A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Antioxidant and antitumor activity of enzymatic extracts from *Ecklonia cava*

Kil-Nam Kim

Department of Marin Biotechnology
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

2005. 12.

Antioxidant and antitumor activity of enzymatic extracts from *Ecklonia cava*

Kil-Nam Kim
(Supervised by professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2005. 12.

This thesis has been examined and approved.

Thesis director, Moon-Soo Heo, Prof. of Marine Biotechnology

Gi-Young Kim, Prof. of Marine Biology

You-Jin Jeon, Prof. of Marine Biotechnology

Date

Department of Marin Biotechnology
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

Contents

국문초록 iv
LIST OF FIGURES vii
LIST OF TABLES xi

INTRODUCTION 1

Part Ⅰ. Protective effect of *Ecklonia cava* enzymatic extracts on H_2O_2-induced cell damage 4
ABSTRACT 5
MATERIAL AND METHODS 5
Materials 5
Preparation of enzymatic extracts from *E. cava* 6
Molecular weight fractionation of enzymatic extract 9
Total polyphenolic compounds 9
Cell culture 9
Hydrogen peroxide scavenging activity 9
Assessment of cell viability 10
Lipid peroxidation inhibitory activity 10
Nuclear staining with Hoechst 33342 12
Flow cytometry analysis 12
RESULTS 13
Hydrogen peroxide scavenging activity 13
Total polyphenolic compounds 14
H_2O_2-induced of cell viability by >30 kDa fraction 14
Lipid peroxidation inhibitory activity 19
Reduction of H_2O_2-induced nuclear fragmentation by >30 kDa fraction 19
DISCUSSION 23

Part Ⅱ. Protective effect of ECUL H_2O_2-induced cell

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

damages......26
ABSTRACT......27
MATERIAL AND METHODS......27
Materials......27
Preparation of Celluclast extracts from *E. cava*......28
Separation of hydrophilic antioxdant......28
Hydrogen peroxide scavenging activity......28
DPPH radical scavenging activity......29
Cell culture......29
Assessment of cell viability......29
Nuclear staining with Hoechst 33342......30
Determination of DNA damage (Comet assay)......31
Assay for antioxidant enzymes......31
Statistical Analysis......32
RESULTS......32
Hydrogen peroxide scavenging activity......32
DPPH radical scavenging activity......33
H_2O_2-induced of cell viability by ECUL......33
Reduction of H_2O_2-induced apoptotic body by ECUL......33
Effect of ECUL on DNA damage of H_2O_2-induced cells......38
Effect of ECUL on antioxidant enzyme activities......38
DISCUSSION......42

Part Ⅲ. Antitumor activity and induction of apoptosis by *Ecklonia cava* enzymatic extract on tumor cells......44
ABSTRACT......45
MATERIAL AND METHODS......45
Materials......46
Preparation of Celluclast extracts from *E. cava*......46
Molecular weight fractionation of enzymatic extract......46
Cell culture......46
Cell growth inhibition assay......47

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Determination of DNA damage (Comet assay)......................................47
Nuclear staining with Hoechst 33342...48
Flow cytometry analysis...48
Statistical analysis..50
RESULTS...49
Inhibition of tumor cell growth by difference molecular weigh fractions of *E. cava* Celluclast extract..49
Cytotoxic effects of >30 kDa fraction in normal cells........................52
DNA damage of U937 cells by 30 kDa fracition.................................52
>30 kDa fraction induced apoptosis in U937 cells...............................52
DISCUSSION..59

SUMMARY..61
REFERENCES...63
ACKNOWLEDGEMENT..73

국문초록

대부분의 생물은 호기성 호흡에 의해 생존에 필요한 에너지인 산소 (O_2)를 체내에 받아 인다. 그러나 이 산소의 생물 체내 대사과정에서 생체에 독성을 나타낼 수 있는 부산물이 생성되기도 하는데 이들을 활성산소(O_2^-, O^-, H_2O_2, 및 OH^- 등)라 한다. 이런 활성산소들은 생체 내에서 생성될 뿐만 아니라 방향성 탄화수소, 담배, 살충제, 유기용매, 튀김 음식, 술 및 공기오염 등을 통하여 생체에 들어오는데 활성산소들은 생체 내 제거 기작에 의하여 대부분이 소멸되나 생성과 소멸의 균형이 깨어질 때 생체 내에서는 각종 질환이 발생한다. 즉, 류마티스성 관절염, 세균성 혹은 바이러스성 감염, 심장병, 파킨스씨병, Alzheimer's disease 그리고 암과 같은 질병을 유발한다고 알려지고 있다. 이런 활성산소들을 제거하기 위해 탁월한 항산화 효과와 경제성 때문에 인공합성항산화제가 많이 이용되었으나, 안전성에 논란이 있고 이로 인하여 소비자의 거부감이 날로 심해지고 있다. 따라서 근래에는 인간이 안전하게 오랫동안 먹어 왔던 천연물로부터 항산화 효과가 있는 물질을 분리, 이용하려는 시도가 활발히 이루어지고 있다.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2001년 통계에 의하면 암은 우리나라 전체 사망원인의 23.9%를 차지하는 주된 사망원인이다. 암은 조기에 발견되지 못할 경우 완치가 거의 불가능하고, 병이 진전 될수록 환자와 가족의 고통과 경제적 손실이 막대하여, 의료보험의 재정고갈 등 사회적으로도 큰 비용을 초래한다. 그러므로 단순한 수명연장보다 즐겁고 건강한 삶을 원하는 삶의 질이 중요한 관심사로 대두되고 있는 요즘, 암의 예방과 조기치료의 중요성은 그 어느 때 보다 높다고 할 수 있다. 암의 발병에는 유전적 소인도 깊이 관여하지만 환경적 요인 또한 큰 영향을 미치며 선진국일수록 암의 발병률이 증가하는 경향을 보인다. 그 이유로는 농약, 살충제 등의 사용량 및 식품내 잔유량 증가, 식품 보존제, 방부제, 착색제 등의 첨가된 가공식품의 소비 증가, 수질, 토양, 대기오염의 증가, 현대인의 스트레스의 증가, 활동량의 감소 그리고 풍요로운 식생활을 통한 비만 등을 들 수 있다. 현대 의학의 눈부신 발전으로 암을 진단하고 치료하는 다양한 방법들이 개발되어 인류의 건강 증진에 기여하였으나 아직도 해결해야 할 많은 문제점들이 남아있다. 암 치료에 있어서 항암제 요법은 매우 중요한 부분을 차지하고 있지만 항암제 자체의 비특이성 및 독성으로 인한 정상세포의 손상은 해결해야 될 시급한 과제로 남아있다. 그래서 오늘날 보다 안전한 천연항암제 개발이 절실히 필요한 실정이다.

해조류는 소화 흡수율이 낮아 영양학적인 측면에서 관심을 끌지 못하였으

나 최근 혈관내 콜레스테롤 침착 방지 및 장관 운동을 원활히 하고, 중금속 배출을 촉진시키며 고지혈증의 개선에 유효하다는 등 식용 해조류의 생리활성 물질들이 확인되면서 기능성 식품으로서의 개발에 관심이 모아지고 있다. 해조류는 육상식물에 비해 생육하는 환경이 현저한 차이가 있어 구성성분이 다를 뿐만 아니라 미네랄과 비타민이 매우 풍부하게 함유되어 있고, 또 어떤 특정적인 해조류의 성분은 항균, 항산화, 항바이러스 활성을 비롯하여 혈압 및 혈중 콜레스테롤 조절, 항종양활성, 면역활성 등을 나타내는 것으로 알려져 있다. 제주도는 지리적인 조건과 청정해역이라는 천해의 조건을 두루 갖추고 있는 지역으로서 특히 다양한 해조류들이 풍부하게 서식하고 있다. 해조류를 이용한 항산화제 및 항암제의 연구는 해조류를 많이 섭취하는 일본에서 특히 많은 연구가 이루어져 있으며 국내에서도 꾸준히 이루어지고 있다. 최근 들어 해조류를 비롯한 여러 천연물에서 항산화 및 항암 물질을 탐색하는 이유는, 국민소득이 증대됨에 따라 식생활이 서구화되면서 고혈압, 당뇨병, 고지혈증, 비만 및 대장암 등 각종 성인병이 유발되고, 사회가 점차 고도화됨에 따라 고령화인구가 늘어나게 되면서 건강문제에 대한 인식이 바뀌게 되었기 때문이다. 또한 합성 항산화제 및 항암제를 다량 섭취했을 때의 독성문제가 야기되면서 인체에 무해하고 활성이 우수한 천연 항산화제 및 항암제를 찾기 위한 노력이 계속되고 있다. 지금까지 연구되었던 대부분의 연구결과는 유기용매 추출물을 이용해 항산화 활성을 검색하였다. 하지만 이것은 수율이 아주 낮고, 그들이 원래 내재하고 있는 유독성에 의한 인체독성이나 혹은 양적, 경제적인 이유로 인해 효과적인 항산화제 및 항암제를 개발한 경우는 극히 드물다.

이에 본 실험은 제주도에 서식하고 있는 갈조류의 한 종류인 감태로부터 수용성 활성물질을 제조하여 항산화활성 및 항암활성을 검토하였다. 감태로부터 수용성 활성물질 제조는 여러 종류의 단백질분해효소와 당분해효소를 이용하여 효소적 추출방법을 이용하였다. 감태로부터 수용성 효소추출물의 항산화활성을 검토하기 위하여 정상세포인 V79-4 cell에 과산화수소를 처리하였을 때의 과산화수소 소거활성을 조사하였으며, 또한 과산화수소에 의한 세포의 산화적 손상으로부터 감태추출물의 보호효과를 조사한 결과, 감태 효소추출물이 우수한 세포 보호효과에 의한 DNA 손상을 억제하였다. 감태 효소추출물의 항암효과를 알아보기 위해 백혈병 세포(U937과 HL60 cells), 자궁암 세포(HeLa cells) 및 피부암 세포(B16 cells)를 이용하여 MTT 방법을 통해 암세포의 세포 증식억제활성을 알아본 결과, 감태 효소추출물의 30 kDa 이상 분획물은 대부분의 암세포에 대해서 세포 증식 억제 활성을 보였으며, 특히 U937 cell에 대해 가장 높은 항암활성을 나타

내었다.

이러한 결과를 종합해 볼 때, 감태의 효소적 추출물이 식품에서 사용가능한 안전한 수용성 물질임에도 불구하고 활성산소종의 하나인 과산화수소를 효과적으로 제거시키고, 또한 암세포의 증식을 억제하는 것으로 볼 때 산업적 용도가 매우 다양할 것으로 추정되며, 특히 식품산업으로서의 이용 가능성을 높일 수 있을 것이라 판단된다. 또한 기존의 항산화제 및 항암제가 갖고 있던 인체 안전성 문제도 충분히 극복할 수 있을 것으로 기대된다.

LIST OF FIGURES

Fig. 1-1. Scheme for preparation of enzymatic extracts from seaweeds.
Fig. 1-2. Ultrafiltration membrane system for fractionation of *E. cava* enzymatic extract according to the molecular weight of constituents.
Fig. 1-3. Hydrogen peroxide scavenging activity of enzymatic extracts of *E. cava*. Samples were tested at the concentration of 100 ㎍/ml. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.
(P, protamex; N, Neutrase; K, Kojizyme; A, Alcalase; F, Flavourzyme; C, Celluclast; T, Termamyl; AMG, AMG; V, Viscozyme; U, Ultraflo)
Fig. 1-4. Hydrogen peroxide scavenging activity of the different molecular weight fractions of *E. cava* enzymatic extract obtained by enzymatic hydrolysis with Celluclast for 12 h. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.
Fig. 1-5. Total polyphenolic content in the different molecular weight fractions of *E. cava* Celluclast extract. Total polyphenolic content was calculated as the gallic acid equivalents (mg) extracted from 100 g.
Fig. 1-6. Protective effect of unfraction and >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ±S.D. -■- >30 kDa fraction and -▲- unfraction.
Fig. 1-7. Lipid peroxidation inhibitory activity of >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.
Fig. 1-8. Protective effect of >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced apoptosis in V79-4 cells. Cellular

제주대학교 중앙도서관

morphological changes were observed using fluorescence microscope. Photomicrographs of V79-4 cells treated with (A) untreated (B) 1 mM H_2O_2 (C) 50 ㎍/ml sample + 1 mM H_2O_2 (D) 100 ㎍/ml sample + 1 mM H_2O_2. Apoptotic bodies are indicated by arrows.

Fig. 1-9. Effect of >30 kDa fraction on cell cycle pattern and apoptotic portion in V79-4 cells with H_2O_2 (1 mM) by flow cytometric analysis. (A) Control (B) H_2O_2 (C) H_2O_2 + >30 kDa fraction (D) Bar graph for sub G1 peak patterns of V79-4 cells H_2O_2 with treated by 100 ㎍/ml concentration of >30 kDa fraction. (1) Control; (2) H_2O_2 treated; (3) H_2O_2 + >30 kDa fraction

Fig. 2-1. Hydrogen peroxide scavenging activity (%) of ECUL and ECDL. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 2-2. DPPH radical scavenging activity (%) of ECUL and ECDL. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 2-3. Protective effect of ECUL against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 2-4. Protective effect of ECUL against H_2O_2-induced apoptosis in V79-4 cells. Cellular morphological changes were observed using fluorescence microscope. Photomicrographs of V79-4 cells treated with (A) vehicle only; (B) 1 mM H_2O_2 (C) 5 ㎍/ml sample + 1 mM H_2O_2 (D) 25 ㎍/ml sample + 1 mM H_2O_2. Apoptotic bodies are indicated by arrows.

Fig. 2-5. The effect of supplementation in vitro with different concentrations of ECUL on DNA damage of H_2O_2-induced L5178 cell. Values are means with standard errors of duplicate experiments. (□: % Fluorescence in tail, -◆-: Inhibitory effect of cell damage)

Fig. 2-6. Comet images of L5178 cells: (a) negative control; (b) 50 ㎛ H_2O_2 (c) 1 ㎍/ml sample + 50 ㎛ H_2O_2 (d) 5 ㎍/ml sample + 50

μM H_2O_2 (e) 10 μg/ml sample + 50 μM H_2O_2 (f) 25 μg/ml sample + 50 μM H_2O_2

Fig. 2-7. Effect of ECUL on antioxidant enzyme activities (A) SOD activity, (B) CAT activity were measured, as described in MATERIALS AND METHODS. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 3-1. Effect of difference molecular weight fractions of *E. cava* Celluclast extract on tumor cell growth inhibition activity in tumor cells (U937, HL60, B16 and HeLa cells). Cells were treated with 100 μg/ml of different molecular weight fractions and measured for viability by MTT assay at 72 h after the sample treatment. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 3-2. Effect of >30 kDa fraction of *E. cava* Celluclast extract in U937 cells. Cells were treated with various concentrations of >30 kDa fraction and measured for viability by MTT assay at 12, 24, 48 and 72 h after the sample treatment. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

Fig. 3-3. Comet images of U937 cells after the treatments of different concentrations of >30 kDa fraction: (A) untreated sample; (B) U937 cells treated with 50 μg/ml; (C) U937 cells treated with 100 μg/ml; (D) U937 cells treated with 200 μg/ml

Fig. 3-4. The effect of >30 kDa fraction on nucleolus DNA damage in U937 cells. Values are means with standard errors of duplicate experiments.

Fig. 3-5. Morphological changes of U937 cells treated with different concentrations of >30 kDa fraction. Cells were treated with >30 kDa fraction for 24 h. (A) untreated cell; (B) U937 cells treated with 50 μg/ml; (C) U937 cells treated with 100 μg/ml; (D) U937 cells treated with 200 μg/ml

Fig. 3-6. Effect of >30 kDa fraction on cell cycle pattern and apoptotic portion in U937 cells by flow cytometric analysis. Histogram

patterns of U937 cells treated with various concentrations of >30 kDa fraction for 24 h by cell cycle analysis. (A) untreated sample; (B) U937 cells treated with 50 ㎍/ml; (C) U937 cells treated with 100 ㎍/ml; (D) U937 cells treated with 200 ㎍/ml.

Fig. 3-7. Bar graph for cell cycle patterns of U937 cells treated by various concentrations of >30 kDa fraction.

