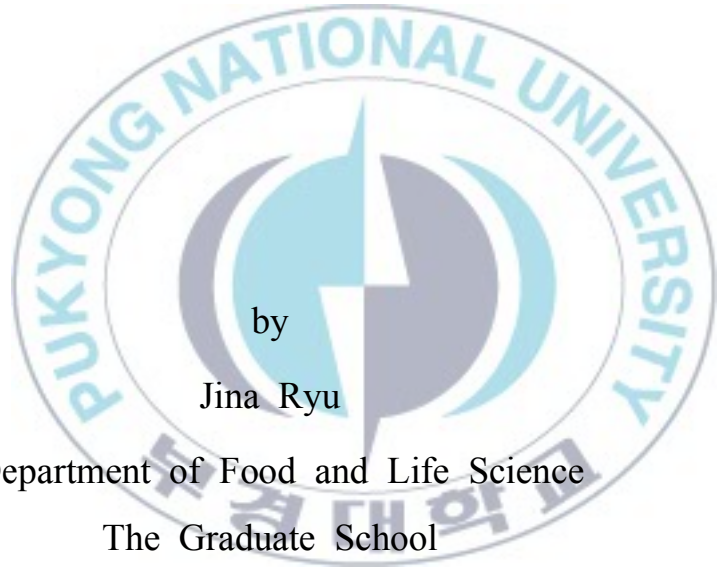
Thesis for the Degree of Doctor of Philosophy

Protective effect of porphyra-334 on UVA-induced photoaging in human skin fibroblasts



by

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February 2014

Protective effect of porphyra-334 on UVA-induced photoaging in human skin fibroblasts

(Porphyra-334의 광노화 보호효과)

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by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Department of Food and Life Science, The Graduate School,
Pukyong National University

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February 21, 2014

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Abbreviations

ABC	avidin-biotin-peroxidase complex
ANOVA	analysis of variance
AP-1	activator protein-1
ATCC	American Type Culture Collection
ATG	autophagy-related gene
BCA	bicinchoninic acid
BSA	bovine serum albumin
CBP	Creb-binding-protein
COX-2	cyclooxygenase-2
CYR61	cystein-rich 61
DAD	diode array detector
DCF-DA	2'-7'-dichlorofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
ESI-MS	electrospray ionisation mass spectrometry
FBS	fetal bovine serum
GAG	glycosaminoglycans
GST	glutathione-S-transferase

HA	Hyaluronan
HO-1	heme oxygenase 1
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-jun N-terminal kinase
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
MAAs	mycosporine-like amino acids
MAPKs	mitogen-activated protein kinases
MMPs	matrix metalloproteinases
mTOR	mammalian target of rapamycin
mTORC1	rapamycin-sensitive mTOR complex Ⅰ
mTORC2	rapamycin-resistant mTOR complex Ⅱ
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NF-κB	nuclear factor kappa B
Nrf 2	nuclear erythroid 2-related factor 2
p38	protein kinase
p70S6K	phosphorylation of the ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PBS-T	50% Tween-20 in PBS
PDK1	phosphoinositide-dependent kinase-1
PI3K	phosphatidylinositol 3-kinase

PIP	Procollagen Type I C-Peptide
PMS	phenazine methosulfate
POD	horseradish peroxidase
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
RT	room temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SA-β-gal	senescence-associated beta-galactosidase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STANA	N-Succinyl-Ala-Ala-Ala-p-nitroanilide
TBS-T	0.1% Tween-20 in 10 mM Tris-HCl, 150 mM NaCl [pH 7.5]
TGF-β	Transforming growth factor beta
TIMPs	tissue inhibitors of metalloproteinases
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
UV	ultraviolet

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류 진 아

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요 약

자외선은 피부노화의 주요한 외인성 요인으로서 특히 UVA는 진피에 침투하여 막단백질 분해, 산화적 스트레스 및 염증을 유발하여 광노화를 일으킨다. 본 연구에서는 방사무늬돌김(*Porphya yezoensis*)으로부터 mycosporine-like amino acids (MAAs) 3종을 HPLC를 이용하여 분리하였으며 (palythine [RT 2.29], asterina-330 [RT 2.493] 및 porphyra-334 [RT 11.53]), 이들의 LC/MS $[M+H]^{+1}$에서의 분자량은 각각 *m/z* 245.1, 289.1, 347.1으로 확인하였다. 이 중 방사무늬돌김의 대부분을 차지하는 porphyra-334의 광노화에 미치는 영향을 알아보고자 인체 유래 피부섬유아세포(CCD-986sk)에 UVA 10 J/cm^2을 조사한 후, 농도별 porphyra-334를 24시간 처리하여 이후의 세포생존율, MMPs생성에 의한 ECM 단백질 분해 및 cytokines의 변화를 관찰하였다. MTS assay 결과, porphyra-334에 대한 세포독성은 나타나지 않았으며, UVA로 유도된 세포독성을 효과적으로 억제하였다. 또한 RT-PCR과 Western blot analysis로 유전자 및 단백질 발현을 확인한 결과, UVA에 의해 생성된 matrix metalloproteinases (MMPs)는 피부 결합조직 내 콜라겐과 다른 ECM 단백질 분해를 촉진하였으나, porphyra-334의 콜라겐 생합성 촉진, 엘라스틴 및 hyaluronan 분해 억제, MMP-1 감소를 통해 주름과 탄력저하와 같은 피부 광손상에 대한 보호효과를 나타내었다. ELISA, RT-PCR, Western blot analysis를 통해 porphyra-334에 의한 염증성 cytokines (TNF-α. IL-1β, IL-6)의 농도 의존적인 생성 감소를 확인하였으며, 특히 TNF-α에서 porphyra-334 처리 전후의 변화가 유의한 수준으로 회복되었으므로, UVA로 유도된 염증 반응에 대한 억제 효과를 기대할 수 있을 것으로 사료된다.

UVA에 대한 porphyra-334의 광노화 보호효과는 3가지의 cell signaling pathway를 통해 조절되어짐을 RT-PCR 및 Western blot analysis를 통해 확인하였다. UVA에 의해 활성화된 ERK, JNK MAP kinase는 porphyra-334에 의해 억제되었으나 p38 MAPK는 자외선에 대한 변화를 보이지 않았다. JNK MAPK의 인산화를 통해 AP-1의 활성화가 유도되며 이로 인한 MMPs 및 c-jun의 증가는 UVA에 의해 광노화가 진행되는 과정으로 보여진다. 콜라겐 합성을 조절하는 TGF-β/Smad singaling pathway는 TGF-βRⅡ의 인산화를 통해 TGF-βRⅠ을 활성화하며 downstream의 receptor-regulated Smads (R-Smads)인 Smad2와 Smad3의 인산화로 인해 common-partner Smads (Co-Smads)인 Smad4와 복합체를 형성, 핵으로 전위되어 전사를 조절함을 확인하였다. 또한, UVA에 의한 EGFR의

활성화는 PI3K/Akt/mTOR signaling pathway를 통해 광노화가 유도되며 porphyra-334에 의해 유의적인 감소를 보였다. 단백질 합성, 세포증식, 대사와 autophagy에 관여하는 mTORC1은 mTOR(Ser2481), GβL, Raptor로 이루어져있는 mTOR complex로서 downstream의 p70S6K, eIF4B, RPS6 역시 porphyra-334의 광노화억제효과를 보였다. Akt kinase인 mTORC2의 경우, mTOR(Ser2448), GβL, Rictor로 구성되어 있으며, 이들 역시 UVA에 의해 과발현되며 porphyra-334에 의해 조절됨을 확인하였다.

앞서 살펴본 UV, ROS, MMPs는 광노화의 주요 요인으로 작용하며 이는 lysosomal proteinases인 cathepsins과도 상호작용을 한다. 따라서 UVA에 따른 cathepsins의 변화를 확인하고자 RT-PCR과 Western blot analysis를 통해 mRNA 및 단백질 발현을 확인하였다. 그 결과, UVA 조사시 노화의 주요조절자로 작용하는 cathepsin B, 세포분화에 관여하는 cathepsin D, ECM 항상성 유지에 관여하는 cathepsin K가 감소하였으며, ECM 손상에 관여하는 cathepsin G와 ECM 단백질 분해에 관여하는 cathepsin L가 증가하며 이를 porphyra-334가 조절하는 것으로 나타났다. Lysosomal pathway를 통한 Autophagy 역시 세포내 손상 복원 및 제거를 통해 항상성을 유지하는 조절 기작으로 노화를 방지하고 노화에 따른 각종 질환을 예방하는 필수적 작용으로 보여진다. 본 연구 결과, porphyra-334는 LC-3Ⅱ 증가, Beclin-1 증가, p62/SQSTM1 감소, ATG7 증가에 의해 autophagy가 유도됨이 규명되었으며, 이는 세포 내 손상을 제거함으로서 세포를 보호하고 노화를 지연시키는 역할을 하는 것으로 보여진다.

Ⅰ. INTRODUCTION

1. Marine organisms

There is growing interest in marine organisms to explore the bioactivity of various marine compounds associated with human life. A variety of marine natural products with their specific activities, such as antimicrobial (Karabay-Yavasoglu *et al*., 2007; Nair *et al*., 2007), anticoagulant (Pushpamali *et al*., 2008; Matsubara *et al*., 2001), antileishmanial (Freile-Pelegrin *et al*., 2008), antitrichomonal (Moo-Puc *et al*., 2008), antitumor (Olano *et al*., 2009), anti-HIV-1 (Wang *et al*., 2007), immunomodulatory (Ahn *et al*., 2008), antioxidant activites (Kim *et al*., 2009; Devi *et al*., 2008; Ananthi *et al*., 2010) and anti-photoaging (Thomas and Kim, 2011) have been reported from diverse marine organisms such as cyanobacteria, macroalgae, phytoplankton and animals.

Several species of marine algae are sources of food, fodder, fertilizer, chemicals and medicine. About 20,000 marine algae species are distributed throughout the world, out of which only 221 species are utilized commercially. These include 145 species for food and 110 species for phycocolloid production (Chandini *et al*., 2008). Currently, more than 900 marine algae are listed for Korean waters. Many species are familiar to Korean people because of their use as sea vegetables, such as *Capsosiphon fubscens*, *Codium fragile*, *Porphyra* spp., *Saccharina japonica*, *Sargassum fusiforme*, *Undaria pinnatifida* and so on, which are cultivated in seawater farms (Lee, 2013). Besides their food value, many macroalgal species that are

commonly exposed to elevated solar radiation synthesize and accumulate high concentrations of mycosporine-like amino acids (MAAs) as UV-sunscreen compounds.

Porphyra is a genus of red algae and nearly 133 species have been reported from all over the world, which includes 28 species from Japan, 30 from North Atlantic coasts of Europe and America and 27 species from the Pacific coast of Canada and United States (Yoshida *et al*., 1997). Several *Porphyra* species, including *P. yezoensis* and *P. tenera*, have been employed as edible seaweeds and have been cultivated in aquaculture industries in many countries of the Asia-Pacific region, in particular Korea, Japan and China (Hwang *et al*., 2005). *Porphyra* cultivation in Korea is a US$200-million industry with an annual production of about 320,000 metric ton which is equivalent to more than ten billion sheets of dried laver (Park and Hwang, 2013). Under natural conditions, most *Porphyra* inhibits in the intertidal zone where the algal body is entirely out of the sea and exposed to sunlight directly when the tide is at ebb, and is expected to have a strong defence system against UV irradiation (Yoshiki *et al*., 2009; Pallela *et al*., 2010; Sahoo *et al*., 2002; Davison and Pearson, 1996).

2. Mycosporine-like amino acids (MAAs)

As with land organisms, marine organisms have a variety of mechanisms to protect themselves from UV irradiation. UV absorbing compounds that are nearly ubiquitous among marine organisms (Ingalls and Whitehead, 2010) and inhibit the penetration of UV light into their tissues or

cells. The major type of UV absorbing substance found in red algae was identified as MAAs. MAAs are a class of water-soluble compounds characterized by aminocyclohexenone or aminocyclohexenimine rings conjugated with the nitrogen or imino alcohol substituents (Carreto *et al*., 1990; Singh *et al*., 2008). Because of maximum UV absorption between 310 to 360 nm, MAAs are favored as photo-protective compounds that consist of an average molecular weight of around 300 Da. Their high molar extinction coefficients (ε=28,100-50,000 $M^{-1}cm^{-1}$) support the hypothesis of a photo-protective role. Presently, about 21 MAAs have been reported from terrestrial, freshwater and marine organisms. They live in shallow water and normally are exposed to intense radiation, have evolved a variety of photo adaptive mechanisms and strategies for successful growth. It has been speculated that MAAs act as a natural UV sunscreen in these marine organisms. Furthermore, MAAs are the most common compounds with a potential role as UV sunscreens in marine organisms such as cyanobacteria, algae, and heterotrophic bacteria (Karentz *et al*., 1991). Many animals such as arthropods, rotifers, mjolluscs, fish, tunicates, eubacteriobionts, poriferans, nemertineans, echinodermates, platyhelminthes, polychaetes, bryozoans and protozoans have been reported to protect themselves from UV radiation by MAAs (Sinha *et al*., 2007).

Several species of red algae, such as *Acanthophora*, *Bangia*, *Bostrychia*, *Caloglossa*, *Catenella*, *Devaleraea*, *Ceramium*, *Chondrus*, *Corallina*, *Devaleraea*, *Gelidiella*, *Gelidium*, *Gracilaria*, *Iridea*, *Palmaria*, *Phyllophora*, *Polysiphonia*, *Porphyra*, *Stictosiphonia* have also been reported

to produce high concentrations of various MAAs (Sinha *et al*., 2007). Among all of these, Rhodophyta *Porphyra yezoensis* was gaining more attention in this study.

Besides their function as photo-protective compounds, it has been suggested that MAAs also have antioxidant activity (Lee and Shiu, 2009), osmotic functions (Oren, 1997) and a regulatory role in reproduction (Shick and Dunlap, 2002). In order to understand the mechanism by which these compounds may act as photoprotectors in living organisms, undertook a detailed study of their photophysical and photochemical properties.

3. Human skin

Human skin is the largest organ as first line of defence against damaging sunlight, unsafe chemicals, harsh temperatures, harmful bacteria and other organisms that changes dramatically over the course of human lifetime. However, because skin is an external organ, it can be easily damaged and disfigured. The appearance of skin varies considerably from person to person. Even within families, there can be obvious differences in the skin of two siblings. The color of skin is determined by melanin, a pigment produced in the outer layer of skin to protect from the sun UV rays. Dark-skinned people produce more melanin than fair skinned people. Not surprisingly, dark skin is very common in people who originated from hot, sun-exposed regions where a lot of melanin is required to protect the body from the sun UV rays. Fair skin on the other hand, is typically found in people who originated from northern Europe where the sun UV rays are much weaker. In northern

Europe, where people need to produce vitamin D from limited sunshine, fair skin is of greater benefit than dark skin.

Human skin is has two main layers: the dermis, the thick layer on the bottom; and the epidermis, the thin layer of cells on the surface above (Fig. 1). The outermost layer of the skin, epidermis, is attached to the dermis at the dermal-epidermal junction by the basement membrane, an intricate network of proteins that extend from inside basal keratinocytes into the papillary layer of the dermis. The basement membrane is absolutely critical for adhesion of the epidermis to the dermis, but also contributes to cell migration and signaling cascades. Keratinocytes constitute approximately 80% of epidermal cells and provide structure, barrier formation and inflammation.

The epidermis also contains melanocytes that produce melanin, an endogenous sunscreen that gives skin color. The dermis is the supporting matrix of the skin, consist of connective tissue with a high capacity for water retention. Fibroblasts are the major cellular component of the dermis that produces the extracellular matrix (ECM) proteins, as well as structural proteins (collagen and elastin), adhesive proteins (laminins and fibronectin), glycosaminoglycans (GAG) and proteoglycans. The most abundant structural protein in skin connective tissue is type Ⅰ collagnen (90% of ECM in dermis), which is synthesized primarily by fibroblasts and is responsible for conferring strength and resiliency to cells (Gelse *et al*., 2003). Elastin is an essential part of various human tissues that depend on elasticity, including the skin, lung and arteries. Elastin provides these elastic tissues with the ability to stretch and recoil and plays a critical role in supporting and maintaining

healthy cells (Alamine *et al*., 2010). Hyaluronic acid is an ECM glycosaminoglycan polysaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine with retaining water, stimulating collagen synthesis, repairing tissue and maintaining intercellular space activities. In aged skin, a dramatic decrease associated with increased skin dryness and decreased skin elasticity, dermal thickness and skin collagen content.

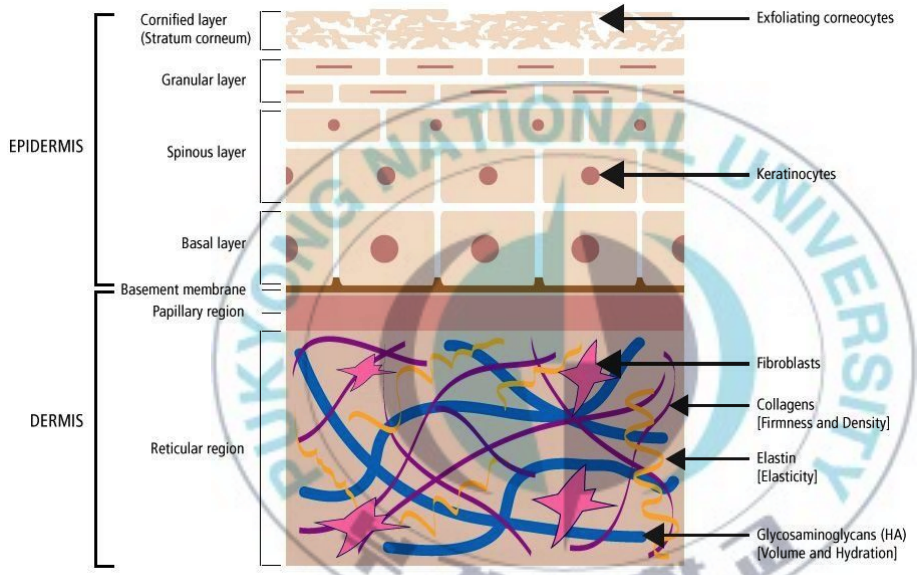

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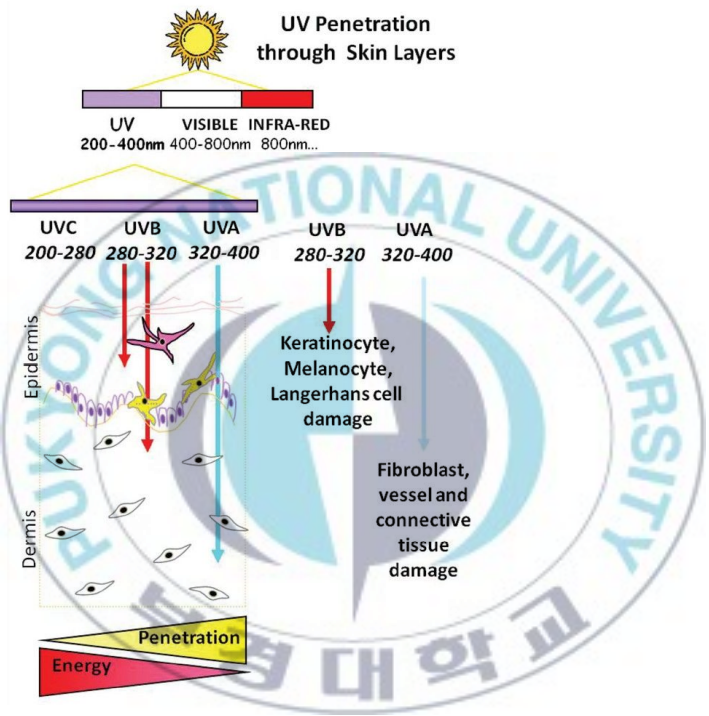
Fig. 1. A diagram of human skin layer.

4. UV irradiation

UV is one of the most harmful exogenous operators and may affect a number of biological functions in all sunlight-exposed living organisms (Green *et al*., 2011). The ultraviolet (UV) spectrum is divided into UVC (200-280 nm), UVB (280-320 nm) and UVA (320-340 nm). Of these, only UVB and UVA are of environmental significance since UVC is the shortest in wavelength and effectively absorbed by ozone in the earth's atmosphere (Fig. 2). UVB and UVA irradiation of cells induces the transcription of many genes encoding multiple proteins, which are responsible for skin cell apoptosis, cancer transformation, collagen degradation, senescence and skin aging. Up to 50% of UVA can reach the depth of melanocytes and the dermal compartment, whereas only 14% of UVB reaches the lower epidermis. It has been estimated that the total photon energy delivered into the lower epidermis and upper dermis is 100 fold higher in the UVA region than in the UVB reagion. Thus, UVB has a major action on the epidermis and UVA can also target the dermis. In response to intense solar radiation, organisms have evolved certain mechanisms such as avoidance, repair and protection by synthesizing or accumulating a series of photo-protective compounds. Photobiological research has preferentially focused on the deleterious involvement of UVB and UVC radiation in skin photo-carcinogenesis. However, the role of UVA and near visible solar irradiation in photodamage, photoaging and carcinogenesis and the need for effective UVA skin photo-protection are now rapidly emerging as important areas of skin photobiology (Gasparro, 2000). Transcription controlling the activity of these

genes is activated by multiple protein kinases or signal pathways (Cao and Wan, 2009). UV irradiation causes alternations of dermal collagen through two primary pathways: 1) stimulation of collagen breakdown, resulting in fragmented, disorganized collagen and 2) inhibition of procollagen biosynthesis, resulting in loss of collagen content. UVA exposure causes near-complete loss of procollagen synthesis, which persists for 24 h, followed by recovery 48-72 h post exposure (Fisher *et al*., 2000).



Indian J. Dermatol. Venereol. Leprol. 2012. 78, 9-14

Fig. 2. Diagram showing depth of UV penetration into the skin and photon associated energy according to wavelength.

5. Cytokine and growth factor receptor

Cytokines are produced from almost all nucleated cells, when stimulated or activated, including keratinocytes, melanocytes, Langerhans cells in the epidermis, and fibroblasts, endothelial cells, smooth muscle cells, mast cells, lymphocytes and other inflammatory cells in the dermis. One of the earliest detectable responses of human skin cells to UV irradiation is activation of multiple cytokine and growth factor cell surface receptors, including epidermal growth factor receptor (EGFR; Sachsenmaier *et al*., 1994, Warmuth *et al*., 1994), insulin receptor (Coffer *et al*., 1995), interleukin (IL)-1 receptor (Brink *et al*., 2000), IL-6 receptor (Urbanski *et al*., 1990), and tumor necrosis factor (TNF)-α receptor (Leverkus *et al*., 1998). Activation of growth factor and cytokine receptors by UV irradiation, leads to clustering and internalization of the receptors. Exposure of the skin to UV radiation is known to enhance the levels of proinflammatory cytokines. As the elevated levels of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, contribute to the tumor promotion process, this effect would be expected to result in an earlier occurrence of tumors and more rapid progression (Mukhtar and Elmets, 1996). Co-administration of EGF, IL-1, IL-6 and TNF-α results in a strong synergic response similar to that caused by exposure to UV irradiation. Thus, cytokines cooperate to produce a proper physiological response that is needed by the organism during physiological and pathophysiological remodeling.

