며 같은 길을 걸어가고 있는 친구이자 동기인 이영득, 김윤범, 전봉근, 곁에는 없지만 언제나 힘이 되어 주는 문현식과 항상 자식처럼 아껴주시는 문현식의 아버님, 어머님께 감사의 마음을 전합니다. 또한 멀리서 저에게 많은 관심과 조언을 해주신 CHA의과학대학 이부용 교수님, 조선대학교 정원교 교수님께 감사의 마음을 전하며, 학위논문 연구에 많은 도움을 주었던 강릉원주대 이정권 학생, CHA의과학대학 전희전, 서민정 학생에게 감사의 마음을 전합니다.

철없던 학창시절부터 많은 시간이 흐른 지금까지 언제나 함께하고 힘들 때 부담 없이 술잔을 기울일 수 있는 소중한 친구들에게 항상 고맙고 앞으로 더욱 발전된 모습으로 함께 하자는 말을 전합니다. 아들 하나만을 잘되기만을 간절히

바라시며 눈물과 헌신적인 사랑으로 오늘의 저를 있게 해주신 어머니께 깊은 감사를 드리며 평생 갚아도 부족할 은혜 꼭 보답하겠습니다. 철없는 남동생을 항상 격려해주는 큰누나, 작은누나 내외분들에게 감사의 마음을 전합니다.

이 학위논문이 인생에 있어서 아주 작은 결실이라면, 앞으로 가꾸고 수확할 열매가 더욱 풍성할 것입니다. 항상 낮은 자세로 앞으로도 학문과 연구에 더욱 정진하며 언제 어디에서더라도 당당하고 자신 있는 제 자신을 다짐하며 이 글을 마칩니다.