LIST OF TABLES

Table 1-1. Optimum hydrolysis conditions and characteristics of the enzymes used for preparation of enzymatic extracts

Table 3-1. Comparison with cell viability by >30 kDa fraction in leukemia and normal cells

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2), are unwanted metabolic by-products of normal aerobic metabolis. High levels of ROS are regarded to be responsible for a variety of pathological conditions, including cardiovascular disease, cancer and aging (Harman, 1994; Cox and Cohen, 1996; Ames, 1998; Finkel and Holbrook, 2000). Among those ROS, H_2O_2 especially plays a pivotal role because it is generated from nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues (Hallivell and Aruoma, 1991).

Synthetic antioxidants have been identified as mutagens and tumor promoters at high dosages (Kahl and Kappis, 1993; Kahl, 1994). Natural antioxidants impart promising safety even at higher dosages. Marine bioresources are known to be attractive as they sometimes include new compounds showing several kinds of bioactivities. Among them, seaweeds or their extracts have been studied as potential natural antioxidants during the last decade (Anggadiredja et al., 1997; Matsukawa et al., 1997; Yan et al., 1998; Tutour et al., 1998; Duval et al., 2000; Hirata et al., 2000; Kovatcheva et al., 2001; Xue et al., 2001; Ruberto et al., 2001; Ruperez et al., 2002; Lim et al., 2002). Seaweeds are rich in vitamins, minerals, natural bioactive compounds and various functional polysaccharides. One of them, polysaccharides plentifully present in brown seaweeds, such as alginates, fucans and laminarans are water-soluble dietary fibers and possess various bioactive properties.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Cancer is a disease manifested by uncontrolled cell growth that presents over 100 distinct clinical pathologies (Kufe et al., 2003). Cancer is the largest single cause of death in human, claiming over 6 million lives each year in the world. In the last few decades, basic cancer researches have produced remarkable advances in understanding of cancer biology and cancer genetics (Lowe and Lin, 2000). Recently, many anti-cancer drugs have been developed and applied in clinical

trials. Chemotherapeutic agents and radiation which cause DNA mutation in actively dividing cells, were intended to selectively kill cancer cells while having limited effect on normal cells. Unfortunately these cytotoxic agents were limited in their utility due to their toxicity on normal dividing cell populations resulting in adverse side effects. Therefore, the researches and developments of new and safe drugs have become one of the interest areas in the pharmaceutical industry (Yang et al., 2000). Marine bioresources are known to be attractive as they sometimes consists new compounds showing several kinds of different bioactivities which are not possible in land plants. Screening of algal extracts for biologically active compounds began in the 1950s with simple antibiotic assays and expanded to include testing for product with antiviral, antibacterial, antifungal, anti-mitotic or anti-tumorigenic activities (Kashiwagi et al., 1980; Gonzalez et al., 1982; Kosovel et al., 1988; Glombitza and Koch, 1989). Studies on antitumor effects of algal species have been reported by a number of researchers (Yamamoto et. al. 1982; Noda et. al. 1989; Jolles et. al 1963; Yamamoto et. al. 1974; Mizukoshi et. al. 1992).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In this study, it was tried to obtain water-soluble enzymatic extract from seaweeds by using some carbohydrases and proteases, which are enzymes derived from microorganisms. These enzymes can convert water-insoluble raw materials of seaweeds into water-soluble ones. Enzymatic extracts from seaweeds possess high-solubility in water and safety, as this method does not adapt any organic solvent or other toxic chemicals. It was previously reported that enzymatic extraction gains a high yield and high radical scavenging activity in comparison with organic extracts (Heo et al., 2003).

A brown seaweed *Ecklonia cava* which is rich in the coast of Jeju Island in Korea but is not useful for food diet due to puckery taste induced by tannins was enzymatically hydrolyzed with five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo) and five proteases (Protamex, Kojizyme, Neutrase, Flavourzyme and Alcalase) to prepare water-soluble seaweed extracts, and then we investigated effect of *E. cava* enzymatic extracts on the H_2O_2

scavenging activity, lipid peroxidation inhibitory activity, cell viability and we measured the protective effect of these enzymatic extracts on H_2O_2-induced apoptosis. In addition, apoptosis-inducing effects of *E. cava* enzymatic extracts were investigated in four types of tumor cells (U937, HL60, B16 and HeLa cells).

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Part Ⅰ

Protective effect of *Ecklonia cava* enzymatic extracts on H_2O_2-induced cell damage

제주대학교 중앙도서관

Part Ⅰ

Protective effect of *Ecklonia cava* enzymatic extracts on H_2O_2-induced cell damage

1. ABSTRACT

In this study, *Ecklonia cava* was enzymatically hydrolyzed to prepare water-soluble extracts by using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultaraflo) and five proteases (Protamex, Kojizyme, Neutase, Flavourezyme and Alcalase) and evaluated their potential antioxidant activity. The Celluclast and Viscozyme extracts of *E. cava* exhibited better hydrogen peroxide (H_2O_2) scavenging activities (73.25% and 72.92%, respectively) compared to other enzymatic extracts. Cellucalast extract was separated into four different molecular weight fractions (<1 kDa, 1~10 kDa, 1~30 kDa and >30 kDa). Among all the fractions, >30 kDa fraction showed the highest H_2O_2 scavenging activity with an IC_{50} of 13 ㎍/ml. Also, >30 kDa fraction strongly enhanced cell viability against H_2O_2-induced oxidative damage and exhibited relatively higher lipid peroxidation inhibitory activity in the chinese hamster lung fibroblast (V79-4) cell line. Furthermore, the fraction reduced the proportion of apoptotic cells induced by H_2O_2, as demonstrated by decreased sub-G_1 hypodiploid cells and decreased apoptotic body formation in flow cytometry assay. These results illustrate that >30 kDa fraction of Celluclast extract from *E. cava* exhibits a good antioxidant activity over H_2O_2 mediated cell damage *in vitro*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. MATERIALS AND METHODS

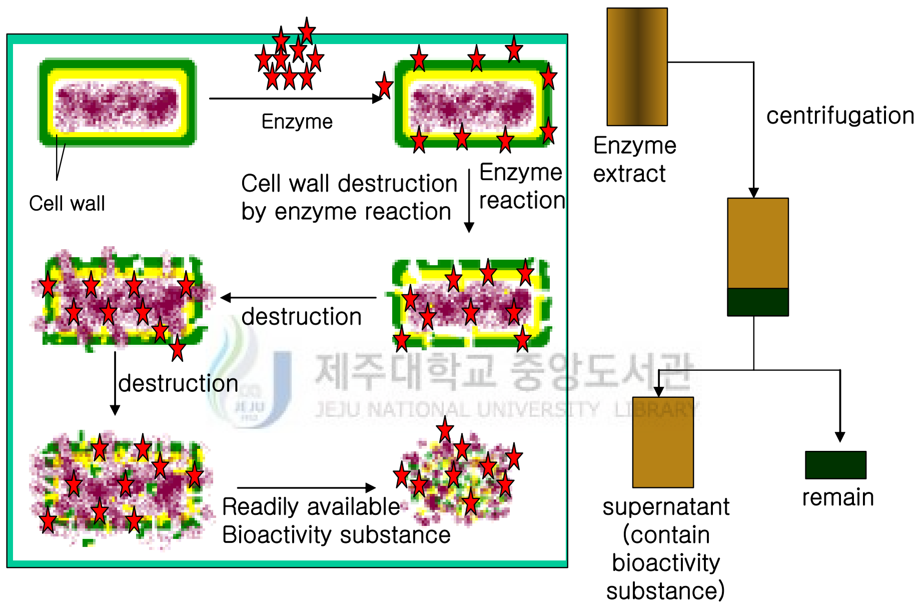
2. 1. Materials

E. cava was collected along Jeju Island coast of Korea during October 2003 to March 2004. Salt, epiphytes and sand from sample were removed using tap water. Finally, *E. cava* were rinsed carefully in freshwater and stored in a medical refrigerator at -20°C. The frozen sample was lyophilized and homogenized with a grinder before extraction. Five carbohydrase including Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L, and the five proteases including Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). The optimum pH, optimal temperature and characters of those enzymes were summarized in Table 1-1. 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), thiobarbituric acid (TBA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte- trazolium bromide (MTT), and Hoechst 33342 were purchased from sigma Co. (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

제주대학교 중앙도서관

2. 2. Preparation of enzymatic extracts from *E. cava*

The enzymatic extracts from *E. cava* were obtained according to the method used by Heo et al. (2003). The freeze dried *E. cava* were pulverized into powder using a grinder. One gram of the dried *E. cava* sample was homogenized with 100 ml of buffer and mixed with 100 mg (or 100 μl) of enzymes. Each reactant was adjusted to be within the optimum pH and temperature range of the respective enzyme and enzymatic reactions were performed for 12 h. Finally, each sample was clarified by centrifugation at 3000 × *g* for 20 min to remove the unhydrolyzed residue. Enzymatic extract of the seaweed was obtained after filtering the supernatant and was stored for further experiments (Fig. 1-1).



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-1. Scheme for preparation of enzymatic extracts from seaweeds

Table 1-1. Optimum hydrolysis conditions and characteristics of the enzymes used for preparation of enzymatic extracts

Enzyme	Optimum condition		Buffer used[a]	Enzyme charateristics
	pH	Temperature (℃)		
Viscozym (V)	4.5	50	0.1N AB[b]	Arababase, celluiase, β-glucanase, hemicelluase and xyianase
Celluclast (C)	4.5	50	0.1N AB	Catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymer
AMG	4.5	60	0.1N AB	An exo-1,4-α-D-glucosidase
Termamyl (T)	6.0	60	0.1N PB[c]	A heat-stable-α-amylase
Ultraflo (U)	7.0	60	0.1N PB	A heat-stable multi-activity β-glucanase
Protamex (P)	6.0	40	0.1N PB	Hydrolysis of food proteins
Kojizyme (K)	6.0	40	0.1N PB	An endoprotease
Neutrase (N)	6.0	50	0.1N PB	Containing both endoprotease and exopeptidase activities
Flavourzyme (F)	7.0	50	0.1N PB	Exopeptidase activities
Alcalase (A)	8.0	50	0.1N PB	A endoprotease

[a] In enzymatic hydrolysis

[b] Acetate buffer

[c] Phosphate buffer

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. 3. Molecular weight fractionation of enzymatic extract

Cellulclast extract was fractionated using millipore's Labscale TFF system with ultrafiltration membrane (1, 10 and 30 kDa) (Fig. 1-2). The resultant fractions were collected according to the molecular weight (<1 kDa, 1~10 kDa, 10~30 kDa and >30 kDa) and investigated for hydrogen peroxide scavenging activity, lipid peroxidation inhibitory activity and cell viability.

2. 4. Total polyphenolic compounds

Total polyphenolic compounds were determined according to Chandler and Dodds (1983) with some modification. One milliliter of *E. cava* hydrolysate was mixed in a test tube containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1 ml of 5 % Na_2CO_3 was added. Thoroughly mixed mixture was placed in a dark room for 1 h and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Ltd., Korea). A gallic acid standard curve was obtained for the calculation of polyphenolic content.

2. 5. Cell culture

Chinese hamster lung fibroblast line (V79-4) was maintained at 37°C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml), and penicillin (100 unit/ml)

2. 6. Hydrogen peroxide scavenging activity

For detection of intracellular H_2O_2, V79-4 cells were seeded in 96-well plates at a concentration of 1.0×10^5 cells/ml. After 16 h, the

cells were treated with various concentrations of the enzymatic extract and fraction samples (1, 10, 50 and 100 μg/ml), and incubated at 37℃ in a humidified atmosphere. After 30 min, 1 mM H_2O_2 was added, and then cells were incubated for an additional 30 min at 37°C. Finally 5 μg/ml DCFH-DA was introduced to the cells and detected at 485 nm excitation and at 535 nm emission using a PerkinElmer LS-5B spectrofluorometer.

2. 7. Assessment of cell viability

Cell viability was estimated by the MTT assay, which is a test of metabolic competence based upon assessment of mitochondrial performance. It is colorimetric assay relying on the conversion of yellow tetrazolium bromide to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmaann, 1983). Cells were seeded in a 96-well plate at a concentration of 1.0×10^5 cells/ml. After 16 h, cells were treated with enzymatic extracts at difference concentrations (1, 10, 50 and 100 μg/ml). Then 1 mM H_2O_2 was to reach added to the cell culture medium and incubated for 24 h at 37 ℃. MTT stock solution (50 μl; 2 mg/ml) was then added to each well for a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of DMSO and absorbance was measured using an ELISA at 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective control.

2. 8. Lipid peroxidation inhibitory activity

The ability of extract to inhibit lipid peroxidation was assayed by

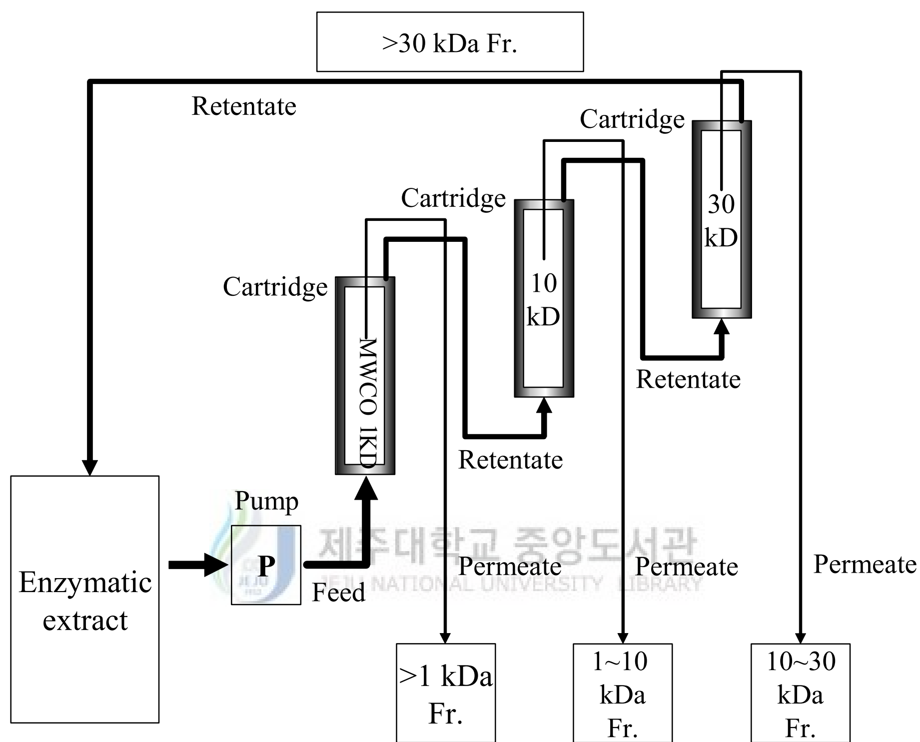


Fig. 1-2. Ultrafiltration membrane system for fractionation of *E. cava* enzymatic extract according to the molecular weight of constituents.

measuring malondialdehyde (MDA) according to the method of Ohkawa et al. (1979). The V79-4 cells were seeded at a concentration of 1.0 × 10^5 cells/ml, and at 16 h after plating they were treated with various concentrations (10, 50 and 100 ㎍/ml) of fraction sample. One hour later, 1 mM H_2O_2 was added to the plate, and was incubated for a further 1 h. The cells were then washed with cold phosphate buffer saline (PBS), scraped and homogenized in ice-cold 1.15% KCl. Samples containing 100 ㎕ of the cell lysates were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% TBA. The mixture was brought to a final volume of 4.0 ml with distilled water and heated for 120 min at 95°C. After cooling down sample into room temperature, 5 ml of a n-butanol and pyridine mixture (15:1, v/v) was added to each sample and vortexed. Then mixture was centrifuged at 1000 × g for 10 min, the supernatant fraction was separated and the absorbance was measured at 532 nm.

2. 9. Nuclear staining with Hoechst 33342

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

The nuclear morphology of cells was studied by using the cell-permeable DNA dye Hoecht 33342. Cell with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The V79-4 cells were placed at a concentration of 1.0 × 10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentration of fraction sample, and further incubated for 1 h before expose to H_2O_2 (1 mM). After 24 h, 1.5 ㎕ of Hoechst 33342 (stock 10 mg/ml), a DNA-specific fluorescent dye, were added to each well, followed by incubation for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2. 10. Flow cytometry analysis

Flow cytometry analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The V79-4 cells were placed in a 6-well plate at a concentration of 1.0×10^5 cells/ml, and at 16 h after plating they were treated with fraction sample (100 µg/ml). After further incubation of 1 h H_2O_2 (1 mM) was added to the culture. After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS and incubated in the dark in 1 ml of PBS containing 100 µg propidium iodide (PI) and 100 µg RNase A for 30 min at 37℃. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit (Wang et al., 1999).