6. Skin aging

Skin aging can be divided into two basic processes, intrinsic and extrinsic aging. Extrinsic againg is generally referred to as photoaging and is characterized by severe wrinkling and pigmentary changes, such as solar lentigo and mottled pigmentation on exposed areas such as the face, neck and forearm. Solar UV irradiation is a major environmental hazard that generates reactive oxygen species (ROS), induces DNA damage, and leads ultimately results in skin inflammation, photoaging, and cancer development (Ichihashi *et al*., 2003). The major changes in photo-induced skin are seen in the dermis. The histologic hallmark of photoaging is a massive accumulation of elastotic material in the upper and mid-dermis, and this phenomenon is known as solar elastosis. This solar elastotic material is composed of elastin, fibrillin and other extracellular matrix components. Dermal changes in photoaging require extracellular matrix turnover and deposition.

Oxidative stress is considered a primary feature in aging and age-related illnesses including cataracts, atherosclerosis, diabetes and alzheimer disease. Aging has been considered the consequence of free radical damage by various endogenous ROS, according to the original free radical theory of aging. ROS form as a natural by-product of the normal cellular metabolism and have important roles in cell signalling and homeostasis. ROS production and release can be influenced by environmental factors such as UV radiation and exogenous toxins. Oxidative damage can compromise cell survival, proliferation, differentiation and metabolism. These cellular events are accompanied or preceded by the release of a wide variety of proinflammatory

mediators, including certain enzymes and cytokines. Antioxidant enzymes including heme oxygenase 1 (HO-1) and glutathione-S-transferase (GST) provide protection against the deleterious effects of ROS (Banerhee *et al*., 1999). The induction of antioxidnat enzymes is one of the most important determinants of cancer susceptibility and is related to both cancer chemopreventive and cytoprotective effects. The induction of antioxidant proteins is mediated by nuclear erythroid 2-related factor 2 (Nrf 2), which is translocated from the cytosol to the nucleous. Consequently, cytoprotective response are characterized by an upregulation of antioxidnat enzymes and decreased sensitivity to oxidative stress damage (Jaiswal, 2004). Various compounds with differential antioxidant proterties are found in plants, which may be applicable as therapeutics to decrease and prevent free radical damage. Several medical plants have been screened and assessed for properties in antagonizing free-radical-induced oxidative stress, and their natural products are used to treat 87% of all classified human diseases (Marchioli *et al*., 2001; Chin *et al*., 2006).

Cystein-rich 61 (CYR61) is an extracellular matrix-associated signaling molecule that belongs to the CCN family (Lau and Lam, 1999; Perbal, 2004). The CCN family plays a fundamental biological role in differentiation, growth, migration, and extracellular matrix regulation (Chen and Lau, 2009). Up-regulation of CYR61 in human skin fibroblasts also causes the alterations of type Ⅰ collagen homeostasis that mimic those observed in chronologically aged and photoaged human skin (Quan *et al*., 2006). Furthermore, elevated CYR61 induced transcription factor activator

protein-1 (AP-1), which acts to stimulate matrix metalloproteinase-1 (MMP-1) expression. In non-irradiated human skin, AP-1 transcription factor complexes contain predominantly c-fos and JunD. Trascription of c-jun and c-fos is induced by a variety of growth factors, cytokines, and environmental stimuli, including UV irradiation. The c-jun and c-fos transcription is largely dependent on activation of the mitogen-activated protein kinases (MAPK) pathways, which directly and indirectly activate transcription factors that drive transcription of the c-jun and c-fos genes.

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are enzymes involved in both inflammatory processes and tumor development. Recent studies have demonstrated the UVA-induced expression of COX-2 and iNOS through the activation of nuclear factor kappa B (NF-κB), and the targeted inhibition of COX-2 or iNOS and the modulation of NF-κB upregulation have been identified as the molecular basis of photoprotection (Chen *et al*., 2000).

7. Protein metalloproteinases and lysosomal proteases

Recently, it was suggested that excessive matrix degradation by UV-induced matrix metalloproteinases (MMPs), which are secreted by various cells including kerationcytes, fibroblasts and inflammatory cells, contributes to connective tissue damage during photoaging. MMPs are a family of enzymes responsible for degrading connective tissue. They are structurally related endopeptidases that mediate degradation of different macromolecular components of the extracellular matrix and the basement membrane, including

collagen. MMPs are synthesized by a variety of cell types and most of them are secreted from cells as latent forms. Activation of proMMPs is primarily brought by the action of proteolytic cascades, mainly catalysed by neutral proteinases. Various types of UV-induced the synthesis of MMPs present on dermal fibroblasts contribute to the breakdown of dermal interstitial collagen and other connective tissue components. UVA is known to induce the overexpression of MMP-1, -2, -8 and -13 in the normal human dermis *in vitro*. These results indicate that MMP-mediated degradation of collagen in photodamaged skin. In particular, in photodamaged aging skin, increased ROS leads to the induction of AP-1 and NF-κB transcription factors, which consequently induce collagen degradation by MMPs upregulation. These properties make MMP-1 an attractive target for anti-photoaging compounds. Under normal physiological conditions, MMPs are regulated by endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). Though, UV disrupts the balance between MMPs and TIMPs (Pillai *et al*., 2005). ECM degrades naturally over time due to intrinsic aging, but this breakdown is accelerated by external factors (espacially UV irradiation) and the resultant oxidative stress, as well as increases in MMP activity.

Cathepsins, belonging to the lysosomal proteinases, are mainly classified into cysteine protease (cathepsin B, K, L), serine protease (cathepsin G), and aspartate protease (cathepsin D). Cathepsins have been recognized for their important biological functions such as proenzymatic proteolysis processing, intracellular protein degrading, antigen presenting, tissue remodeling, inflammation processing and bone matrix reabsorbing (Conus and

Simon, 2008). Additionally, cathepsins are involved in human skin physiological mechanisms such as extracellular matrix (ECM) turnover, dermal and epidermal cell proliferation, and senescence (DeJesus *et al*., 2002). These studies indicate their close relationship with various dermatitides and skin tumors. Since studies demonstrate that the loss of structural integrity of the dermal ECM, abnormal cycles of fibroblasts and keratinocytes, inflammatory reaction of dermatitis, and the increased rate of skin tumors are closely related to photoaged human skin. Therefore, this study investigates the role of cathepsins in prematurely senescent human skin fibroblasts induced by UVA irradiation.

8. Signaling cascade

The elucidation of UV signaling pathways that lead to matrix destruction responsible for premature skin aging has identified targets for photoaging prevention strategies. Among the cell surface receptors, epidermal growth factor receptor (EGFR) appears to be critical, as its activation is necessary for a wide variety of UV-induced responses (Singh *et al*., 2009; Huang *et al*., 1996). EGFR activation is synonymous with activation of its intrinsic tyrosine kinase activity, which results in phosphorylation of specific tyrosine residues within this intracellular C-terminal domain. Thus, the level of tyrosine phosphorylation is a well-characterized and universally accepted quantitative measurement of the activation state of EGFR.

In addition to cell surface receptor activation, generation of ROS appears to be critical for MAPKs-mediated signal trasduction triggered by UV

irradiation (El-Abaseri *et al*., 2005; Muthusamy and Piva, 2010). The MAPKs regulate essential cellular events including the cell proliferation and differentiation. The MAPKs signal pathway includes the extracellular signal-regulated kinase (ERK), the c-jun N-terminal kinase (JNK) and protein kinases (p38) signaling pathways. ROS drive MAPK phosphorylation which recruits c-Fos and c-Jun to the nucleus and NF-κB activation, and the gene related to proimflammatory would be upregulated subsequently (Bickers and Athar, 2006). Thus it is reasonable to assume that the ROS generation and mitochondrial damage induced by UVA irradiation can provoke MAPKs signal pathway.

Akt (also known as "protein kinase B", PKB) is a well-known regulator of mammalian target of rapamycin (mTOR) signaling pathway. mTOR exists in a large multi-protein complex, at least two mTOR complexes have been identified so far, including rapamycin-sensitive mTOR complex Ⅰ (mTORC1) and rapamycin-resistant mTOR complex Ⅱ (mTORC2). Cutaneous activation of Akt during photoaging could affect mTOR activation and it phosphorylated differentially when associated with mTORC1 and mTORC2. mTORC1 has recently been shown to consist of three proteins (mTOR Ser2448, Raptor and mLST8), is sensitive to rapamycin and regulates cell growth, in part, by phosphorylation of the ribosomal protein S6 kinase (p70S6K). mTORC2 also contains mLST8, but instead of Raptor, mTORC2 contains Rictor and mTOR (Ser2481) which is not rapamycin sensitive and modulates cell survival and proliferation by direct phosphorylation of Akt on Ser473 and by facilitating Akt phosphorylation on Thr308 by PDK1 in vitro

(Bayascas and Alessi, 2005).

Transforming growth factor beta (TGF-β) mediates signals through pairs of specific plasma membrane receptors (Ⅰ and Ⅱ). TGF-β binds activates the type Ⅱ receptor, which then phosphorylates the receptor Ⅰ. The active receptor complex then phosphorylates Smad2 or Smad3. The phosphorylation of Smad2/3 propagates the signal by binding with Smad4. This complex translocates into the nucleus and binds transcriptional co-activators such as p300 and Creb-binding-protein (CBP), which regulate the transcriptional activity of various TGF-β target genes.

9. Autophagy

Autophagy is an evolutionarily conserved catabolic process, by which cytoplasmic organelles and proteins are engulfed in autophagosomes and degraded after fusion with lysosomes (Fig. 3). This degradation pathway permits the cell to eliminate unwanted or unnecessary organelles and to recycle the components for re-use (Kim and Klionsky, 2000). Biological roles of autophagy can be illustrated in a variety of physiological and pathophysiological conditions, such as starvation, adaptation, clearance of damaged proteins, cell survival and death, tumor suppression, and antigen presentation. In addition, it has been suggested that autophagy can have a pro-longevity effect in organisms from yeast to flies, although this hypothesis remains controversial. Furthermore, several lifespan extension interventions, including calorie restriction as well as treatment with resveratrol, rapamycin, or spermidine, have exploited their effects through activation of autophagy.

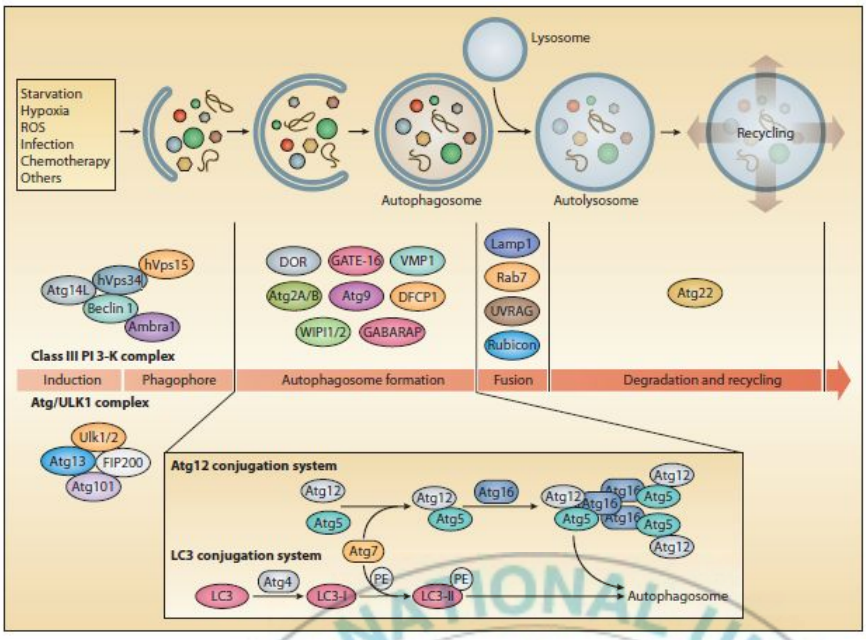


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Fig. 3. Autophagy pathway. A portion of cytoplasm, including organelles, is enclosed by a phagophore or isolation membrane to form an autophagosome by the ULK1 complex and PI3KⅢ complex. The conjugation of ATG12 to ATG5 conjugate and ATG16L. This complex assists the lipidation of LC3 proteins at the phagophore. Selective autophagy depends on binding of substrates to the inner surface of the growing phagophore, and this can be achieved by cargo receptors (p62 and NBR1). The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome.

Although the roles of autophagy in age-related diseases involving neurodegenerative dysfunction, such as alzheimer's disease, parkinson's disease, huntington's disease, macular degeneration, hypercholesterolemia, and cardiomyopathy, have been implicated, the effect of autophagy impairment by the homozygous knockout of autophagy-related gene (ATG) 5, ATG7 or Beclin-1 genes revealed embryonic lethality or postnatal death (Cecconi and Levine, 2008). In contrast, overexpression of ULK3, an isoform of ULK1, the mammalian homolog of ATG1, enhances autophagy activation but results in premature senescence in human fibroblasts (Young *et al*., 2009). This equivocal result brought confusion to the precise role of autophagy in senescence. Therefore, this study examined the effect of UVA-induced autophagy impairment involved in human dermal fibroblasts in order to clarify the role of autophagy during cellular senescence. In this study found that inhibition of autophagy can induce premature senescence in human dermal fibroblasts in a ROS dependent manner.

10. Puropose of this study

While data exists on the MAAs profiles of *P. yezoensis*, although studies have been reported on the photo-protective effect of MAAs on algae and tissue, little has been investigated about its effects on aging process of skin cells. Thus, the objectives of this study were elucidate the isolation and identification of MAAs in *P. yezoensis*, especially porphyra-334 was checked. Futhermore, isolated porphyra-334 was investigated the influence of UVA-induced cellular senescence-like characteristics of human skin fibroblasts and the possible mechanisms involved in the activity.

Ⅱ. MATERIALS AND METHODS

1. Extraction and isolation of water-soluble MAAs from *Porphyra yezoensis*

MAAs extraction method was performed as described by Tao *et al*. (2008) with minor modifications. Briefly, dried *P. yezoensis* (100 g) was ground and extracted in hydrophilic solvent consisting of 80% aqueous methanol (v/v) at 45 ℃ for 2 h. The extract was filtered (No. 3, 90 mm, Advantec, Tokyo, Japan) to remove the particles of the powder and the residual aqueous suspension was evaporated to dryness under vacuum at 41 ℃ (EYELA N-N series, Tokyo rikakikai Co., Ltd, Japan). The dried crude extract of *P. yezoensis* was redissolved in 150 mL of ultrapure water and transferred to a separating funnel containing 666 mL of chloroform-methanol-ultrapure water (2:1:1, v/v/v). The upper layer containing crude MAAs was collected, filtered through 0.2 μm pore sized syringe filters (Woongki science, Seoul, Korea) and loaded onto a Strata C18-E cartridge (Phenomenex, Torrance, CA, USA) (previously equilibrated with ultrapure water) for analysis (Fig. 4).

2. UV spectroscopic analysis

Ultraviolet absorption spectra were obtained using a UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) at room temperature (RT). All experiments were repeated at least three times with consistently the same results.

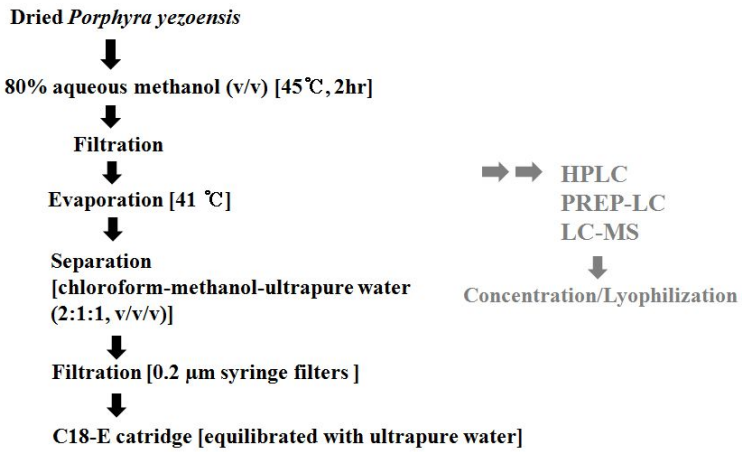

Fig. 4. Extraction procedure for the isolation and identification from *Porphyra yezoensis*. Dried *P. yezoensis* was ground and extracted with 80% methanol (v/v). The crude extracts were filtered, evaporated to dryness and partitioned into chloroform-methanol-ultrapure water (2:1:1, v/v/v). The upper layer was subjected to C18-E catridge and HPLC. Purified MAAs were readily identified by a combination of spectroscopic analysis.

3. High-performance liquid chromatography (HPLC) analysis

MAAs were purified using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD, Agilent Technologies, Palo Alto, CA, USA). The HPLC conditions used were as follows: column, Gemini-NX 5μ C18 (i.d. 250×21.20 mm, Phenomenex, Torrance, CA, USA); column

temperature, RT; flow rate, 30 mL/min; mobile phase, 0.1% acetic acid in H_2O; and wavelength for detection, 334 nm. Identification of MAAs was performed using UV absorption spectra and mass spectrometry.

4. Electrospray ionization mass spectrometry (ESI-MS) analysis

To identify the peaks of the fingerprints, Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) ion-trap mass spectrometer with electrospray ionization (ESI) source was used for the HPLC/MS method. ESI-MS conditions of each HPLC peak were set as follows: scan mass range, *m/z* 100−600; fragmentor voltage, 70 V; drying gas N_2 flow rate, 12 L/min; sheath gas flow rate, 60 arbitrary units; the drying gas temperature 350 ℃; and capillary voltage 3,000 V.

5. Cell culture

Human skin fibroblasts (CCD-986sk) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% (v/v) penicillin−streptomycin (Gibco, Grand Island, NY, USA) under a humidified atmosphere of 5% CO_2 at 37 °C. Cells were sub-cultured at about 80% confluence by detaching with trypsin−EDTA solution, and used between the tenth and fifteenth passages.

6. UVA irradiation and treatment

Prior to UVA irradiation, the cells were washed with phosphate buffered saline (PBS) and exposed to a radiation dose of 10 J/cm^2 of UVA light (Vilber Lourmat, France) in PBS. After irradiation, the treated cells were washed with PBS and replaced with different concentrations of porphyra-334 for 24 h. In parallel, no-irradiation control cells were treated in the same manner, but the wells were covered with aluminum foil to prevent irradiation.

7. Cell viability

Cell viability was determined by CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay (Promega, Madison, WI, USA) following manufacturer's instructions. The cells plated at 2 × 10^4 cells/well and allowed to attach in 96-well plates were exposed to serial concentrations of porphyra-334 for 24 h. At the end of 24 h, solution of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS) were added. After 30 min at 37 °C in humidified, 5% CO_2 atmosphere, the quantity of the soluble formazan product as measured by the amount of 490 nm absorbance was recorded using multiplate reader (SpectraMAX 340-pc, Molecular Devices, Sunnyvale, CA, USA) as directly proportional to the number of living cells in culture (Fig. 5). Relative cell viability was calculated as the percentage of viability of untreated cells that were considered as the control. Each experiment was performed in triplicate.

8. SA-β-gal staining

Senescence-associated beta-galactosidase (SA-β-gal) activity was determined at 24 h after UVA irradiation. Cellular senescence assay kit (Cell Biolabs, Inc., San Diego, CA, USA) was used according to the manufacturer's instructions. Briefly, cells were twice washed in PBS and then incubated at room temperature for 5 min with fixing solution. The cells were washed three times with PBS, aspirate the final wash, and completely cover cells by adding freshly prepared cell staining working solution incubated at 37 ℃ protected from light for overnight. Removed the cell staining working solution, the stained cells were washed twice with PBS and observed blue stained senescence cells using light microscope.

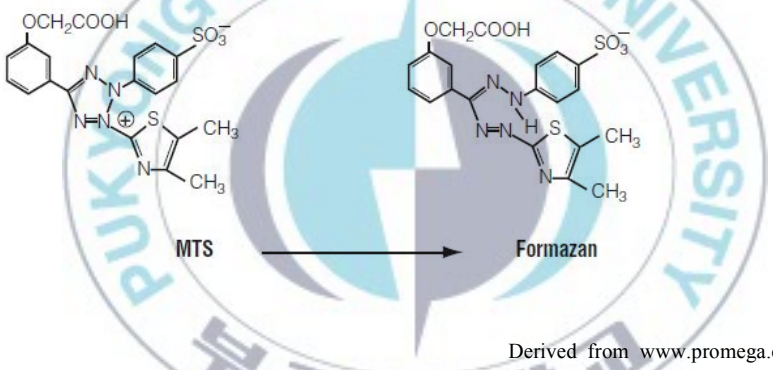

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Fig. 5. Structures of MTS tetrazolium salt and its formazan product.

9. Determination of intracellular ROS production

The production of intracellular ROS by CCD-986sk human skin fibroblasts was measured using the redox-esnsitive fluorescent dye 2'-7'-dichlorofluorescein diacetate (DCF-DA [$C_{24}H_{14}C_{l2}O_7$]; Sigma-Aldrich, Inc., Missouri, USA). The capacity of cells to produce ROS was measured by fluorescence. The cells were treated with 25 μM DCF-DA for 30 min at 37 ℃, washed twice in PBS. Representative images and fluorescence intensity were detected using a fluorescence microscope (Olympus Microscope System IX51, Olympus, Tokyo, Japan) and spectrophotometer.

10. Measurement IL-6 production

Quantitative detection of human IL-6 production was based on standard sandwich enzyme-linked immunosorbent assay technology using human IL-6 ELISA kit (Ab frontier, Seoul, Korea) according to the manufacture's instruction. In brief, added 0.1 mL of each properly diluted cell culture supernatants to each empty well and incubated at 37 ℃ for 90 min. Following the incubation step, 0.1 mL of biotinylated anti-human IL-6 antibodies were added to the wells subsequently at 37 ℃ for 60 min and unbound conjugates were washed away three times with PBS, and each time let washing buffer stay in the wells for 1 min. After the washing step, added 0.1 mL of prepared avidin-biotin-peroxidase complex (ABC) working solution into each well and incubated the plate at 37 ℃ for 30 min and then followed by washing with PBS again. Added 90 μL of tetramethylbenzidine (TMB) color developing agent into each well and incubated plate at 37 ℃ in

dark for 30 min. TMB was catalyzed by horseradish peroxidase (HRP) to produce a blue color product that changed into yellow after adding TMB stop solution (1N solution of sulfuric acid [H_2SO_4]). The density of yellow is proportional to the human IL-6 amount of porphyra-334 captured in plate, measured by the O. D. absorbance at 450 nm in a microplate reader within 30 min after adding the stop solution.

11. Measurement of TNF-α and IL-1β production

Quantitative detection of human TNF-α and IL-1β production was based on standard sandwich enzyme-linked immunosorbent assay technology using human TNF-α and IL-1β ELISA kit (KomaBiotech Inc., Seoul, Korea) according to the manufacture's instructions. Briefly, 100 μL of standard or sample was added and incubated at room temperature for at least 2 h. The plate aspirated the wells to remove liquid and washed 4 times with PBS-T (Tween-20 in PBS). After that 100 μL of diluted detection antibody (Biotinylated antigen-affinity purified rabbit anti-human TNF-α and goat anti-human IL-1β) was added and incubated at room temperature for 2 h. The plate washed 4 times and 100 μL of color development solution was added at room temperature for proper color development. To stop the color reaction, added 100 μL of the stop solution (2 M H_2SO_4) to each well and the amount of human TNF-α and IL-1β density was measured by O. D. absorbance at 450 nm in a microplate reader.

12. Measurement of elastase activity

Elastase activity using the synthetic substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide (STANA, Sigma-Aldrich, Inc., Missouri, USA) was measured as previously described by Nakagawa *et al*. (1987). In brief, 100 μL of enzyme solution was dispensed into 96 well plates, which were pre-incubated for 15 min at 37 ℃. After addition of 2 μL 55.3 mM STANA, the plates were further incubated for 1 h at 37 ℃. The release of p-nitroaniline was measured by absorbance at 410 nm and enzymatic activity is expressed as %.