3. RESULTS

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

3. 1. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging ability of the enzymatic extracts of *E. cava* is exhibited on Fig. 1-3. As shown in Fig. 1-3, all the enzymatic extracts of *E. cava* showed more than >40% H_2O_2 scavenging activity at same concentration. Comparatively, enzymatic extracts prepared by cabohydrases showed better activities than those by proteases extracts. Especially Celluclast and Viscozyme extracts showed the highest activities (~73 and ~72%) respectively than other counterparts.

Therefore Celluclast extract was selected for further experiments. In order to characterize the molecular weight, the fraction was passed through ultraflltration membranes and consequently fractions (unfractionated sample, <1 kDa, 1~10 kDa, 10~30 kDa and >30 kDa) were investigated for their relevant H_2O_2 scavenging activities. The H_2O_2 scavenging activities of four molecular weight fractions of

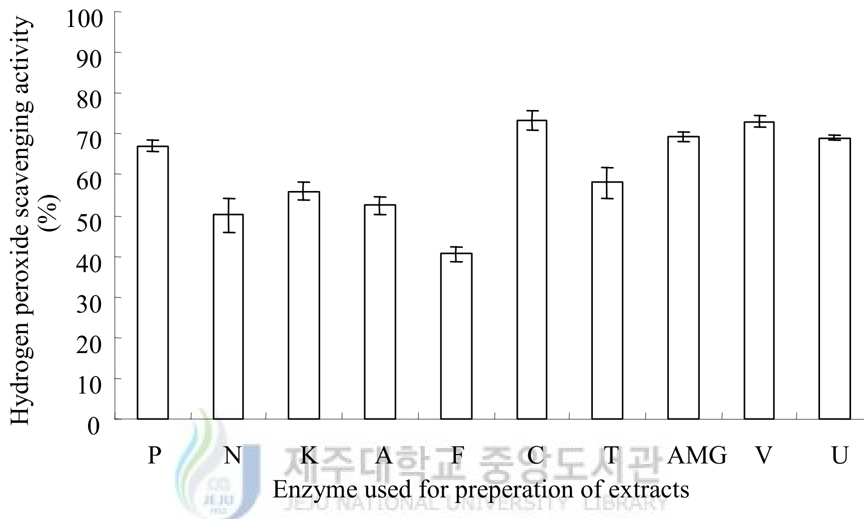
Celluclast extract are as well as unfractionated sample shown in Fig. 1-4. The >30 kDa fraction of Celluclast extracts had the highest H_2O_2 scavenging activity with an IC_{50} value of 13.0 μg/ml. However, the IC_{50} values of 10～30 kDa, 1～10 kDa, unfractionated sample and <1 kDa sample increased in the order of 22.2, 30.9, 38.9 and 51 μg/ml respectively.

3. 2. Total polyphenolic compounds

All molecular weight fractions were subjected to total polyphenolic content assay to get an idea about the polyphenolic content of the each extract. The total polyphenolic amount of each extract is exhibited in Fig. 1-5. The highest polyphenolic content was recorded from >30 kDa fraction (2.23 g/100 ml) while the lowest content was exhibited from <1 kDa fraction (0.86 g/100 ml). The other three fractions including unfractionated sample showed almost similar polyphenolic content (1.88 ～1.98 g/100 ml).

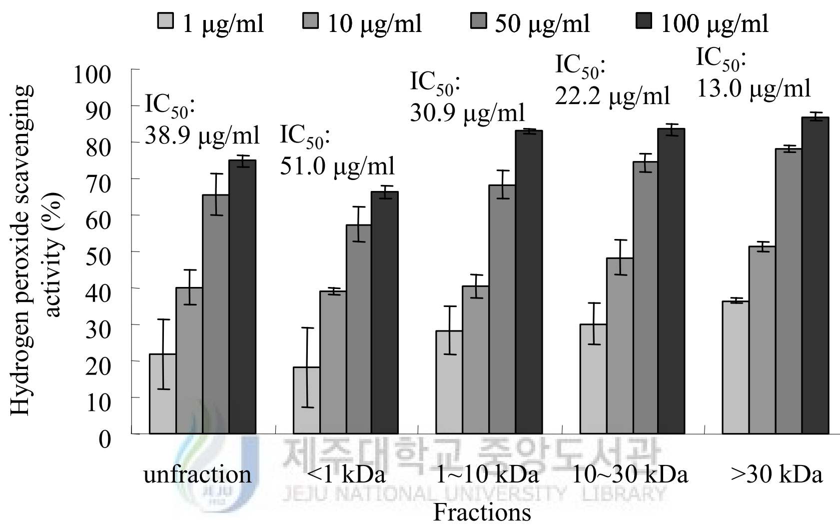
3. 3. H_2O_2-induced of cell viability by >30 kDa fraction

As >30 kDa fraction possessed the highest hydrogen peroxide scavenging ability, this fraction was further evaluated for its protecting ability against H_2O_2-induced cell damages. The protective effect of the unfractionated sample and >30 kDa fraction of *E. cava* on H_2O_2-induced cell damage on V79-4 cells is exhibited in Fig. 1-6. In this study, both samples dose-dependently controlled H_2O_2-induced cellular damage. The addition of H_2O_2 to the cell culture medium, without algal extracts rendered only 33% cell survival rate but addition of extracts along with H_2O_2 to the medium dose-dependently increased cell survival rate. However, the unfractionated sample and >30 kDa fractions with concentrations of 1～50 ug/ml indicated almost similar activity enhancement. Especially, the highest cell viability with 67% was recorded on >30 kDa at a concentration of 100 μg/ml.



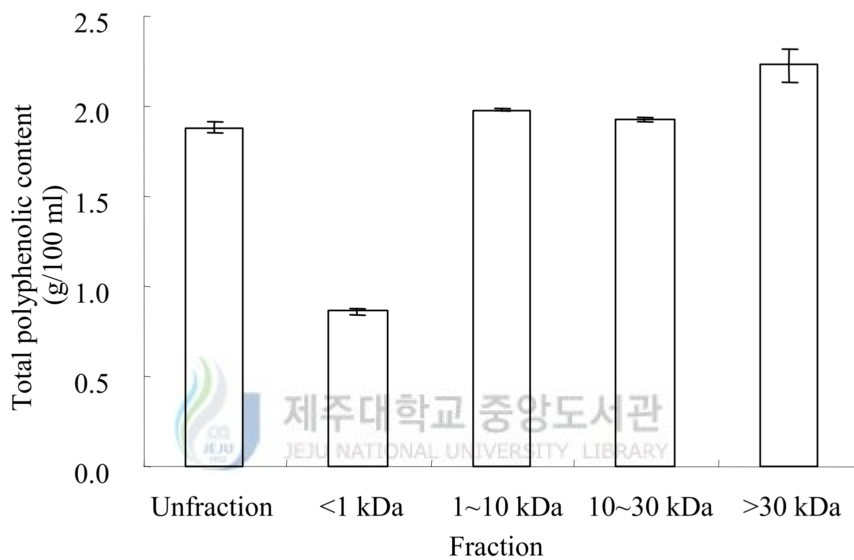
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-3. Hydrogen peroxide scavenging activity of enzymatic extracts of *E. cava*. Samples were tested at the concentration of 100 ㎍/ml. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.
(P, protamex; N, Neutrase; K, Kojizyme; A, Alcalase; F, Flavourzyme; C, Celluclast; T, Termamyl; AMG, AMG; V, Viscozyme; U, Ultraflo)



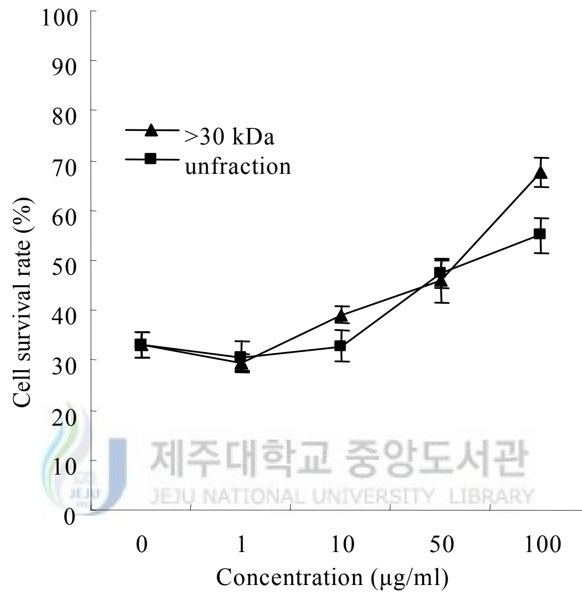
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-4. Hydrogen peroxide scavenging activity of the different molecular weight fractions of *E. cava* enzymatic extract obtained by enzymatic hydrolysis with Celluclast for 12 h. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-5. Total polyphenolic content in the different molecular weight fractions of *E. cava* Celluclast extract. Total polyphenolic content was calculated as the gallic acid equivalents (mg) extracted from 100 g.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-6. Protective effect of unfraction and >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ±S.D. -■- >30 kDa fraction and -▲- unfraction.

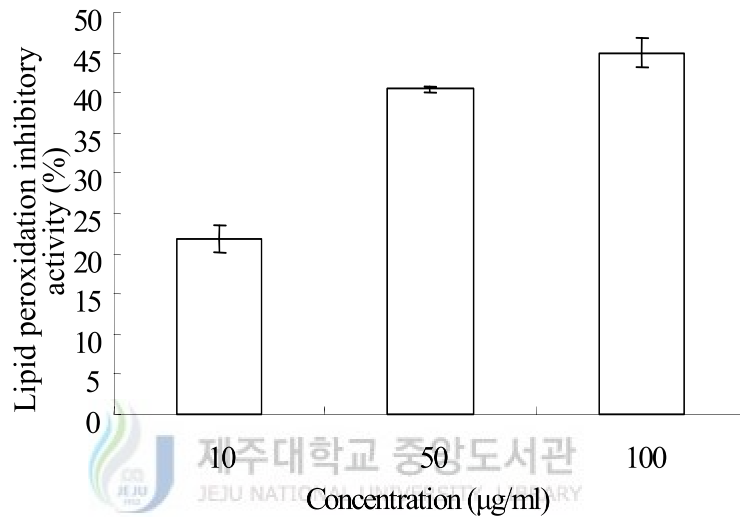
3. 4. Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity of >30 kDa fraction which contained highestly antioxidant active compounds was investigated in H_2O_2-treated V79-4 cells (Fig. 1-7). The generation of MDA, and the related substances that react with TBA, was nicely controlled by >30 kDa fraction. Especially, >30 kDa fraction delayed lipid peroxidation in a dose-dependant manner, and the activity reached to a maximal at the highest dosage of 100 μg/ml (44%).

3. 5. Reduction of H_2O_2-induced nuclear fragmentation by >30 kDa fraction

Typical fluorescence photographs of shrunken nuclei, chromatin condensation and appearance of apoptotic bodies were shown in V79-4 cell after 1 mM H_2O_2 treatment for 24 h (Fig. 1-8B). The negative control, treated without the sample and H_2O_2, showed clear image and exhibited no DNA damage (Fig. 1-8A). However, Obvious cell damage was observed in the cells treated with H_2O_2. Cells treated with >30 kDa fraction 1 h prior to H_2O_2 treatment, a dramatic reduction in apoptotic bodies. Therefore, the photographs (Fig. 1-8C and D) clearly suggest the ability of >30 kDa fraction to protect cell damage against H_2O_2 attack. The addition to the morphological evaluation, the protective effects of >30 kDa fraction were confirmed by flow cytometry. Analysis of the DNA contents following H_2O_2 treatment of V79-4 cells revealed an increase in the proportion of cells with sub-G_1 DNA content, to 22% (Fig 1-9). This result indicates that apoptosis was induced by H_2O_2. However, cells that were pretreated with >30 kDa fraction showed significantly reduced sub-G_1 DNA content (11%).

제주대학교 중앙도서관



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-7. Lipid peroxidation inhibitory activity of >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

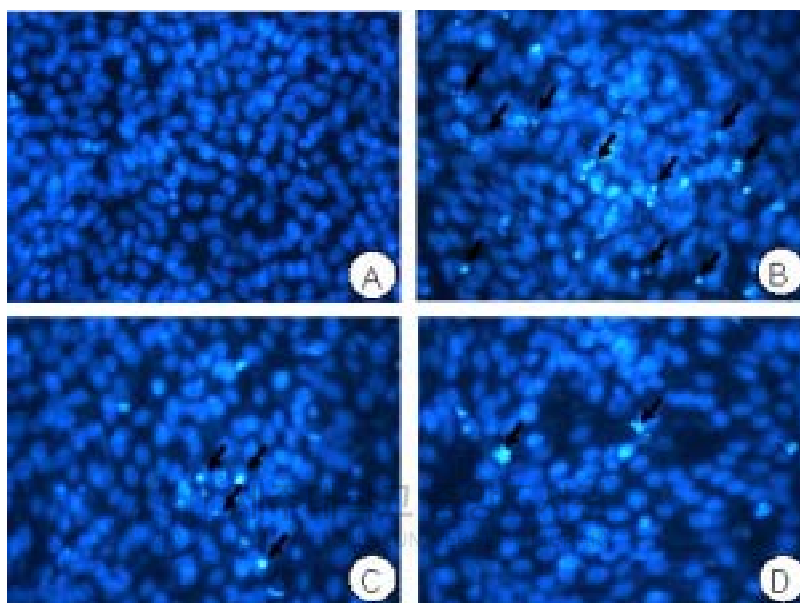
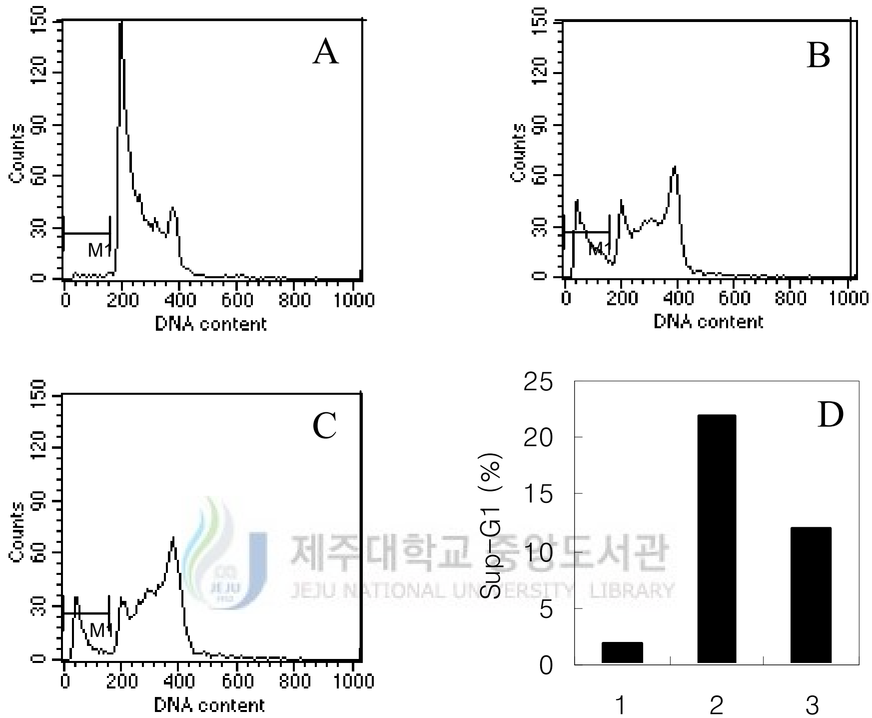


Fig. 1-8. Protective effect of >30 kDa fraction from *E. cava* Celluclast extract against H_2O_2-induced apoptosis in V79-4 cells. Cellular morphological changes were observed using fluorescence microscope. Photomicrographs of V79-4 cells treated with (A) untreated (B) 1 mM H_2O_2 (C) 50 μg/ml sample + 1 mM H_2O_2 (D) 100 μg/ml sample + 1 mM H_2O_2. Apoptotic bodies are indicated by arrows.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 1-9. Effect of >30 kDa fraction on cell cycle pattern and apoptotic portion in V79-4 cells with H_2O_2 (1 mM) by flow cytometric analysis. (A) Control (B) H_2O_2 (C) H_2O_2 + >30 kDa fraction (D) Bar graph for sub G1 peak patterns of V79-4 cells H_2O_2 with treated by 100 µg/ml concentration of >30 kDa fraction. (1) Control; (2) H_2O_2 treated; (3) H_2O_2 + >30 kDa fraction

4. DISCUSSION

ROS are unwanted metabolic by-products of normal aerobic metabolism under high level of O_2 pressure. High levels of ROS create oxidative stress, which leads to a variety of biochemical and physiological lesions. Such cellular damage often impairs metabolic function and leads cell death (Finkel and Holbrook, 2000). Cells are protected from ROS-induced damage by a variety of endogenous ROS scavenging enzymes, chemical compounds and natural products. Recently there has been an increasing interest in the therapeutic use of natural plants as antioxidants in reducing such free radical-induced tissue injury, suggesting that many plants have antioxidative compounds that could be therapeutically useful. In the present study, we focused on natural water-soluble enzymatic extracts from *E. cava*, which were prepared by enzymatic hydrolysis using different carbohydrases and proteases, and investigated antioxidant effect of these *E. cava* enzymatic extracts on the H_2O_2 treatment.