13. Measurement of total collagen

Total collagen synthesis in fibroblasts was measured by the Procollagen Type I C-Peptide (PIP) EIA kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Briefly, transfer 100 μL of antibody-POD conjugate solution into appropriate wells and added 20 μL cell culture medium to the wells within 5 min, and incubated 3 h at 37 ℃. Removed contents by suction and washed the wells 4 times with 300 μL of washing buffer. Added 100 μL of substrate solution to each well and incubated at room temperature for 15 min. Added 100 μL of stop solution to all wells, and read at 450 nm.

14. Measurement of Hyaluronic acid (HA)

The level of Hyaluronic acid in culture supernatant was measured using Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (HA-ELISA,

Echelon Biosciences Inc., UT, USA) according to the manufacture's instruction. In brief, 100 μL of standards and samples were transferred into incubation plate, added 50 μL of working HA detector and incubated for 1 h at 37 ℃. Following the incubation step, the standards and samples were transferred into detection plate complete, mixed the detection plate by gently tapping, then incubated at 4 ℃ for 30 min to competitive binding. The plate were washed with wash buffer, added 100 μL of working enzyme to each well, and incubated at 37 ℃ for 30 min. Routinely, the plates were incubated with 100 μL of working substrate solution at 37 ℃ for 30 min development, measured the absorbance of each well at 405 nm.

15. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA from each sample was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's instructions, total RNA (1 μg) was subjected to first strand cDNA synthesis using Reverse Transcriptase PreMix Kit (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea). PCR amplification of the cDNA products were performed with 2X TOPsimple^{TM} DyeMIX(aliquot)-*n*Taq (Enzynomics, Daejeon, Korea) and primer pairs. Amplified products were seperated by 1% agarose gel electrophoresis and visualized with 1 mg/mL ethidium bromide. All mRNA levels were normalized using GAPDH as an internal control. The primers used in amplification are shown in Table 1.

16. Western blot analysis

After treatment, the cells were washed twice with PBS, harvested and lysated in RIPA buffer (50 mM Tris [pH 7.4], 1 mM ethylene glycol tetraacetic acid [EGTA], 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate) containing protease inhinitor cocktail (Geno Technology Inc., Saint Louis, MO, USA). The lysates were centrifuged at 13,475×g for 15 min at 4 ℃ (Smart-R17, Hanil Science Industrial, Incheon, Korea). Supernatants were collected and their protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, Illinois, USA). Equal amounts of protein (30 μg) were boiled for 10 min and then subjected on a 7.5-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked by incubation in 1% BSA in TBS-T (10 mM Tris-HCl, 150 mM NaCl [pH 7.5] containing 0.1% Tween-20) at room temperature for 1 h and incubated with specific primary antibody (Table 2) for 3 h. The membranes were washed three times with TBS-T and incubated 2 h with the appropriate HRP-conjugated goat anti-rabbit, goat anti-mouse or rabbit anti-goat secondary antibody (Santa Cruz Biotechnology, CA, USA) used at 1:10,000 in TBS-T containing 1% BSA. The respective proteins were detected with SuperSignal® West Pico (Thermo, Rockford, IL, USA). Equal protein loading was examined by GAPDH

detection. The blots were quantified by MultiGauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

17. Statistical Analysis

The results were presented as means ± SEM of at least three independent expreriments. All data were analyzed by one-way analysis of variance (ANOVA) with student's t-test using SPSS 10.0 (Chicago, IL, USA). Differences were considered significant at $p<0.05$.

Table 1. Oligonucleotide primer sequences used in RT-PCR

Gene	Primer sequence (5'-3')	
	Forward primer	Reverse primer
Akt	CAACTTCTCTGTGGCGCAGTG	GACAGGTGGAAGAACAGCTCG
Cathepsin B	CAGCGTCTCCAATAGCGA	AGCCCAGGATGCGGAT
Cathepsin D	TCTGTGGAGGACCTGATTGC	GCTGGACTTGTCGCTGTTGTA
Cathepsin G	CATCCGCCACCCTCAATA	CAGTCCCTCCTGGGCTCTA
Cathepsin K	CACGATGGTGCAGTGTAACG	TCAGGGGCAGGCTGTAGTC
Cathepsin L	GCACGGCTTTTCCATGGA	CCACCTGCCTGAATTCCTCA
c-fos	GGAGAATCCGAAGGAAAGG	GCTTGGGCTCAGGGTCATTG
c-jun	ATGACTGCAAAGATGGAAACG	TCAAAATGTTTGCAACTGCTG CG
COL1A1	AGAGGTTTCAGTGGTTTGGA	CCAGGAGCACCATTGGCACC
Elastin	GGCCATTCCTGGTGGAGTTCC	AACTGGCTTAAGAGGTTTGCCT CCA
ERK1	GATTGCCCCTGAGCAC	GGGGGCCTCTGGTGCC
ERK2	GCCCGGAGATGGTCCGC	ATGGTCTGGATCTGCAACA
GAPDH	CAGCCGAGCCACATCG	TGAGGCTGTTGTCATACTTCTC
HAS1	ACCATCGCCTTCGCCCTGCTC ATCC	CCCGCTCCACATTGAAGGCTAC CCA
HAS2	TTTCTTTATGTGACTCATCTGTC TCACCGG	ATTGTTGGCTACCAGTTTATCC AAAGGG

HAS3	CAGAAGGCTGGACATATAGAGGAGGG	ATTGTTGGCTACCAGTTTATCCAAACG
IL-1β	CTGTCCTGCGTGTTGAAAGA	TTCTGCTTGAGAGGTGCTGA
IL-6	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT
MMP1	TCTGCAAGGTTATCCCAAGG	TATTCCTGGAAAGGCACCTG
MMP13	TGAAGGTCGGTGTGAACGGA	CATGTAGCCATGAGGTCCACCAC
MMP8	GCTGCTTATGAAGATTTTGACAGAG	ACAGCCACATTTGATTTTGCTTCAG
mTOR	CGCTGTCATCCCTTTATCG	ATGCTCAAACACCTCCACC
p38	GTG CCC GAG CGT TAC CAG ACC	CTA TAA GCT TCT GAC ATT TC
p70S6K	TACTTCGGGTACTTGGTAA	GATGAAGGGATGCTTTACT
PDK1	CCCACGTGATGGACTCAAAGA	AAGGGTACGGGCCTCTCAAA
PI3K	AGGAGCGGTACAGCAAAGAA	GCCGAACACCTTTTTGAGTC
PTEN	CATAGCGCCTCTGACTGGGA	CATAGCGCCTCTGACTGGGA
RPS6	AAGGAGAGAAGGATATTCCTGGAC	AGAGAGATTGAAAAGTTTGCGGAT
Smad2	TAGGTGGGGAAGTTTTTGCTGA	CGTCTGCCTTCGGTATTCTG
TGF-β1	GCA GAA CCC AAA AGC CAG AGT G	CCA TAA CTA CCG TGG AGG TTG A
RhoA	CTC ATA GTC TTC AGC AAG GAC CAG TT	ATC ATT CCG AAG ATC CTT CTT ATT
Smad3	GGAGGGCAGGCTTGGGGAAAATG	GGGGAGGGTGCCGGTGGTGTAATA
Smad4	AAGGTGAAGGTGATGTTTG	GAGCTATTCCACCTACTG AT

TG2	GGAGGATATCACCCACACCTACA	CGTAAGGCAGTCACGGTATTTC
TIMP-1	TGGGGACACCAGAAGTCAAC	TTTTCAGAGCCTTGGAGGAG
TIMP-2	GTCAGTGAGAAGGAAGTGGACTCT	ATGTTCTTCTCTGTGACC
TNF-α	TGCACCACAGTTTAAACCCA	GACTCCTTCAGGTGCTCAGG

Table 2. Primary antibodies used in western blot analysis

1_{st} Antibody	company	dilution rate
Akt	Santa Cruz Biotechnology, Inc.	1:500
ATG12	Santa Cruz Biotechnology, Inc.	1:1000
ATG7	Santa Cruz Biotechnology, Inc.	1:1000
BECN1	Santa Cruz Biotechnology, Inc.	1:1000
Cathepsin B	Santa Cruz Biotechnology, Inc.	1:1000
Cathepsin D	Santa Cruz Biotechnology, Inc.	1:1000
Cathepsin G	Santa Cruz Biotechnology, Inc.	1:1000
Cathepsin K	Abcam	1:1000
Cathepsin L	Santa Cruz Biotechnology, Inc.	1:1000
c-fos	Santa Cruz Biotechnology, Inc.	1:1000
c-jun	Santa Cruz Biotechnology, Inc.	1:1000
COL1A	Santa Cruz Biotechnology, Inc.	1:1000
Cox2	Santa Cruz Biotechnology, Inc.	1:1000
Cyr61	Santa Cruz Biotechnology, Inc.	1:1000
EGFR	Santa Cruz Biotechnology, Inc.	1:1000
Elastin	Santa Cruz Biotechnology, Inc.	1:1000
elf4B	Bethyl Laboratories, Inc.	1:1000
ERK	Santa Cruz Biotechnology, Inc.	1:1000

GAPDH	Santa Cruz Biotechnology, Inc.	1:1000
GST	Abcam	1:1000
HAS1	Santa Cruz Biotechnology, Inc.	1:1000
HAS2	Santa Cruz Biotechnology, Inc.	1:1000
HAS3	Santa Cruz Biotechnology, Inc.	1:1000
HO-1	Santa Cruz Biotechnology, Inc.	1:1000
IL-1β	Santa Cruz Biotechnology, Inc.	1:1000
IL-6	Abcam	1:1000
JNK	Santa Cruz Biotechnology, Inc.	1:1000
LAMP-1	Santa Cruz Biotechnology, Inc.	1:1000
LC3	Cell Signaling TECHNOLOGY	1:1000
MMP-1	Enzo Life Sciences	1:1000
MMP-13	Santa Cruz Biotechnology, Inc.	1:1000
MMP-2	Santa Cruz Biotechnology, Inc.	1:1000
MMP-8	Santa Cruz Biotechnology, Inc.	1:1000
Nrf2	Enzo Life Sciences	1:1000
p38	Santa Cruz Biotechnology, Inc.	1:1000
p62/SQSTM1	Cell Signaling TECHNOLOGY	1:1000
p70S6K	Bethyl Laboratories, Inc.	1:1000
p-Akt (Ser473)	Santa Cruz Biotechnology, Inc.	1:1000
PDK1	Bethyl Laboratories, Inc.	1:1000

p-ERK	Santa Cruz Biotechnology, Inc.	1:1000
PI3K	Santa Cruz Biotechnology, Inc.	1:1000
p-JNK	Santa Cruz Biotechnology, Inc.	1:1000
p-mTOR (Ser2448)	Bioworld Technology, Inc.	1:500
p-p38	Santa Cruz Biotechnology, Inc.	1:1000
p-p70S6K	Bethyl Laboratories, Inc.	1:1000
p-Smad2	Santa Cruz Biotechnology, Inc.	1:500
p-Smad3	Santa Cruz Biotechnology, Inc.	1:500
Raptor	Santa Cruz Biotechnology, Inc.	1:500
Rictor	Santa Cruz Biotechnology, Inc.	1:500
RPS6	Bethyl Laboratories, Inc.	1:1000
Smad2	Santa Cruz Biotechnology, Inc.	1:500
Smad3	Bioworld Technology, Inc.	1:500
Smad4	Santa Cruz Biotechnology, Inc.	1:500
TG2	Abcam	1:1000
TGFβ RI	Santa Cruz Biotechnology, Inc.	1:1000
TGFβ RII	Santa Cruz Biotechnology, Inc.	1:1000
TGF-β1	Santa Cruz Biotechnology, Inc.	1:1000
TNF-α	Santa Cruz Biotechnology, Inc.	1:1000

Ⅲ. RESULTS

1. Purification and identification of MAAs

A major impediment to further research investigating the potential bioactivities of MAAs is the absence of commercially available standards to confirm the identity of MAAs based on LC retention times and molar extinction coeffcients alone, or LC/MS methods. More recent reports have elucidated the MS stectra of the principal MAAs by electrospray ionisation mass spectrometry (ESI-MS; Whitehead and Hedges, 2002). In order to establish optimum condition to separate a HPLC chromatogram of crude extract, various types of columns and mobile phase conditions were examined for the crude extract of *Porphyra yezoensis*. From the optimized HPLC chromatogram, three known compounds previously identified from *P. yezoensis* were successfully identified by direct comparison of their molecular weight and retention order with reported data (Mushir and Fatma, 2011; Takano *et al*., 1979; Tao *et al*., 2008; Whitehead and Hedges, 2002).

The absorption sectrum of the crude extract of *P. yezoensis* indicated that the MAAs presented a metabolite with absorption in the UVA region (315 ~ 400 nm). Fig. 6. shows the absorption spectra of a purified peaks from a *P. yezoensis.* The absorption spectrum was characterized by a sharp absorption maximum at 334 nm. HPLC analysis revealed that the *P. yezoensis* contain a complex mixture of MAAs. HPLC chromatograms showed that the three peaks of the crude extract had retention time of 2.29 min (peak 1), 2.493 min (peak 2) and 11.53 min (peak 3).

From UV-absorbance maxima (Fig. 7) and ESI-MS molecular ion spectra (Fig. 8 and 9) was employed to confirm that MAAs were present in the crude extract of *P. yezoensis*. Peak 1 eluted at 2.29 min with a λ_{max} of 320 nm and $[M+H]^+$ molecular ion of *m/z* 245.1. Peak 2 exhibited at 2.493 min with a λ_{max} of 330 nm and $[M+H]^+$ molecular ion of *m/z* 289.1. Peak 3 exhibited at 11.53 min with a λ_{max} of 334 nm, and $[M+H]^+$ molecular ion of *m/z* 347.1 and $[M-H]^-$ molecular ion of *m/z* 345.1. The UV spectrum and ESI-MS molecular ion spectrum, therefore, verified peak 1 as palythine, peak 2 as asterina-330 and peak 3 as porphyra-334 in the crude extract of *P. yezoensis*.

The data presented in this study suggest that porphyra-334 can serve as a UVA protecting sunscreen by providing wide protection against UV radiation. Furthermore, this MAA dissipated UV energy without creating any reactive species which might cause a phototoxic effect on living organisms.

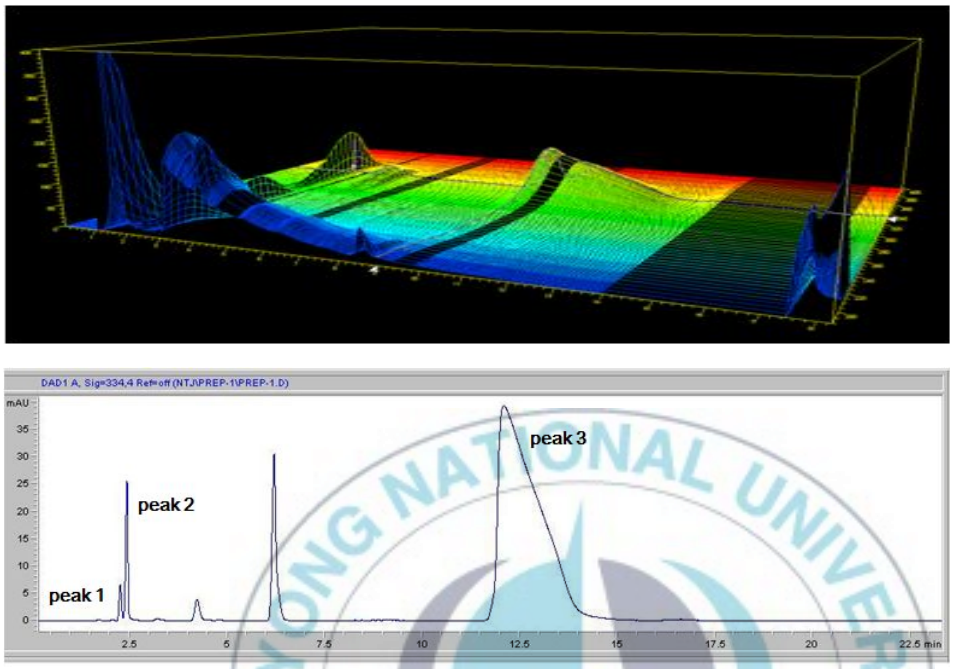


Fig. 6. HPLC chromatogram of the crude extract of *Porphyra yezoensis* monitored at 334 nm. Whole 3-dimensional chromatogram was represented in the upper panal. Three characterizing MAAs were idntified by UV spectrum of each peak, retention order, and exact molucular weight recorded from ESI-MS analyser, respectively.

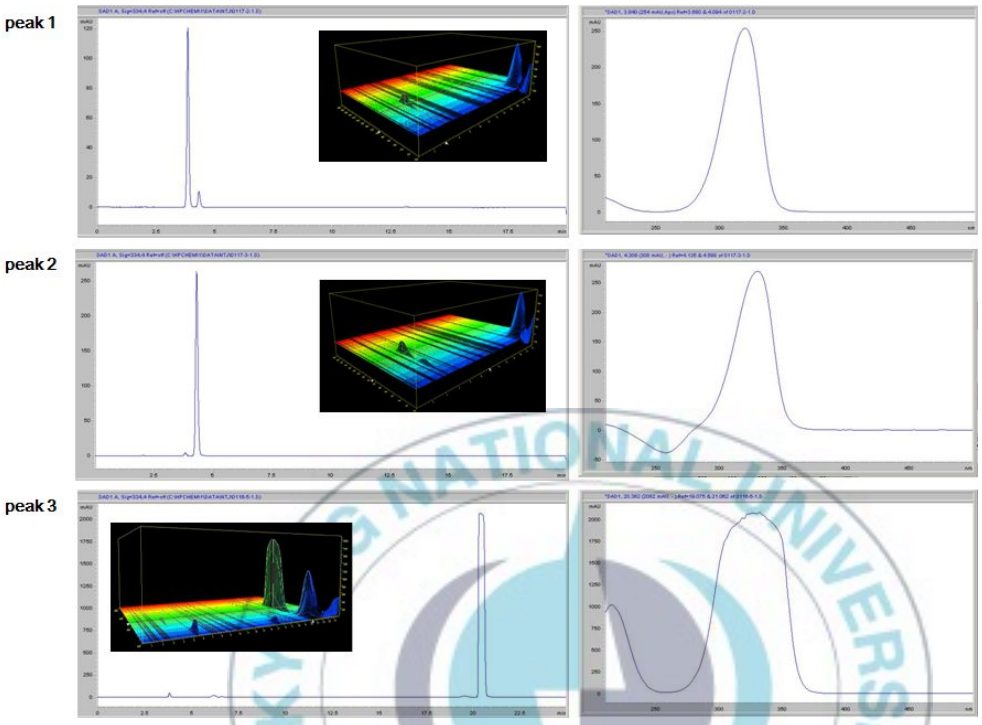
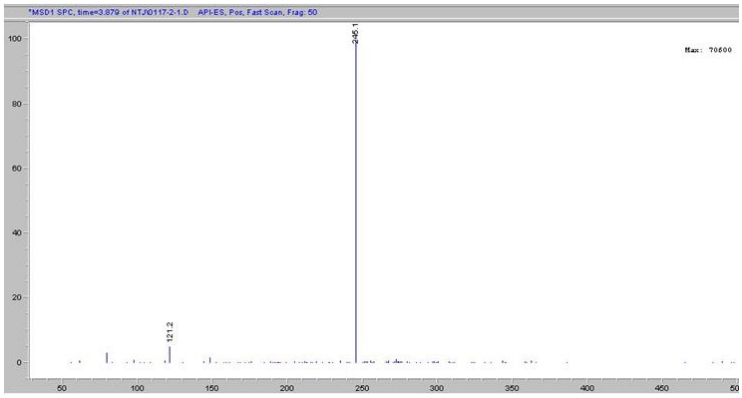


Fig. 7. HPLC chromatograms of individual peaks (1, 2, and 3) of MAAs showing the retention times of MAAs in *Porphyra yezoensis* (334 nm) for indicated time periods.

(A)



(B)

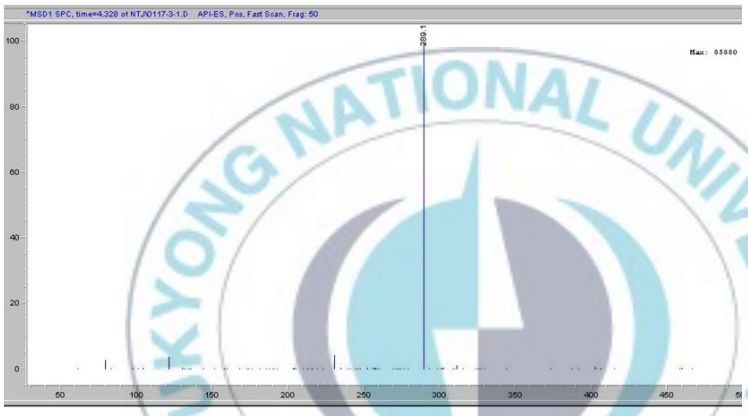
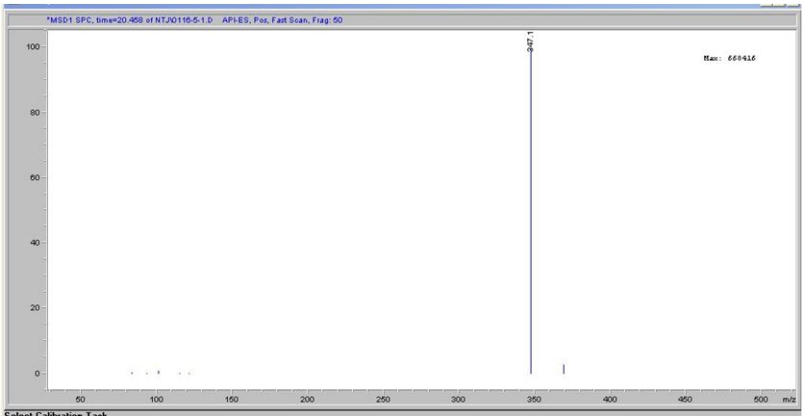


Fig. 8. ESI-MS spectra of major MAAs present in *Porphyra yezoensis* crude extract (peak 1 and peak 2). The crude extract was subjected to HPLC-DAD-ESI-MS. The MS scans were performed for positive molecular ions (*m/z* 100 to *m/z* 600). (A) Mass spectra of peak 1 at *m/z* 245.1 (RT 2.29 min), peak 2 at *m/z* 289.1 (RT 2.493 min) were identified as palythine, asterina-330.

(A)



(B)

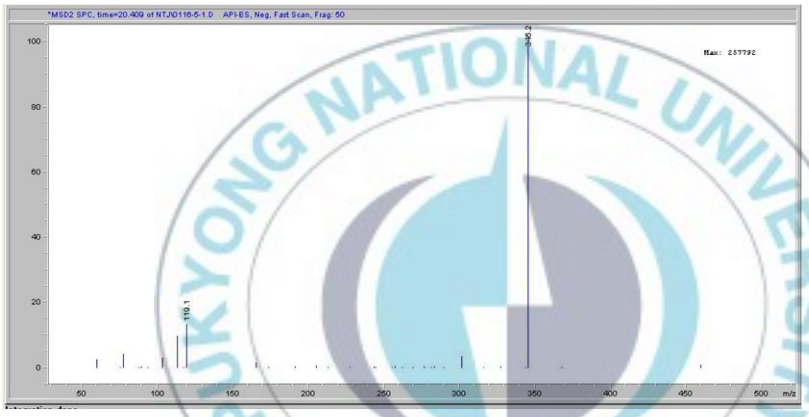
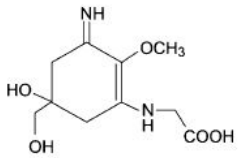
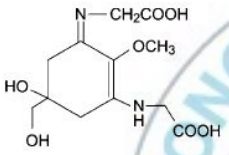
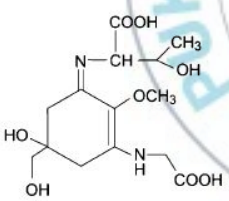


Fig. 9. ESI-MS spectra of major MAAs present in *Porphyra yezoensis* crude extract (peak 3). The crude extract was subjected to HPLC-DAD-ESI-MS. The MS scans were performed for positive and negative molecular ions (*m/z* 50 to *m/z* 500). (A) Mass spectra of peak 3 at positive molecular ion *m/z* 347.1 and (B) negative molecular ion *m/z* 345.1 were identified as porphyra-334.

Table 3. Molecular structure, extinction coefficients and molecular weights for isolated MAAs

peak no.	molecular structures	λmax	Extinction coefficient (per mol.cm)	Molecular weight (g/mol)	References
1	Palythine 	320	36200	244.11	Dunlap and Chalker, 1986
2	Asterina-330 	330	43500	288.13	Dunlap and Yamamoto, 1995
3	Porphyra-334 	334	42300	346.14	Takano *et al*., 1979

2. Effect of UVA irradiation on cell cytotoxicity

The effect of UVA irradiation on cell viability of human skin fibroblasts were exposed to various UVA at different intensities, and the results were displayed in Fig. 10. The cell cytotoxicity was measured by MTS assay and cells exposed to UVA irradiation reduced the cell viability in a time-dependant manner. Cell viability showed that cells were significantly damaged after 24 h incubation followed by UVA irradiation. Therefore, incubation period for 24 h was chosen as the optimum condition. In case of UVA irradiation dosage, 10 J/cm^2 was chosen as th optimum condition. 5 J/cm^2 did not initiate the cell damage, while 15 J/cm^2 irradiation caused higher cell damage than the optimum irradiation level which initiates the cell damage with no significant cell loss.

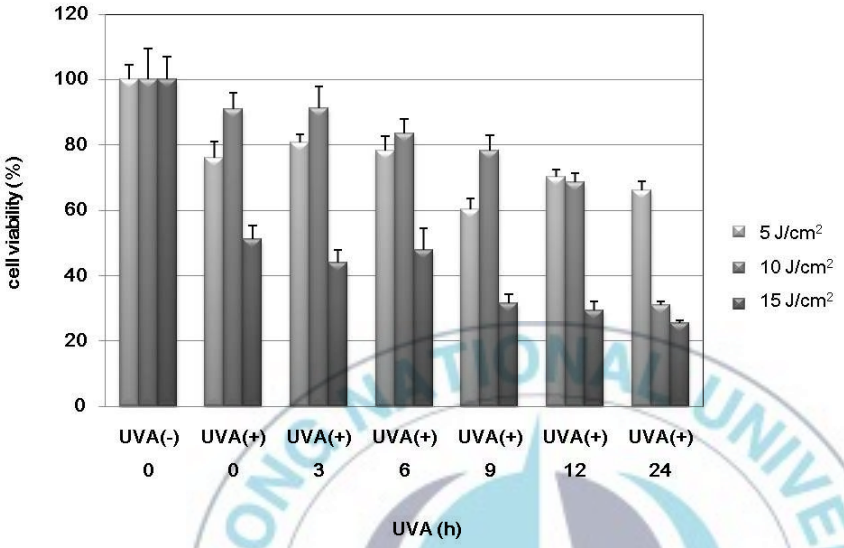


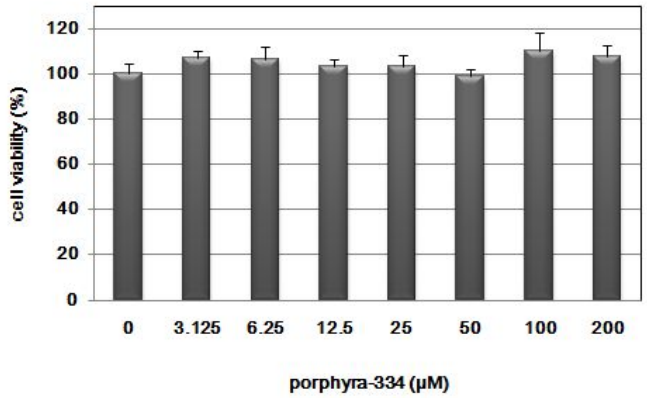
Fig. 10. Dose-dependent effect of UVA irradiation on human skin fibroblasts cell viability. Cells were exposed to UVA irradiation, the cytotoxicity levels were determined by MTS assay in a time dependent manner.

3. Effect of porphyra-334 on cell viability

To evaluate the cytotoxic effect of porphyra-334 on human skin fibroblasts, MTS assay was carried out. Human skin fibroblasts were 24 h incubated with or without porphyra-334 at concentrations of 0 to 200 μM, respectively. As shown in Fig. 11(A), any significant toxic effect was not observed on the cells treated with porphyra-334 incubated with 24 h. Furthermore, Human skin fibroblasts were 40 h incubated with porphyra-334 at concentrations of 0 to 200 μM, up to 20% proliferation effect was observed in Fig. 11(B). As shown in Fig. 12, observation of morphological changes caused by porphyra-334 was observed by light microscope, any significant change was not observed on human skin fibroblasts.

In order to determine the protective effect of porphyra-334 on UVA-induced cell damage, human skin fibroblasts were incubated with various concentrations of porphyra-334 (0-40 μM) which were previously stimulated with UVA irradiation. The cell viability was evaluated using MTS assay. As shown in Fig. 13, treatment with porphyra-334 was significantly protected cell damage in a dose-dependent manner.

(A)



(B)

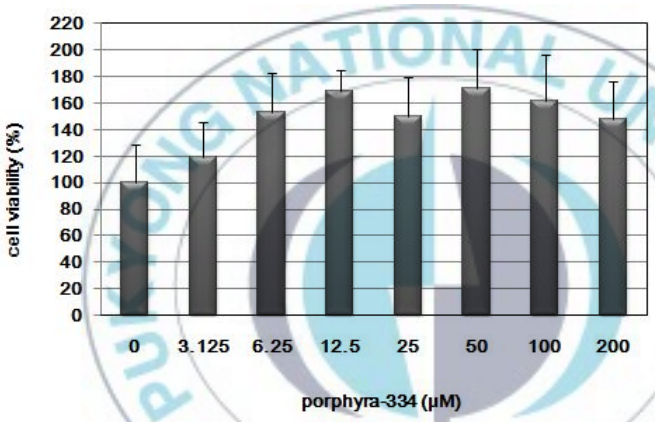


Fig. 11. Effect of porphyra-334 on viability in human skin fibroblasts. The viability of cells was determined by the MTS assay as described in materials and methods. (A) Cell viability was not affected with porphyra-334 treatment (3.125-200 μM) at 24 h (compared with 0 μM group, $p < 0.05$). (B) Increased cell viability was observed with porphyra-334 treatment at 40 h.

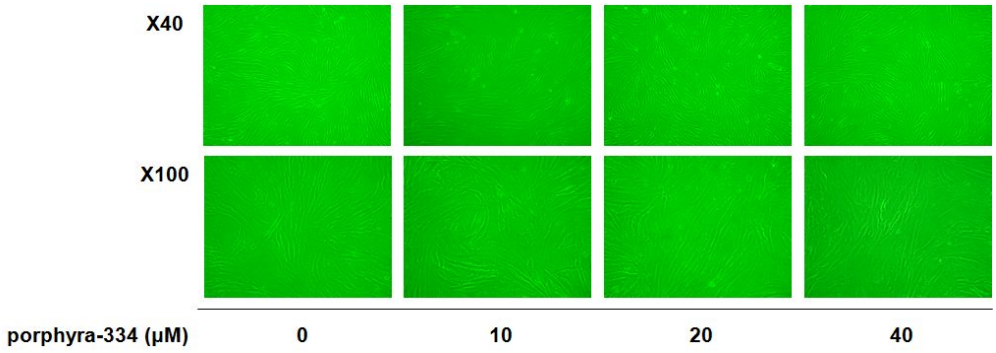
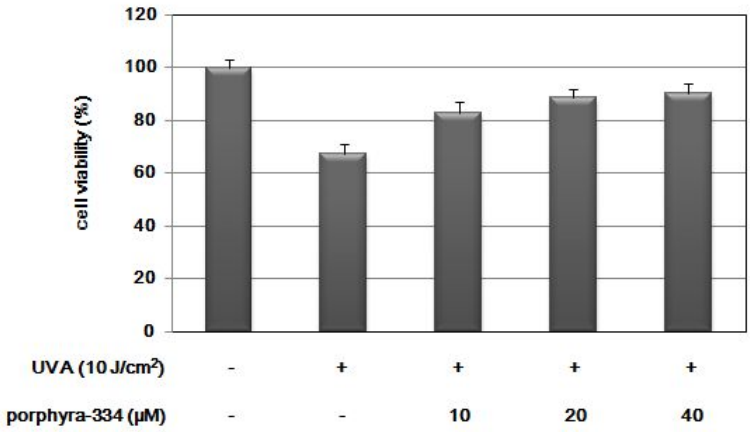
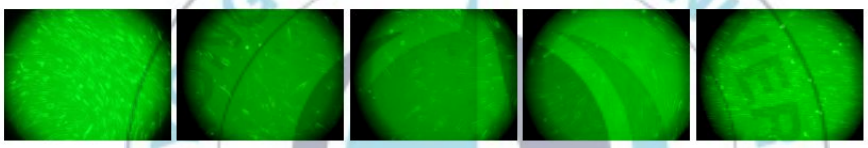


Fig. 12. Effect of porphyra-334 on phenotypic characteristics of cultured human skin fibroblasts. Cell viability was not affected with porphyra-334 treatment (0-40 μM) at 24 h.

(A)



(B)

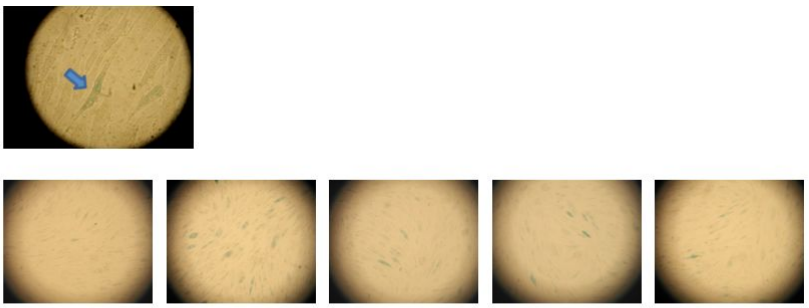


UVA (10 J/cm²)	-	+	+	+	+
porphyra-334(μM)	-	-	10	20	40

Fig. 13. Effect of porphyra-334 on UVA-induced human skin fibroblasts viability. Cells were exposed to UVA (10 J/cm^2) irradiation, the cell viability (%) were determined by MTS assay (A) and morphology (B) in a various concentration of porphyra-334 after UVA treatment.

4. Inhibitory effects of porphyra-334 on UVA-induced SA-β-gal activity

The SA-β-gal staining was performed to observe the SA-β-gal activity, one of the biomarkers of senescence. Images clearly indicated that cells were induced to a senescence-like state by UVA irradiation. As shown in Fig. 14, the inhibitory activity of porphyra-334 was also observed and found to effectively suppress the expression of SA-β-gal in a dose-dependent manner.

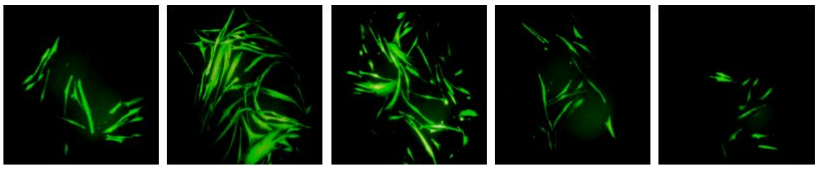


UVA (10 J/cm^2)	-	+	+	+	+
porphyra-334(μM)	-	-	10	20	40

Fig. 14. UVA irradiation induces senescence-associated beta-galactosidase (SA-β-gal) expression in human skin fibroblasts and stress-induced premature senescence. The expression of SA-β-gal was detected using cytochemical staining method as described in materials and methods, and cell in blue was considered as positive for SA-β-gal staining. Porphyra-334 treatment for 24 h inhibits the activity of SA-β-gal in a dose-dependent manner in HDFs, and a significant inhibitory effect on SA-β-gal activity was observed at doses of 10, 20 and 40 μM (compared with UVA group).

5. Effect of porphyra-334 on UVA induced intracellular ROS levels

To determine if porphyra-334 functions as a scavenger of UVA-induced ROS, intracellular ROS levels were measured. As shown in Fig. 15, The level of ROS in UVA-irradiated cells were increased when compared with non-irradiated cells. Treatment with porphyra-334 led to a significant reduction of DCF-DA fluorescence intensity, resulting in increased scavenging activity against intracellular ROS formation in a dose-dependent manner compared to non-irradiated control. Furthermore, UVA-activation of the cellular antioxidant response was substantiated by immunodetection of increased protein levels of the trascription factor Nrf2 and its downstream target heme oxygenase-1 (HO-1) (Fig. 16).



UVA ($10\ J/cm^2$)	-	+	+	+	+
porphyra-334(μM)	-	-	10	20	40

Fig. 15. Effect of porphyra-334 on UVA-induced ROS generation in human skin fibroblasts.

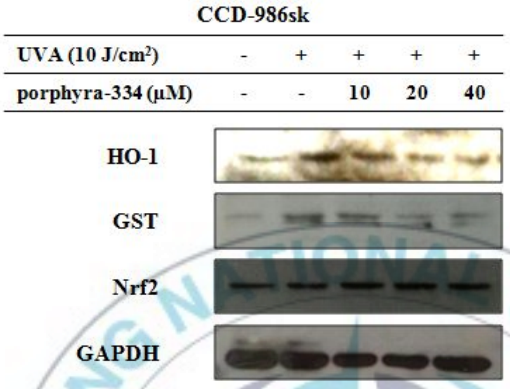


Fig. 16. UVA-induced up-regulation of oxidative stress response in human skin fibroblasts. Cells were exposed to 10 J/cm^2 of UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of HO-1, GST and Nrf2 were determined by Western blot analysis. GAPDH was used as an internal standard.

6. Effect of porphyra-334 on UVA-induced pro-inflammatory cytokines production

UVA activation of human skin fibroblasts induced pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 plays important role in photoaging (Rabe, 2006). Levels of TNF-α, IL-1β and IL-6 in human skin fibroblasts culture supernatants after UVA irradiation with or without porphyra-334 were measured using ELISA. The levels of TNF-α, IL-1β and IL-6 in the cell supernatant were markedly increased after 10 J/cm^2 UVA stimulation for 24 h. UVA-induced porphyra-334 treatment produced a dose-dependent inhibition of TNF-α (Fig. 17), IL-1β (Fig. 18) and IL-6 (Fig. 19) production. The concentration dependent effect of porphyra-334 treatment on pro-inflammatory cytokines level in cultured media was significantly lower than in the UVA-irradiated control. The amount of TNF-α, IL-1β and IL-6 measured after UVA irradiation was 42.47±2.10, 137.5±15.43 and 165.94±15.85 pg/mL, which was higher than the level observed for non-irradiated cells. porphyra-334 significantly protected the cells against the secretion of pro-inflammatory cytokines after UVA exposure, and porphyra-334 treatment reduced pro-inflammatory cytokines production (TNF-α; 24.9±0.27 pg/mL at 10 μM, 22.54±0.51 pg/mL at 20 μM and 22.18 pg/mL at 40 μM, IL-1β; 118.95±2.76 pg/mL at 10 μM, 98.79±4.82 pg/mL at 20 μM and 78.63±2.05 pg/mL at 40 μM, IL-6; 82.40±11.12 pg/mL at 10 μM, 62.81±2.65 pg/mL at 20 μM and 56.88±2.21 pg/mL at 40 μM. Pro-inflammatory cytokines secretion by UVA-irradiated human skin fibroblasts was reduced by porphyra-334 treatment in a concentration-dependent manner. These results suggested that the presence of

porphyra-334 effectively attenuated the secretion of inflammatory cytokines by UVA irradiation in human skin fibroblasts.

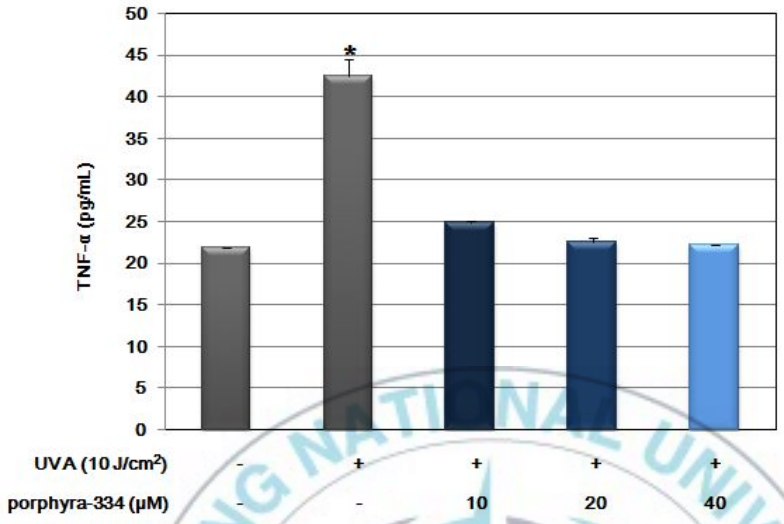


Fig. 17. Effect of porphyra-334 on TNF-α production in UVA irradiated human skin fibroblasts. Cells were stimulated with 10 J/cm^2 of UVA irradiation and then treated with various concentrations of porphyra-334 for 24 h. The concentration of cytokines in the supernatants was determined by ELISA. Vlaues are the mean ± S.D. of triplicate experiments. *$p < 0.05$.

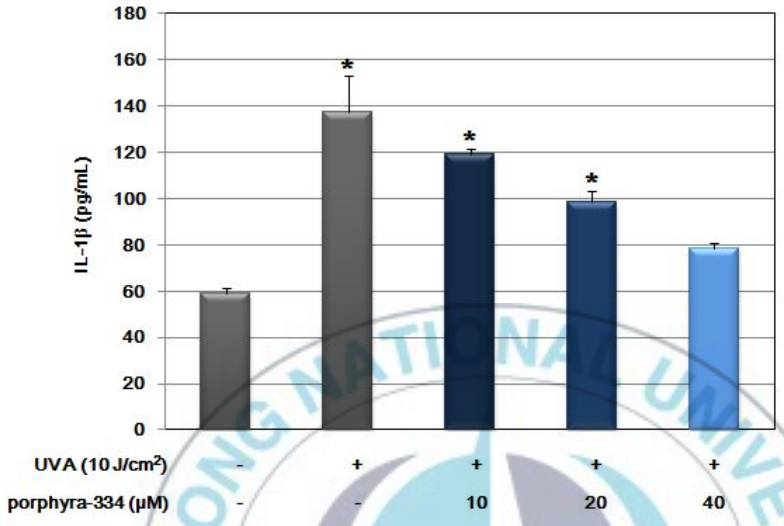


Fig. 18. Effect of porphyra-334 on IL-1β production in UVA irradiated human skin fibroblasts. Cells were stimulated with 10 J/cm^2 of UVA irradiation and then treated with various concentrations of porphyra-334 for 24 h.

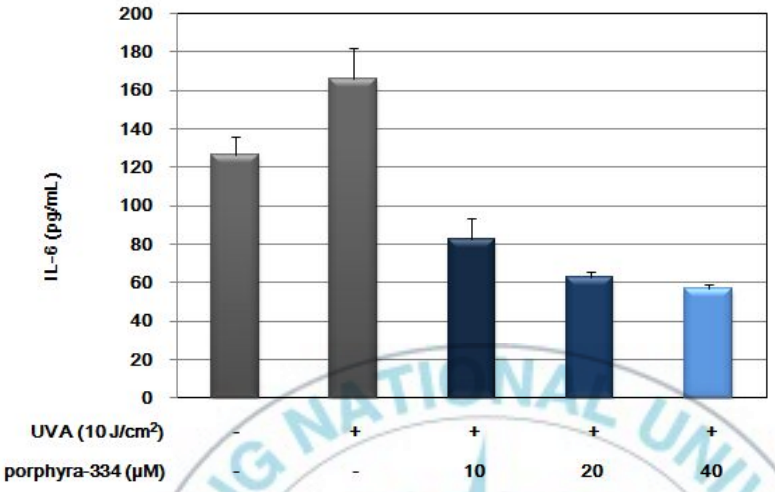


Fig. 19. Effect of porphyra-334 on IL-6 production in UVA irradiated human skin fibroblasts. Cells were stimulated with 10 J/cm^2 of UVA irradiation and then treated with various concentrations of porphyra-334 for 24 h.

7. Effect of porphyra-334 on UVA-induced inflammatory response

In order to evaluate the effect of porphyra-334 on UVA-induced inflammation, the basal and UVA-modulated production of TNF-α, IL-1β and IL-6 in human skin fibroblasts were determined by RT-PCR and western blot analysis, respectively. As shown in Fig. 20, TNF-α mRNA expression was increased by UVA irradiation treatment when compared to basal levels. The UVA induced TNF-α mRNA expression was markedly prevented by a treatment of porphyra-334 at doses of 10, 20 and 40 μM in comparison to the group with UVA irradiation only. By western blot analysis, porphyra-334 was shown to reduce the up-regulated expression of TNF-α in UVA treated human skin fibroblasts (Fig. 21), which corresponded to the RT-PCR results. In accordance with the previous ELISA assay results for cytokine secretion, porphyra-334 showed the highest efficiency to reduce the expression of pro-inflammatory cytokine TNF-α mRNA and protein levels.

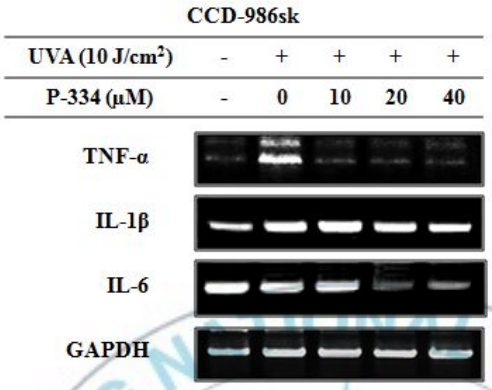


Fig. 20. Effect of porphyra-334 on mRNA expression levels of TNF-α, IL-1β and IL-6 in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.