In this study, Celluclast and Viscozyme extracts of *E. cava* showed strong H_2O_2 scavenging activity (>70%) As it has been previously observed, there are some reports, about high H_2O_2 scavenging ability of seaweeds (Athukorala et al., 2003a; Sriwardhana et al., 2003). Especially *E. cava* enzymatic extract have exhibited high H_2O_2 scavenging and DPPH radical scavenging activities previously (Heo et al., 2005).

Therefore, Celluclast extract was selected for further experiments. In order to characterize the molecular weight distribution of the active compounds, the enzymatic extract was passed through ultrafliltration membranes and consequently fractions (unfractionated sample, <1 kDa, 1~10 kDa, 10~30 kDa and >30 kDa) were separately investigated for their relevant H_2O_2 scavenging activities. The total phenolic content and H_2O_2 scavenging activities of >30 kDa fraction were higher than those of other fraction (unfractionated sample, <1 kDa, 1~10 kDa and 10~30 kDa). The >30 kDa fraction from *E. cava* Celluclast extract increased lipid peroxidation inhibition in a dose-dependent manner. The >30 kDa fraction also enhanced viability in V79-4 cells exposed to H_2O_2. As it

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

has been mentioned in previous recorded (Siriwardhana et al., 2004), there is a correlation between total polyphenolics and radical scavenging activity of the seaweed extracts and this was again confirmed in this study. Many researchers have reported positive correlation between ROS scavenging activity and total phenolic compound. Polyphenolic compounds are widely distributed in plants or seaweeds and are known to exhibit higher antioxidative activities. The activities have been reported through various methods of ROS scavenging activity and the inhibition of lipid peroxidation (Yan et al., 1999; Athukorala et al., 2003a,b; Heo et al., 2003a,b; Siriwardhana et al., 2003, 2004). Some researches observed that the hydrogen peroxide scavenging activity increased with the increase of phenolic compound content (Oki et al., 2002; Siriwardhana et al., 2003; Heo et al., 2005.

Due to high antioxidative activity of >30 kDa fraction, the fraction was further evaluated on its protecting ability for DNA damage. DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress representing the imbalance between free radical generation and efficiencies of the antioxidant system (Gutteridge, 1995; Kassie et al., 2000). Hoechst 33342 specifically stains DNA and is widely used to detect shrinkage of the nuclei (chromatin condensation, nuclear fragmentation, appearance of apoptotic bodies are indicative of apoptosis) (Kerr et al., 1995). In addition to the morphological evaluation, the protective effects of the >30 kDa fraction were confirmed by flow cytometry. Cell exposed to H_2O_2 exhibit distinct morphological features of programmed cell death, such as nuclear fragmentation and an increase in the percentage of cells with a sub-G_1 DNA content. However, treatment with >30 kDa fraction of Celluclast extract from *E. cava* reduced the appearance of the morphological features characteristic of apoptotic cells and sub-G_1 content.

Several studies have shown that increasing in materials including environmental pollutants, radiation, dietary habits and various chemicals induced DNA damage, which can lead to the diseases such as cancer and heart disease (Hertog et al., 1993; Hartmann et al., 1995; and Singh

et al., 1995), Also many researchers have investigated inhibition of DNA damage by food materials such as tea (Zhang et al., 2002), juice (Park et al., 2003), plant extract (Yen et al., 2001; Zhu and Loft, 2001), flavonoid (Senthilmohan et al., 2003) and aquatic animals (Janssens et al., 2002). The cells of the human body are continuously attacked by physical agents (such as solar radiation), a variety of chemical compounds and ROS. These substances can induce DNA damage. If DNA damages are not repaired, it can initiate cascade of biological consequences at the population and also could promote cancer development via several mechanisms (Bagchi et al., 2000).

Antioxidative activity of a brown seaweeds *E. cava* can be due to carotenoid pigments (fucoxanthin), polyphenolics (phlorotannins), vitamin (vitamin C and E), sulfated polysaccharides, their breakdown products (laminarin and fucoidans) and proteins or their breakdown products (Nardella et al., 1996; Yan et al., 1999; Ramos and Xiong, 2002; Burtin, 2003). In the enzymatic hydrolysis, polyphenolics, vitamin C, sulfated polysaccharides and protein of the above potential antioxidative materials can be the most effective due to their native water soluble character. Polyphenolics are present in foods as polyphenolics sugar compounds, which are called glycosides in general or more specifically glucosides, rutinosides, or xylosides depending on their sugar moiety. The sugar polyphenolics bond is a β-glycosidic bond which is resistant to hydrolysis by pancreatic enzymes (Kühnau, 1976; Hollman et al., 1997). Generally, a major part of brown seaweed polyphenols is high molecular weight (>10 kDa) and potentially bind with polysaccharide. Therefore the breakdown of sugar may enhance the availability of free polyphenols to scavenging free radical or any other oxidative materials. thus, It can be assumed that higher H_2O_2 scavenging activity is noticed from >30 kDa fraction because of high molecular weight of polyphenolics and polysaccharides which are potential bioactive compounds. However, the correlation between scavenging activity and those bioactive compounds of *E. cava* are still unknown. Therefore further experiments are need to purify the compounds responsible for scavenging effects.

제주대학교 중앙도서관

Part Ⅱ

Protective effect of ECUL on H_2O_2-induced cell damages

Part Ⅱ

Protective effect of ECUL on H2O2-induced cell damages

1. ABSTRACT

Celluclast extract of *Ecklonia cava* was separated by millipore′s Labscale TFF system (Millipore, Billerica, USA) to obtain more than 30 kDa molecular weight fraction which showed the highest antioxidant activity in the previous results. The >30 kDa fraction was sub-fractionated by liquid-liquid partition using a mixture of methylene chloride and methanol (1:1, v/v), and then the resultant upper layer (ECUL) and down layer (ECDL) were investigated for H_2O_2 and DPPH radical scavenging activity, as well as protective effect against H_2O_2-induced cell damage. In addition, we measured the protective effect of these fraction on H_2O_2-induced apoptosis. H_2O_2 and DPPH radical scavenging activity of ECUL was increased in a dose-dependent manner. ECUL strongly enhanced cell viability against H_2O_2-induced oxidative damage in the chinese hamster lung fibroblast (V79-4) cell line. Furthermore, ECUL reduced the proportion of apoptotic cells induced by H_2O_2, as demonstrated by decreased sub-G_1 hypodiploid cells and decreased apoptotic body formation in flow cytometry assay. According to the result, ECUL of *E. cava* Celluclast extract exhibits a good antioxidant activity against H_2O_2 mediated cell damage *in vitro*

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. MATERIALS AND METHODS

2. 1. Materials

1-Diphenyl-2-pricrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzthia-zoline-6-sulfonic acid (ABTS), thiobarbituric acid (TBA), 3-(4,5-

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33342 were purchased from sigma (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

2. 2. Preparation of Celluclast extracts from *E. Cava*

The Celluclast extract was obtained according to the method used by Heo et al. (2003). The freeze dried *E. cava* were pulverized into powder using a grinder. One gram of the dried *E. cava* sample was homogenized with 100 ml of buffer (Table 1-1) and mixed with 100 μl of Celluclast. Sample was adjusted to be within the optimum range of the respective enzyme and enzymatic reactions were performed for 12 h. Finally, the reaction was clarified by centrifugation at 3000 × *g* for 20 min to remove the unhydrolyzed residue. Celluclast extract of the seaweed was obtained after filtering the supernatant and was stored for further experiments (Fig. 1-1).

제주대학교 중앙도서관 LIBRARY

2. 3. Separation of hydrophilic antioxidant

In order to obtain hydrophilic antioxidants, Celluclast extract of *E. cava* was separated into two different molecular weight fractions (<30 kDa and >30 kDa) by millipore's Labscale TFF system (Millipore, Billerica, USA). After >30 kDa fraction was sub-fractionated by liquid-liquid partition using a mixture of methylene chloride and methanol (1:1, v/v) then the resultant upper layer (ECUL) and down layer (ECDL) were investigated for H_2O_2 and DPPH radical scavenging activity, and protective effect against H_2O_2-induced cell damage. In addition, we measured the protective effect of these fraction on H_2O_2-induced apoptosis

2. 4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller et al. (1985). One hundred microliter of 0.1 M

phosphate buffer (pH 5.0) and sample solution were mixed in a 96-well plate. And 20 ㎕ of H_2O_2 was added to the mixture, and then incubated at 37℃ for 5 min. After incubation, 30 ㎕ of 1.25 mM ABTS and 30 ㎕ of peroxidase (1 unit/ml) were added to the mixture, then incubated for 10 min at 37℃. The absorbance was read with an ELISA reader at 405 nm.

2. 5. DPPH radical scavenging activity

Free radical scavenging activity was determined by using a stable free radical, DPPH, according to a slightly modified method of Blois (1958). DPPH solution was prepared at the concentration of 4×10^{-4} M in dimethyl sulfoxide (DMSO). During the assay, a 100 ㎕ seaweed extract and 100 ㎕ of freshly prepared DPPH solution were thoroughly mixed. The reaction mixture was incubated in the room temperature for 1 h. After standing for 1 h, the absorbance was recorded at 517 nm by ELISA reader (ELX tek Instrument Inc). The percentage inhibition was calculated as $[1-(A_i-A_j)/A_c]$ 100; A_i is the absorbance of extract mixed with DPPH solution, A_j is the absorbance of same extract mixed with 100 ㎕ DMSO, A_c is the absorbance of control with particular solvent (without seaweed extract).

2. 6. Cell culture

Chinese hamster lung fibroblast line (V79-4) was maintained at 37°C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 ㎍/ml), and penicillin (100 unit/ml)

2. 7. Assessment of cell viability

Cell viability was estimated by the MTT assay, which is a test of metabolic competence based upon assessment of mitochondrial

performance. It is colorimetric assay relying on the conversion of yellow tetrazolium bromide to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmaann, 1983). Cells were seeded at a concentration of 1.0 × 10^5 cells/ml. After 16 h, cells were treated with enzymatic extracts at difference concentration (1, 5, 10 and 100 ㎍/ml). Then 10 ㎕ of 1 mM H_2O_2 was to reach added to the cell culture medium and incubated for 24 h at 37℃. MTT stock solution (50 ㎕; 2 mg/ml) was then added to each well for a total reaction volume of 200 ㎕. After incubating for 4 h, the plate was centrifuged at 800 × g for 5 min and the supernatants were aspirated. The formazan crystals were dissolved in 150 ㎕ of DMSO and absorbance was measured using an ELISA at 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability. Data are mean percentages of viable cells versus the respective control.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. 8. Nuclear staining with Hoechst 33342

The nuclear morphology of cells was studied by using the cell-permeable DNA dye, Hoecht 33342. Cell with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The V79-4 cells were placed at a concentration of 1.0 × 10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentration of the fraction samples and further incubated for 1 h before exposed to H_2O_2 (1 mM). After 24 h, 1.5 ㎕ of Hoechst 33342 (stock 10 mg/ml) was added to each well, followed by incubation for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2. 9. Determination of DNA damage (Comet assay)

The alkaline comet assay was conducted according to Singh et al. (1995) with a little modification. The L5179 cell suspension was mixed with 75 ㎕ of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 75 ㎕ of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium laurylasarcosine, 1% Triton X-100 and 10% DMSO) for 1 h at 4℃. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4℃. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4℃, and then treated with ethanol for another 5 min before staining with 50 ㎕ of ethidium bromide (20 μg/ml). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2. 10. Assay for antioxidant enzymes

The V79-4 cells were seeded at 1 × 10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of the ECUL for 1h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and lysed on ice by sonicating them twice for 15 sec. One percentage of Triton X-100 was then added to the lysates and the mixure was incubated for 10 min on ice. The lysates were separated by centrifugation at 5000 × *g* for 10 min at 4℃ to remove the cellular debris. The protein content of the supernatant was determined using the Bradford method with bovine serum albumin as the standard.

The SOD activity was used to detect the level of epinephrine auto-oxidation inhibition (Misra and Fridovich, 1972). Fifty micrograms of the protein were added to 500 mM of the phosphate buffer (pH 10.2)

and 1 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrom, which is a pink colored product that can be measured at 480 nm using a UV/VIS spectrophotometer in kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm and one unit of enzyme activity was defined as the amount of enzyme required to produce 50% inhibition. The SOD activity is expressed as units/mg protein.

For catalase (CAT) activity fifty micrograms of protein was added to 50 mM of a phosphate buffer (pH 7) and 100 mM (v/v) H_2O_2; this mixture was then incubated for 2 min at 37℃ and the absorbance was monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of H_2O_2 (Carrillo et al., 1991), and one unit of enzyme activity was defined as the amount of enzyme required for the breakdown of 1 µM H_2O_2. The CAT activity is expressed as units/mg protein.

2. 11. Statistical Analysis

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Data were analysed using the SPSS package for Windows (Version 10). Values were expressed as mean±standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-value of less than 0.05 was considered significant.

3. RESULTS

3. 1. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activities of ECUL and ECDL were shown in Fig. 2-1. As shown in the results, ECUL showed higher activities than ECDL. ECUL increased H_2O_2 scavenging activity in a dose-dependant manner and the activity reached to a maximum at the highest dosage of 500 µg/ml (90.47%).

3. 2. DPPH radical scavenging activity

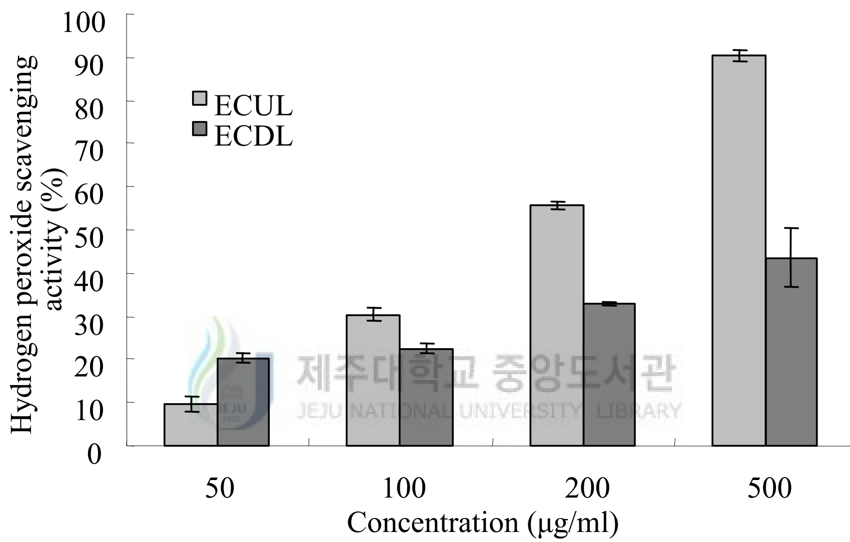
The DPPH radical scavenging activities of ECUL and ECDL are shown in Fig. 2-2. ECUL had the highest DPPH radical scavenging activity (84%) at 100 ㎍/ml. Whereas at the same concentration ECDL showed 67% DPPH radical scavenging effect. ECUL and ECDL showed increased DPPH radical scavenging activity in a dose-dependent manner.

3. 3. H_2O_2-induced cell viability by ECUL

The protective effect of ECUL against H_2O_2-induced cell damage in V79-4 cells is exhibited in Fig. 2-3. In this study, although the oxidative stress induced by 1 mM H_2O_2 caused about 50% V79-4 cell damage ECUL treatment for 1 h markedly suppressed the damage of H_2O_2 in a dose-dependent manner (Fig. 2-3). Hydrogen peroxide reduced cell viability up to ~50%, but 50 ㎍/ml ECUL enhanced cell viability up to 86%. Therefor, ECUL has high ability to reduce the toxicity induced by H_2O_2 in V79-4 cells.

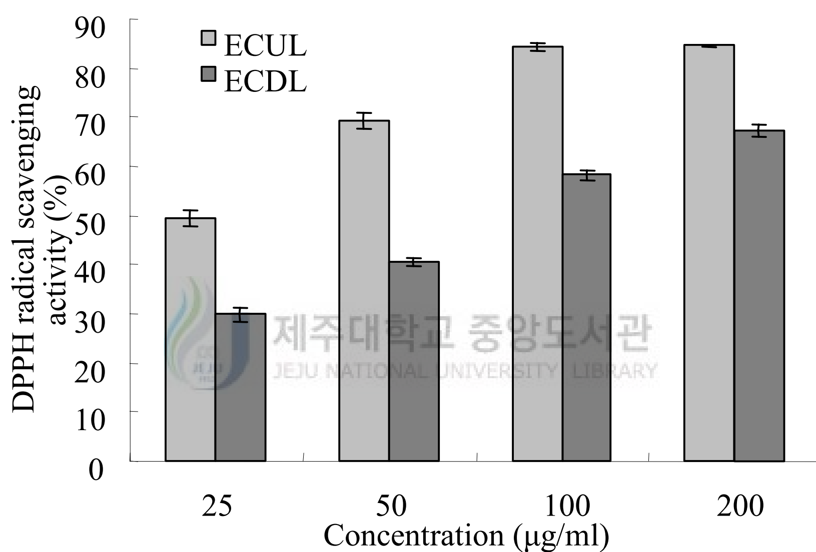
3. 4. Reduction of H_2O_2-induced apoptotic body by ECUL

In order to analyze the protective effect of ECUL on H_2O_2-induced apoptosis, the author stained nuclei of V79-4 cells treated with H_2O_2 in the absence or presence of ECUL. Typical fluorescence photographs of shrunken nuclei, chromatin condensation and appearance of apoptotic bodies were shown in V79-4 cell after the treated with H_2O_2 (1 mM) for 24 h (Fig. 2-4 B). The negative control, treated without the sample and H_2O_2, showed clear image and exhibited no DNA damage (Fig. 2-4 A). However, Obvious cell damage was observed in the cells treated with H_2O_2. Cells treated with ECUL 1 h prior to H_2O_2 treatment, a dramatic reduction in apoptotic bodies. Therefore, the photographs (Fig. 1-8C and D) clearly suggest the ability of ECUL to protect cell damage against H_2O_2 attack.



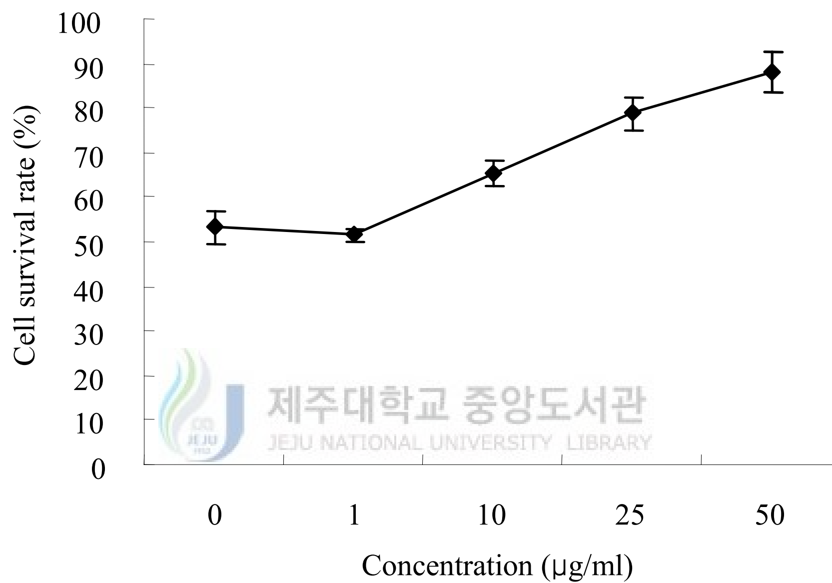
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2-1. Hydrogen peroxide scavenging activity (%) of ECUL and ECDL. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2-2. DPPH radical scavenging activity (%) of ECUL and ECDL. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2-3. Protective effect of ECUL against H_2O_2-induced oxidative damage in V79-4 cells. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

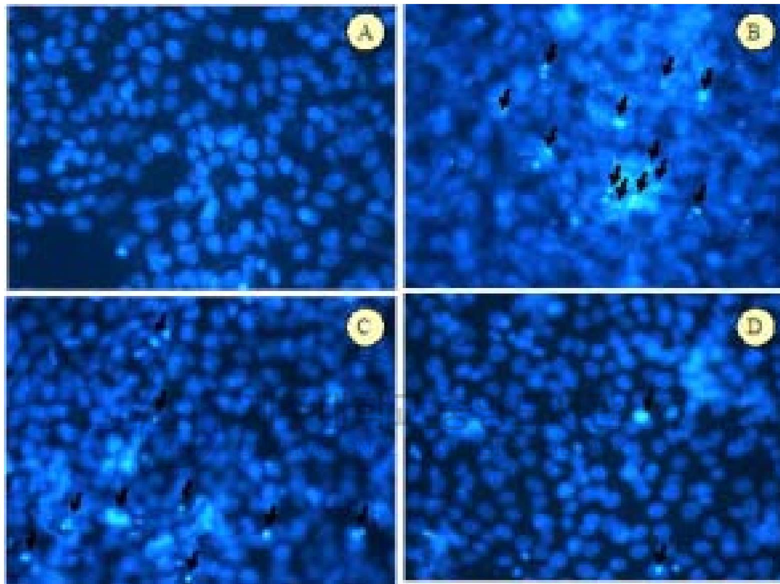


Fig. 2-4. Protective effect of ECUL against H_2O_2-induced apoptosis in V79-4 cells. Cellular morphological changes were observed using fluorescence microscope. Photomicrographs of V79-4 cells treated with (A) vehicle only; (B) 1 mM H_2O_2 (C) 5 μg/ml sample + 1 mM H_2O_2 (D) 25 μg/ml sample + 1 mM H_2O_2. Apoptotic bodies are indicated by arrows.

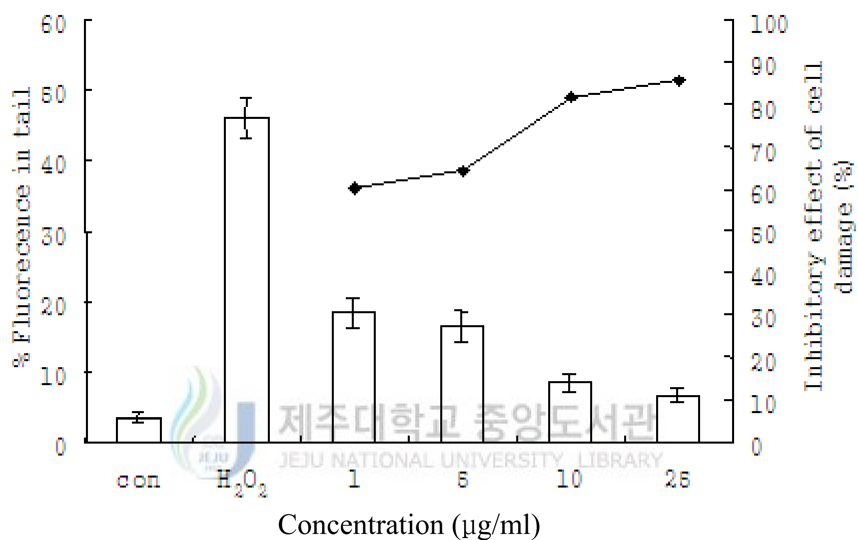
3. 5. Effect of ECUL on DAN damage of H_2O_2-induced cells

Inhibitory effect of ECUL against DNA damage was investigated using comet assay (Fig. 2-5 and 6). The percent fluorescence in tail DNA intensity of V79-4 cells was significantly increased in cells treated with only H_2O_2. This level of DNA damage induced by H_2O_2 was significantly controled dose-dependently by preincubating H_2O_2 together with the ECUL at the concentrations of 1, 5, 10 and 25 ㎍/ml in PBS (Fig. 2-5), and the activity reached to a maximum at the highest dosage of 25 ㎍/ml (about 86%). Fig. 2-6 showed photomicrographs of different DNA migration profiles obtained from V79-4 cells, with the presence of different concentrations of ECUL. In the cells exposed to only H_2O_2, the DNA was completely damaged but the addition of ECUL with H_2O_2 effectively suppressed DNA damage.

3. 6. Effect of ECUL on antioxidant enzyme activities

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

In order to examine whether these antioxidant properties of ECUL are mediated by an increase in antioxidant enzyme activities, SOD and CAT activities in V79-4 cells treated with ECUL were measured (Fig. 2-7). The SOD activity of control cells was 19.2 ± 1.4 U/mg protein. Treatment with the ECUL at 1, 5, 10 and 25 ㎍/ml increased 22%, 25%, 43%, and 48% SOD activity respectively (Fig. 2-7A). One the other hand, CAT activity in untreated control cells was 15.4 ± 1.4 U/mg protein (Fig. 2-7B). The presence of ECUL at the doses of 1, 5, 10 and 25 ㎍/ml increased 12%, 20%, 32% and 41% CAT activity respectively. Therefore, addition of the algal extract induced SOD and CAT enzyme production dose dependently.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2-5. The effect of supplementation in vitro with different concentrations of ECUL on DNA damage of H_2O_2-induced L5178 cell. Values are means with standard errors of duplicate experiments. (□: % Fluorescence in tail, -◆-: Inhibitory effect of cell damage)

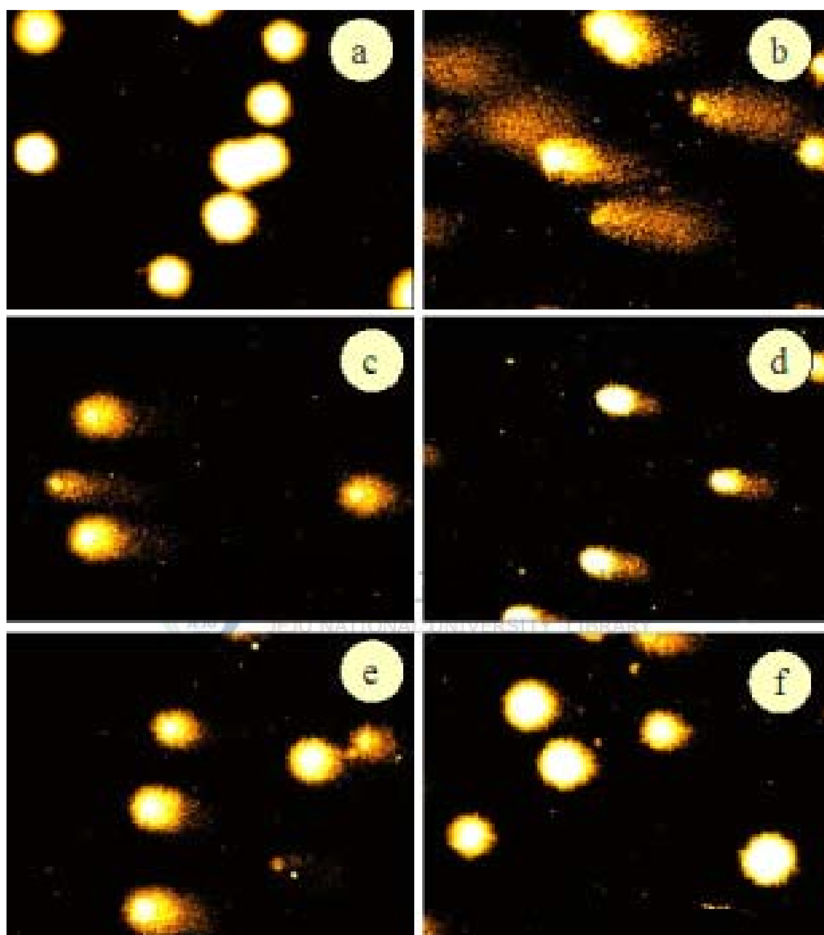
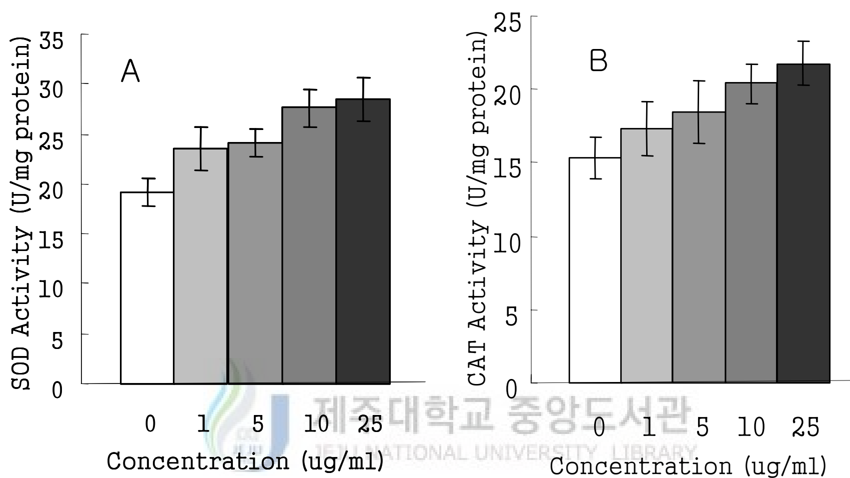


Fig. 2-6. Comet images of L5178 cells: (a) negative control; (b) 50 µM H_2O_2 (c) 1 µg/ml sample + 50 µM H_2O_2 (d) 5 µg/ml sample + 50 µM H_2O_2 (e) 10 µg/ml sample + 50 µM H_2O_2 (f) 25 µg/ml sample + 50 µM H_2O_2



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 2-7. Effect of ECUL on antioxidant enzyme activities (A) SOD activity, (B) CAT activity were measured, as described in MATERIALS AND METHODS. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

4. DISCUSSION

High levels of free radicals or ROS create oxidative stress, which leads to a variety of biochemical and physiological lesions and often results in metabolic impairment and cell death. (Ames, 1998). Among a variety of ROS, H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues (Hallivell and Aruoma, 1991). Cells are protected from ROS-induced damage by a variety of endogenous ROS scavenging enzymes, chemical compounds and natural products. Recently there has been increasing interest in the therapeutic potential of natural plants as antioxidants in reducing such free radical-induced tissue injury, suggesting that many plants have antioxidant activities that could be therapeutically useful. In the present study, more than 30 kDa fraction was separated from *E. cava* Celluclast extract and the fraction was sub-fractionated by liquid-liquid partition with a mixture of methylene chloride and methanol (1:1, v/v) then the resultant upper layer (ECUL) and down layer (ECDL) were investigated on the H_2O_2 and DPPH radical scavenging activity, and protective effect against H_2O_2-induced cell damage.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

ECUL from *E. cava*, increased H_2O_2 and DPPH radical scavenging activities and then enhanced the viability of V79-4 cells exposed to H_2O_2. The protective effect of ECUL on H_2O_2-induced apoptosis was observed under microscopy. The cells exposed to H_2O_2 exhibited distinct morphological features of apoptosis, such as nuclear fragmentation and apoptoic body. However, the cells pretreated with ECUL had significantly reduced H_2O_2-mediated apoptosis in V79-4 cells. Effect of ECUL on H_2O_2-induce DNA damage in V79-4 cells was increase of DNA damage induced by H_2O_2, in contrast, V79-4 cells with H_2O_2 and ECUL treatment significantly inhibited in a dose-dependent manner. Several studies have shown that increasing materials (environmental pollutants, radiation, dietary habits and various chemicals) induced DNA damage, which can lead to the diseases such as cancer and heart

disease (Hertog et al., 1993; Hartmann et al., 1995; and Singh et al., 1995).

In the present study, ECUL showed the inhibitory effect on H_2O_2-induced DNA damage in V79-4 cells. Therefore, the ECUL have the potential to inhibit the H_2O_2-induced DNA damage. The author observed that ECUL increased activities of all antioxidant enzymes examined, including SOD and CAT. These enzymes are modulated in various diseases by free radical attack (Ames, 1998). Thus, maintaining the balance between the rate of radical generation and the rate of radical scavenging is an essential part of biological homeostasis. It is of particular interest to note that SOD catalyzes the breakdown of $O_2^{\cdot}$ to $O_2^{\cdot -}$ and H_2O_2, and thus prevents the formation of ·OH, and thereby, has been implicated as an essential defense against the potential oxygen toxicity. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT, as SOD generates H_2O_2, which needs to be further scavenged by CAT. ECUL activited SOD and CAT, indicating that this fraction can effectively scavenge $O_2^{\cdot -}$ and H_2O_2. The effects of ECUL on cell viability might involve dual actions: direct oxygen radical scavenging of ECUL, and indirectly through the induction of the antioxidant enzymes, SOD and CAT by ECUL.