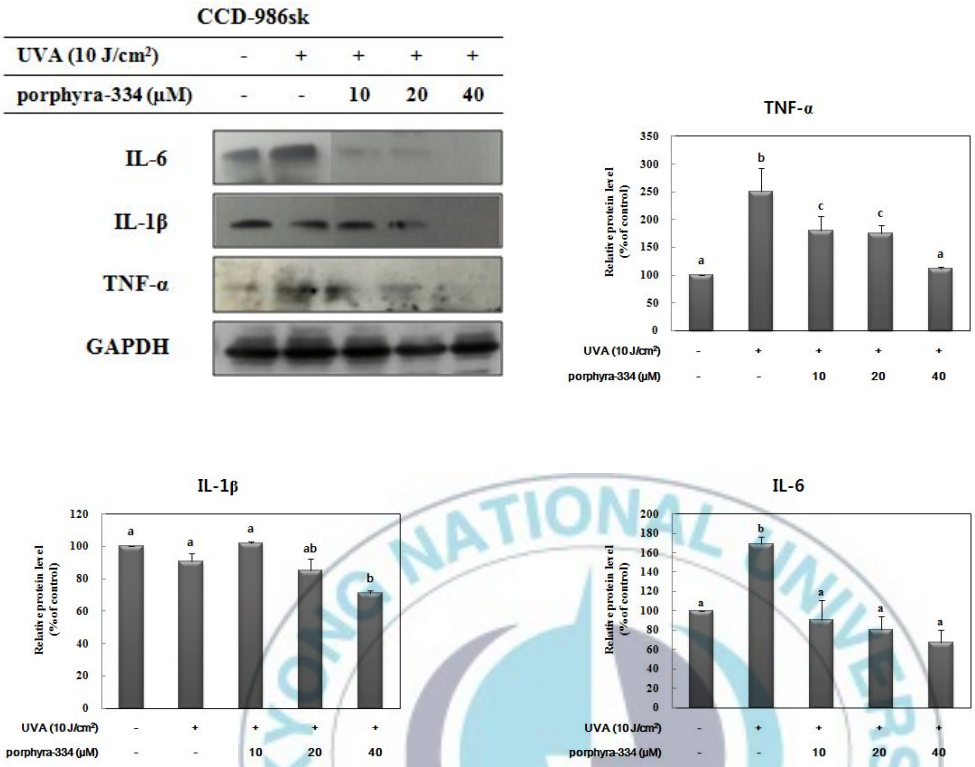


Fig. 21. Effect of porphyra-334 on protein expression of TNF-α, IL-1β and IL-6 in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

8. Effect of porphyra-334 on UVA-induced COX-2 and iNOS expression

To assess the effects of porphyra-334 in human skin fibroblasts, the cells were exposed to various concentrations of porphyra-334 for 24 h after stimulation with UVA irradiation. The cells were prepared for RT-PCR and western blot analysis. As shown in Fig. 22, UVA-irradiation significantly increase the level of COX-2 mRNA expression when compared with the UVA-induced COX-2 mRNA expression was prevented by porphyra-334 in concentration-dependent manner. Western blot demonstrated that UVA-irradiation caused significant increase in the level of COX-2 expression when compared with the UVA-untreated control, and porphyra-334 tended to block this effect.

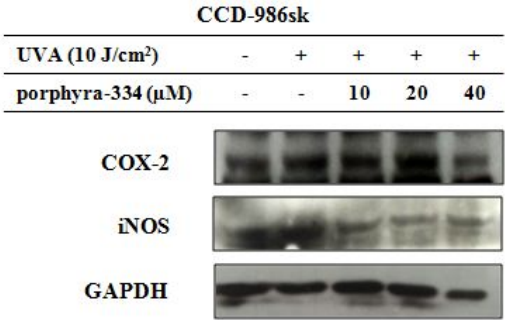


Fig. 22. Effect of porphyra-334 on the levels of iNOS and COX-2 in UVA-induced human skin fibroblasts. Cells were exposed to 10 J/cm^2 of UVA irradiation and incubated with porphyra-334 for 24 h. The protein levels of COX-2 and iNOS were determined by western blot analysis. GAPDH was used as a loading control.

9. Effect of porphyra-334 on UVA-induced intracellular procollagen

To evaluate the effect of porphyra-334 on the synthesis of collagen, human skin fibroblasts were treated with various concentrations of porphyra-334 (0-40 μM) over 24 h. The procollagen secretion level was determined in the culture medium by ELISA assay. As shown in Fig. 23, the collagen content was 319.5 ± 0.069 ng/mL in the non-irradiated cells, but collagen was 217.333 ± 0.177 ng/mL in UVA-irradiated cells. Further, procollagen secretion level was increased by 269.167 ± 9.090, 253.833 ± 1.464 and 271.833 ± 7.224 ng/mL in the presence of porphyra-334 at the concentration of 10, 20 and 40 μM, respectively. As a result, porphyra-334 increased collagen synthesis in a dose-dependent manner. Thus, porphyra-334 have protective effect on collagen degradation by enhancement of collagen synthesis in photo-damaged human skin fibroblasts.

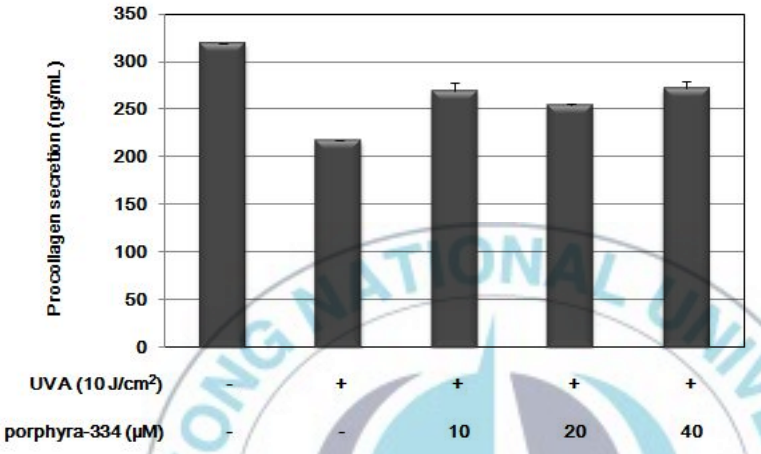


Fig. 23. Effect of porphyra-334 on UVA-induced production of procollagen levels in human skin fibroblasts.

10. Effect of porphyra-334 on UVA-induced elastase activity

Elastin also plays an important role in human skin fibroblasts (Bernstein *et al*., 1994). UV irradiation has been shown to cause elastin degradation by activating elastase (Seite *et al*., 2006) and loss of skin elastin caused by UVA irradiation results in wrinkle formation. In this study, elastase activity of human skin fibroblasts as a reponse to porphyra-334 was confirmed by released p-nitroaniline. As shown in Fig. 24, elastase activity was elevated 51.9% found in supernatant and intracellular respectively after UVA irradiation compared to non irradiation cells. This increase in elastase inhibitory effect was effectively decreased by porphyra-334 treatment at 10, 20 and 40 μM after UVA irradiation by 51.9%, 51.9% and 82.5% compared to UVA irradiation cells respectively.

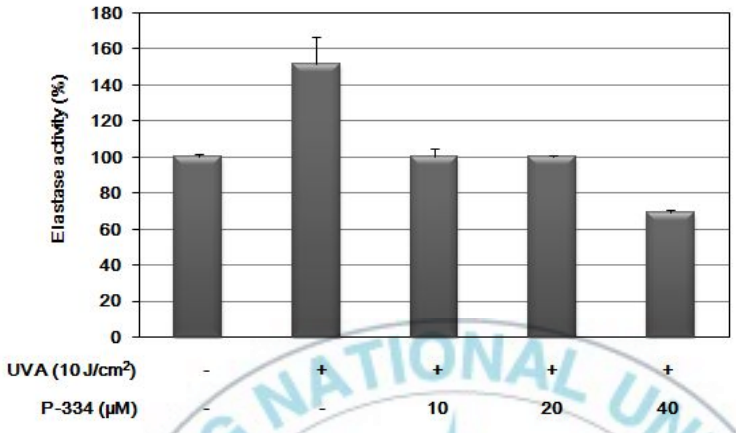


Fig. 24. Effect of porphyra-334 on UVA irradiation induced elastase activity in cultured human skin fibroblasts.

11. Effect of porphyra-334 on UVA-induced collagen and elastin degradation

In order to examine the effect of porphyra-334 on elastin and collagen degradation, human skin fibroblasts were incubated with various concentrations of porphyra-334 (10-40 μM) which were previously stimulated with UVA irradiation. The expressions of the specific elastin and type Ⅰ collagen at mRNA and protein levels were determined by western blot and RT-PCR analysis.

In fibroblasts, the collagen molecules are synthesized and secreted into the extracellular space as procollagens. Until now many of collagen has been identified, but the most abundant collagen component of skin is type Ⅰ collagen (Rittié and Fisher, 2002). Also, elastin is an important in connective tissue that allows many tissues in the body to resume their shape after stretching or contracting. The mRNA transcription and protein level of type Ⅰ collagen and elastin was shown in Fig. 25. Expression of type Ⅰ collagen was decreased in UVA alone irradiated cells whereas these decreased cellular levels of collagen result in UVA exposure were enhanced dose dependent manner in the presence of porphyra-334. Similarly, Expression of elastin was enhanced followed by porphyra-334 treatment after UVA irradiation. Western blot analysis (Fig. 26) showed parallel results with the RT-PCR. These result indicated that porphyra-334 might be involved in collagen synthesis by regulation of collagen degrading MMP expressions and elastin activity.

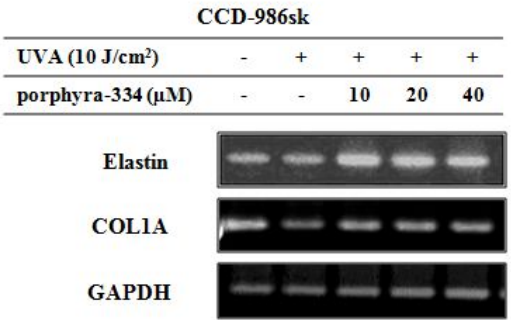


Fig. 25. Effect of porphyra-334 on mRNA expression levels of elastin and collagen in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.

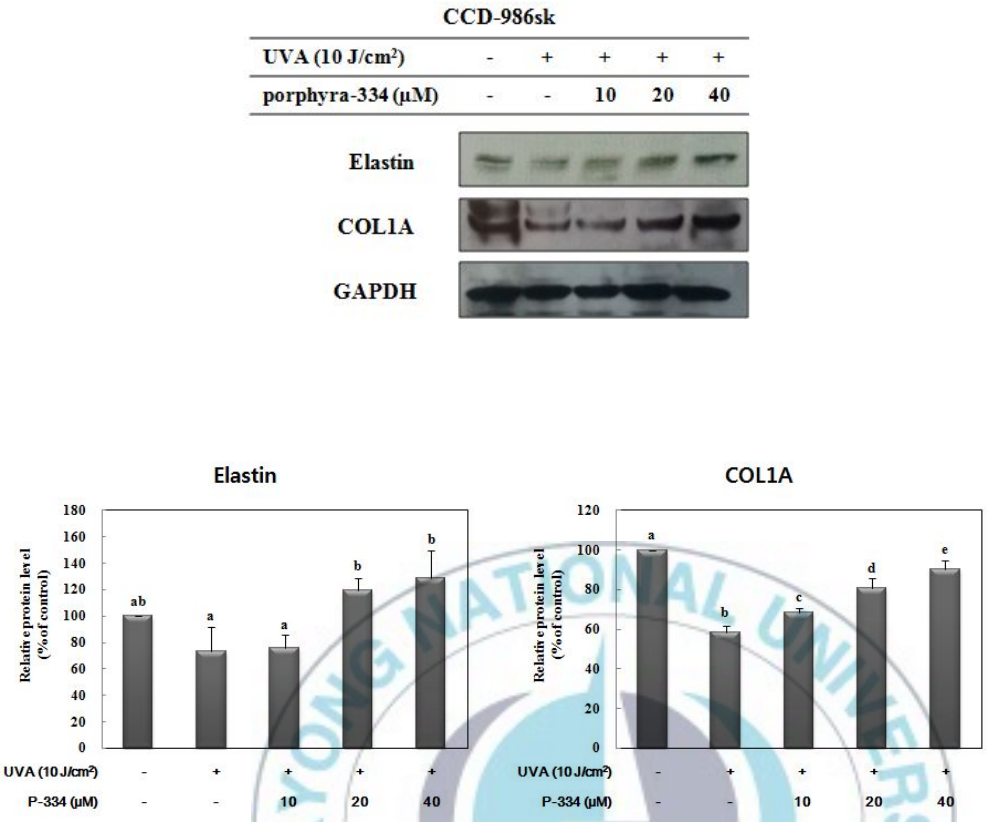


Fig. 26. Effect of porphyra-334 on protein expression of elastin and collagen in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

12. Effect of porphyra-334 on UVA-induced hyaluronan secretion

It has been reported that the exposure to UVA causes degradation of collagen and fibronectin in the extracellular matrix (Scharffetter *et al*, 1991). This decomposition of extracellular components is followed by increased levels of hyaluronan synthase in UVA irradiation. In this study, UVA-induced hyaluronan secretion was determined by ELISA. As shown in Fig. 27, UVA irradiation caused accumulation of hyaluronan in the human skin fibroblasts culture medium. Hyaluronan secretion level caused by UVA irradiation was successfully reduced by treatment with porphyra-334 at the concentration of 10 μM. At the porphyra-334 concentration of 40 μM, the highest reduction of hyaluronan synthase amount which is again at the same level with UVA non irradiated control. Therefore, treatment of porphyra-334 might be an effective strategy for the protection of UVA induced hyaluronan degradation.

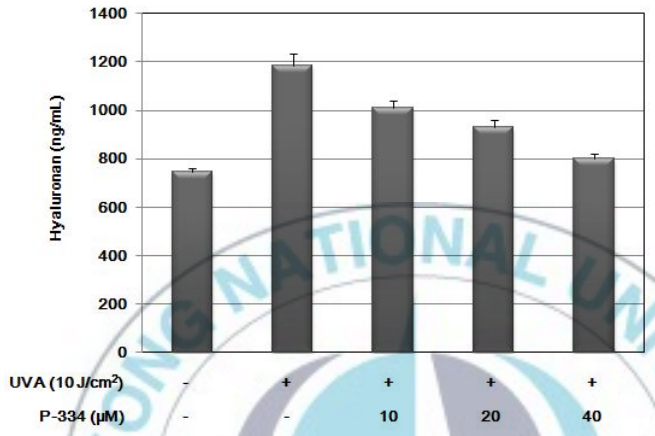


Fig. 27. Effect of porphyra-334 on UVA irradiation induced hyaluronan secretion in cultured human skin fibroblasts.

13. Effect of porphyra-334 on UVA-induced hyaluronan synthases expression

To examine the effect of porphyra-334 on UVA-induced hyaluronan synthases, human skin fibroblasts were incubated with various concentrations of porphyra-334 (10-40 μM) which were previously stimulated with UVA irradiation. The expressions of the specific HAS (HAS1, HAS2 and HAS3) at mRNA and protein levels were determined by western blot and RT-PCR analysis.

As shown in Fig. 28, the mRNA expression levels of hyaluronan synthases such as HAS1, HAS2 and HAS3 were significantly increased 10 J/cm^2 of UVA irradiation after 24 h. The expressions of HAS2 and HAS3 mRNA were increased by UVA irradiation, while HAS1 also increased, however, not as efficient as the HAS2 and HAS3. Collectively, protein levels of HAS1, HAS2 and HAS3 demonstrated by western blot analysis (Fig. 29), followed the same pattern with mRNA expression levels. porphyra-334 treatment significantly decreased the protein levels of HAS2 and HAS3 but failed to cause any change in protein level of HAS1.

The results revealed that HAS2 and HAS3 upregulation was a major factor in the fibroblast response and mRNA and protein expression levels of hyaluronan synthases were down-regulated after treatment with porphyra-334 in UVA irradiated human skin fibroblasts.

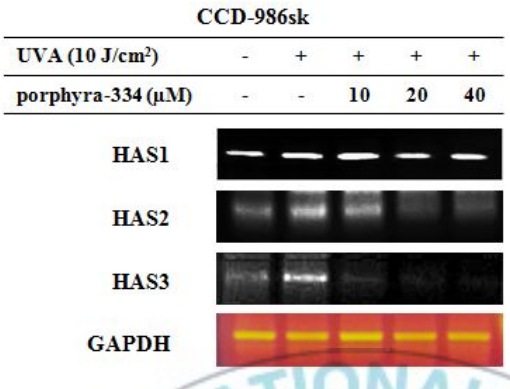


Fig. 28. Effect of porphyra-334 on mRNA expression levels of HAS1, HAS2 and HAS3 in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.

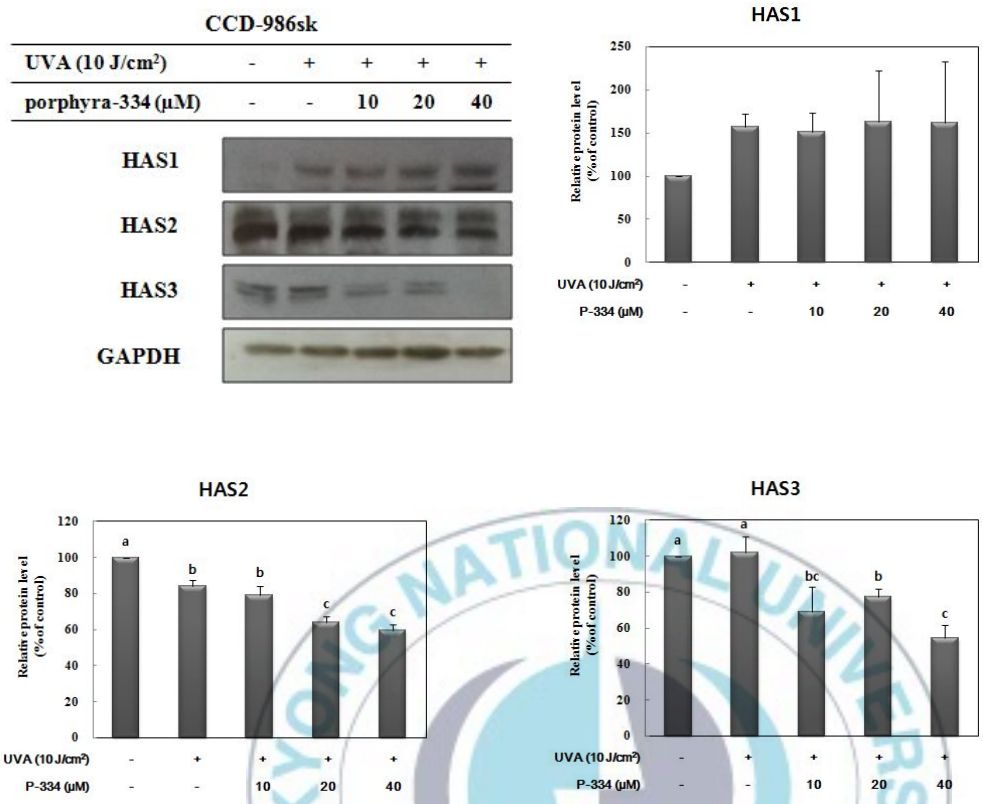


Fig. 29. Effect of porphyra-334 on protein expression of HAS1, HAS2 and HAS3 in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

14. Effect of porphyra-334 on UVA-induced MMP expression

In photoaging process, decomposition of dermal collagen is closely related to UVA irradiation by activating the matrix degrading enzymes as well as MMPs (Herrmann *et al*., 1993). Among MMPs, gelatinase (MMP-2) and collagenase (MMP-1, -8 and -13) which are considered to be involved in photoaging in relation to winkle formation (Kondo, 2000).

The effects of porphyra-334 on collagenase and gelatinase MMPs levels in UVA irradiated human skin fibroblasts were evaluated by RT-PCR (Fig. 30) and western blot analysis (Fig. 31). Irradiation of UVA on human skin fibroblasts increased the expression level of MMP-1 in the culture medium, significantly. The inhibitory effect of porphyra-334 on MMP-1 expression seemed not to originate from its cytotoxicity because porphyra-334 showed similar proliferation rate of human skin fibroblasts as control. To further investigate its dose-dependent effect, cells treated different concentrations of porphyra-334 ranging from 10 to 40 μM. The highest concentration (40 μM) of porphyra-334 inhibited MMP-1 expression level in UVA irradiated human skin fibroblasts up to 56.2% and the inhibition was concentration-dependent. The results of gelatinase MMP-2 and -8 resembled those of MMP-1 but not MMP-13. In case of effect of porphyra-334 on MMP-13 expression was observed to have a similar effect to that of UVA irradiation control. porphyra-334 exhibited a reducing effect on the elevated MMPs expression (except MMP-13) both in the gene and protein levels compared to control groups which was irradiated only without any treatment. Among these three MMPs, MMP-1 was the most active one in respect to

positive control.

According to these results, porphyra-334 was exhibited protective effect on UVA induced collagen degradation via negative regulation of MMPs expression.

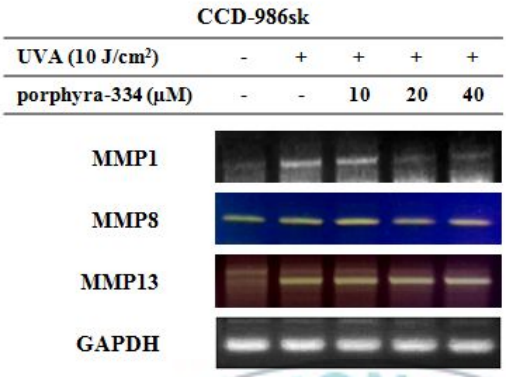
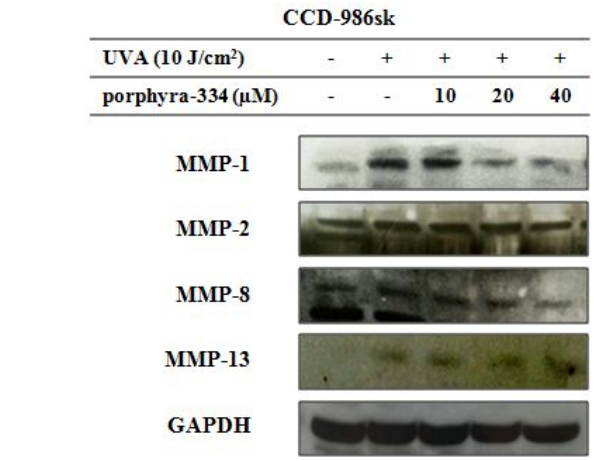


Fig. 30. Effect of porphyra-334 on mRNA expression levels of collagenases in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.



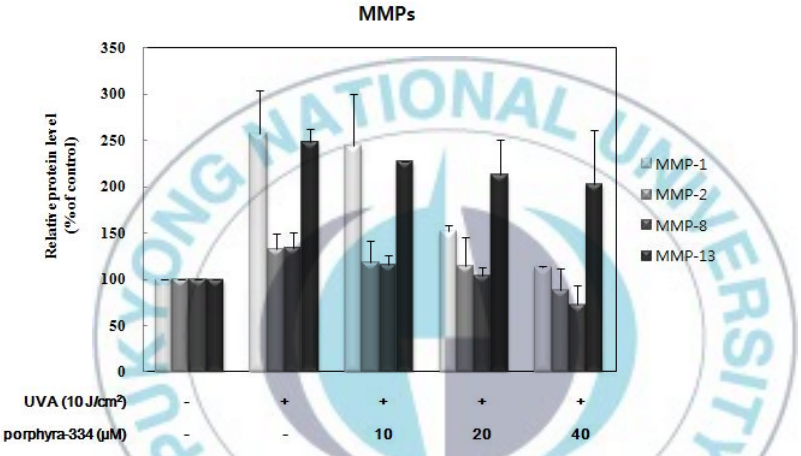


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Fig. 31. Effect of porphyra-334 on protein expression of MMPs in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

15. Effect of porphyra-334 on UVA-induced TIMPs expression

The changes of mRNA expression of TIMP-1 and TIMP-2 were evaluated in human skin fibroblasts under the treatment of UVA by RT-PCR (Fig. 32). TIMPs are a specific inhibitor of matrix metalloproteinases which is the key regulator of MMP activity and ECM degradation (Nagase *et al*., 2006). Thus, TIMPs are key mediators for decreased collagen synthesis through inhibiting collagenase enzyme activity and the expression of TIMP-1 and TIMP-2 were also evaluated. Following UVA irradiation, specific mRNAs of MMPs 1, 2, 8 and 13 were induced concomitantly up to 5-fold compared to mock irradiated controls. In contrast, TIMP-1 mRNA levels remained unaltered. The expression of TIMP-2 in human skin fibroblasts treated with UVA in the absence or presence of porphyra-334 was differ to that of TIMP-1. In case of TIMP-2, UVA irradiation significantly decreased the mRNA expression and protein level which were highly elevated after porphyra-334 treatment. Western blot analysis and RT-PCR demonstrated similar patterns.