Part Ⅲ

Antitumor activity and induction of apoptosis by *Ecklonia cava* enzymatic extract on tumor cells

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Part Ⅲ

Antitumor activity and induction of apoptosis by *Ecklonia cava* enzymatic extract on tumor cells

1. ABSTRACT

Celluclast extracts of *Ecklonia cava* were examined for a potential antitumor activity against four tumor cell lines such as U937 (human monoblastoid leukemia cell line), HL60 (human promyelocytic leukemia cell line), B16 (murine melanoma cell line) and HeLa (woman cervical carcinoma cell line). Cellulclast extract was fractionated using millipore's Labscale TFF system with ultrafiltration membrane (5 and 30 kDa). The resultant fractions were collected according to the molecular weight (<5 kDa, 5~30 kDa and >30 kDa). The >30 kDa fraction inhibited cell growth on the four tumor cells than the other fractions. Especially the fraction significantly showed inhibition of cell growth against U937 cell. Therefore the dose-dependent effect of >30 kDa fraction on U937 cells was further investigated and the results are showed clear dose-dependent antitumor activity on U937 cells Moreover the activity clearly increased with the incubation time, the highest activity (~63%). It was also revealed that >30 kDa fraction increased DNA fragmentation, apoptotic body and sub-G_1 DNA contents in U937. These results indicate that >30 kDa fraction of *E. cava* Celluclast extract can control U937 cells through apoptosis. Therefore, >30 kDa fraction has a potential anticancer activity.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. MATERIALS AND METHODS

2. 1. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

propidium iodide (PI), and Hoechst 33342 were purchased from sigma (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

2. 2. Preparation of Celluclast extract from *E. cava*

The Celluclast extract was obtained according to the method used by Heo et al. (2003). The freeze dried *E. cava* were pulverized into powder using a grinder. One gram of the dried *E. cava* sample was homogenized with 100 ml of buffer (Table 1-1) and mixed with 100 μl of Celluclast. Sample was adjusted to be within the optimum range of the respective enzyme and enzymatic reactions were performed for 12 h. Finally, sample was clarified by centrifugation at 3000 × *g* for 20 min to remove the unhydrolyzed residue. Celluclast extract of the seaweed was obtained after filtering the supernatant and was stored for further experiments (Fig. 1-1).

제주대학교 중앙도서관

2. 3. Molecular weight fractionation of enzymatic extract

Cellulclast extract was fractionated using millipore's Labscale TFF system with ultrafiltration membrane (5 and 30 kDa) (Fig. 1-1). The resultant fractions were collected according to the molecular weight (<5 kDa, 5~30 kDa and >30 kDa).

2. 4. Cell culture

U937 (human monoblastoid leukemia cell line), HL60 (human promyelocytic leukemia cell line), B16 (murine melanoma cell line) and HeLa (woman cervical carcinoma cell line) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in 5 % CO_2 incubator.

2. 5. Cell growth inhibition assay

The cytotoxicity of different molecular weight fractions from E. cava Celluclast extract against the tumor cells (U937, HL60, B16 and HeLa cells) was determined by a colorimetric MTT assay. Suspension cells (U937 and HL60 cells) were seeded together with the extracts and incubated up to 72 h before MTT treatment. Attached cells (HeLa and CT26 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells/ml. Sixteen hours after plating, the cells were treated with the extract samples. The cells were then incubated for an additional 72 h at 37°C. MTT stock solution (50 μl; 2 mg/ml in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h, the plate was centrifuged at 2,000 rpm for 5 min and the supernatant was aspirated. The formazan crystals in each well were dissolved DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

2. 6. Determination of DNA damage (Comet assay)

The alkaline comet assay was conducted according to Singh *et al.* with a little modification. The cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 75 μl of 0.5% LMA and then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100) for 1 h at 4℃. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4℃. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4℃, and then treated with ethanol for another 5 min before staining with 50 μl of ethidium bromide (20 μg/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence

microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2. 7. Nucleare staining with Hoechst 33342

The nuclear morphology of cells was studied by using the cell-permeable DNA dye Hoecht 33342. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The U937 cells were placed in 24-well plate at a concentration of 1.0 × 10^5 cells/ml. The cells were treated with various concentration (50, 100, and 200 ㎍/ml) of the fraction samples. After 24 h, 1.5 ㎕ of Hoechst 33342 (stock 10 mg/ml) and a DNA-specific fluorescent dye were added to each well (1.5 ml), followed by incubation for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2. 8. Flow cytometry analysis

Flow cytometry analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The U937 cells were placed in a 6-well plate at a concentration of 1.0 × 10^5cells/ml. The cell were treated with various concentration of fraction sample (50, 100 and 200 ㎍/ml). After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with phosphate buffered saline (PBS) and incubated in the dark in 1 ml of PBS containing 100 ㎍ PI and 100 ㎍ RNase A for 30 min at 37 ℃. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by

histograms generated by the computer program Cell Quest and Mod-Fit (Wang et al., 1999).

2. 9. Statistical Analysis

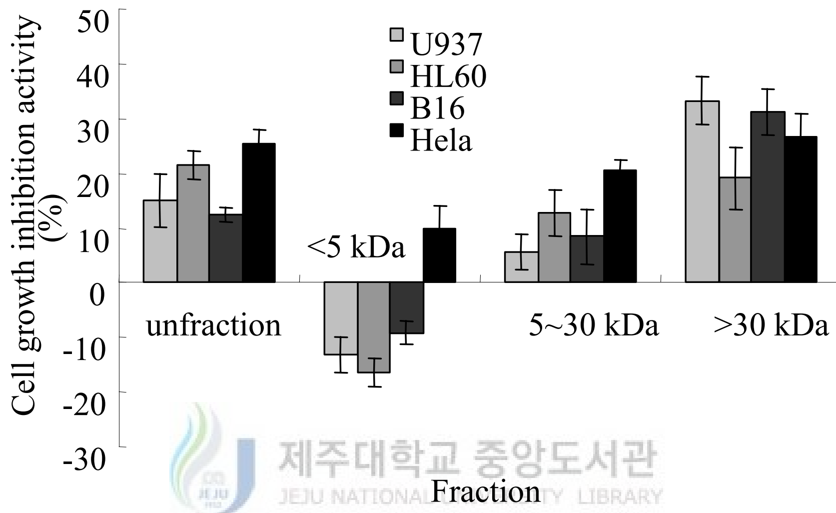
Data were analysed using the SPSS package for Windows (Version 10). Values were expressed as mean±standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-value of less than 0.05 was considered significant.

3. RESULTS

3. 1. Inhibition of tumor cell growth by difference molecular weight fractions from *E. cava* Celluclast extract

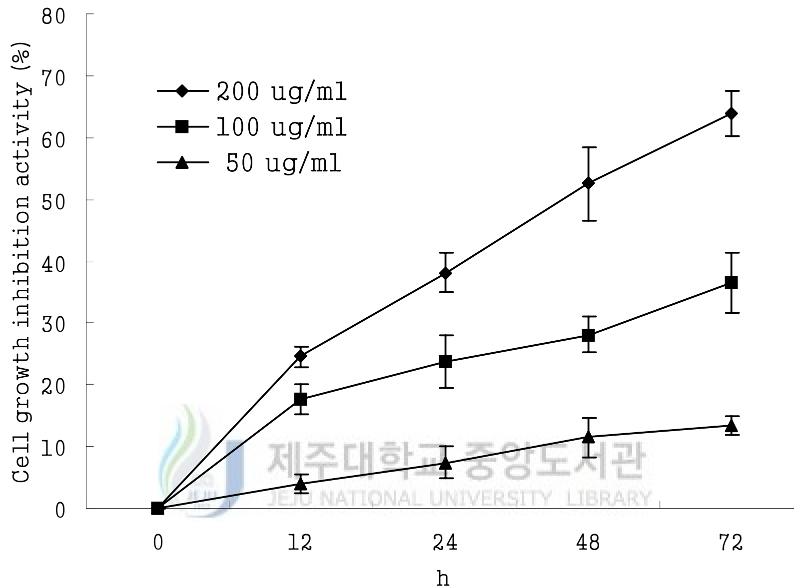
제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Initially, MTT assay was used as an indirect measure to evaluate cell growth inhibition activity on tumor cell lines in the presence of molecular weight fractions of Celluclast extract. The cell growth inhibitory activity was shown in Fig 3-1. Of the tested molecular weight fractions, >30 kDa fraction significantly suppressed the growth of cell the tumor cellss the lowest molecular weight fraction of Celluclast extract showed the poorest activity, however 5~30 kDa fraction showed moderate activity. Compared to unfractionated sample, the antitumor activity of >30 kDa recorded the highest activity, especially against U937 cells the sample showed the highest cell growth inhibition. Therefore the dose-dependent effect of >30 kDa fraction on U937 cells was further investigated and the results are shown in Fig. 3-2. The highest molecular weight fraction (>30 kDa) showed clear dose-dependent antitumor activity on U937 cells, moreover the activity clearly increased with the incubation time, the highest activity (~63%) was recorded at 200 µg/ml after 72 h incubation time.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3-1. Effect of difference molecular weight fractions of *E. cava* Celluclast extract on tumor cell growth inhibition activity in tumor cells (U937, HL60, B16 and HeLa cells). Cells were treated with 100 μg/ml of different molecular weight fractions and measured for viability by MTT assay at 72 h after the sample treatment. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3-2. Effect of >30 kDa fraction of *E. cava* Celluclast extract in U937 cells. Cells were treated with various concentrations of >30 kDa fraction and measured for viability by MTT assay at 12, 24, 48 and 72 h after the sample treatment. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

3. 2. Cytotoxic effects of >30 kDa fraction in normal cells

We compared to cytotoxic effects of >30 kDa fraction in leukemia and normal cells (Table 3-1). In U937 cells, a dose-dependent cell viability was observed. A slight reduction in cell viability of normal cell (V79-4) was observed at 200 ㎍/ml of >30 kDa fraction after 72 h incubation period.

3. 3. DNA damage of U937 cells by >30 kDa fraction

In this study, the ability of >30 kDa fraction from *E. cava* Celluclast extract to damage nuclerous DNA of U937 cells was investigated by comet assay. The cells in th control showed very low DNA strand breakages (Fig. 3-3A) therefore % of fluorescence in tail was minimum (about 5%) (Fig 3-4). In contrast, presence of the fraction in the medium dose-dependently enhanced DNA damage of the U937 cells (Fig 3-3B, C, and D). Addition of >30 kDa fraction to the medium triggered 15%, 20% and 28% fluorescence in tail at the sample concentrations of 50, 100, and 200 ㎍/ml, respectively. Hence, the presence of the extract in the medium effectively damage the unclear DNA of tumor cells.

3. 4. >30 kDa fraction induced apoptosis in U937 cells

The nuclear morphology changes of U937 cells were investigated by using the cell-permeable DNA dye Hoechst 33342. Number of apoptotic bodies were observed after the treatment of the fraction sample in a dose-dependent manner (Fig 3-5). The cytotoxicity of >30 kDa fraction on U937 cells was due to the induction of apoptosis, cell cycle analysis was performed. >30 kDa faction dose-dependently decreased DNA contents of the G_0/G_1, S and G_2/M phase, Compared to untreated control it increased the apoptotic portion of sup-G_1 peaks to 8%, 9%, and 23% at the concentrations of 50, 100 and 200 ㎍/ml, respectively. (Fig. 3-6 and 3-7).

Table 3-1. Comparison with cell viability by >30 kDa fraction in leukemia and normal cells

Treatment	Concentration (ug/ml)	Viability (%)	
		U937	V79-4
None (PBS)	—	100.0±3.6	100.0±4.1
>30 kD fraction	50	86.3±1.4	105.3±2.7
	100	63.6±4.3	104.8±2.9
	200	36.2±3.5	92.4±3.7

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

U937 and V79-4 cells were treated with various concentrations of >30 kDa fraction and the cell were tested for viability by MTT assay after the treatment of >30 kDa fraction for 72 h. Experiments were performed in triplicates and data are expressed as average percent change from control ± S.D.

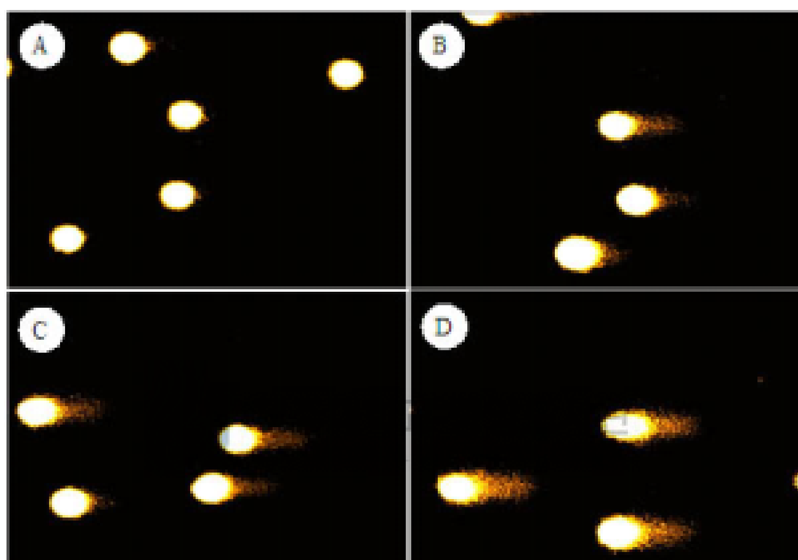
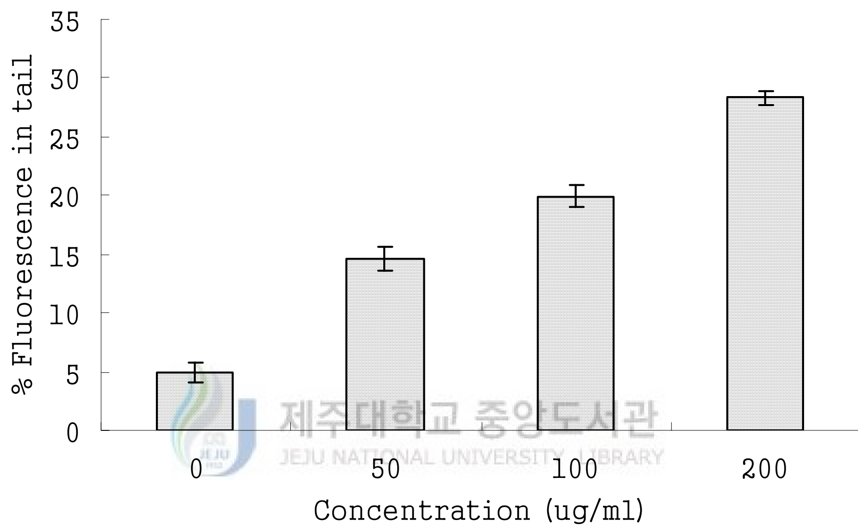


Fig. 3-3. Comet images of U937 cells after the treatments of different concentrations of >30 kDa fraction: (A) untreated sample; (B) U937 cells treated with 50 μg/ml; (C) U937 cells treated with 100 μg/ml; (D) U937 cells treated with 200 μg/ml



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3-4. The effect of >30 kDa fraction on nucleolus DNA damage in U937 cells. Values are means with standard errors of duplicate experiments.