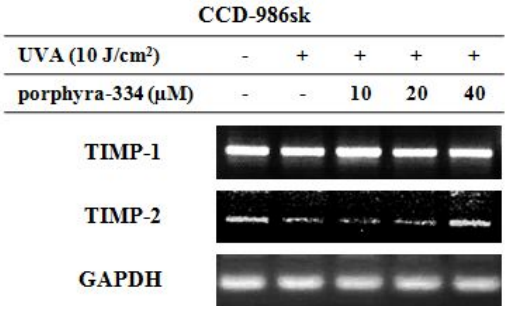

Fig. 32. Effect of porphyra-334 on mRNA expression levels of TIMP-1 and TIMP-2 in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these genes were detected using RT-PCR. GAPDH was used as an internal standard.

16. Effect of porphyra-334 on UVA-induced c-jun and c-fos expression

Oxidative stress caused by UV irradiation, ozone, hydrogen peroxide and free radicals may lead to activation of AP-1, thereby increasing MMPs expression and causing collagen degradation (Watanabe *et al*., 2004). MMP-1 promoter contains AP-1 binding site, and UV increased activation of AP-1 has been reported to increase MMP-1 expression (Kim *et al*., 2005). Because AP-1 complex includes c-jun and c-fos, to confirm the role of porphyra-334 in the regulation of c-jun and c-fos expressions, mRNA levels were measured by RT-PCR analysis in UVA irradiated human skin fibroblasts with or without porphyra-334. As shown in Fig. 33, UVA exposure significantly increased c-jun mRNA expression and porphyra-334 (10, 20, 40 μM) showed decreases as compared to the UVA-irradiated control. Also, UVA exposure increased c-fos mRNA significantly and porphyra-334 (10, 20, 40 μM) showed decreases as compared to the UVA-irradiated control.

AP-1 protein expression was examined by western blot analysis (Fig. 34). UVA irradiation of human skin fibroblasts upregulated the expressions of CYR61, c-jun and c-fos compared to the basal. The UVA induced upregulation of CYR61 protein level was markedly prevented by 20 μM of porphyra-334 treatement. The UVA induced c-fos protein level was significantly suppressed by 10 μM and higher concentration of porphyra-334 treatment. Though, there is no dose-depentent manner observed in the c-jun protein expression but importantly, there were significant reduced mRNA level detected in a dose-dependent manner.

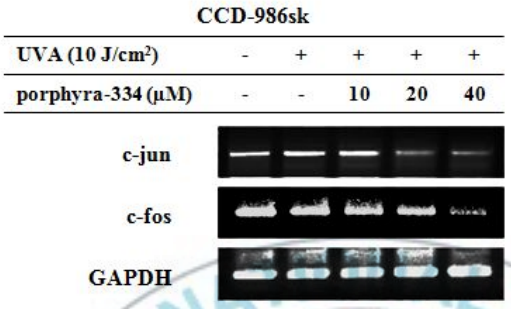

Fig. 33. Effect of porphyra-334 on mRNA expression levels of c-jun and c-fos in human skin fibroblasts. Cells were exposed to UVA irradiation (10 J/cm^2) and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR).

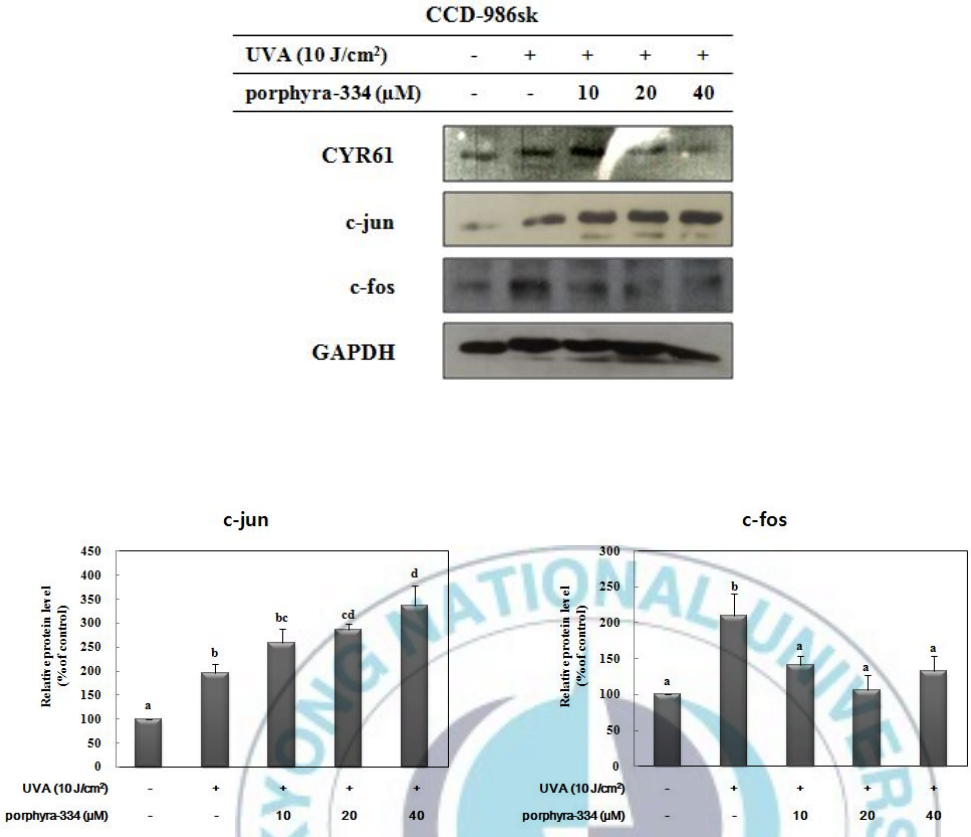

Fig. 34. Effect of porphyra-334 on AP-1 signaling cascade in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

17. Effect of porphyra-334 on UVA-induced MAP kinase

To determine whether porphyra-334 inhibited the activation of MAPK induced by UVA irradiation, the mRNA levels of ERK, JNK and p38 MAPK were measured by RT-PCR.

As shown in Fig. 35, the mRNA expressions of ERK2 and JNK MAPK after UVA irradiation were significantly higher than the control. The inhibitory effect of porphyra-334 on ERK2 and JNK mRNA levels were dose-dependent and the mRNA expression level of ERK2 returned to the basal level at 20 μM. In addition, treatment with porphyra-334 at a dose of 20 μM suppressed the mRNA expression of JNK but there was no dose-dependent manner observed in p38 MAPK mRNA level. To confirm these results, the effect of porphyra-334 on the phosphorylation of ERK, JNK and p38 MAPK in human skin fibroblasts were measured by western blot analysis (Fig. 36). Treatment of porphyra-334 (10-40 μM) with UVA induced the rapid phosphorylation of ERK, JNK, p38 MAPK and porphyra-334 blocked the activation of all the tested ERK and p38 MAPK.

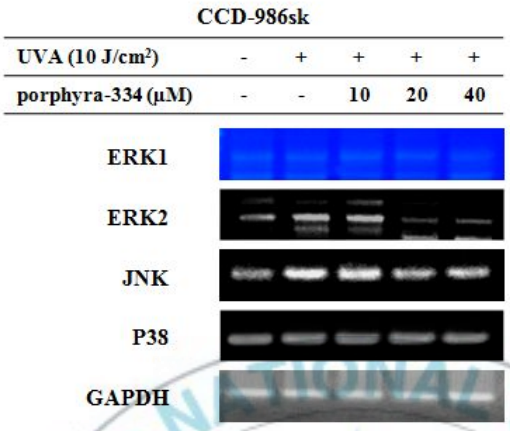

Fig. 35. Effect of porphyra-334 on mRNA expression levels of ERK1/2, JNK and p38 in human skin fibroblasts. Cells were exposed to UVA irradiation (10 J/cm^2) and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR).

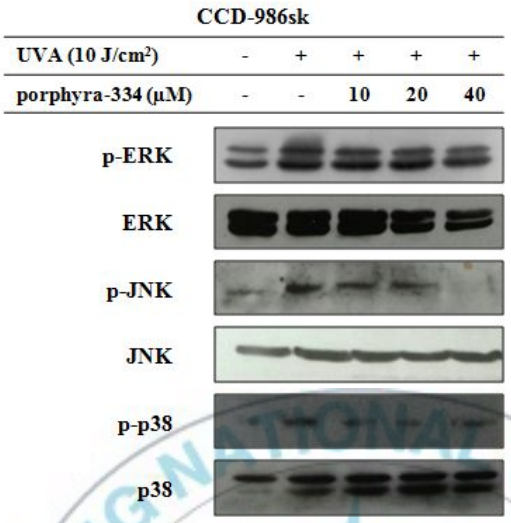

Fig. 36. Effects of porphyra-334 on UVA-induced MAPK phosphorylation in human skin fibroblasts. Representative western blot analysis of JNK, ERK1/2 and p38 in fibroblasts pre-treated with UVA (10 J/cm^2) and, then exposed to porphyra-334 (10-40 μM) for 24 h.

18. Effects of porphyra-334 on UVA-induced TGF-β/Smad signaling pathway

Effects of porphyra-334 on the levels of TGF-β receptors, TGF-β and Smads protein and mRNA expressions were determined by western blot analysis and RT-PCR, respectively. UVA mediated down-regulation of collagen synthesis also occurs via signaling pathways involving TGF-β. As a major profibrotic cytokine, TGF-β regulates various cellular functions involving differentiation, proliferation and enhancement of synthesis of extracellular matrix such as collagen and elastin (Gosain *et al*., 2004).

The mode of action of Smad4 differs from those of other members of the Smad family. After ligand stimulation and phosphorylation of pathway-restricted Smads, Smad4 forms hetero-oligomers with pathway-restricted activate transcriptional responses. In human skin fibroblasts, Smad4 forms complexes with Smad2 and Smad3 after activation of TGF-β receptors. TGF-β binding leads to the assembly of a heterotetrameric receptor complex in which the type Ⅱ recpetor phosphorylates and activates the type Ⅰ receptor. Pathway restricted Smad2 and Smad3, which may be anchored in the cytoplasm in homotrimeric forms, are phosphorylated, which leads to heteromerization with Smad4, a common-mediator Smad. The hetero-oligomeric complex was then translocated to the nucleus, where it binds directly to DNA and affects transcription of specific genes.

RT-PCR analysis was conducted to find the effects of porphyra-334 on mRNA expression level of TGF-β receptors, TGF-β and Smad 2, 3, 4 on human skin fibroblasts stimulated by UVA irradiation (Fig. 37). The porphyra-334 treatment for 24 h inhibited the UVA-induced expression of

TGF-β receptors, TGF-β and Smad 2, 3, 4 mRNA. In the western blot analysis, it was clear that UVA irradiation sufficiently increased TGF-β receptors, TGF-β and Smad 2, 3, 4 protein expression compared to the UVA non-treated control (Fig. 38). UVA-induced TGF-β receptors, TGF-β and Smad 2, 3, 4 protein expression levels were evidently decreased with porphyra-334 treatment (10-40 μM) for 24 h. Collective analysis of this data showed that the porphyra-334 decreased TGF-β receptors, TGF-β and Smad 2, 3, 4 gene expression and protein transcription in a dose dependent manner which were observed in by RT-PCR and western blot analysis, significantly demonstrated similar patterns. According to these results, porphyra-334 causes an impairment of the TGF-β/Smad signaling cascade in UVA irradiation (Fig. 39).

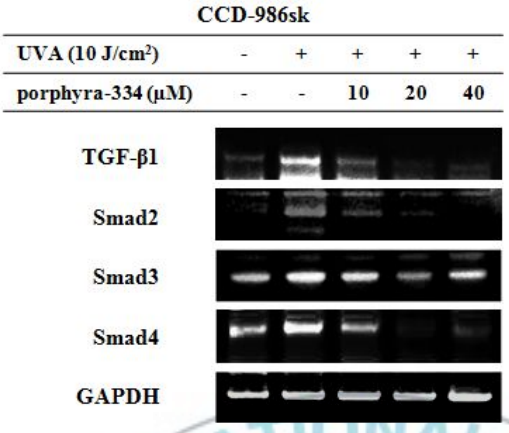

Fig. 37. Effect of porphyra-334 on mRNA expression levels of TGF-β/Smad signaling cascade in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR). Results of the evaluation for c-jun and c-fos mRNA expression/GAPDH ratio densitometry were detected by RT-PCR.

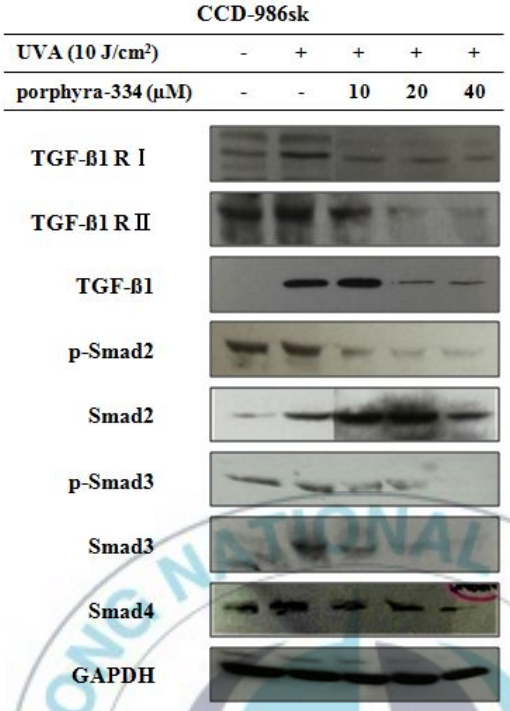

Fig. 38. Effect of porphyra-334 on TGF-β/Smad signaling cascade in UVA irradiated human skin fibroblasts. Cells were exposed to 10 J/cm^2 of UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of TGF-β receptors and signaling cascades were determined by Western blot analysis. GAPDH was used as an internal standard.

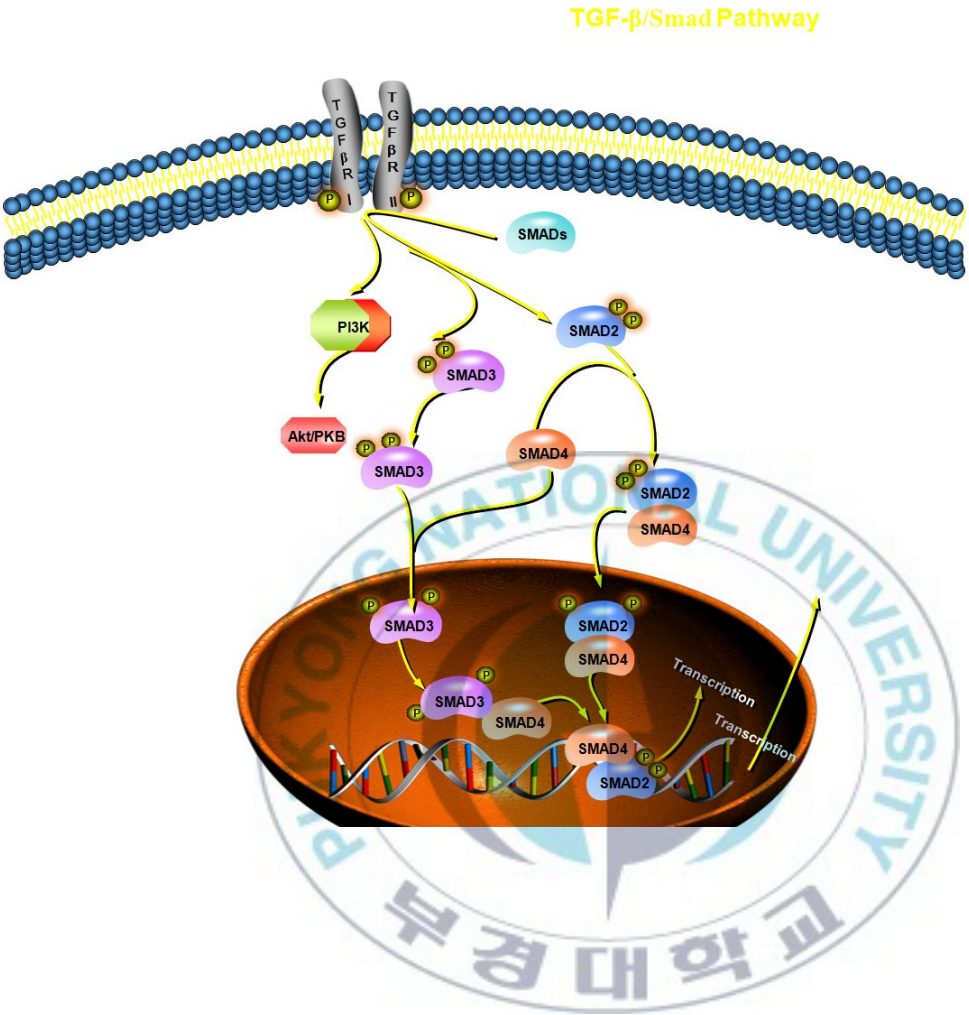

Fig. 39. Smad proteins in TGF-β signaling.

19. Effect of porphyra-334 on UVA-induced PI3K/Akt/mTOR signaling pathway

Signaling through the PI3K/Akt/mTOR pathway can be initiated by several mechanisms and propagated to various substrates, including mTOR (mammalian target of rapamycin), a master regulator of protein translation (Sarbassov, 2005). As shown in Fig. 40 and 41, UVA-induced initial activation of this pathway occurs at the cell membrane, where the signal for pathway activation was propagated through PI3K. PI3K activation occurs through tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR). Tyrosine phosphorylation, a marker of EGFR activation (Ullrich and Schlessinger, 1990), more than tripled over nonirradiated control levels, and treatment with porphyra-334 almost completely blocked EGFR phosphorylation after UV irradiation.

As shown in Fig. 42 and 43, mRNA expression and phosphorylation protein of mTOR was detected in human skin fibroblasts after UVA irradiation. UVA-induced activation of Akt (Thr308) phosphorylates and inhinits TSC2, and destabilizes the TSC1/2 complex, thus losing the ability of TSC2 to inhibit mTORC1. The characterized substrate of mTORC2 includes AKT kinase (Ser473), which suggests mTORC2 downstream in the PI3K pathway to regulate photo-damage process. The expression of mTOR and activation of mTORC1 and mTORC2 protein levels were increased in UVA-irradiated human skin fibroblasts. Compared to non-irradiated control, 142.55% increases were shown in total protein level of phospho-mTOR (Ser2448), respectively in UVA irradiated human skin fibroblasts. The level of Raptor and Rictor, a major component of mTORC1 and mTORC2 was

affected by UVA irradiation. Densitometric analysis revealed significant increases in expression and activation of mTOR during UVA alone exposed in human skin fibroblasts. Both phosphorylated forms of mTOR at Ser2488 which is phosphorylated by Akt and Ser2481 autophosphorylation were increased by UVA irradiation.

mTORC1 was sensitive to porphyra-334 in UVA irradiation, in part, by phosphorylation of p70S6K and subsequently RPS6 and elF4B, thus promoting protein translation (Fig. 44). Futhermore, mTORC2 directly phosphorylates Akt on Ser473 and facilitates Thr308 phosphorylation by PDK1 and mTOR is directly phosphorylated by p70S6K at Ser2448. Taken together, these data suggest that there was a positive feedback loop from p70S6K to mTOR and from mTOR to Akt that possibly accelerates effects of porphyra-334 on PI3K/Akt/mTOR signaling pathway in UVA irradiation. porphyra-334 blocked UVA-induced phosphorylation of p70S6K, the downstream target of mTORC1, but had no effect on cell death.

Therefore, the result demonstrated that mTOR was generally considered a downstream substrate of Akt, mTOR can also phosphorylated Akt when bound to Rictor in mTORC2. Also, downstream mTOR effector p70S6K can also regulate the pathway by PI3K, thereby enhancing activation of Akt (Fig. 45).

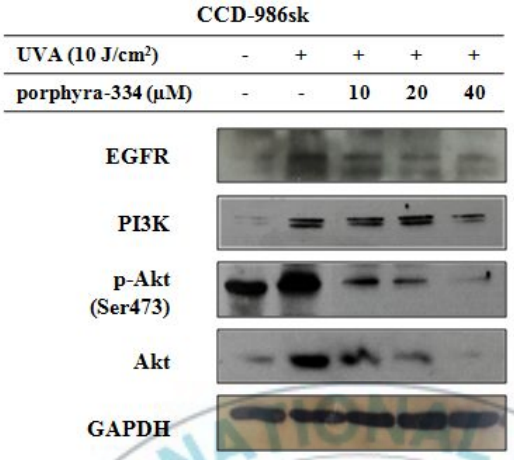

Fig. 40. Effect of porphyra-334 on PI3K and Akt in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

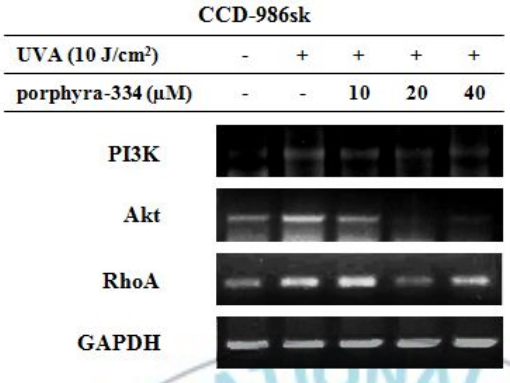

Fig. 41. Effect of porphyra-334 on mRNA expression levels of PI3K, Akt and RhoA in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR).

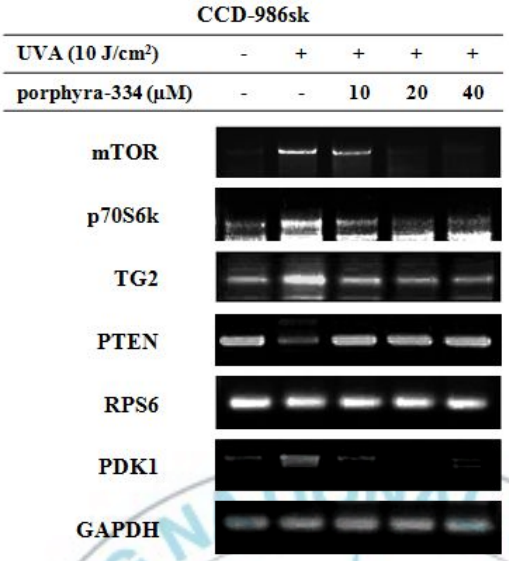

Fig. 42. Inhibition of mTOR up-regulates activation of its downstream effectors. human skin fibroblasts were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR).