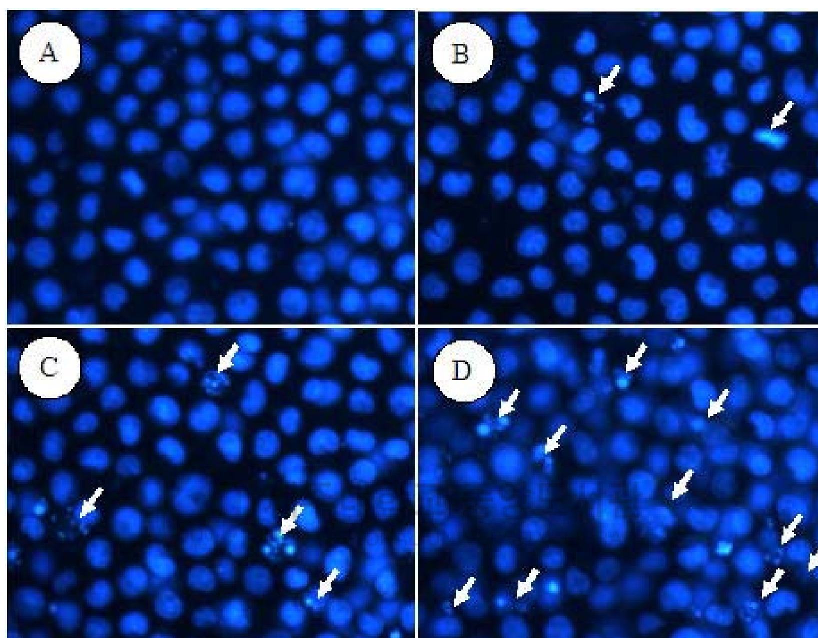
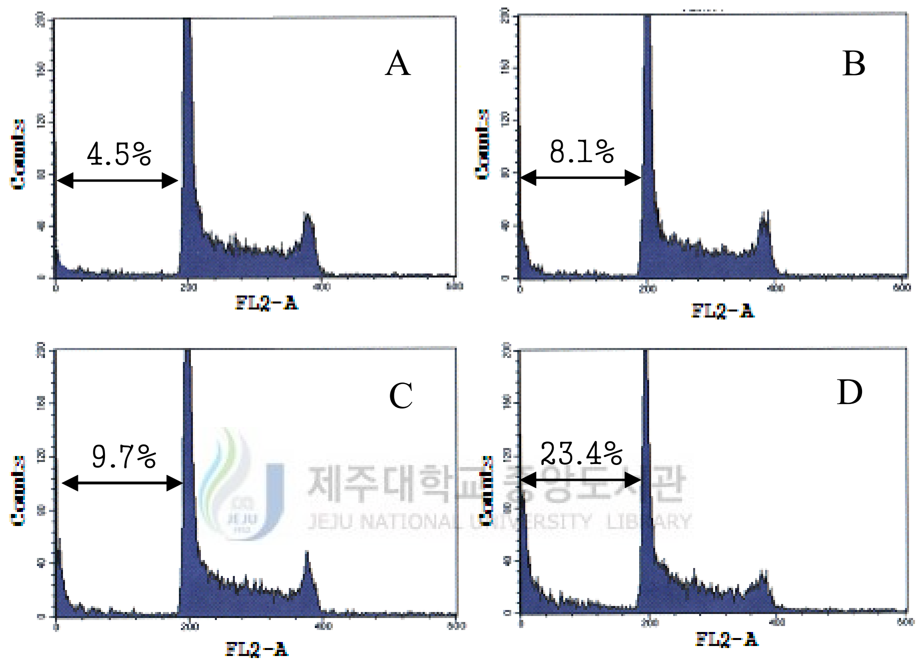
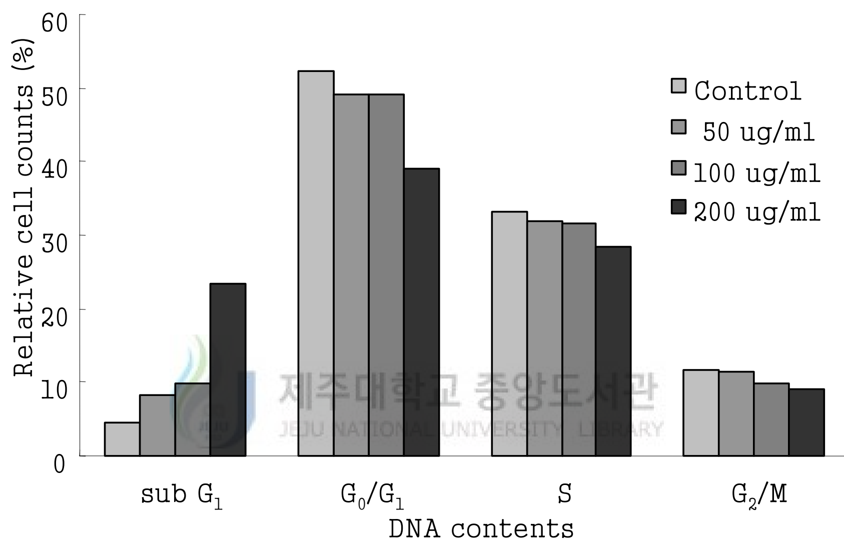


Fig. 3-5. Morphological changes of U937 cells treated with different concentrations of >30 kDa fraction. Cells were treated with >30 kDa fraction for 24 h. (A) untreated cell; (B) U937 cells treated with 50 ㎍/ml; (C) U937 cells treated with 100 ㎍/ml; (D) U937 cells treated with 200 ㎍/ml



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3-6. Effect of >30 kDa fraction on cell cycle pattern and apoptotic portion in U937 cells by flow cytometric analysis. Histogram patterns of U937 cells treated with various concentrations of >30 kDa fraction for 24 h by cell cycle analysis. (A) untreated sample; (B) U937 cells treated with 50 μg/ml; (C) U937 cells treated with 100 μg/ml; (D) U937 cells treated with 200 μg/ml.



제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Fig. 3-7. Bar graph for cell cycle patterns of U937 cells treated by various concentrations of >30 kDa fraction.

4. DISCUSSION

While many anticancer agents have been developed and used for patients, side effects and resistance to anticancer drugs are serious problems to be overcome in the treatment of cancer (Panchal, 1998). Therefore, the research and development of safer and better therapeutic drugs have become necessary. Recently, there is a growing interest in the use of marine bioresources for the treatment of various human disease including cancer and microbial infection (Kashiwagi et al., 1980; Gonzalez et al., 1982; Kosovel et al., 1988; Glombitza and Koch, 1989). In the present study, *E. cava* Celluclast extract was prepared by using enzymatic hydrolysis and into three different molecular weight fractions (>5 kDa, 5~10 kDa, and <30 kDa). These fractions were investigated for tumor cell growth inhibition activity, cell damage, morphologic change and cell cycle in U937 cells.

Among different molecular weight fractions of *E. cava*, the >30 kDa fraction of Celluclast extract was significantly recorded high cell growth inhibition activity on four tumor cells than the other fractions, especially against U937 cells. Therefore the dose-dependent effect of >30 kDa fraction on U937 cells were further investigated and showed dose-dependent antitumor activity on U937 cells. Moreover the activity clearly increased with the incubation time, the highest activity (~63%) was recorded at 200 μg/ml after 72 h incubation time. The >30 kDa fraction-induced dose-dependantly DNA damage of U937 cells. It may be well known that the growth inhibitory activities of the aqueous extracts of brown seaweeds can be attributed to the phlorotannis and fucoxanthin. Previous studies have reported that fucoxanthin would causes tumoral cell growth inhibition of human neuroblastoma GOTO cells, human leukemia cells, and prostate cancer cells (Okuzumi et al., 1990; Nishino et al., 1992; Hosokawa et al., 1999 and Kotake-Nara et al., 2001). Several phlorotannins of brown seaweeds have been identified for their potential bioactivities such as antioxidant, antibacterial and antihyluranase (Fukuyama et al., 1985; Fukuyama et al., 1989a; Fukuyama et al., 1989b; Fukuyama et al., 1990; Nakayama et al., 1989;

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

Nakamura et al., 1996; Alena and Steinberg, 1992; Hay and Fenical, 1988; Boettcher and Targett, 1993; Targett et al., 1995; Steinberg, 1992; Jennings and Steinberg, 1997).

In present study, we have shown >30 kDa fraction-induced cell death via an apoptotic pathway in U937 cells. Apoptosis is cellular suicide or programmed cell death that is mediated by the activation of an evolutionary conserved intracellular pathway (Bold et al., 1997). Apoptosis include cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies (Buttke and Sandstrom, 1994). These apoptotic bodies are rapidly cleaned from the local tissue by macrophages (Wyllie, 1985). After U937 cells were treated with >30 kDa fraction for 24 h, the microscopic observations demonstrated the apoptotic characters such as DNA fragmentation and apoptotic bodies. (Fig 3-5). The apoptotic cells were observed after the treatment of >30 kDa faction (Fig 3-6 and 7). Apoptosis gives some clues about effective anticancer therapy, and many chemotherapeutic agents were reported to exert their antitumor effects by inducing apoptosis of cancer cells (Kamesaki, 1998). Recent years have brought an increased understanding of the complexity of cellular responses to drug interactions with a variety of cellular targets that ultimately result in cell death (Kaufmann and Earnshaw, 2000) Both DNA damage and protein damage can promote apoptosis. While DNA damage initiates death signaling, protein damage is known to distort the cell redox homeostasis, which facilitates apoptosis execution. Our present findings indicate >30 kDa fraction may use control leukemia cells through apoptosis and have a possibility of potential anticancer activities. Further studies are needed to better understand whether PHWE had effects on DNA damage (such as p53, Bcl-2, Bax, cytochrome c, and caspases) or protein damage (such as thioredoxin an glutathione) in cancer cells.

SUMMARY

Reactive oxygen species (ROS) are unwanted metabolic by-products of normal aerobic metabolism under high level of O_2 pressure. High levels of ROS create oxidative stress, which leads to a variety of biochemical and physiological lesions. Such cellular damage often impairs metabolic function and leads cell death. Cells are protected from ROS-induced damage by a variety of endogenous ROS scavenging enzymes, chemical compounds and natural products. Recently there has been attracted much interest in the therapeutic potential of natural plants as antioxidants in reducing such free radical-induced tissue injury, suggesting that many plants have antioxidant activities that could be therapeutically useful.

While many anticancer agents have been developed and used, side effects and resistance to anticancer drugs are serious problems to be overcome in the treatment of cancer. Therefore, the research and development of safer and better therapeutic drugs have become necessary. Recently, there is a growing interest in the use of marine bioresources for the treatment of various human disease including cancer.

In the present study, natural water-soluble enzymatic extracts from *E. cava*, which were prepared by enzymatic hydrolysis using five different carbohydrate degrading enzymes, carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultaraflo) and proteases (Protamex, Kojizyme, Neutase, Flavourezyme and Alcalase), were investigated for antioxidant and antitumor activity. Celluclast extract of *E. cava* indicated hydrogen peroxide scavenging activity, which enhanced the viability of V79-4 cells exposed to H_2O_2. The protective effect of the enzymatic extract on H_2O_2-induced apoptosis was observed by microscopy and flow cytometry. Cells exposed to H_2O_2 exhibited distinct morphological features consistent with apoptosis, such as nuclear fragmentation and an increase in the percentage of sub-G_1 hypodiploid cells. However, cells that were pretreated with the enzymatic extract

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

had significantly reduced characteristics of apoptotic cells, as shown by their morphology and sub-G_1 DNA contents. >30 kDa fraction of *E. cava* enzymatic extract had the strong cytotoxic effect on U937 cells. The number of live U937 cells was less than 37% after exposure to 200 μg/ml >30 kDa fraction for 72 h. Therefore enzymatic extracts from *E. cava* can be used in food and pharmaceutical industry. Further studies are required in order to identify the antioxidant and antitumor compounds being responsible for the results observed in this study.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

REFERENCES

Abourriche, A., Charrouf, M., Berrada, M., Bennamara, A., Chaib, N. and Francisco, C. 1999. Antimicrobial activities and cytotoxicity of the brown alga *Cystoseira tamariscifolia*. *Fitoterapia* 70: 611-614.

Altena, A. and Steinberg, D. 1992. Are differences in the responses between North American and Australian marine herbivores to phlorotannins due to differences in phlorotannin structure. *Biochem. Syst. Ecol.* 20: 493-499.

Ames, B. 1998. Micronutrients prevent cancer and delay aging. *Toxicol. Lett.* 102, 5-18.

Anggadiredja, J., Andyani, R. and Muawanah, H. 1997. Antioxidant activity of *Sargassum polycystum* (Phaeophyta) and *Laurencia obtusa* (Rhodophyta) from Seribu Islands. *J. Appl. Phycol.* 9: 477-479.

Athukorala, Y., Lee, K. W., Shahidi, F., Heu, M. S., Kim, H.T., Lee, J. S. and Jeon, Y. J. 2003a. Antioxidant efficacy of extracts of an edible red alga (*Grateloupia filicina*) in linoleic acid and fish oil. *J. Food Lipids* 10: 313-327.

Athukorala, Y., Lee, K. W., Song, C. B., Shin, T. S., Cha, Y. J., Shahidi, F. and Jeon, Y. J. 2003b. Potential antioxidant activity of marine red alga *Grateloupia filicina* extract. *J. Food Lipids* 10: 251-265.

Bagchi, D., Bagchi, M., Stohs, S. J., Das, D. K., Ray, S. D., Kuszynski, C. A., Joshi, S. S. and Pruess H. G. 2000. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicology* 148: 187-197.

Boettcher, A. and Targett, M. 1993. Role of polyphenolic molecular size in reduction of assimilation efficiency in *xiphister mucosus*. *Ecology* 74: 891-903.

Bold, R. J., Termuhlen, P. M. and McConkey, D. J. 1997. Apoptosis, cencer and cancer therapy. *Surgi. Oncol.* 6: 133-142.

Burtin, P. Nutritional value of seaweeds. *Electron. J. Environ. Agric. Food Chem.* (http://ejeafeche.uvigo.es/2(4)2003/017242003F.htm).

제주대학교 중앙도서관 JEJU NATIONAL UNIVERSITY LIBRARY

2003.
Buttke, T. M. and Sandstrom, P. A. 1994. Oxidative stress as a mediator of apoptosis. *Immunol. Today* 15: 7-10.
Carrillo, M. C., Kanai, S., Nokubo, M. and Kitani K. 1991. (-) deprenyl induces activities of both superoxide dismutase and catalase but not of glutathione peroxidase in the striatum of young male rats. *Life Sci*. 48: 517-521.
Chandler, S. F. and Dodds, J. H. 1983. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. *Plant Cell Rep.* 2: 105-108.
Cox, D. A. and Cohen, M. L. 1996. Effects of oxidized low density lipoproteins on vascular contraction and relaxation. *Pharmacol. Rev.* 48: 3-9.
Duval, B. S. hetty, K. and Thomas, W. H. 2000. Phenolic compounds and antioxidant properties in the snow alga *Chlamydomonas nivalis* after exposure to UV light. *J. Appl. Phycol.* 11: 559-566.
Finkel, T. and Holbrook, N. J. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247.
Fukuyama, Y., Kodama, M., Miura, I., Kinzyo, Z., Kido, M., Mori, H., Nakayama, Y. and Takahashi, M. 1989. Structure of an anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* OKAMURA and inhibitory activities of its derivatives on plasma plasmin inhibitors. *Chem. Pharm. Bull.* 37: 349-353.
Fukuyama, Y., Kodama, M., Miura, I., Kinzyo, Z., Mori, H., Nakayama, Y. and Takahashi, M. 1989. Anti-plasmin Inhibitor. V. Structure of novel dimeric eckols isolated from the brown alga *Ecklonia kurome* OKAMURA. *Chem. Pharm. Bull.* 37: 2438-2440.
Fukuyama, Y., Kodama, M., Miura, I., Kinzyo, Z., Mori, H., Nakayama, Y. and Takahashi, M. 1990. Anti-plasmin Inhibitor. VI. Structure of phlorofucofuroeckol A, a novel phlorotannin with both dibenzo-1,4-dioxin and dibenzofuran elements, from *Ecklonia kurome* OKAMURA. *Chem. Pharm. Bull.* 38: 133-135.
Glombitza, K. W. and Koch, M. 1989. Secondary metabolites of pharmaceutical potential. In: Algal and Cyanobacterial

Biotechnology (Cresswell, R.C., Rees, T.A., Shah, N., Eds.). Longman Scientific & Technical. pp. 161-238.

Gonzalez, A. G., Darias, V. and Estevez, E. 1982. Chemotherapeutic activity of polyhalogenated terpenes from Spanish algae. *Planta Med.* 44: 44-46.

Gschwind, M. and Huber, G. 1995. Apoptotic cell death induced by β-amyloid 1-42 peptide is cell type dependent. *J. Neurochem.* 65: 292-300.

Gutteridge, J. M. C. 1995. Lipid peroxidation and antioxidants as biomarker of tissue damage. *Clin. Chem.* 41: 1819-1828.

Halliwell, B. and Aruoma, O. I. 1991. DNA damage by oxygen-derived species. *FEBS Lett.* 281: 9-19.

Halliwell, B. and Gutteridge, J. M. C. 1998. Free radicals in biology and medicine. 3rd Ed., Oxford University.

Harman, D. 1994. Free radical theory of aging, increasing the functional life span. *Ann. N.Y. Acad. Sci.* 717, 1-15.