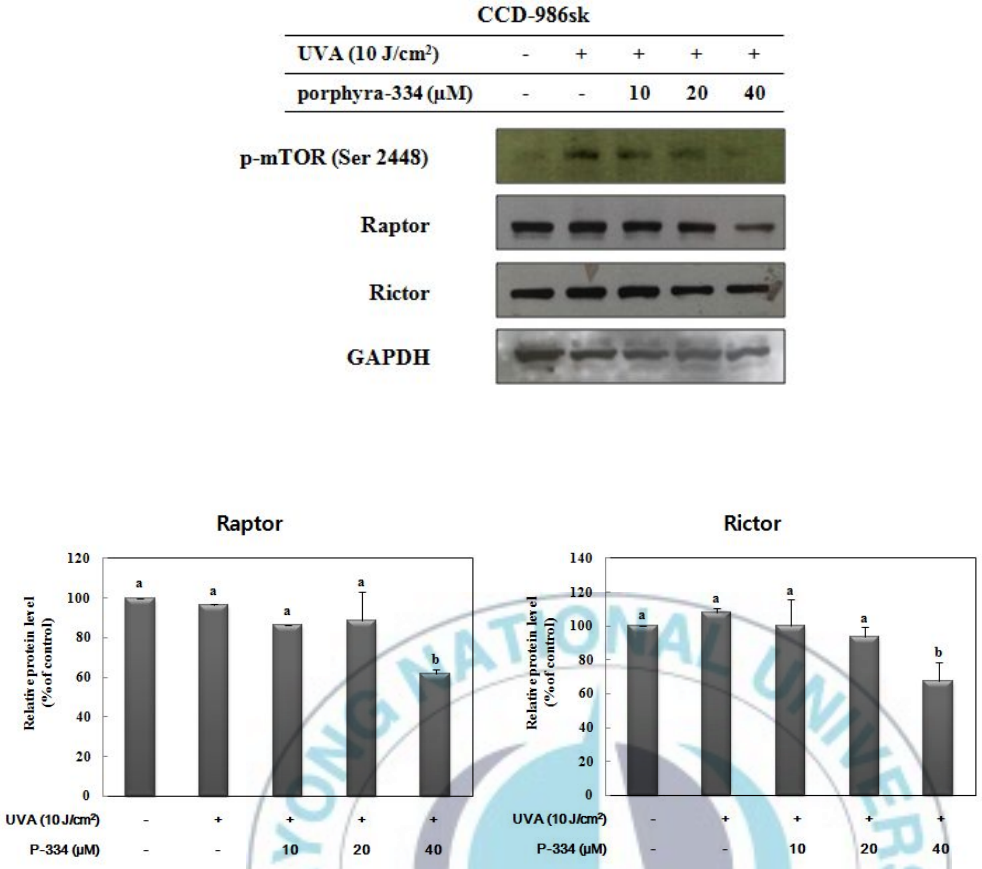

Fig. 43. Changes of the expression and activities of mTOR complexes in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard. Activities of mTOR complex 1 and 2 which were represented by phosphorylated forms of mTOR at Ser2448 and Ser2481 respectively were measured by western blot analysis using their specific antibodies.

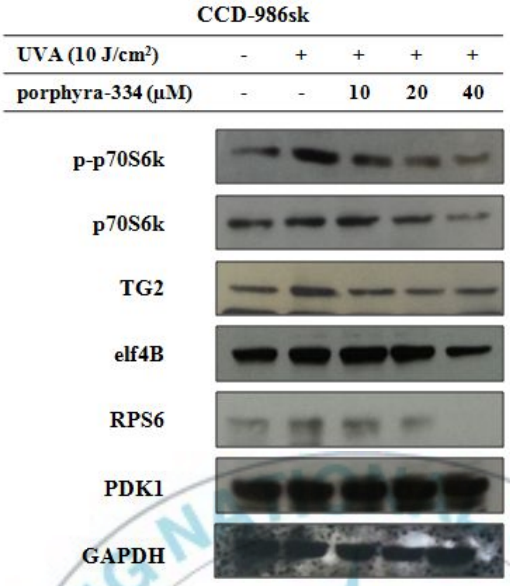

Fig. 44. Activation status of mTOR signaling proteins in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Western blot analysis after subcellular fractionation showed that UVA-irradiated human skin fibroblasts express activated p70S6K, TG2, elf4B, RPS6 and PDK1. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

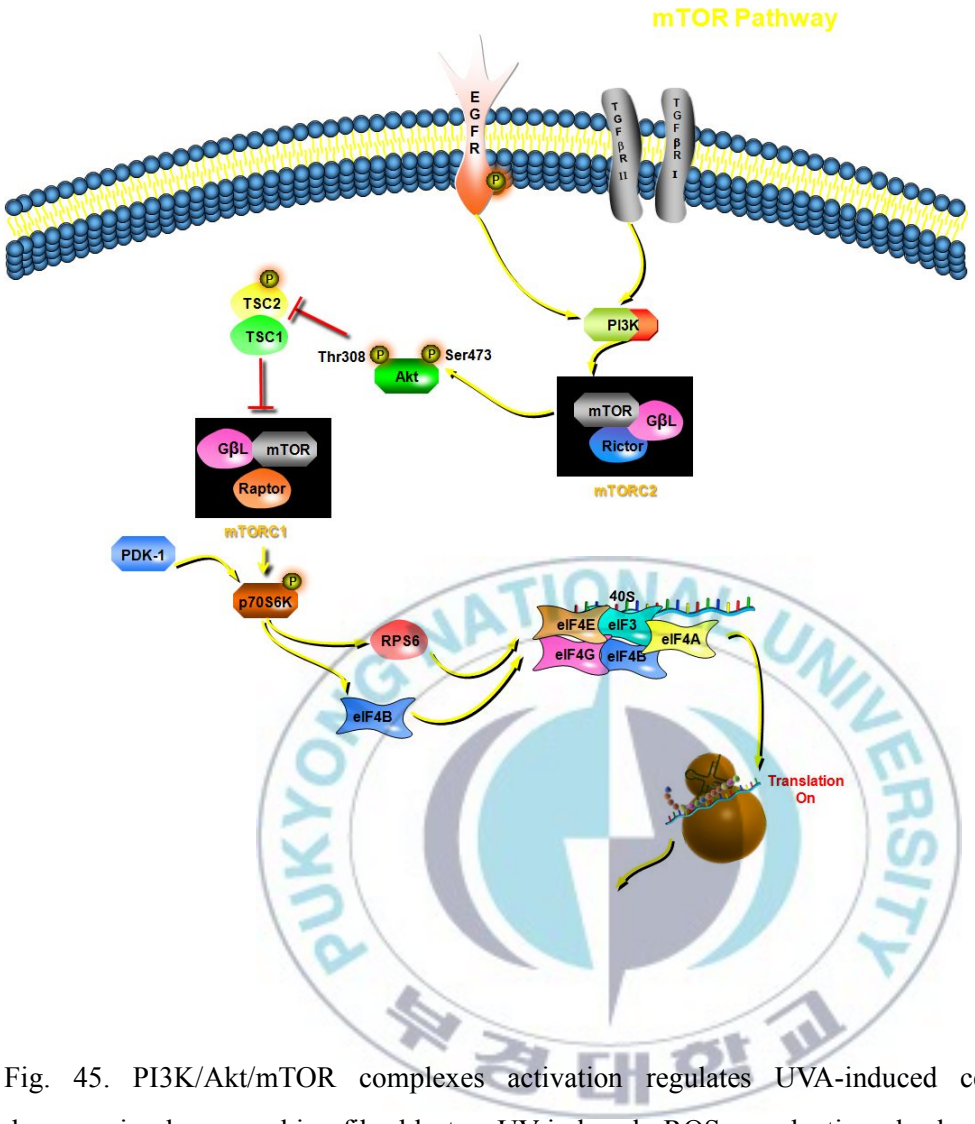

Fig. 45. PI3K/Akt/mTOR complexes activation regulates UVA-induced cell damage in human skin fibroblasts. UV-induced ROS production leads to trans-activation of cell surface receptors including EGFR, and activated cell surface receptors mediating downstream signaling activation including PI3K/Akt/mTOR signaling pathway.

20. Effect of porphyra-334 on UVA-induced cathepsins expression

UVA-induced cathepsins protein level changes in human skin fibroblasts in response to chronic UVA exposure were examined using western blot analysis (Fig. 46). Densitometric analysis indicated down-regulation of cathepsin B, D and K in UVA-irradiated human skin fibroblasts by approximately 93.08%, 71.25% and 73.99% compared to UVA-non irradiated control. On the contrary, UVA-irradiation increased the amount of cathepsin G and L protein levels (113.4% and 189.16%) compared to UVA-non irradiated control and treatment with porphyra-334 impairs the increased cathepsins. A significant increased of cathepsin L was detectable after exposure to UVA (10 J/cm^2) and inhibition by approximately 33.07% was observed when treatment with 20 μM porphyra-334 after UVA irradiation. Total RNA extracted from the human skin fibroblasts was detected separately using RT-PCR. After UVA exposure of HDFs, mRNA expression of Cathepsin B, D and K was reduced compared to non-treated control (Fig. 47).

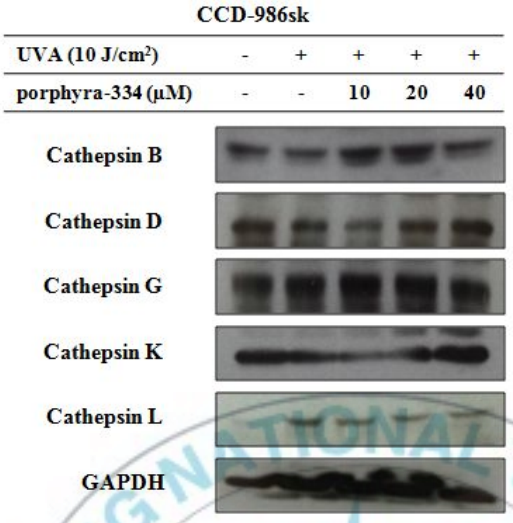

Fig. 46. Effect of porphyra-334 on cathepsin B, D, G, K and L in human skin fibroblasts stimulated with 10 J/cm^2 of UVA irradiation. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The expression levels of these proteins were determined by western blot analysis. GAPDH was used as an internal standard.

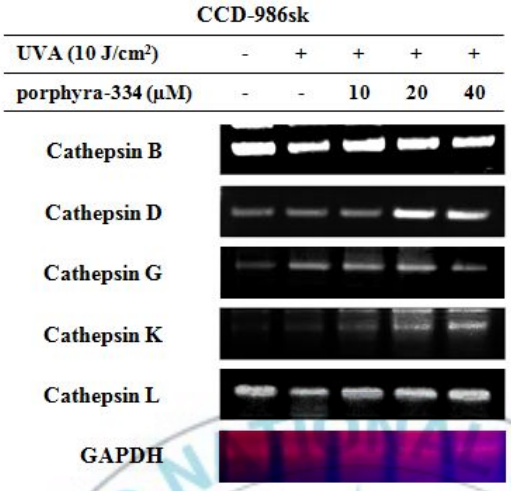

Fig. 47. Effect of porphyra-334 on mRNA expression levels of cathepsin B, D, G, K and L in human skin fibroblasts stimulated with 10 J/cm^2 of UVA. Cells were exposed to UVA irradiation and incubated with porphyra-334 for 24 h. The mRNA expression was quantified by semi-quantitiative reverse transcriptase polymerase chain reaction (RT-PCR). Results of the evaluation for c-jun and c-fos mRNA expression/GAPDH ratio densitometry in human skin fibroblasts were detected by RT-PCR.

21. Autophagy impairment induces premature senescence

To determine whether porphyra-334 treatment triggered autophagy and induces impairment of premature senescence in human skin fibroblasts, CCD-986sk cells were treated with porphyra-334 after UVA irradiation for 24 h and then established set of protein markers (Beclin-1, p62/SQSTM1, LC3). LC3, the mammalian equivalent of yeast Atg8, is one method that can be used to monitor autophagy (González-Polo, 2005). A hallmark of mammalian autophagy is the conversion of LC3-I to LC3-II via proteolytic cleavage and lipidation. This modification of LC3 is essential for the formation of autophagosomes and for the completion of macroautophagy (Tanida, 2005). To verify whether LC3 (autophagosomes) is redistributed after porphyra-334 treatment, human skin fibroblasts observed after UVA irradiation.

Immunoblot analysis revealed accumulation of Beclin-1 UVA-treated cells. Similarly, consistent with autophagic-lysosomal impairment downstream of cathepsin inactivation, increased cellular levels of the autophagic cargo receptor and substrate p62/SQSTM1 and the autophagosome component LC3-Ⅱ were increased in UVA-treated cells, changes that occurred exclusively at the protein level (Fig. 48). The C-terminal glycine of LC3 is attached to phosphatidylethanolamine (PE), and the C-terminal glycine of ATG12 is attached to ATG5. The expression level of ATG12-ATG5 conjugation was slightly increased in UVA-treated cells, even though the level of ATG7 was not changed. Moreover, porphyra-334 treatment after UVA irradiation for 24 h, the level of beclin-1 was gradually increased and ATG7 was also modestly induced in cells undergoing premature senescence.

Therefore, the results demonstrated that UVA not only inflict primary photo-oxidative insult on cellular components but also causes dual inactivation of autophagic-lysosomal proteolytic clearance of damaged molecules and organelles, a novel molecular pathway as envisioned in Fig. 49.

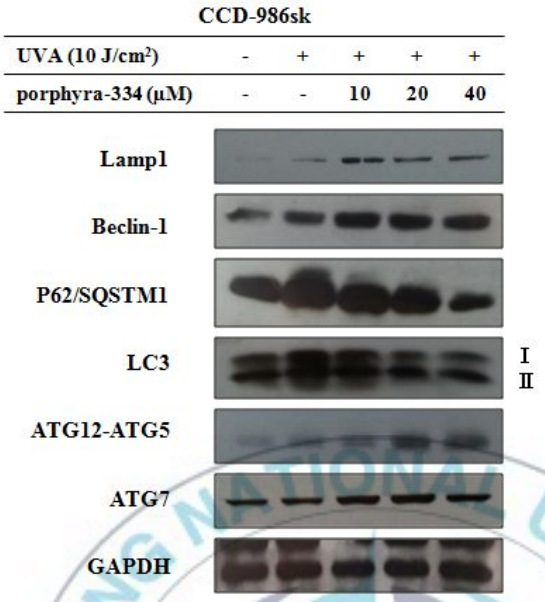

Fig. 48. Effect of porphyra-334 on induction of autophagy in human skin fibroblasts (western blot analysis). Cells were exposed to 10 J/cm^2 of UVA irradiation and incubated with porphyra-334 for 24 h. Expression of autophagic-lysosomal market proteins (Beclin-1, p62/SQSTM1, LC3) and ATG12-ATG5 conjugates, ATG7 were determined by Western blot analysis. GAPDH was used as an internal standard.

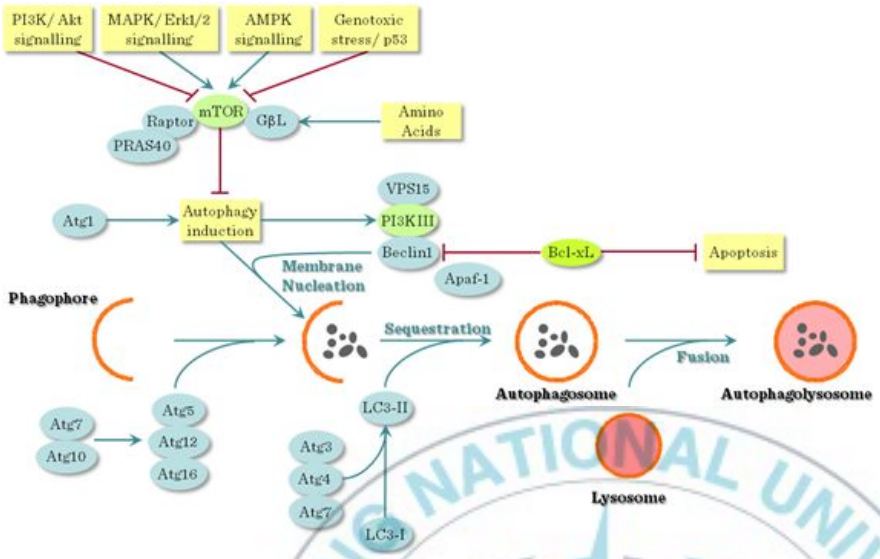

Fig. 49. UVA-induced cutaneous photooxidative stress impairing autophagic-lysosomal function. UVA-induced oxidative stress mediated through reactive oxygen species (ROS) causing autophagic-lysosomal blockade in human skin fibroblasts.

Ⅳ. DISCUSSION

In recent years, natural products from marine organisms have gained increasing research awareness, and a number of novel marine compounds of potential economic importance have been reported from different marine organisms (Mayer *et al*., 2013; Erwin *et al*., 2010). A number of photo-protective compounds, such as phenylpropanoids (Tavares *et al*., 2013), flavonoids (Agati and Tattini, 2010), melanins (Nagiller and Sommaruga, 2009), mycosporines (Brandão *et al*., 2011), MAAs (Rastogi and Incharoensadki, 2013), scytonemin (Singh *et al*., 2010), parietin (Solhaug and Gauslaa, 1996), usnic acid (Fernández *et al*., 2006), carotenoids (Yong and Lee, 1991), phycobiliproteins (Lohscheider *et al*., 2011), and several other UV-absorbing compounds of obscure chemical structure (Gröniger *et al*., 2000), have been established from different organisms. There have been a number of reviews (Mayer and Hamann, 2004; Yasuhara-Bell and Lu, 2010; Schumacher *et al*., 2011; Rocha *et al*., 2011; Orhan *et al*., 2010; Rastogi *et al*., 2010) about various classes of compounds from natural sources including marine habitats, but the existence of photo-protect materials from marine sources has only partially been explained.

It has known that mycosporine-like amino acids (MAAs) possess significant chemoprotective effects against photo-induced skin senescence. MAAs found in and isolated from a number of marine organisms, such as cyanobacteria, algae, and heterotrophic bacteria, have attracted a great deal of interest, especially for potential UV protection. Recent studies further

suggested that MAAs have antioxidant properties and UV absorbance activity (Andreguetti *et al*., 2013). An important MAA is porphyra-334, which has been reported to act mainly in photoprotection, but it also has anti-oxidation abilities. Recent studies revealed that algae extract prevent UV-induced photodamage in human keratinocytes (Piao *et al*., 2012).

Although *Porphyra* sp. has been used as an effective materials with protective and curable roles in a variety of diseases, porphyra-334 are not yet fully understand for its intracellular signaling and mechanism against UVA-induced photo-damage in human skin fibroblasts. Numberous studies have been conducted on the impact of UV irradiation on skin cells, the beneficial effect of photochemoprotective agents on UVA-induced damage on human skin is less well defined. UVA has been shown to be a potent inducer of various reactive oxygen species, as well as a cause of lipid peroxidation in cell membranes. Antioxidant defense mechanisms may be overwhelmed by excessive free-radical generation, with result damage to the cells and increased chances of photocarcinogenesis. Development of new antioxidant strategies by supplementing the natural defense mechanism operating in the skin may be an important strategy in inhibiting UV-induced effects in the skin. This study shown that porphyra-334, a rich source of mycosporine-like amino acid (MAA), derived from *Porphyra yezoensis* is effective in reducing the adverse effects of UVA-mediated cutaneous damage.

Photoaging associated with UV irradiation is thought to play a central role in initiating and driving the signaling events that lead to cellulcar response. UV radiation of skin decreases anti-oxidant enzymes (Yamamoto,

2001) and increases hydrogen peroxides and other reactive oxygen species (ROS; Masaki *et al*., 1995, Yasui and Sakurai, 2000). UV irradiation initiates the generation of reactive oxygen species (ROS) alters gene and protein structure and function, leading to skin damage. Furthermore, ROS can activate mitogen-activated protein kinases that phosphorylate AP-1, which in turn results in up-regulation of MMPs (Rittié and Fisher, 2002). Porphyra-334 was shown to dose-dependently decrease intracellular UVA-induced ROS in human skin fibroblasts using a modified DCF-DA fluorescence assay. Since the generation of MMPs is significantly induced by ROS (Nalson and Melendez, 2004), this study shown that the ROS scavenging of porphyra-334 was comparable to that of UV-unirradiated control. This result provide that porphyra-334 could control the expression levels of MMPs by scavenging excess ROS in damaged skin fibroblasts.

UV irradiation enhances decomposition of skin connective tissue by activating MMPs that are responsible for the degradation of skin collagen and inhibition of collagen synthesis of extracellular matrix in connective tissues (Wlaschek *et al*., 1994). In this study, these features alters MMPs, especially MMP-1, upregulation and the degradation of dermal collagen. Also, porphyra-334 was not only a potent suppressor of UVA-induced MMPs generation, but that it also inhibited the MMP-1 initiated degradation of type Ⅰcollagen. Therefore, inhibition of collagenase MMPs expression by activating collagen synthesis might be an effective strategy to prevent wrinkle formation after UVA irradiation. This mechanism predicts that a free radical scavenger might prevent UV-induced dermal damage by inhibiting the

induction of MMPs. These findings suggest that the regulation of MMPs and type Ⅰ collagen levels in UV-irradiated fibroblasts might be related to the inhibition of ROS generation by porphyra-334. MAAs from red algae was also reported to effectively decrease MMPs and increase type Ⅰ collagen expression levels against premature skin-aging (Pallela, 2010). This study implicated that porphyra-334 could increase procollagen production by suppressing MMPs gene expression and thus reducing MMPs production, which can be involved in reduced cytokines secretion by human skin fibroblasts. Many algae has been screened to provide potential agent for MMPs inhibitors. For example, *Corallina pilulifera* (Ryu *et al*., 2009), *Ecklonia cava* (Zhang *et al*., 2010), *Laurencia undulata* (Li *et al*., 2009) and *Amphiroa dilatata* (Khan *et al*., 2010) showed inhibitory effects on MMPs activities. In this study, porphyra-334 was not only safe to human skin fibroblasts, but also significantly decreased the expression of MMPs over-expressed by UVA irradiation.

In conjunction with the upregulation of MMP activity, UV irradiation also induces a specific inhibitor of MMP, TIMPs, which helps to counter balance the degradative effects of the MMPs. Despite this, UV exposure clearly encourages a more degradative environment within the dermal tissue resulting in the destruction of both the collagen and elastin.

Wrinkle formation in the skin is closely associated with the degradation of extracellular matrix (ECM) and UV irradiation is known to induce degradation of ECM (Rittié and Fisher, 2002). UV irradiation enhances collagenase activity and contributes to wrinkle formation through degradation

of collagen in the dermal extracellular matrix (Uitto, 2008; Uitto *et al*., 1989). Hence, collagenase inhibitors have been identified as potential therapeutic agents that can protect against photoaging and wrinkle formation (Inomata *et al*., 2003). Collagen is the main component of the ECM of dermal connective tissue, and its concentration decreases with photoaging. Once collagen is initially cleaved by MMP-1, MMP-13 and other MMPs, collagen breakdown is further promoted. The enzyme mainly responsible for collagen breakdown in skin is MMP-1, which cleaves types Ⅰ, Ⅲ, Ⅶ, Ⅷ and Ⅹ collagen. Chung *et al*. (2001) reported that, with increasing photoage, MMP-1 levels rise and collagen synthesis declines for sun-protected human skin in vivo. In this study, the exposure of human skin fibroblasts to UVA resulted in a dramatic reduction in type Ⅰ collagen and a marked increase in MMP secretion, both of which were reversed by porphyra-334. Elastin also plays an important role in dermis (Labat-Robert and Robert, 1988). UV exposure has been shown to cause elastin degradation by activating elastase (Getie *et al*., 2005). As shown in Fig. 24, the inhibitory effect of porphyra-334 on elastase activity was significant at a concentration of 10 μM and higher. Hyaluronic acid is a moisturizer in the skin. The result indicated that porphyra-334 elevated the hyaluronic acid content in human skin fibroblasts. Therefore, porphyra-334 may play an important role in skin hydration for preventing skin photoaging.

CYR61 upregulates MMP-1 by activating transcription factor AP-1, consists of homodimers and heterodimers of members from the fos (c-fos, fosB, fra1 and fra2) and jun (c-jun, junB and junD) family proteins (Karin *et*

al, 1997). UV irradiation activates multiple cell surface growth factor and cytokine receptors, and these activated signaling cascades activate target transcription factor including AP-1 in skin fibroblasts (Quan *et al*., 2005). Furthermore, suggesting that human dermal fibroblasts attenuates UVA-induced MMPs expression is regulated by components of AP-1 complex. Activated JNK phosphorylates c-jun, enhancing its transcriptional activity. In this study, the inhibitory effects of porphyra-334 on UVA-induced CYR61, c-jun and c-fos expression may further contribute to the protective effects of porphyra-334 on UVA-induced MMP-1 and on UVA-diminished collagen gene and protein expression. This is supported by Fisher *et al*. (2000) finding that inhibition of collagen production by UV irradiation is mediated in part by UV-irradiation induction of AP-1. Exposure of human skin to UV radiation in vivo activates multiple cell-surface cytokine and growth factor receptors as well as MAPK signaling pathways and rapidly up-regulates AP-1 and NF-κB activity (Vile *et al*., 1995). However, the mechanisms by which porphyra-334 inhibit photoaging have not yet been determined. This study investigated porphyra-334 block MMP-mediated photoaging by influencing MAPK, NF-κB, AP-1 signaling, modulated by UVA irradiation. I found that porphyra-334 down-regulated the activation of ERK, p38 MAPK, which act upstream of MMP-1 in UVA-irradiated human skin fibroblasts. It also reduced the activation of NF-κB and c-fos, downstream targets of ERK and p38 MAPK.

Recently, there has been considerable interest in the cathepsin family of proteases. The lysosomal protease, cathepsin K, is a potent mammalin elastase and may play a role in formation of solar elastosis. UVA activation

of this potent elastase has been observed in human fibroblasts from young donors both *in vitro* and *in vivo* (Codriansky *et al*,, 2009). The enzymatic activity of cathepsin B, a lysosomal cysteine protease is substantially reduced by repeated UVA irradiation which also compromised cathepsin B maturation and impaired autophagy downstream of cathepsin B inactivation. Another lysosomal protease, cathepsin L, which degrades matrix proteins was not modified by acute exposure of fibroblasts to UVA but chronic irradiation using repeated exposure led to significant extracellular release of the protein. Furthermore, this was associated with a reduction in intracellular processed cathepsin L and an accumulation of unprocessed cathepsin. This research showed that the expressions of cathepsin B, D, G, K and L were decreased in photoaged human skin fibroblasts while cathepsin G was increased. During this process, the relation of cathepsin B, D, K, L and G might have a part in cell senescence, fibrosis and matrix degradation and inflammation of the photoaged skin. This suggest that cathepsin B, D, K, L and G act as potential biomarkers in photo-damaged human skin fibroblasts.

One of the earliest detectable responses of human skin cells to UV irradiation is activation of multiple cytokine and growth factor cell surface receptors, including epidermal growth factor receptor (EGFR; Warmuth et al., 1994), tumor necrosis factor (TNF)-α receptor (Dy *et al*., 1999), interleukin (IL)-1 receptor (Rosette and Karin, 1996), and platelet derived growth factor (PDGF) receptor (He *et al*., 2001). Rosette and Karin (1996) demonstrated that activation of growth factor and cytokine receptors (EGF, IL-1 and TNF-α) by UV irradiation, leads to clustering and internalization of the receptors.

Interestingly, whereas activation of EGFR, IL-1R or TNF-α receptor alone by its cognate ligand resulted in relatively modest activation of downstream signaling pathway, co-administration of EGF, IL-1 and TNF-α results in strong synergic response similar to that cause by exposure to UV irradiation. COX-2 is a primarily upregulated gene in response to UVA exposure in human skin (Soriani *et al*,, 1998), and it is thought that TNF-α may be the major intermediated regulator that transduces UVA-induced signals in human skin fibroblasts. This results implicate that porphyra-334 could repair effect due to the inhibition of TNF-α and COX-2 protein expression in human skin fibroblasts.

MAPK signaling plays an important role in regulating MMP gene expression, cell motility, cell proliferation and cell survival. Three distinct groups of MAPKs (ERK, JNK and p38) have been identified in mammalian cells. (Robinson and Cobb, 1997). MAP kinases are upstream regulators of MMP activity (Yang *et al*., 2009). It has been reported that Tiotropium bromide inhibits TGF-β-induced MMP production by MAPK pathways in vitro (Asano *et al*., 2010). In addition, UV light leads to phosphorylation of c-Fos by MAPK (Tanos *et al*., 2005). In this study reported that oxidative stress due to ROS accumulation initiates the MAPK signaling cascade by phosphorylation of MAPK proteins. The level of the corresponding phosphorylated kinases was significantly higher in all cases, meaning that all the three MAPKs signal pathways were activated upon UVA treatment. Moreover, the highest difference in between non irradiated and irradiated UVA was p-ERK. In addiation, porphyra-334 significantly inhibited

UVA-induced p-ERK and phosphorylated p38 expression at doses of 10 μM, respectively.

TGF-β is a member of a superfamily of multifunctional cytokines which initiates its signals by interacting with its receptors. The binding of TGF-β to TGF-βRⅡ results in the formation of a heteromeric complex between TGF-β to TGF-βRⅡ, which results in phosphorylation of TGF-βRⅠ by TGF-βⅡ. Binding of TGF-βRⅡ is the first critical step in the TGF-β singnaling cascade, because TGF-βRⅠ does not bind TGF-β directly. TGF-β RⅠ phosphorylates and activates the transcription factors Smad2 and Smad3, which then bind with their common partner, Smad4, to form a heteromeric complex that translocates to the nucleus, where it acts as a transcription factor (Verrecchia and Mauviel, 2002). UVA stimulated inhibitory Smad leading to receptor-activated Smad protein phosphorylation causing inhibition of Smad complex translocating into the nucleous, thereby reducing expression of type Ⅰ collagen and elastin. In order to verify this fact in our system, both the TGF-β/Smad signaling pathways were investigated by assessing the mRNA and protein levels by RT-PCR and western blot analysis in UVA-induced human skin fibroblasts. Cells increased TGF-β mRNA and protein level after UVA irradiation, and decreased as non-irradiated control level after porphyra-334 treatment. The protein of the TGF-β receptors Ⅰ and Ⅱ, which connect the extracellular TGF-β singal and the intracellular effectors, also undergone a tremendous increase, and this further proved the important role played by the TGF-β/Smad pathway in response to porphyra-334. Futhermore, investigated evaluation of the Smads signaling

pathway, R-Smads (Smad2/3) and Co-Smad (Smad4) protein and mRNA levels significantly increased, showing a stimulated transcription activity. All these results demonstrated that UVA stimulated and activated the TGF-β /Smad signaling pathway which trigger the photoaging. Also, the expression level of TGF-β/Smads found decrease in the mRNA and protein level compared to the UV irradiation.

It has been shown that PI3K is an important driver of oncogenesis (Bader *et al*., 2005). However, paradoxically, hyperactivated PI3K can actually promote cellular senescence. On activation of PTEN, a negative regulator of the PI3K/Akt pathway, mediates senescence in mouse prostate epithelium and embryonic fibroblasts, indicating that the PI3K-Akt pathway is involved in cellular senescence (Chen *et al*., 2005). Activation of Akt, a downstream mediator of PI3K signaling, also induces premature senescence in mouse embryonic fibroblasts by increasing intracellular ROS levels through elevation of oxygen consumption and inhibition of FoxO transcription factors (Nogueira *et al*, 2008; Astle *et al*., 2011). Here, this study showed that UVA irradiation induced senescence through activation of the PI3K-Akt-mTOR pathway. UVA irradiation stimulated phosphorylation of Akt, mTOR and p70S6K and treatment with porphyra-334 blocked this phosphorylation event in human skin fibroblasts. Therefore, this study suggests that the PI3K/Akt/mTOR signaling pathway is involved in UVA-induced senescence.

Autophagy is a vacuolar, self-digesting mechanism responsible for the removal of long-lived proteins and damaged organelles by the lysosome. Autophagy is a dynamic process of subcellular degradation that is critical for

cell survival under nutrient-deprived conditions. The mechanism by which macroautophagy, another form of autophagy, decreases with aging is not yet known. This study findings indicated that during skin aging autophagic activity was decreased, as evidenced by an accumulation of p62/SQSTM1. UVA irradiation also decreased autophagic flux and autophagosome formation as shown in data using RT-PCR and western blot analysis. p62/SQSTM1 was reported as an important NF-κB mediator in cell survival, plasticity, and disease (Mattson and Meffert, 2006). Therefore, the inhibition of autophagy can be linked to NF-κB activation, molecular inflammation. Beclin-1 is the humanhomologue of Atg6/Vps30, which is a key regulator of autophagy in yeast. The involvement of beclin-1 is necessary for the induction and initiation of autophagosome formation in mammalian cells. During autophagy, the autophagic market LC3 is cleaved and covalently conjugated to phosphatidyl-ethanolamine, yielding LC3-Ⅱ, which is specifically targeted to elongated, Atg12-Atg5 associated autophagosome precursors, thereafter remaining associated with autophagosomes (Vellai, 2008; Tanida and Kominami, 2004). Moreover, a conditional knockout of ATG7 in mice, which is an essential gene for autophagy, caused pathological defects in the liver, neuro-degradation, and early death (Komatsu *et al*., 2006). These studies suggest that autophagy-activating interventions might be useful tools for promoting longevity. The RT-PCR and immunoblotting results were comfirmed by the detection of LC3Ⅱ accumulation and autophagic flux in the presence of porphyra-334 after UVA irradiation. Thus, this data suggest that the stimulation of UVA irradiation in human skin fibroblasts actively

induces autophagosome formation and regulates the maturation of autophagolysosomes.

Ⅴ. CONCLUSION

Skin aging is influenced by several factors, and solar UV irradiation is considered one of the most important causes of skin photoaging. To study the anti-photoaging role of porphyra-334 from *Porphyra yezoensis*, a mycosporine-like amino acid (MAA), high performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESI-MS) and UV absorbance maxima were applied. The protective effect of porphyra-334 against UVA radiation has been studies in human skin fibroblasts.

This study reveal the photoprotective actions of porphyra-334 in UVA irradiation suppressed collagen, elastin degradation and MMPs production related to photoaging. The MAPK family and transcription factors NF-κB and AP-1 appear to be responsible for cell survival during UVA-induced oxidant injury. The MAPKs, PI3K/Akt/mTOR and TGF-β/Smad related signaling pathways involved in the photodamage by the porphyra-334. Furthermore, prophyra-334 mediated through reactive oxygen species (ROS) causing autophagic-lysosomal blockade in human skin fibroblasts. The human skin fibroblasts against UV irradiation protected signaling pathways may be causally linked to cellular antioxidant signaling that is readily responseve to porphyra-334 (Fig. 50).

The results suggest that porphyra-334 can act as a UVA protecting sunscreen, furthermore, this MAA depletes UV energy without creating any reactive species might cause a phototoxic effect on living organisms. Porphyra-334 can be inferred to have various uses in cosmetics and toiletries

as a anti-inflammatory and anti-photoaging activities, indicating that could be a potential anti-aging agent.

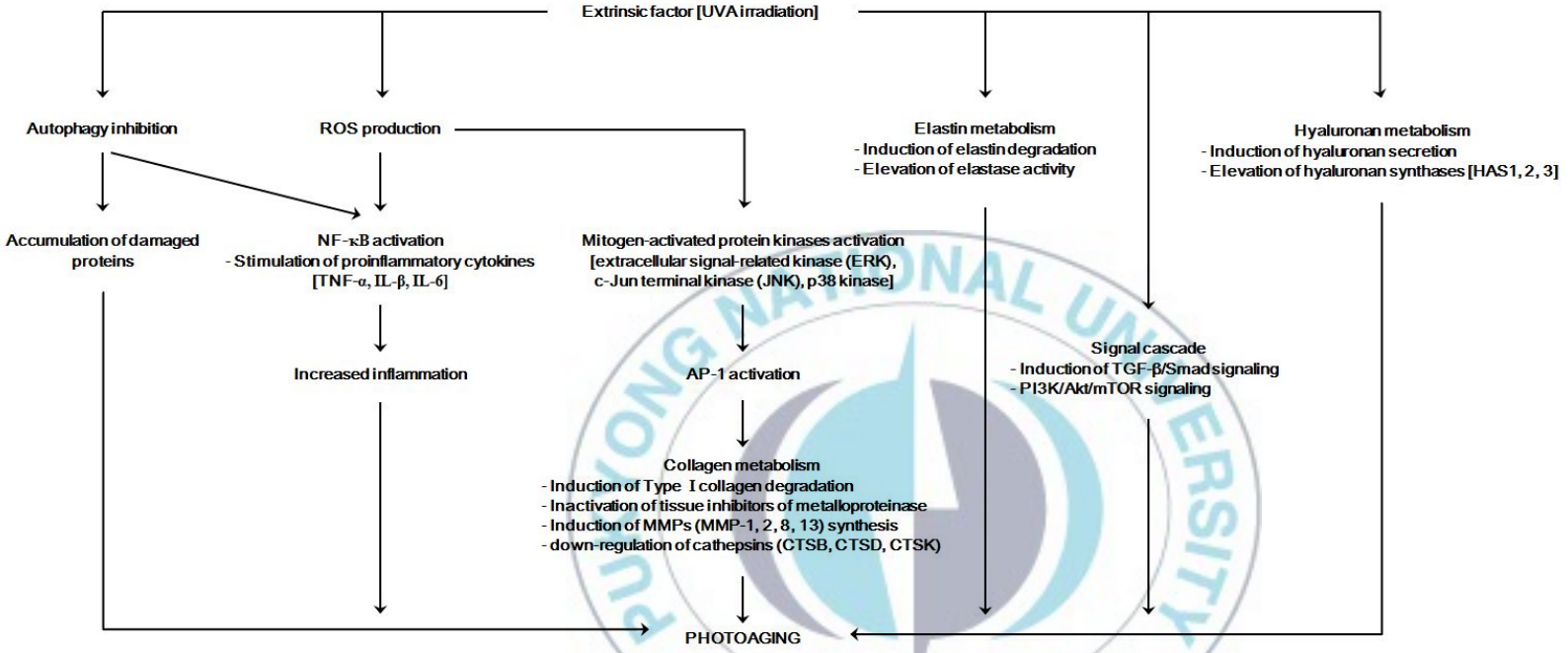

Fig. 50. A model for the pathophysiology of photoaging. Exposure of human skin fibroblasts to UVA results in the activation of growth factor and cytokine receptors that leads to activation of MAP kinases. UVA irradiation also results in production of ROS, which are critical for MAP kinase activation. The MAP kinase signal transduction pathway leads to increased expression of the transcription factor AP-1, which in turn upregulates MMPs protein and gene expression. MMPs degrade the extracellular matrix of the dermis. This matrix damage is followed by repair, which cannot be perfect, resulting in a deficit in the dermal structure, skin wrinkling or photoaging.

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Acknowledgment

2014년 2월. 드디어 제 인생에 큰 과제를 해결하였습니다. 새로운 전공으로 공부하고자했던 저에게 흔쾌히 그 길을 열어주신 남택정 지도교수님, 믿고 지켜봐주시는 마음을 알기에 더욱더 열심히 공부할 수 있었습니다. 사랑으로 이해해주시고 때로는 다그쳐주셨기에 좋은 결실을 맺게 되었습니다. 감사합니다. 그리고 논문심사를 위해 애써주신 최재수 교수님, 바쁘신 중에도 부족한 제 논문을 처음부터 큰 관심으로 글자 하나까지 확인해주시던 그 모습에 감동하였고, 진심으로 감사했습니다. 류홍수 교수님, 몇 년 전 처음 교수님을 마주할 때 긴장감은 어느새 사라지고 흥미로운 강의를 기대하는 학생이자 팬이 되었습니다. 따뜻한 감성을 지니신 교수님의 서체에서 느껴지는 또 다른 모습이 아름다웠고, 삭막한 연구환경에서 지쳐있는 우리에게 보여주시는 글귀와 사진들, 행복했습니다. 마주치면 고운 목소리로 반겨주시는 미소가 아름다운 류은순 교수님, 학위논문 마무리까지 관심있게 확인해주시고, 늘 격려해주심에 감사했습니다. 멋진 목소리로 아낌없는 조언을 해주셨던 변대석 교수님, 장난스레 내뱉으시는 말 한마디에도 핵심을 집어주시는 김형락 교수님, 어려운 교수님들 사이에서 늘 학생들이 편히 대할 수 있도록 먼저 배려해주셨던 김재일 교수님, 그리고 바쁘신 중에도 논문심사를 위해 심사때마다 자리하셔서 조언해주신 경성대 김동수 교수님께도 감사의 말씀을 전하고자 합니다.

우리 영양학 연구실의 많은 선후배들의 도움 또한 큰 힘이 되었습니다. 전공이 다르던 저에게 첫 실험을 가르쳐주셨던 황혜정 선생님, 무슨일이든 적극적으로 하시는 최윤희 선생님, 육아와 학업을 훌륭히 해내시는 앞서가신 선배의 모습을 보며 많이 깨우칩니다. 소중한 시간을 함께 보낸 김인혜 선생님, 그리고 연구실의 모든 일을 너무도 꼼꼼히 챙겨주시는 멋진 여인 조인선 선생님 감사합니다. 먼저 사회로 진출한 서현이와 히로애, 밝은 성격으로 모두를 기분 좋게 만들어주는 희경, 졸업후에도 잊지않고 이쁜 웃음 들려주는 미경, 입 무거운 괜찮은 남자 정욱, 석박사과정을 훌륭히 끝내는 멋쟁이 영민, 긍정마인드가 매력적인 미혜와 너무도 귀여운 애교쟁이 따뜻한 막내 민경, 타국에서 열심히 생활하는 소마에게 감사하고 함께 지낸 시간 내내 즐거웠습니다. 또한 언니들의 방대한 실험량으로

하루에도 몇 번씩 세척하느라 힘들었을 학부생 은진, 혜영, 예진, 새봄, 윤정, 미진에게도 진심으로 감사함을 전합니다. 그리고 옆자리 수진이, 닮은 듯 하지만 너무도 다른 취향을 가진 서로에게 의지하며 같이 공부하고, 고민하고, 이겨내며 함께 학위를 받게 되었습니다. 고맙고, 사랑해.

논문의 첫 단추이자 연구실의 첫 시도했던 분리. 너무도 적극적으로 도와주신 나의 은인 인제대 BPRC 안은영 선생님, 덕분에 포기하지 않고 지난해 겨울 내내 김해를 출퇴근했습니다. 진심으로 감사합니다. 그리고 누구보다 끝까지 잘할 수 있다며 응원해주던 경화언니와 나에게 늘 큰 언니인 순정언니, 후배 수영, 동기 종욱, 인구 그리고 남기완 교수님 외 조류학 연구실 가족들에게도 감사함을 전합니다. 사랑하는 세실리아 경희와 안드레아 신부님, 기도해주신 덕분입니다. 가까이 있지 않아도 서로 사랑하는 마음은 늘 간직하고 감사하며 살아가겠습니다. 그리고 나의 사랑하는 여우야 친구들 희진, 하나, 재경, 효영, 현정, 인미와 언제나 먼저 걱정해주고 연락해주는 선영, 같은 고민으로 서로를 다독이며 열심히 공부하고 있는 은정, 타국에 있어 더 보고픈 보아와 보라색 교복의 추억을 함께하는 혜정, 동희, 영애 그리고 18년을 함께하는 부가고연 21명 친구들과 2001년 여름을 함께 영국에서 행복했던 난영언니, 승목선배, 유라, 은경, 효진에게도 그동안 바쁘단 핑계로 자주 연락하지 못한 미안함과 늘 걱정해주고 응원해줘서 감사한 마음 전합니다.

개인적으로도 학위과정 중에 많은 변화가 있었습니다. 하나에서 시작한 학위과정은 셋이 되어 마무리가 되었습니다. 세상에서 가장 멋진 분을 만나 결혼하였고 흑룡띠의 예쁜 딸이 태어났습니다. 사랑하는 가족이 많이 늘어서 더 힘을 낼 수 있었나봅니다. 늘 내 편이 되어주는 여름미남 문석오빠, 하고 싶은 연구할 수 있도록 도와주고 응원해주는 당신이 곁에 있어서 감사합니다. 그리고 예서. 태어나서 누워만 있던 아기가 어느새 “공부 열심히 하고 와, 예서는 잘놀고 있을게, 사랑해”라며 아침마다 응원해줍니다. 많은 시간을 함께 보내지 못해 미안한 마음 가득하지만 훗날 아이가 컸을 때 자랑스러운 엄마가 되기 위한 과정이라 여기며 더 열심히 나아가겠습니다.

제 공부를 흔쾌히 지원해주시며 늘 응원해주시는 사랑하는 우리 어머니, 아버지 너무 감사합니다. 멋쟁이 시아버지께서 신혼여행 후 처음 말없이 잡아주신 제 손

에 느껴진 그 말로는 표현 못할 따스함을 평생 잊을 수 없을 것 같습니다. 그리고 사랑하는 어머니, 이 세상에 이런 시어머니는 없으십니다. 당신의 가족을 향한 무한한 사랑과 내재된 성품에 매일매일 감동합니다. 힘들다며 지친다고 울기도 했던 부족한 며느리에게 늘 사랑한다며 안아주시고 먼저 토닥여주시며 달래주셨던 많은 날들. 따뜻하기만 한 그 사랑을 저도 닮아가고 가족들에게 베풀며 살아가도록 노력하겠습니다. 그리고 저에게 맡겨진 많은 일들에 힘겨워 무심해보일 수 있었던 올케를 늘 웃음으로 맞이해주는 따뜻한 지원언니, 제 몫까지 부모님은 물론이고 예서까지 신경써주셔서 너무도 감사해요. 조금씩 더 다가갈게요. 우리 예서의 세 번째 할머니, 윤 스콜라 선생님. 사랑으로 가득하신 선생님 덕분에 우리 가족 모두 제자리에서 열심히 생활할 수 있었습니다. 진심으로 감사합니다.

사랑하는 엄마, 아빠. 왜 미리 알지 못했을까 후회가 많이 됩니다. 천천히 가는 딸에게 다그침없이 그저 지켜보기만 하시고. 누구보다 잘 아는 길이기에 더 말할 수밖에 없었을텐데도 오히려 묵묵히 기다려주심을 이제야 헤아립니다. 늦은 시작이 되었지만 두 분을 따라 행복한 마음으로 이 길을 가겠습니다. 우리 세 가족을 너무도 사랑으로 맞이해주시는 두 분이 있어서 행복하고 든든합니다. 제가 그랬듯 훗날 아이가 마음으로 따를 수 있는 좋은 부모가 되도록 노력하며 살아가겠습니다. 사랑해요.

돌이켜보면, 공부도 하고 사람도 얻은 행복한 학위과정이었습니다. 그동안 좋은 일에 함께 기뻐해주고 지칠 때 힘이 되어주었던 많은 분들에게 짧게나마 감사의 인사를 전하며, 앞으로 더 열심히 제 자리를 지키며 살아가겠습니다.