Hartmann, A., Herkomme,r K., Gluck M. and Speit G. 1995. DNA-damaging effect of cyclophosphamide on human blood cells *in vivo* and *in vitro* studied with the single-cell gel test (Comet assay). *Environ. Mol. Mutagen.* 25: 180-187.

Hay, M. E. and Fenical, W. 1988. Marine plant-herbivore interactions: the ecology of chemical defence. *Annu. Rev. Ecol. Syst.* 19: 111-145.

Heo, S. J., Jeon, Y. J., Lee J., Kim, H. T. and Lee, K. W. 2003a. Antioxidant effect of enzymatic hydrolyzate from a Kelp, *Ecklonia cava*. *Algae* 18: 341-347.

Heo, S. J., Park, E. J., Lee, K. W. and Jeon, Y. J. 2005. Antioxidant activities of enzymatic extracts from brown seaweeds. *Biores. Tech.* 96: 1613-1623.

Heo, S. J., Lee, K. W., Song, C. B. and Jeon, Y. J. 2003b. Antioxidant activity of enzymatic extracts from brown seaweeds. *Algae* 18: 71-81.

Hertog, M. G. L., Feskens, E. J. M. Hollman, P. C. H., Katan, M. B. and Kromhout D. 1993. Dietary antioxidants flavonoids and the

제주대학교 중앙도서관

risk of coronary heart disease: the zutphen elderly study. *Lancet* 342: 1007-1011.

Hirata, T., Tanaka, M., Ookie, M., Tsunomura, T. and Sakaguchi, M. 2000. Antioxidant activities of phycocyanobilin prepared from *Spirulina platensis*. *J. Appl. Phycol.* 12: 435-439.

Hiroishi, S., Sugie, K., Yoshida, T., morimoto, J., Taniguchi, Y., Imai, S. and Kurebayashi, J. 2001. Antitumor effects of *Marginisporum crassissimum* (Rhodophycere), a marine red alga. *Cancer latters* 167: 145-150.

Hollman, P. C., van Trijp, J. M., Buysman M. N., van der Gaag M. S., Mengelers, M. J., de Vries, J. H. and Katan M. B. 1997. Relative bioavailability of the antioxidant flavonoid quercetin from variousfoods in man. *FEBS Lett.* 418, 152-156.

Hosokawa, M., Wanezaki, S., Miyauchi, K., Kurihara, H., Kohno, H., Kawabata, J., Odashima, S. and Takahashi, K. 1999. Apoptosis-inducing effect of fucoxanthin on human leukemia cell HL-60. *Food Sci. Technol. Res.* 5: 243-246.

Janssens, B. J., LeGall, R. and Rees, J. F. 2002. Peroxide-triggered erythrocytes haemolysis as a model for the study of oxidative damage in marine fishes. *J. Fish Biol.* 61: 71-84.

Jennings, J. G. and Steinberg P. D. 1997. Phlorotannins versus other factors affecting epiphyte abundance on the kelp *Ecklonia radiata*. *Oecologia* 109: 461-473.

Jolles, B., Remington, M. and Andrews, P. S. 1963. Effects of sulphated degraded laminarin on experimental tumor growth. *Br. J. Cancer* 17: 109-115.

Kahl, R. and Kappus, H. 1993. Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E. *Z. Lebensm. Unters. Forsch.* 196: 329-338.

Kahl, R. 1994. Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology* 33: 185-228.

Kamesaki, H. 1998. Mechanism involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *Int. J*

Hematol.. 68: 29-43.

Kashiwagi, M., Mynderse, J. S., Moore, R. E and Norton, T. R. 1980. Antineoplastic evaluation of pacific basin marine algae. *J. Pharm. Sci.* 69: 735-738.

Kassie, F., Parzefall, W. and Knasmuller, S. 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. *Mutat Res.* 463: 13-31.

Kaufmann, S. H. and Earnshaw, W. C. 2000. Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* 256: 42-49.

Kerr, J. F., Gobe, G. C., Winterford, C. M. and Harmon, B. V. 1995, Anatomical methods in cell death. In: L.M. Schwartz and B.A. Osborne, Editors, Methods in Cell Biology. V. Cell Death vol. 46, Academic, New York pp 1-27.

Kosovel, V., Avanzini, A., Scarcia, V. and Furlani, A. 1988. Algae as possible sources of antitumoral agents. Preliminary evaluation of the *in vitro* cytostatic activity of crude extracts. *Pharmacol. Res. Commun.* 20: 27-31.

제주대학교 중앙도서관

Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K. and Nagao, A. 2001. Carotenoids affect proliferation of human prostate cancer cells *J. Nutr.* 131: 3303-3306.

Kovatcheva, E. G., Koleva, I. I., Ilieva, M., Pavlov, A., Mincheva, M. and Konushlieva, M. 2001. Antioxidant activity of extracts from *Lavandula vera* MM cell cultures. *Food Chem.* 72: 295-300.

Kufe, D. W., Pollock, R. E., Weichselbaum, R. R., Bast Jr, R. C., Gansier, T. S. and Holland, J. F. 2003. Cancer medicine, 6th ed. Hamiton, Ontario: American Cancer Society Inc. and B.C. Decker, Inc.

Kuhnau, J. 1976. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* 24: 117-191.

Lim, S. N., Cheung, P. C. K., Ooi, V. E. C. and Ang, P. O. 2002. Evaluation of antioxidative activity of extracts from a brown seaweed, *Sargassum siliquastrum. J. Agric. Food Chem.* 50: 3862-3866.

Lizard, G., Fournel, S., Genestier, L., Dhedin, N., Chaput, C., Flacher,

M., Mutin, M., Panaye G. and Revillard, J. P. 1995. Kinetics of plasma membrane and mitochondrial alterations in the cells undergoing apoptosis. *Cytometry* 21: 275-283.

Lowe, S. W. and Lin, A. W. 2000. apoptosis in cancer. *Carcinogenesis* 21: 485-495.

Matsukawa, R., Dubinsky, Z., Kishimoto, E., Masaki, K., Masuda, Y., Takeuchi, T., Chihara, M., Yamamoto, Y., Niki, E. and Karube, I. A. 1997. comparison of screening methods for antioxidant activity in seaweeds. *J. Appl. Phycol.* 9: 29-35.

Midgely, O. R. and Kerr, D. 1999. Colorectal cancer. *Lancet* 353: 391-399.

Misra, H. P. and Fridovich, I. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170-3175.

Mizukoshi, S., Matsuoka, S., Nakamura, K., Katou, H. and Noda, H. 1992. Search for bioactive substances from marine algae. *Bull. Faculty Biosources, Mie Univ.* 8: 27-34.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.*J. Immunol. Methods* 65: 55-63.

Nakamura, T., Nagayama, K., Uchida, K. and Tanaka, R. 1996. Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fish. Sci.* 62: 923-926.

Nakayama, Y., Takahashi, M., Fukuyama, Y. and Kinzyo, Z. 1989. An anti-plasmin Inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* OKAMURA. *Agric. Biol. Chem.* 63: 3025-3030.

Nardella, A. Chaubet, F. Boisson-vidal, C. Blondin, C. Durand, P. and Jozefonvicz J. 1996. Anticoagulant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from the brown seaweed *Ascophyllum nodosum*. *Carbohydr Res*. 289: 201-208.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani F. and Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J.*

제주대학교 중앙도서관

Immunol. Methods 139: 271-279.
Nishino, H., Tsushima, M., Matsuno, T., Tanaka, Y., Okuzumi, J., Murakoshi, M., Satomi, Y., Takayasu, J., Tokuda, H., Nishino, A. and Iwashima, A. 1992. Anti-neoplastic effect of halocynthiaxanthin a metabolite of fucoxanthin. *Anticancer Drugs* 3:493-497
Noda, H., Amano, H., Arashima, K., Hashimoto, S. and Nisizawa, K. 1989. antitumor activity of polysaccharides and lipids from marine algae. *Nippon Suisan Gakkaishi* 55: 1265-1271.
Ohkawa, H., Ohishi, N. and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351-358.
Okai, Y. and Okai, K. H. 1997. Potent anti-inflammatory activity of pheophytin a derived from edible green alga, *Enteromorpha prolifera* (Sujiao-nori). *Int.. J. Immuno. pharmacol.* 19: 355-358.
Oki, T., Masuda, M., Furuta, S., Nishibia, Y., Terahara, N. and Suda, I. 2002. Involvement of anthocyanins and other phenolic compounds in radical-scavenging activity of purple-fleshed sweet potato cultivars. *J. Food Sci*. 67, 1752-1756.
Okuzumi, J., Nishino, H., Murakoshi, M., Iwashima, A., Tanaka, Y., Yamane, T., Fujita, Y. and Takahashi, T. 1990. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. *Cancer Lett.* 55: 75-81.
Panchal, R. G. 1998. Novel therapeutic strategies to selectively kill cancer cells. *Biochem. Pharmaclo.* 55: 247-252.
Park, Y. K.. Park, E.. Kim. J. S. and Kang, M. H. 2003. Daily grape juice consumption reduces oxidative DNA damage and plasma free radical levels in healthy Koreans. *Mutat. Res.* 529: 77-86.
Ragan, M. A. and Glombitza, K. W. 1986. Phlorotannins, brown algal polyphenols: In: Round F. E. Chapman D. J. eds. *Progress in phycological Research 4*. Biopress, Bristol, England. pp. 129-241.
Ruberto, G., Baratta, M. T., Biondi, D. M. and Amico, V. 2001. Antioxidant activity of extracts of the marine algal genus *Cystoseira* in a micellar model system. *J. Appl Phycol.* 13:

403-407.
Ruperez, P., Ahrazem, O. and Leal, J. A. 2002. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *J. Agric. Food Chem.* 50: 840-845.
Senthilmohan, S. T., Zhang J. and Stanley, R. A. 2003. Effects of flavonoid extract Enzogenol with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr. Res.* 23: 1199-1210.
Siddhanta, A. K., Shanmugam, M., Mody, K. H., Goswami, A. M. and Ramavat, B. K. 1999. Sulphated polysaccharides of *Codium dwarkense*Boergs.from the west coast of India : Chemical composition and blood anticoagulant activity. *Int. J. Biol. Macromol.* 26: 151-154.
Singh, N. P. Graham, M. M. Singh, V. and Khan, A. 1995. Induction of DNA single-strand breaks in human lymphocytes by low doses of X-rays. *Int.. Radiat. Biol.* 68: 563-569.
Siriwardhana, N., Lee, K. W., Kim, S. H. Ha, J. W. and Jeon, Y. J. 2003. Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. *Food Sci. Tech. Int.* 9: 339-346.
Siriwardhana, N., Lee, K. W., Kim, S. H., Ha, J. W., Park G. T. and Jeon, Y. J. 2004. Lipid peroxidation inhibitory effects of *Hizikia fusiformis* methanolic extract on fish oil and linoleic acid. *Food Sci. Tech. Int.* 10: 65-72.
Steinberg, P. D. 1992. Geographical variation in the interaction between marine herbivores and brown algal secondary metabolites. In: V.J. Paul, Editor, *Ecological Roles of Marine Natural Prodacts*, Cornell Univ. Press, Ithaca pp. 51-92.
Targett, M., Boettcher, A., Targett, E. and Vrolijk, H. 1995. Tropical marine herbivore assimilation of phenolic-rich plants. *Oecologia* 103: 170-179.
Tutour, B. L., Benslimane, F., Gouleau, M. P., Gouygou, J. P., Saadan, B. and Quemeneur, F. 1998. Antioxidant and pro-oxidant activities of the brown algae, *Laminaria digitata*, *Himanthalia elongata*,

제주대학교 중앙도서관

Fuecus veesiculosus, *Fuecus serratus* and *Ascophyllum nodosum*. *J. Appl. Phycol.* 10: 121-129.

Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace A. J. and Harris, C. C. 1999. GADD45 induction of a G_2/M cell cycle checkpoint. *Proc. Natl. Acad. Sci. U. S. A.* 96: 3706-3711.

Wyllie, A. H. 1985. The biology of cell death in tumors. *Anticancer Res.* 5: 131-142.

Xu, N., Fan X., Yan, X., Li, X., Niu, R. and Tseng, C.K. 2003. Antibacterial bromophenols from the marine red alga *Rhodomela confervoides. Phytochemistry.* 62: 1221-1224.

Xue, C. H., Fang, Y., Lin, H., Chen, L., Li, Z. J., Deng, D. and Lu, C. X. 2001. Chemical characters and antioxidative properties of sulfated polysaccharides from *Laminaria japonica*. *J. Appl. Phycol.* 13: 67-70.

Yamamoto, I., Nagumo, T., Yagi, K., Tominaga, H. and Aoki, M. 1974. Antitumor effect of seaweeds. *Jpn. J. Exp. Med.* 44: 543-546

Yamamoto, I., Takahashi, M., Tamura, E. and Maruyama, M. 1982. Antitumor activity of crude extracts from edible marine algae against L-1219 leukemia. *Bot. Mar.* 25: 455-457.

Yan, X., Chuda, Y., Suzuki, M. and Nagata T. 1999. Fucoxanthin as the major antioxidant in *Hizikia fusiformis*, a common edible seaweed. *Biosci Biotechnol. Biochem.* 63: 605-607.

Yan, X., Nagata T. and Fan, X. 1998. Antioxidant activities in some common seaweeds. *Plant. Foods Human. Nutr.* 52: 253-262.

Yang, L L., Lee, C. Y. and Yen, K. Y. 2000. Induction of apoptosis by hydrolysable tannins from *Eugenia jambos* L. on human leukemia cells. *Cancer letter* 157: 65-75.

Yen, G. C., Chen, H. Y. and Peng, H. H. 2001. Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants. *Food Chem. Toxicol.* 39: 1045-1053.

Zhang, H., Spitz,, M. R., Tomlinson, G. E., Schabath, M. B., Minna J. D. and Wu, X. 2002. Modification of lung cancer susceptibility by green tea extract as measured by the Comet assay, *Cancer*

Detect. Prev. 26: 411-418.
Zhu, C. Y. and Loft, S. 2001. Effects of Brussels sprout extracts on hydrogen peroxide-induced DNA strand breaks in human lymphocytes. *Food Chem. Toxicol.* 39: 1191-1197.

ACKNOWLEDGEMENT

진로를 제대로 잡지 못했던 학부시절부터 대학원생활을 거쳐 본 논문이 있기까지 세심한 가르침으로 절 이끌어주신 전유진 교수님께 머리숙여 진심으로 감사드립니다. 또한 바쁘신 중에도 본 논문이 좀 더 나은 방향으로 나아 갈 수 있게 심사해 주시고 관심을 보여주신 허문수 교수님과 김기영 교수님, 학부시절부터 대학원기간 동안 늘 많은 관심과 조언을 해주셨던 이기완 교수님, 송춘복 교수님, 이제희 교수님, 여인규 교수님, 이경준 교수님 그리고 박근태 박사님께도 감사드립니다. 그리고 연구에 전념 할 수 있도록 많은 도움을 주신 김수현 교수님, 하진환 교수님, 김규일 교수님, 강정숙 교수님, 현진원 교수님 그리고 김소미 교수님께 감사의 마음을 전합니다.

대학원생활 동안 항상 옆에서 힘이 되어 주었던 우리 실험실원 수진이 형, 영빈이 형, 선희, 긴내, 승홍, 진희, 성명, 석천, 지혁, 원우, 대승 미흡한 영어실력 때문에 수정 보느라 고생 많이 한 Nalin, Yasantha, Rohan, Mahinda 그리고 본 논문의 실험에 많은 부분을 가르쳐주고 도움을 준 강경아 선생님, 실험에 관한 조언을 해주신 전경임 선생님에게 감사드립니다.

대학원 생활하면서 많은 도움을 준 용욱이 형, 맹진이 형, 철홍이 형, 영건이 형, 문휴 형, 현실, 힘들 때나 즐거울 때나 항상 옆에 있어주었던 동기 만철, 상규, 태형, 철영이 형, 경임, 기정, 민주에게 감사드립니다. 또한 인생선배로서 많은 조언을 해주신 진창남 계장님, 김원석 본부장님, 양현필 차장님, 강도형 선배님, 박경일 선배님, 양병규 선배님께도 감사의 말씀을 드립니다.

끝으로 묵묵히 따뜻한 눈길로 지켜봐주신 아버지, 어머니, 집안일 때문에 고생 많이 한 어미니 같은 누나, 이제야 철이 든 착한 동생 그리고 항상 바쁘다는 핑계로 자주 못 만나도 이해해주는 효정이에게 이 논문을 바칩니다. 이 글을 보고 계시는 모든 분들께 감사드리며 항상 건강하시고 좋은 일만 있기를 바랍니다. 그리고 사랑합니다.

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY