DOCTORAL THESIS

New Skin Brightening Mechanisms by Degradation of Tyrosinase in Melanocytes and Digestion of Melanosome in Keratinocytes

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Chapter 1 Introduction

1.1. Background

1.1.1. Human skin and epidermis

Human skin accounts for 6.3-6.9% of body weight, about 9 kilograms, and an area of about 1.6 square meters. It is the largest organ of the human body, and its main function is to protect the body, wick perspiration and feel. From the outside to the inside are: epidermis, dermis and subcutaneous tissue.

The thickness of the epidermis is about 0.1 to 0.2 mm. Keratinocytes are the main cells of the epidermis, accounting for about 95%. The remaining 5% of cells are Merkel cells, Melanocytes, Inflammatory cells and Langerhans cells. According to the morphological characteristics of cells, the epidermis is divided into four layers from the surface: stratum corneum, stratum granulosum, stratum spinosum and stratum basale. The cells above the spinous layer are derived from the basal cells of the basal layer. Keratinocytes divide only in the basal layer, and differentiate into spinous layer, granular layer and outermost stratum corneum after maturation. In the process of differentiation (keratinization), the barrier function of ecological protection is obtained [1–3]. Pigment cells are located in the basal layer, melanin synthesized near the nucleus of melanocytes accumulates in melanosomes, and the mature melanosomes are transported to the vicinity of the cell membrane via long-range bidirectional microtubules and directly under the cell membrane via short-range unidirectional actin fibers. Subsequently, it combines with the cell membrane, is transferred to the adjacent keratinocytes, and accumulates on the nucleus of the keratinocytes to form a nuclear cap (melanin cap) [4,5], which protects the keratinocyte DNA from UV damage [6].

1.1.2.Skin color determinant-Melanin

Human skin color is determined by the abundance and proportion of melanin, hemoglobin, carotene, etc. in the skin. Among them, melanin contributes

most strongly to the determination of skin color.

Epidermal melanin is important in the regulation of skin color and determines the wide variety of skin colors associated with ethnic diversity [7]. Melanin is produced by melanosomes, which are organelles within melanocytes. Melanosomes are a complex of bilayers in which enzymes involved in melanin production are localized. The melanin produced accumulates in the melanosome complex and fills into a mature melanosome complex. The mature melanosome complex is passed from the tip of the dendritic process of melanocytes to the surrounding keratinocytes. Melanosome complexes are digested within keratinocytes. The size, number and distribution of melanosome complexes are the main factors that determine skin color.

1.1.3. Melanin and Melanosomes

Melanin is synthesized in melanosomes, which are the organelles found in the pigment cells (melanocytes) in the basal layer of the epidermis. In the melanosomes, tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA), and DOPA to dopaquinone, by the action of the enzyme tyrosinase. After dopaquinone formation, the eumelanin pathway proceeds as follows: first, dopachrome is converted to 5,6-dihydroxy-1H-indole (DHI) by spontaneous decarboxylation or to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) catalyzed by dopachrome tautomerase (DCT) [8]. Melanogenesis can culminate in eumelanin by oxidative polymerization reactions following the formation of DHI and DHICA [8]. Tyrosinase, a melanin synthase, is synthesized separately from melanosomes and transported by vesicles to immature melanosomes. The optimum pH of human tyrosinase is 7.5 [9]. Tyrosinase-related protein-1 (TRP-1) is present in mature melanosomes and has been used as a marker for melanosomes [10,11]. The melanin synthesized near the nucleus of melanocytes accumulates in melanosomes, and the mature melanosomes are transported to the vicinity of the cell membrane via long-range bidirectional microtubules and directly under the cell membrane via short-range unidirectional actin fibers. Subsequently, it combines with the cell membrane, is transferred to the adjacent keratinocytes, and accumulates on the nucleus of the keratinocytes to form a nuclear cap

(melanin cap) [4,5].

Melanosomes are specialized lysosomes [12–14]. The first, the protein formally proved to be shared by lysosomes and melanosomes was the lysosomal membrane glycoprotein Lamp-1 [13,15]. Furthermore, Lamp-1 was identified in vesicles implicated in the trafficking of the tyrosinase-related protein family to melanosomes [15,16]. Melanosomes are the major repository in melanocytes for the lysosomal hydrolytic enzymes β-glucuronidase, β-galactosidase, β-hexosaminidase, cathepsin B and cathepsin L [17]. In addition, the high levels of cathepsin D were found to be present in immature melanosomes in retinal pigment epithelial cells [18].

1.1.4. The relationship between pH and pigment

Generally, the three-dimensional structure of an enzyme is prone to change with alteration in conditions such as heat, pH, and salinity, or its solvent. When that alteration is too great, the enzyme degenerates and becomes unable to retain its correct structure and is rendered inactive. This alteration applies to the case of tyrosinase, as shown in Himalayan rabbits and mice. The heads, paws, and tails of these animals are exposed to lower temperatures than other parts of their bodies, and these parts display regions of black fur called Himalayan markings. These animals have regions of dark coloration when they are raised in low-temperature zones, and these regions become lighter when they are raised in high-temperature zones. The Himalayan markings represent a dominant allele of the tyrosine gene called the ch gene. Bearers of this ch gene have a temperature-sensitive tyrosinase mutation and they experience loss of tyrosinase activity, lack melanin formation, and become white at high temperatures. Specifically, the body parts that are kept warm are white and body parts that are exposed to a low temperature are darkly pigmented. Due to this mutation, the animals have a white body trunk, where tyrosinase enzyme activity is lost and no melanogenesis occurs, and this area is kept warm, but their head, paws, and tails, which are exposed to the cold, retain tyrosinase activity and melanin-formation capacity, and these regions have dark pigment as a result [19]. The distinctive markings of Siamese cats are produced by a similar mechanism [20]. The

temperature-sensitive form of oculocutaneous albinism type 1 (OCA1-TS; one of the three categories of this condition together with OCA1A and OCAB1) is characterized by loss of tyrosinase activity at temperatures above 35°C, and the resulting diffused areas of melanin accumulation are related to differential temperatures for parts of the body [21,22].

The actions of tyrosinase and environment-related loss or fluctuation in the activity described above give rise to another question: can changes in pH suppress melanogenesis? Research on this question has advanced with a focus on the pH of the intramelanosomal environment. The optimum pH for tyrosinase is between 7 and 8, and reportedly this pH range does not vary between light-skinned people (Caucasians) and dark-skinned people (people of African descent) [23]. Racial differences in skin color are known to be caused by epidermal melanosome genetic characteristics, with reports that light-skinned people have less melanin and an acidic intramelanosomal pH, whereas dark-skinned people have more melanin and a neutral intramelanosomal pH [24,25].

Melanosome pH is regulated by vacuolar-type H^+-ATPase (V-ATPase), which mediates hydrogen ion (H^+) influx, and sodium-hydrogen (Na^+/H^+) exchangers, which mediate H^+ extrusion. Light-skinned people have plentiful melanosome-membrane V-ATPase, which enables large-scale H^+ influx, and few Na^+/H^+ exchangers, which restrict extrusion of H^+ from the melanosome and preserve large quantities of inwardly-transported H^+ and, thus, retains acidity. This is a stark contrast to the situation in dark-skinned people, who have little melanosome-membrane V-ATPase and limited H^+ influx, and numerous Na^+/H^+ exchangers, with consequently large-scale H^+ extrusion from the melanosome, which has a small quantity of H^+ and retains a neutral pH [23,25].

1.1.5. Lysosome and Cathepsin

Lysosomes are membrane-bound cytoplasmic organelles involved in intracellular protein degradation. They contain an assortment of soluble acid-dependent hydrolases and a set of highly glycosylated integral membrane proteins. Most of the properties of lysosomes are shared with a group of cell type-specific compartments referred to as ‘lysosome-related organelles’, which include

melanosomes, lytic granules, MHC class II compartments, platelet-dense granules, basophil granules, azurophil granules, and Drosophila pigment granules [26]. Lysosomal cathepsins, which are enclosed in the lysosomes, help to maintain the homeostasis of the cell's metabolism by participating in the degradation of heterophagic and autophagic material [27].

Melanosome complexes are known to be degraded by lysosomes in normal human epidermal keratinocytes [28]. It has been reported that aspartic proteases and cysteine proteases are involved in the promotion of tyrosinase degradation in acidic melanosomes [29]. Cysteine proteases are a type of acidic protease, and cathepsin L2 (also known as cathepsin V) is considered to act as a cysteine protease in the degradation of tyrosinase in melanosomes. Studies on racial and skin color differences have reported that melanosomes of light-skinned keratinocytes are degraded faster than those of dark-skinned keratinocytes [30] and the activity of the enzyme cathepsin L2 is reported to be higher in light-skinned keratinocytes than in dark-skinned keratinocytes [31]. The expression level of cathepsin L2 was lower in the epidermal basal layer than those in the stratum corneum in the hyperpigmented region. In addition, melanosome degradation was suppressed in cathepsin L2 knockdown cells [32]. Recent studies have shown that cathepsin L is involved in melanosome degradation in melanocytes through its lysosomal activity during autophagosome-lysosome fusion [33]. Since cathepsin K is also a cysteine protease, it is thought to degrade tyrosinase in melanosomes.

1.1.6. Pigmented spots

The formation of pigment spots is due to cell proliferation of pigment cells, increased melanin production and increased transfer of MS to keratinocytes, and long-term retention of melanin in the epidermis due to decreased keratinocyte differentiation potential [28,34].

In healthy skin, melanosome complexes in the epidermis are digested along with keratinization. senile lentigo is pigment spots that become apparent on the surface of the skin due to chronic exposure to sunlight. Its formation mechanism is currently unclear, however, in senile lentigo, they accumulate as

large melanosome complexes in the basal and spinous layers of the epidermis [5]. At present, there is no cosmetics that has a good therapeutic effect on it.

1.1.7. Parabens

Parabens are used in cosmetic formulations, and the C1 to C4 (side chain) parabens are indicated to reduce tyrosinase activity as the chain length increases. Parabens reported to have a wide antibacterial spectrum and high safety were investigated for anti-microbial action and were found to have a drug effect increasing in intensity in the order of methylparaben < ethylparaben < propylparaben < butylparaben. Accordingly, a very good linear relationship between the number of carbons on the alkyl chain and affinity was established. The carbon chain was determined to be the major player in differences in anti-microbial effects. The minimum growth inhibitory concentrations of benzoic acid and methylparaben at pH 3.50 were measured in yeasts with different resistance to preservatives, and it was reported that the resistance of yeast to benzoic acid was enhanced, but not to methylparaben, and that the minimum pH required for growth was not related to the resistance to weak acids [35]. Propylparaben retains its antibacterial efficacy at pH levels as high as 8.9 [36].

1.1.8. Angelica sinensis

Angelica (scientific name: *Angelica acutiloba*) is a perennial herb belonging to the Apiaceae (Umbelliferae) family. Used as a Chinese herbal medicine. In order to improve women's symptoms such as body cold, anemia, and irregular menstruation, the root is used as a medicinal ingredient and is used as the main medicine in many Chinese herbal medicines for the treatment of gynecological diseases. In addition, in an ancient Chinese story, a wife who had been suffering from gynecological diseases for a long time recovered through a herbal medicine (Angelica sinensis). Because she missed her husband who had been away for a long time, she stood at the door of the house and shouted expectantly: "Husband, you Time to go home." Therefore, this herb was named Angelica sinensis [37–39].The roots contain essential oils such as butylidene phthalide, carbachlor, and camphene, which have a sedative effect on the

cerebral cortex and a mild stimulating effect on the medulla oblongata center, thus increasing blood circulation and having a positive effect on respiration and substance metabolism [40], and are effective in relieving pain caused by hyperemia. It is said to have pus-dissipating and granulation properties. Other active ingredients include sitosterol, palmitic acid, linoleic acid, vitamin B1, and nicotinic acid [40].

1.1.9. Fennel

Fennel (scientific name: *Foeniculum vulgare* Mill.) is a perennial herb belonging to the Apiaceae (Umbelliferae) family. The young leaves and seeds (fennel seeds) have a sweet aroma and bitter taste, and effectively promote digestion and deodorizing. Since ancient times, fennel has been used as a spice and herb for food and medicinal purposes. The constituents of fennel seed oil are (E)-anethole, limonene, methyl chavicol, fenchone, α-pinene, and (Z)-β-ocimene [41,42]. Fennel seed oil reportedly exhibits anti-inflammatory properties [41]. In addition, fennel seed oil and seed extract in methanol or ethanol reportedly display antioxidant properties (DPPH scavenging activity, ferric reducing activity, and inhibition of peroxidation of linoleic acid) [43–45]. The composition of fennel seed methanolic extracts was characterized by its richness in quinic acid, 4-O-caffeoylquinic acid, p-coumaric acid, and 4-O-caffeoylquinic acid [44]. The water extract of fennel fruits also reportedly exhibits strong antioxidant activity (inhibition of peroxidation of linoleic acid) [45–47], and 4-O-β-D-glucosyl sinapyl alcohol, 4, 9-di-O-β-glucosyl alcohol, and 4-β-glucosyloxy benzoic acid were identified as its active components [47].

1.2. Objective

In Japan, medicated brightening cosmetics (“quasi-drugs”) that inhibit tyrosinase activity, including kojic acid, arbutin [48], and rucinol, promote the degradation of tyrosinase, such as linoleic acid [49]. Furthermore, stable vitamin C derivatives, vitamin C ethyl with antioxidant properties [50], and anti-inflammatory properties, such as that in tranexamic acid [51], are being developed [52]. However, agents such as rhododendrol and magnolignan are

currently not used because they release hydroxyl radicals from tyrosinase, are highly cytotoxic to melanocytes, and may induce leukoderma [53].The use of such substances, wherein hydroxyl radicals are generated by tyrosinase to regulate melanin production, poses a risk of causing vitiligo. Furthermore, they are ineffective in reducing the appearance of senile pigmentation.

Therefore, a new brightening mechanism that does not induce leukoplakia needs to be developed. Additionally, there is a growing need for developing skin-brightening ingredients with a mechanism that promotes the degradation of melanosome complexes accumulated in keratinocytes.

This study focused on a new brightening mechanism related to melanosome digestion. Additionally, the brightening effect and mechanisms of propylparaben, *Angelica acutiloba* root extract (touki extract), and *Foeniculum vulgare* Miller fruit extract (fennel extract) were investigated. Chapter 2 investigated the potential for pH-dependent tyrosinase degradation with a focus on the low pH of the intramelanosomal environment-induced suppression of melanogenesis. And also investigated whether the mechanism of the anti-melanogenic agent propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations. Chapter 3 hypothesized that enhancement of lysosomes function as the digestive system in highly pigmented keratinocytes could promote the digestion of melanosome complexes and recover decreased cellular proliferation, which leads to improve hyperpigmentation. The object of this study is to find active ingredients from plant extracts (*Angelica acutiloba* Root extract) for enhancing proliferation of keratinocytes with melanins and for enhancing lysosome function in the digestion of melanosome complexes and to apply their activity to improve hyperpigmentation. Chapter 4 investigated the degradation of melanosome complexes in cultured human keratinocytes by fennel extract, a natural component with reportedly strong antioxidant properties and analyzed the mechanism by focusing on lysosomes effects. In addition, to explore its potential effects on senile lentigo, Chapter 4 also investigated the effect of fennel extract on the expression of cathepsin K and cathepsin L2 in the epidermis (stratum corneum, stratum granulosum, stratum spinosum and stratum basale) by applying a human epidermal 3D model.

Chapter 2 Degradation of Tyrosinase by Melanosomal pH Change and a New Mechanism of Whitening with Propylparaben

2.1. Introduction

Melanin is synthesized in melanosomes, which are organelles found in the pigment cells (melanocytes) of the basal layer of the epidermis. Within melanosomes, the enzyme tyrosinase catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA into dopaquinone. After dopaquinone formation, the order of appearance in the eumelanin formation pathway is, first, dopachrome, which evolves to 5,6-dihydroxy-1H-indole (DHI), through spontaneous decarboxylation, or to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), catalyzed by dopachrome tautomerase (Dct) [8]. Melanogenesis can culminate in eumelanin through oxidative polymerization reactions following the formation of DHI and DHICA [8].

In Japan, active skin-lightening ingredients of formulated medicated brightening cosmetics (quasi-drugs) can suppress melanogenesis by inhibiting tyrosinase activity (as is the case with kojic acid, arbutin, and rucinol) or by accelerating the degradation of tyrosinase (as is the case with linoleic acid) [48,49,52]. However, agents, such as rhododendrol and magnolignan, are no longer used; they release hydroxyl radicals from tyrosinase and are associated with strong cytotoxicity to melanocytes and the potential to induce leukoderma [53,54].

Generally, the three-dimensional structure of an enzyme is prone to change with alteration in conditions such as heat, pH, and salinity, or its solvent. When that alteration is too great, the enzyme degenerates and becomes unable to retain its correct structure and is rendered inactive. This alteration applies to the case of tyrosinase, as shown in Himalayan rabbits and mice. The heads, paws, and tails of these animals are exposed to lower temperatures than other parts of their bodies, and these parts display regions of black fur called Himalayan

markings. These animals have regions of dark coloration when they are raised in low-temperature zones, and these regions become lighter when they are raised in high-temperature zones. The Himalayan markings represent a dominant allele of the tyrosine gene called the ch gene. Bearers of this ch gene have a temperature-sensitive tyrosinase mutation and they experience loss of tyrosinase activity, lack melanin formation, and become white at high temperatures. Specifically, the body parts that are kept warm are white and body parts that are exposed to a low temperature are darkly pigmented. Due to this mutation, the animals have a white body trunk, where tyrosinase enzyme activity is lost and no melanogenesis occurs, and this area is kept warm, but their head, paws, and tails, which are exposed to the cold, retain tyrosinase activity and melanin-formation capacity, and these regions have dark pigment as a result [19]. The distinctive markings of Siamese cats are produced by a similar mechanism [20]. The temperature-sensitive form of oculocutaneous albinism type 1 (OCA1-TS; one of the three categories of this condition together with OCA1A and OCAB1) is characterized by loss of tyrosinase activity at temperatures above 35°C, and the resulting diffused areas of melanin accumulation are related to differential temperatures for parts of the body [21,22].

The actions of tyrosinase and environment-related loss or fluctuation in the activity described above give rise to another question: can changes in pH suppress melanogenesis? Research on this question has advanced with a focus on the pH of the intramelanosomal environment. The optimum pH for tyrosinase is between 7 and 8, and reportedly this pH range does not vary between light-skinned people (Caucasians) and dark-skinned people (people of African descent) [23]. Racial differences in skin color are known to be caused by epidermal melanosome genetic characteristics, with reports that light-skinned people have less melanin and an acidic intramelanosomal pH, whereas dark-skinned people have more melanin and a neutral intramelanosomal pH [24,25].

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Na^+/H^+ exchangers, which restrict extrusion of H^+ from the melanosome and preserve large quantities of inwardly-transported H^+ and, thus, retains acidity. This is a stark contrast to the situation in dark-skinned people, who have little melanosome-membrane V-ATPase and limited H^+ influx, and numerous Na^+/H^+ exchangers, with consequently large-scale H^+ extrusion from the melanosome, which has a small quantity of H^+ and retains a neutral pH [23,25].

Based on this background, we investigated the potential for pH-dependent tyrosinase degradation with a focus on the low pH of the intramelanosomal environment-induced suppression of melanogenesis. We also investigated whether the mechanism of the anti-melanogenic agent propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations. In this report, we describe both of these investigations.

2.2. Materials and Methods

2.2.1. Materials

p-Hydroxybenzoic acid methyl ester (methylparaben), p-hydroxybenzoic acid ethyl ester (ethylparaben) and p-hydroxybenzoic acid butyl ester (butylparaben) were purchased from Wako Pure Chemical Industries (Osaka, Japan). p-Hydroxybenzoic acid propyl ester (propylparaben) was from Ueno Fine Chemicals Industry (Osaka, Japan). Bafilomycin A1, aprotinin from bovine lung, phenylmethane sulfonyl fluoride (PMSF), leupeptin hemisulfate salt, E-64, and pepstatin A were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). DAMP {N-(3-[(2,4-dinitrophenyl)amino]propyl)-N-(3-aminopropyl)methylamine dihydrochloride} was from Oxford Biomedical Research, Inc. (Oxford, MI, USA). The anti-tyrosinase antibody, anti-tyrosinase-related proteins-1 (Trp-1) antibody, and anti-Dct antibody were purchased from Aviva Systems Biology (San Diego, CA, USA). The anti-premelanosome protein 17 (Pmel17) antibody was purchased from Sigma-Aldrich Corp. and the anti-microphthalmia-associated transcription factor (Mitf) antibody (C5) was purchased from Abcam plc. (Cambridge, UK). The anti-dinitrophenyl-KLH, rabbit IgG antibody, Alexa Fluor 488 goat anti-rabbit IgG antibody and Alexa Fluor 546 goat anti-rat IgG antibody

were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other reagents used in experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2.2. Preparation of Melanosome-Fractions and Examination of Melanosomes

Cells were seeded (at 100,000 cells/mL) into ten 10-cm dishes and cultured for three days. B16 melanoma cells that had been cultured for three days were harvested with trypsin-EDTA solution, washed with a phosphate-buffered salt (PBS) solution, and pulverized with a homogenizer to prepare the cellular tissue. The cellular tissue was mixed with 0.25 mol/L sucrose solution to prepare a homogenate. A discontinuous sucrose density gradient was created with sucrose solutions at 1.8 mol/L, 1.6 mol/L, 1.4 mol/L, 1.2 mol/L, 1.0 mol/L, 0.8 mol/L, and 0.4 mol/L by overlaying lower concentrations on higher concentrations. The homogenate was subjected to density-gradient centrifugation at 700× g, and the supernatant was layered and further centrifuged with an ultracentrifugation device (21 h, 100,000× g, 4°C). After centrifugation, fractions were collected by layer descending from the upper layer with a quantitative delivery liquid pump, to prepare melanosome fractions. The prepared fractions were numbered from 1 (fraction 1) for the highest layer (0.25 mol/L sucrose layer) to fraction 8 for the lowest layer (1.8 mol/L sucrose layer). Fractions were examined with an inverted microscope to confirm the presence of melanosomes.

2.2.3. Measurement of Melanosome-Fraction Tyrosinase Activity

Each fraction was added to wells on a 96-well plate (Corning Incorporated, Corning, NY, USA) at 10 μL/well (n = 3). Then, 80 μL of 1% Triton X-100 solution and 10 μL of 10 mmol/L DOPA were added to each well, and the absorbance was measured (wavelength: 475 nm) immediately (0 min) and two hours later using a microplate reader (Multi-Detection Microplate POWERSAN HT; BioTek, Winooski, VT, USA).

2.2.4. Effect of pH on Melanosome-Fraction Tyrosinase Activity

Citric acid–sodium citrate buffers (0.1 mol/L; pH 3, pH 4, and pH 5) and sodium phosphate buffers (0.1 mol/L; pH 6 and pH 7) were prepared. A 45 μL aliquot of each buffer was added to a 1.5 mL micro-tube, and 45 μL of a prepared melanosome fraction (fraction 8) were added to each tube. The cells were then left to stand overnight in an incubator (37°C) and a freezer (−20°C). After overnight incubation, 10 μL aliquots of the mixture in each tube were added to wells on a 96-well plate (n = 3). Then, 80 μL of PBS (pH 6.8) and 10 μL of 10 mmol/L DOPA solution were added to each well, and the absorbance was measured (wavelength: 475 nm) immediately (0 min) and 2 h later using the microplate reader. A 10% volume of glycerin (a 10% volume) was added to a sample of each mixture, which had been left to stand overnight, and 15 μL of each sample was applied to a 4–20% SDS-PAGE mini-gel and subjected to electrophoresis. After electrophoresis, the gel was removed by washing with PBS solution two to three times, and the sample was stained with a 2% DOPA solution. After two hours, the solution was exchanged for fresh DOPA solution and the stained sample was examined for a tyrosinase band.

2.2.5. Effect of pH on Melanosome-Fraction Tyrosinase Proteins

Citric acid–sodium citrate buffers (pH 3, pH 4, and pH 5) and sodium phosphate buffers (pH 6 and pH 7) were added to micro-tubes at 45 μL per tube. A 45 μL aliquot of a melanosome fraction prepared in each buffer was added to each micro-tube, and the tubes were left overnight in the incubator (37°C) and a freezer (−20°C). After being left overnight, a 10% volume of glycerin was added to each sample. A 15 μL aliquot of each sample was then added to a 4–20% SDS-PAGE gel and subjected to electrophoresis. After electrophoresis, each sample was submitted to a Western blotting assay. An anti-tyrosinase antibody was used as the primary antibody. The secondary antibody was a peroxidase-labeled anti-rabbit antibody, which had been prepared by dilution with a tris-buffered salts (TBS) solution.

2.2.6. Effect of Bafilomycin A1 on Melanin Content

B16 melanoma cells were seeded onto 10-cm dishes at 10 mL per dish (100,000 cells/mL) and cultured for one day. A 10 μL aliquot of 1 μmol/L bafilomycin A1 formulation was added to each dish, and the cells were then cultured for three days. Cultured cells were harvested with trypsin-EDTA and centrifuged. The culture medium was discarded, and the cells were repeatedly washed with PBS solution, and then counted with a hemocytometer. After being counted, the cells were centrifuged again, the supernatant was discarded, and the cellular sediment was dissolved in an amount of 1 mol/L NaOH according to the number of cells. Dissolved melanosome content was imaged with a digital camera.

2.2.7. Effect of Enzyme Inhibitors on Tyrosinase Degradation

Citric acid-sodium citrate buffer (pH 4) was prepared and added to 1.5 mL micro-tubes at 50 μL per tube. A 50 μL aliquot of the prepared melanosome fraction was added to each tube followed by 1 μL of the relevant enzyme inhibitor. The cells were left overnight in the incubator (37°C) and a freezer (−20°C). The enzyme inhibitors were 1 mol/L phenylmethane sulfonyl fluoride (PMSF; a serine protease inhibitor), 2.2 mmol/L leupeptin (a serine/cysteine protease inhibitor), 1.4 mmol/L E64 (a cysteine protease inhibitor), and 10 mmol/L pepstatin A (an aspartic proteinase inhibitor). After being left overnight, 10 μL aliquots of each sample were added to wells on the 96-well plate (n = 3). An 80 μL aliquot of PBS solution and a 10 μL aliquot of 10 mmol/L DOPA solution were added to each well, and absorbance was measured (wavelength: 475 nm) with the microplate reader immediately (0 min), 2 h, and one day after to determine tyrosinase activity.

2.2.8. Effects of Parabens on Cell Proliferation

Parabens with methyl, ethyl, propyl, and butyl chains were used. B16 melanoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and subjected to the experiments described below. Cells were seeded into wells of the 96-well plate at 99 μL per

well (30,000 cells/mL) and cultured for one day. Methylparaben, ethylparaben, propylparaben, and butylparaben were dissolved in dimethyl sulfoxide (DMSO) solution to prepare 1, 2, and 4 mg/mL, and these solutions were added to wells at 1 μL per well (n = 3). The samples were cultured for two days, after which each well was washed with PBS solution and the cells were then cultured with DMEM diluted 100-fold with Hoechst 33258 (100 μg/mL DMSO) for 30 min. The relative fluorescence unit (RFU) was then measured with the microplate reader (excitation, 360 nm; emission, 465 nm).

2.2.9. Comparison of Paraben Inhibitory Effects on Intracellular Anti-Tyrosinase Activity

B16 melanoma cells were seeded into wells on a 96-well plate at 99 μL per well (30,000 cells/mL), and cultured for one day. Methylparaben, ethylparaben, propylparaben, and butylparaben were dissolved in dimethyl sulfoxide (DMSO) solution to prepare 1, 2, and 4 mg/mL, and these solutions were added to wells at 1 μL per well (n = 3). The samples were cultured for two days, after which 10 μL of 10 mmol/L DOPA solution and 90 μL of 1 mmol/L Triton X-100 solution were added to each well, and the absorbance was measured (wavelength: 475 nm) immediately (0 min) and 1 h later with the microplate reader.

2.2.10. Comparison of Anti-Melanogenic Effects of Propylparaben, 4-Methoxy Salicylic Acid Potassium Salt, and Arbutin

B16 melanoma cells were seeded onto 10-cm dishes at 9.99 mL per dish (100,000 cells/mL) and cultured for one day. Propylparaben, 4-methoxy salicylic acid potassium salt and arbutin were dissolved in DMSO/H_2O solution to prepare 40 mg/mL solutions. Then 10 μL aliquots of these solutions were added to the dishes, and the dishes were cultured for two days. The cells were imaged with a microscope, and the cultured cells were then harvested with trypsin-EDTA. The cells were centrifuged, the culture medium was discarded, and the cells were repeatedly washed with PBS solution, then counted. After being counted, the cells were centrifuged again, the liquid was discarded, and the cellular sediment

was dissolved in 1 mol/L NaOH and imaged with a digital camera.

2.2.11. Effect of Propylparaben on mRNA Expression of Melanogenesis-Related Proteins

B16 melanoma cells were seeded onto 35-mm dishes at 1 mL per dish (100,000 cells/mL) and cultured for one day. Propylparaben was dissolved in DMSO solution to prepare 2 and 4 mg/mL solutions, 1 μL aliquots of these solutions were added to the dishes, and the cells were cultured for two days. The mRNA was extracted from cultured cells with an mRNA extraction kit (RNeasy Mini-kit, Qiagen K.K., Tokyo, Japan). The mRNA expression was measured for tyrosinase, Trp-1, Dct, Pmel17, Mitf, and glycerol-3-phosphate dehydrogenase (G3pdh) using a real-time polymerase chain reaction (PCR) system (ABI PRISM 7900HT, Applied Biosystems, Foster City, CA, USA).

2.2.12. Effects of Propylparaben on mRNA Expression of Melanogenesis-Related Proteins in Western Blotting

B16 melanoma cells were seeded into 10-cm dishes at 9.99 mL per dish (100,000 cells/mL) and cultured for one day. Propylparaben was dissolved in DMSO to prepare 1, 2, and 4 mg/mL solutions, 10 μL aliquots of these solutions were added to the dishes, and the cells were cultured for two days. The cells were harvested, and the proteins were quantified by the microplate reader measurement (at 560 nm) with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). After quantification, the proteins were separated with electrophoresis and subjected to a Western blot assay. The primary antibodies were anti-tyrosinase, anti-Trp1, anti-Dct, anti-Pmel17, and anti-Mitf antibodies, which were diluted 1000-fold. The secondary antibody was a peroxidase-labelled anti-rabbit IgG antibody, which was diluted 5000-fold in Tris-buffered saline (TBS) solution.

2.2.13. Effect of Propylparaben on mRNA Expression of Melanosomal pH Regulators (Slc24a5, Slc45a2, Atp6v0c-Encoded V-ATPase Sub-Unit, and G3pdh)

Cells were seeded into 35-mm dishes at 0.999 mL per dish (30,000 cells/mL) and cultured for one day. Propylparaben was dissolved in DMSO to prepare 2 and 4 mg/mL solutions, which were added to the dishes at 1 μL per dish, and the cells were cultured for three days (n = 3). mRNA was extracted from the cultured cells with the mRNA extraction kit (RNeasy Mini-kit) mRNA expression was measured for Slc24a5, Slc45a2, Atp6v0c-encoded sub-unit protein, and G3pdh using the real-time PCR system. The primers for Slc24a5, Slc45a2, Atp6v0c, and G3pdh were acquired from Qiagen K.K.

2.2.14. Effect of Propylparaben on Melanosome pH

Cells were added to chamber slides at 0.999 mL per slide (10,000 cells/mL) and cultured for one day. Propylparaben was dissolved in DMSO to prepare 2.5 and 5 mg/mL solutions, which were added to the slides at 1 μL per slide, and the cells were cultured for three days. The chamber slides were washed with serum-free DMEM, and a 30 μmol/L DAMP solution was prepared with serum-free DMEM and added to the slides at 1 mL per slide. The cells were incubated for 40 min and then washed with PBS solution, 1 mL of 4% paraformaldehyde was added, and the slides were left to stand for 20 min to fix the cells in paraformaldehyde. After fixation, the cells were washed with PBS, 1 mL of 50 mmol/L NH_4Cl was added, and the cells were incubated for 10 min. When DAMP is incubated with live cells, its intake is dependent on the intracellular organelle acidity pH. Any acidic granules with DAMP accumulation in fixed cells can be detected with an anti-dinitrophenol (DNP) antibody and a secondary antibody. DAMP is an acidotropic reagent and fluorescence will be stronger when the interior of the cell is acidic. The cells were permeabilized with 0.1% Triton X-100, isothiocyanate Alexa Fluor 488 goat anti-rabbit IgG antibody and an anti-2,4-dinitrophenol antibody diluted 2000-fold with 1% bovine serum albumin (BSA) was added. The cells were incubated for two hours. The cells were washed four times with PBS solution. The primary antibody (rat anti-Trp-1

antibody) was then added, and the cells incubated for two hours at room temperature. The cells were washed with PBS solution three times, then 2000-fold diluted Alexa Fluor 546 goat anti-rat IgG antibody was added and the cells incubated for one hour at room temperature. The cells were stained and examined under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and a confocal laser scanning microscopy (FV3000; Olympus, Tokyo, Japan).

2.2.15. Statistical Analysis

Measured values were analyzed for significant differences using an unpaired t-test function (two-tailed) in Microsoft Excel. $p < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Effect of pH on Tyrosinase Activity and Content

We investigated how the pH of the intramelanosomal environment affects tyrosinase. We made a homogenate from B16 melanoma cells, and we prepared melanosome fractions with sucrose density gradient centrifugation. We examined each fraction for tyrosinase activity, and we found activity in fractions 2, 3, and 8; however, only fraction 8 showed dark organelles under microscopic examination. Accordingly, we considered melanosome collection successful for fraction 8 (the 1.8 mol/L sucrose layer). Prepared melanosome fractions were left overnight in buffers with pH values between 3 and 6. The melanosome fractions were then transferred into pH 7 buffer for measurements of the activity and the amount of tyrosinase. The results demonstrated decreases in both activity and the amount of tyrosinase at pH 3 and pH 4 (acidic conditions; **Figure 2-1**). Next, we investigated fluctuations in melanin content when a low intramelanosomal pH decrease is blocked with bafilomycin A1, which is a V-ATPase inhibitor. B16 melanoma cells were cultured with bafilomycin A1 for three days, and tyrosinase and melanin contents were determined. We found that both tyrosinase and melanin contents decreased in bafilomycin A1-treated cells relative to controls (**Figure 2-2**). Furthermore, we investigated enzyme inhibitors for tyrosinase-degrading effects to determine the mechanism of tyrosinase degradation at a low intramelanosomal pH. The enzyme inhibitors that were used were aprotinin (a serine protease inhibitor), PMSF (a serine protease inhibitor), leupeptin (a serine/cysteine protease inhibitor), E64 (cysteine protease inhibitor), and pepstatin A (an aspartic proteinase inhibitor). Melanosome fractions were incubated overnight with these enzyme inhibitors, and tyrosinase activity was measured. We found that aspartic proteinase inhibitor pepstatin A suppressed the decrease in tyrosinase activity at a low intramelanosomal pH (**Figure 2-3**).

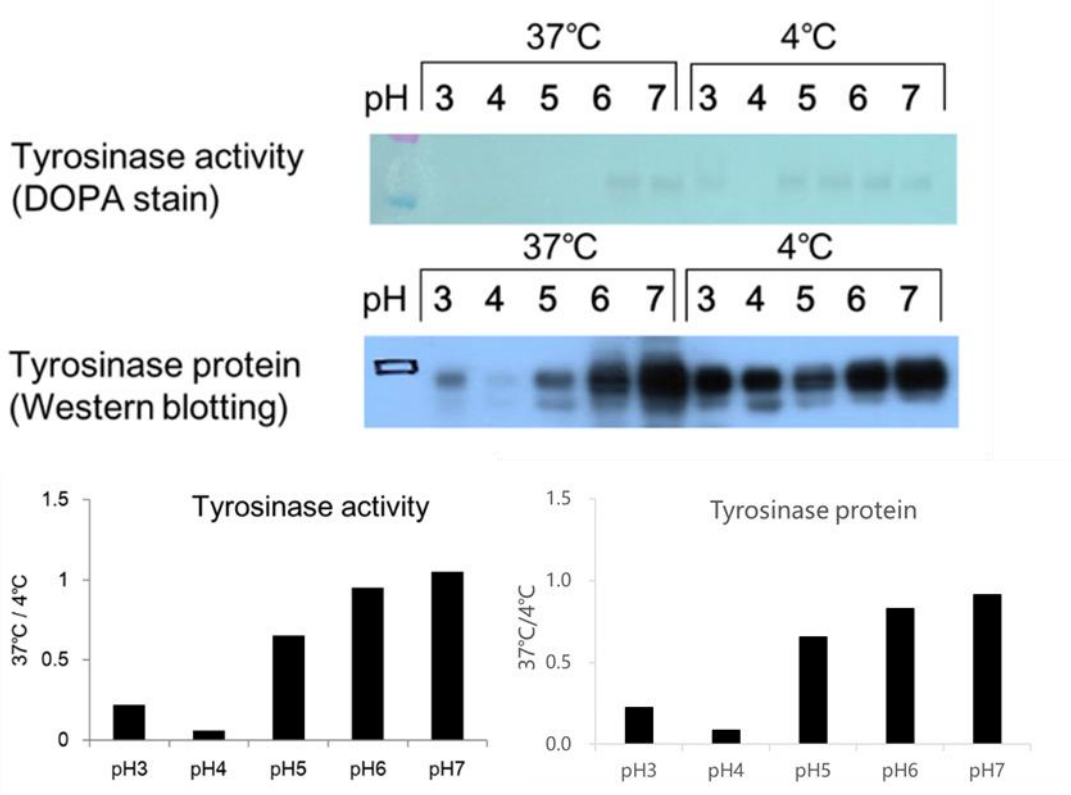


Figure 2-1. Effect of melanosome pH on the activity and amount of tyrosinase.

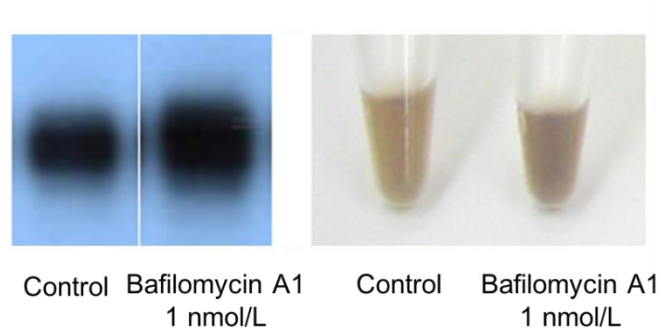


Figure 2-2. Effect of bafilomycin A1 on the amount of melanin.

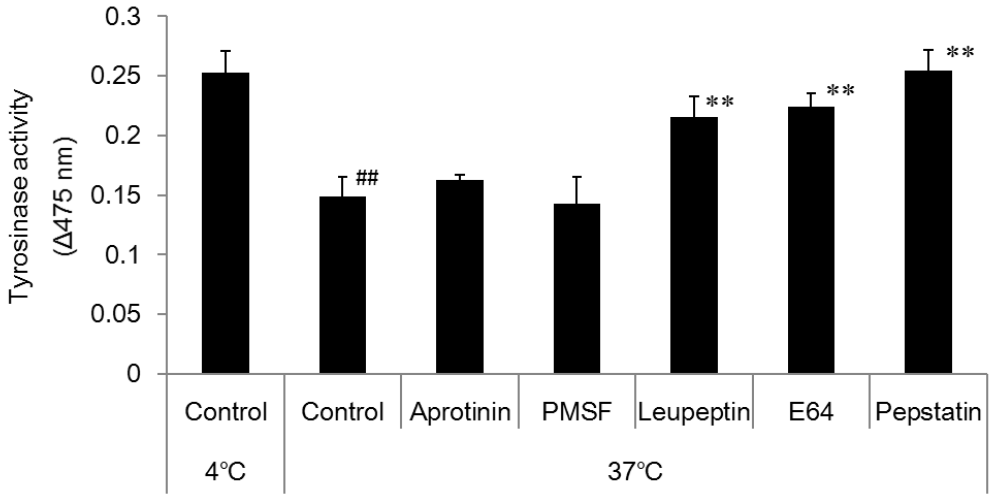


Figure 2-3. Effect of various enzyme inhibitors on the promotion of decomposition of the tyrosinase of melanosomes at pH 4. The results are expressed as the mean ± standard deviation of three experiments. ## $p < 0.01$ vs. control at 4°C, ** $p < 0.01$ vs. control at 37°C.

2.3.2. Inhibitory Effects of Parabens on Intracellular Tyrosinase Activity

We investigated the effects of parabens on cell proliferation in B16 melanoma cells cultured for two days. We found no statistically significant difference from controls for any paraben at any tested concentration (10, 20, and 40 μg/mL), which demonstrated that parabens have no effects on cell proliferation at concentrations of 40 μg/mL or below (**Figure 2-4a**). We also investigated the effects of parabens on intracellular tyrosinase activity at 10, 20, and 40 μg/mL and found that parabens inhibited tyrosine activity depending on concentration. The strength of this effect increased in the following order: methylparaben < ethylparaben < propylparaben < butylparaben (**Figure 2-4b**). The photograph of melanin in cultured B16 melanoma cells was reduced by propylparaben, as shown in **Figure 2-4c**. Particularly noteworthy findings were that propylparaben and butylparaben reduced tyrosinase activity by more than 50% when applied at 40 μg/mL. Propylparaben is widely used in cosmetic products, and we found that propylparaben was more effective than the skin-lightening agents 4-methoxy salicylic acid potassium salt and arbutin when these parabens were applied at 40 μg/mL (**Figure 2-4d**).

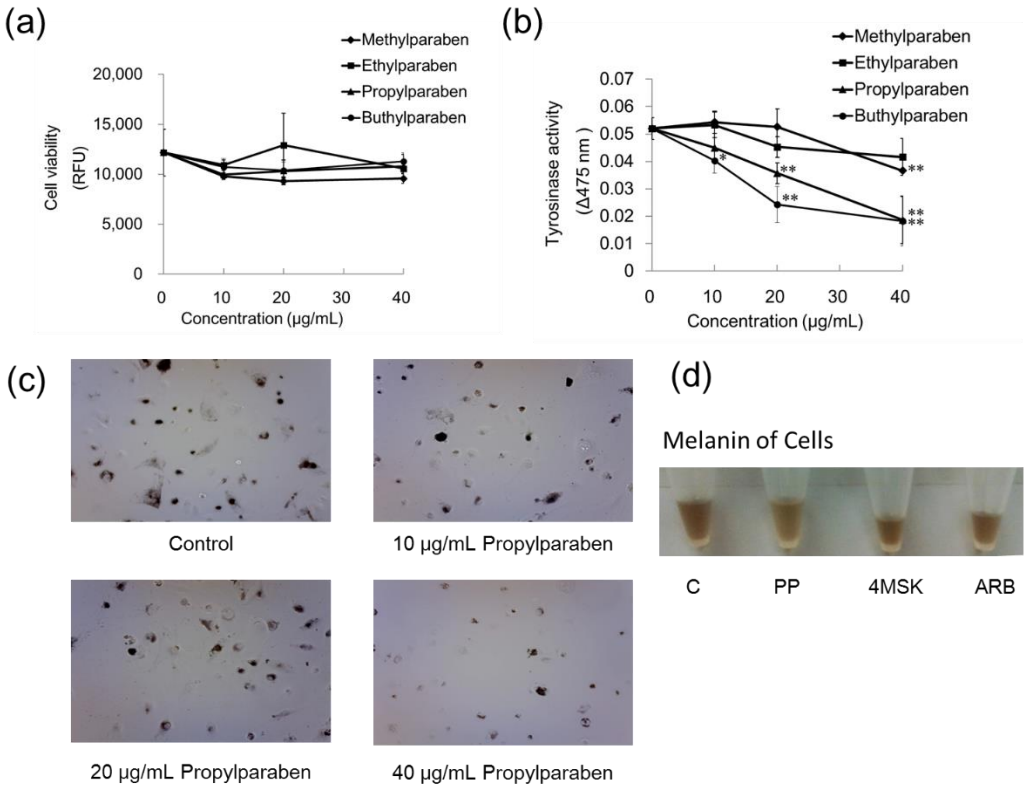


Figure 2-4. (a) Effect of parabens (methyl, ethyl, propyl, and butyl) on the cell number of cultured B16 melanoma cells. The results are expressed as the mean ± standard deviation of three experiments. (b) Effect of parabens (methyl, ethyl, propyl, and butyl) on cellular tyrosinase activity of cultured B16 melanoma cells. The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control. (c) Phase contrast microscope images of B16 melanoma cells cultured with or without propylparaben. (d) Photographs of cellular melanin of cultured B16 melanoma cells with control (C), propylparaben (PP), 4-methoxy salicylic acid potassium salt (4MSK), and arbutin (ARB) at 40 μg/mL. Cellular melanin was normalized according to the number of cells.

2.3.3. Effect of Propylparaben on Melanogenesis-Related Protein mRNA Expression

We ascertained inhibition of tyrosinase and suppression of melanogenesis in propyl paraben-treated cells, and then we investigated the effect of this paraben on mRNA expression levels for the following melanogenesis-related proteins: tyrosinase, Trp-1, Dct, Pmel17, and Mitf. At 40 μg/mL, propylparabens increased Mitf mRNA expression, had no major effect on tyrosinase, Trp-1, or Dct mRNA expression, and increased Pmel17 mRNA expression in a concentration-dependent manner (**Figure 2-5**). In our investigation of propylparaben effects on melanogenesis-related proteins, we found no major effects on the intramelanosomal proteins tyrosinase, Trp-1, and Dct, but there were increases for the premelanosome structural protein Pmel17 and nuclear transcription factor Mitf (**Figure 2-6**).

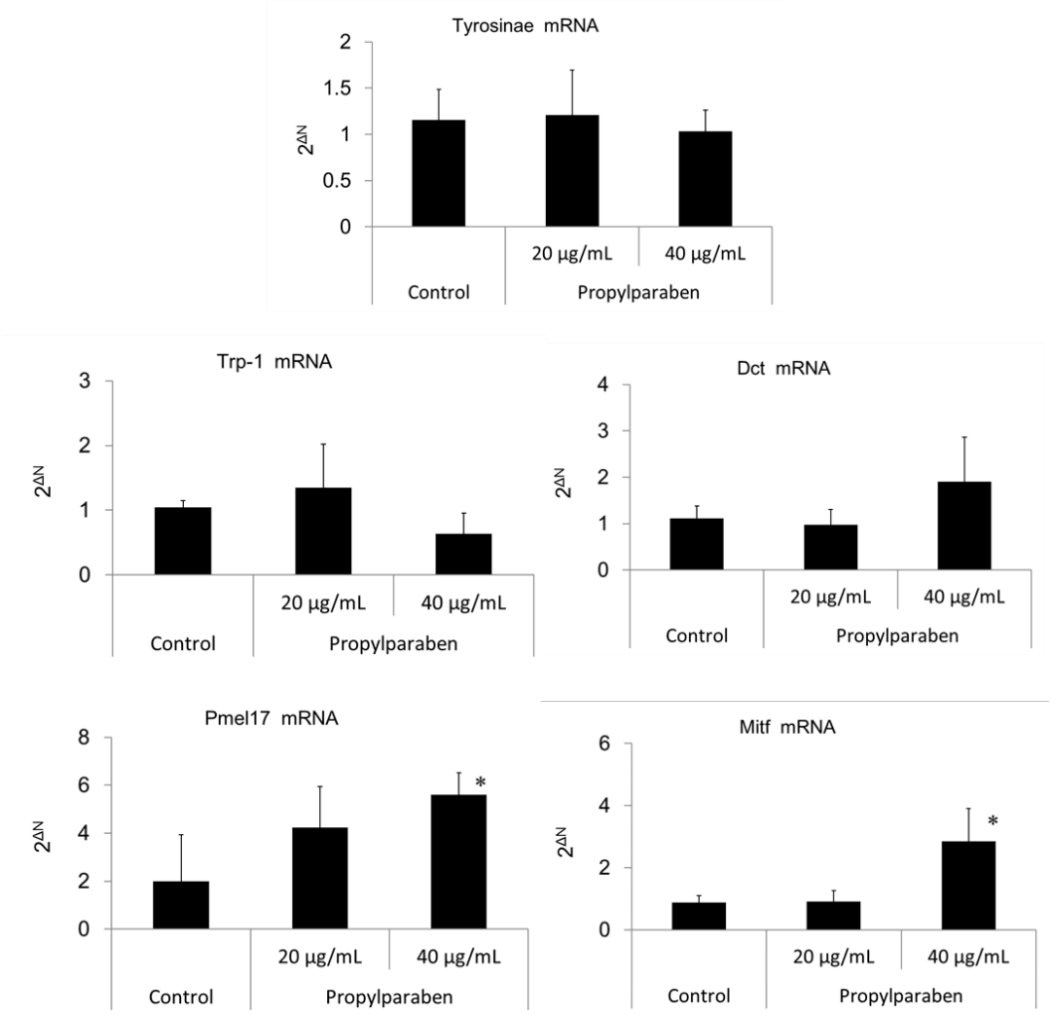


Figure 2-5. Effects of propylparaben on the mRNA expression of melanogenic proteins in cultured B16 melanoma cells. The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control.

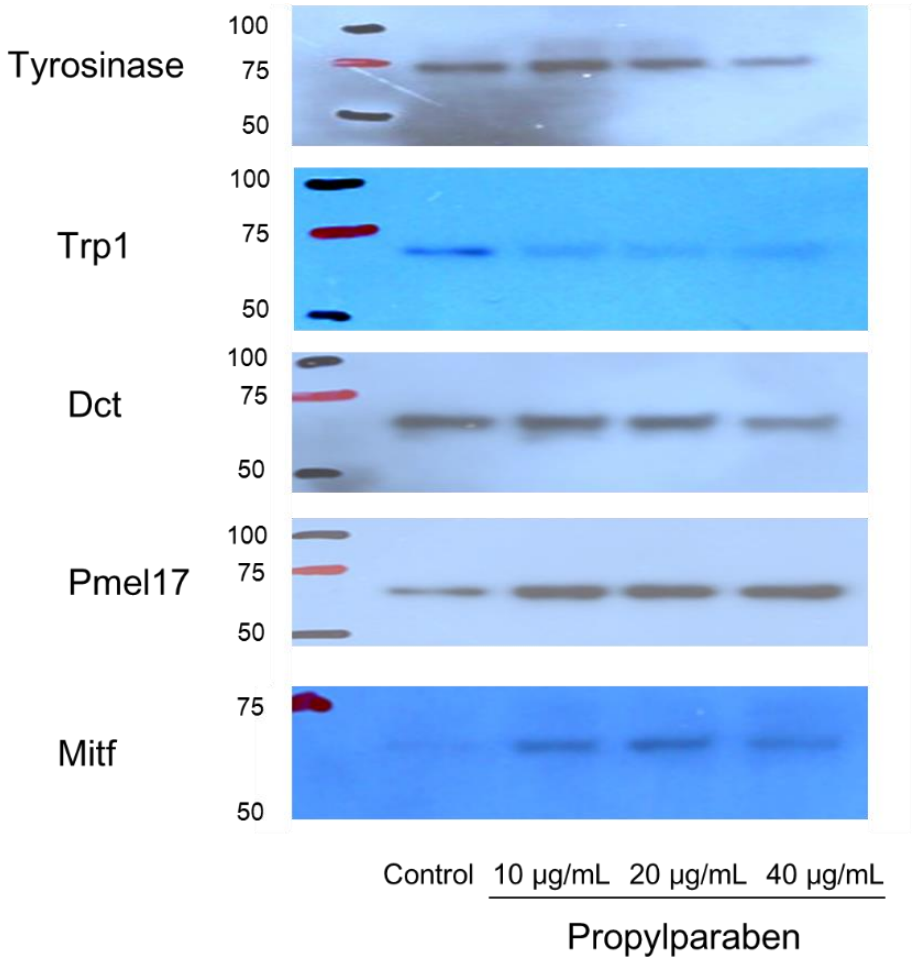


Figure 2-6. Effect of propylparaben on the levels of melanogenic proteins in cultured B16 melanoma cells.

2.3.4. Effects of Propylparaben on Intramelanosomal pH

We investigated the effects of propylparaben on mRNA expression for proteins that regulate intramelanosomal pH. We investigated the effects on mRNA expression for Slc24a5 and Slc45a2, which are proteins that have been shown to be involved in intramelanosomal pH regulation, at concentrations (20 and 40 μg/mL) that would not affect cell proliferation, and an inhibitory effect on tyrosinase activity was observed. We found that mRNA expression was reduced in a concentration-dependent manner for Slc45a2, which is a protein involved in H^+ extrusion, and the results were significantly different from controls at 20 and 40 μg/mL (**Figure 2-7**). Sl24a5, which is an Na^+/Ca^{2+} exchanger, and the

difference from controls, was significant at both concentrations (**Figure 2-7**). Conversely, mRNA expression for the Atp6v0c-encoded V-ATPase sub-unit protein was significantly reduced at 20 μg/mL but unchanged at 40 μg/mL (**Figure 2-7**). We used DAMP, which is an indicator of intracellular pH, and Trp-1, which is specific to melanosomes, to visualize intramelanosomal pH, and we found strong fluorescence at final propylparaben concentrations of 20 and 40 μg/mL, and the propylparaben-induced acidification of intramelanosomal pH was confirmed (**Figure 2-8**). After DAMP staining, melanosomes were stained with an anti-Trp-1 antibody, and the fractions were merged, which revealed concordance between the melanosome position and the band of acidification (**Figure 2-8**).

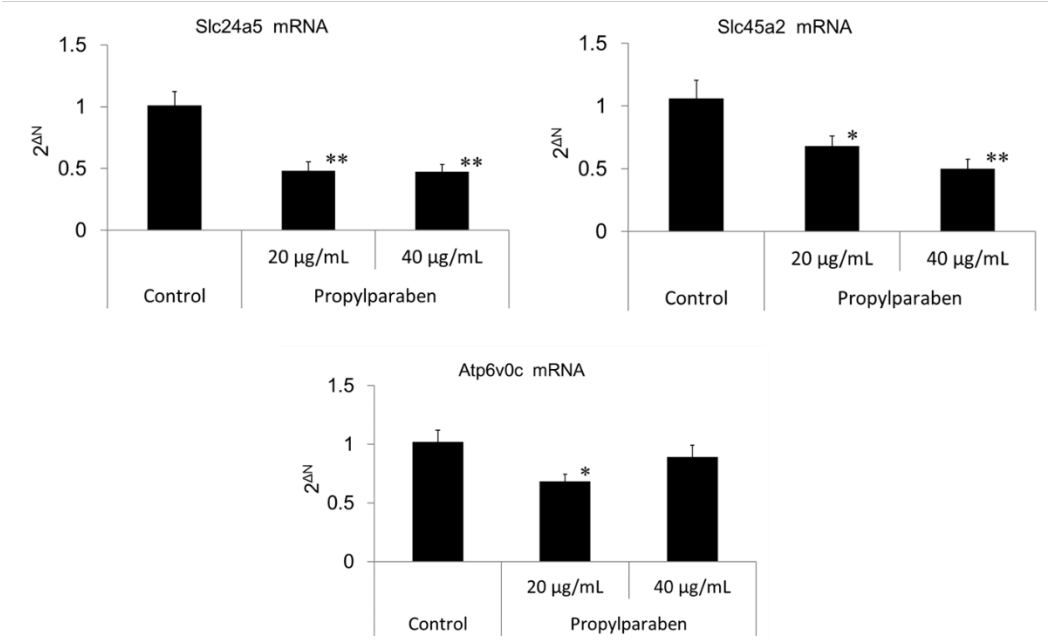


Figure 2-7. Effects of propylparaben on the mRNA levels of the proteins related to pH controls in melanosomes. The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

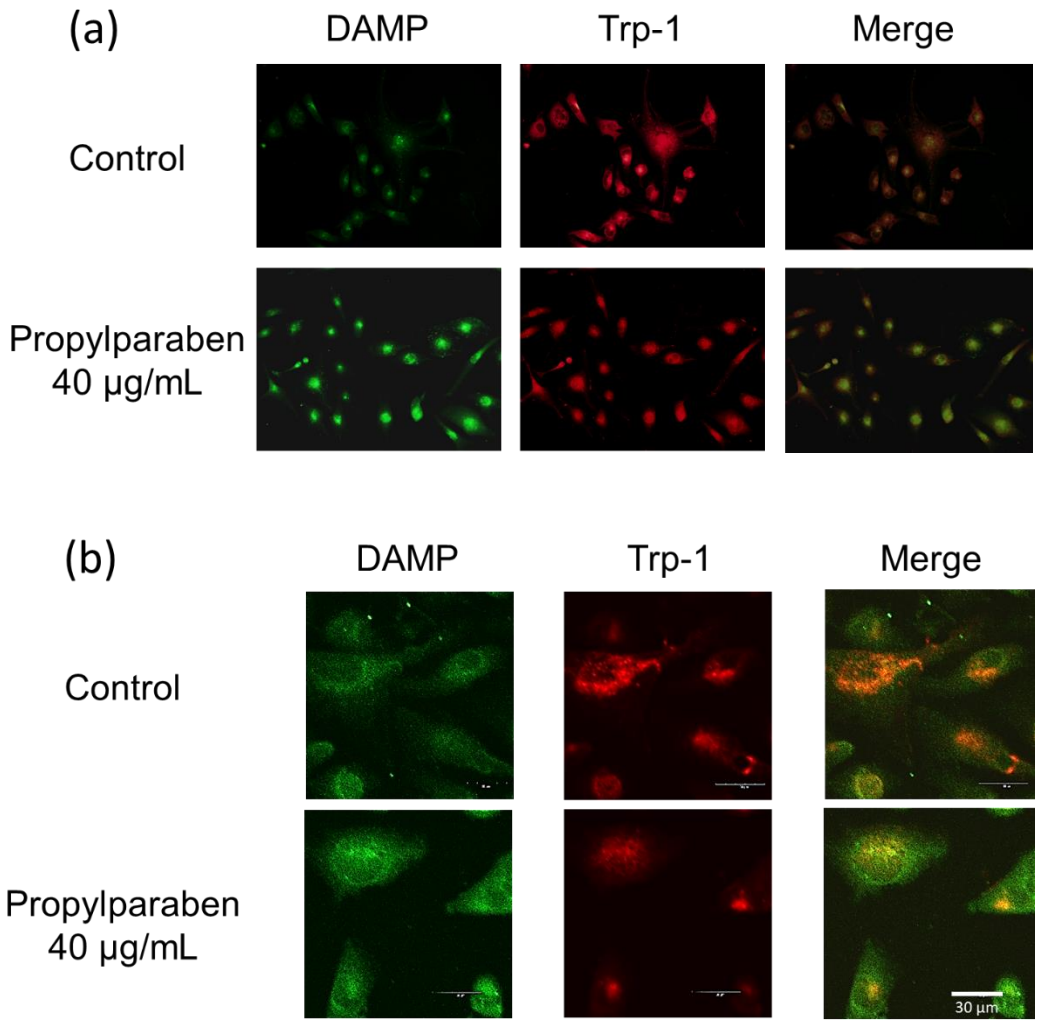


Figure 2-8. Effects of propylparaben on melanosome pH. DAMP is stronger in the intracellular organelles in the pH-dependent acidic areas, which are stained with an anti-DNP antibody and Alexa 488 secondary antibody (green). Trp-1 is a melanosomal protein labeled with the anti-Trp-1 antibody and Alexa 546 secondary antibody (red). pH-dependent acidity and melanosomes are shown in yellow in the merged, colored pictures. (a) observation by the fluorescence microscope, (b) observation by the confocal laser scanning microscopy.

2.4. Discussion

DOPA staining of the collected melanosome fraction revealed that tyrosinase activity decreased when the intramelanosomal pH was acidic. Western blotting revealed a decrease in tyrosinase protein content with acidification of intramelanosomal pH. In other words, we found that acidification of intramelanosomal pH enables tyrosinase degradation, as well as decreases

tyrosinase activity.

Melanosomal pH is regulated by V-ATPase, which mediates H^+ influx and Na^+/H^+ exchangers, which mediate H^+ extrusion. We investigated the fluctuation in melanin content when acidification of pH was blocked with bafilomycin A1, a V-ATPase inhibitor. We found that both melanin and tyrosinase contents increased in bafilomycin A1-treated cells. These results suggested enhanced melanogenesis occurred after blocking intramelanosomal pH acidification. Consequentially, we considered that suppression of melanogenesis is facilitated by an acidic pH, and we, thus, investigated this mechanism with a variety of enzyme inhibitors. We found that both inhibitors of aspartic protease and cysteine protease decreased the degradation of tyrosinase. Accordingly, we consider that aspartic protease and cysteine protease are involved in accelerating tyrosinase degradation in acidic melanosomes. Aspartic proteases are one of the acidic proteases, and cathepsin D and cathepsin E are considered to be aspartic proteases in the degradation of tyrosinase in melanosomes. Cysteine proteases are another type of acidic proteases, and cathepsin L2 is considered to be a cysteine protease in the degradation of tyrosinase in melanosomes. In studies on racial or skin color differences, it has been reported that melanosomes in keratinocytes from light skin are more rapidly degraded than those from dark skin [30] and that the activity of cathepsin L2, which is a lysosomal enzyme, is higher in keratinocytes from lightly-pigmented skin relative to darkly-pigmented skins [23].

Slc45a2 is a known Na^+/H^+ exchanger, and increased Slc45a2 expression intensifies H^+ extrusion. This increase results in greater melanosomal H^+ extrusion and pH-neutralization of the intramelanosomal environment. Decreased Slc45a2 expression weakens melanosomal H^+ extrusion, which leads to greater melanosomal H^+ retention and the resulting acidification of the intramelanosomal environment. A mutation in the Slc45a2 gene induces oculocutaneous albinism type 4 [55]. A comparison of mutant Slc45a2-gene-transfected melanocytes with normal melanocytes showed a lower melanin content in the latter cells, and Slc45a2 mutation abolishes or reduces melanogenesis [56]. Slc24a5 plays a role in potassium-dependent Na^+/Ca^{2+} exchangers. H^+ is transported into the melanosome through V-ATPase, and Na^+

intake is mediated through Na^+/H^+ exchangers through an electrochemical gradient. Na^+ extrusion is conjugated with Ca^{2+} influx through Slc24a5. In other words, decreased Slc24a5 expression results in intramelanosomal accumulation of Na^+ and a cessation of Na^+/H^+ exchange, which results in melanosomal retention of acidity [57,58]. Reduction of melanogenesis from normal levels has been reported to occur in B16 melanoma cells with Slc24a5 knockdown [59].

Parabens are used in cosmetic formulations, and the C1 to C4 (side chain) parabens are indicated to reduce tyrosinase activity as the chain length increases. Parabens reported to have a wide antibacterial spectrum and high safety were investigated for anti-microbial action and were found to have a drug effect increasing in intensity in the order of methylparaben < ethylparaben < propylparaben < butylparaben. Accordingly, a very good linear relationship between the number of carbons on the alkyl chain and affinity was established. The carbon chain was determined to be the major player in differences in anti-microbial effects. Based on this good linear relationship between affinity and the number of carbons on the alkyl chain, proliferation of cells with a high number of carbons, and the relationships between differences in the number of carbons and differences in anti-microbial effects, we surmise that decreases in tyrosinase activity may be related to increases in paraben chain length. Propylparaben has an anti-melanogenic effect, and we consider that the mechanism of its anti-melanogenic effect encompasses intramelanosomal degradation of the protein tyrosinase, Trp-1, and Dct because these protein contents were reduced without any corresponding decrease in mRNA expression. Propylparaben also increased Mitf, but Mitf serves as a survival factor of melanocytes [60], and so we believe that propylparaben will not induce cytotoxicity to melanocytes. On the other hand, we observed acidic changes in the melanosomes due to propylparaben. To determine the mechanism for this phenomenon, we investigated mRNA expression for Slc24a5 and Slc45a2, which are proteins involved in intramelanosomal pH regulation. We found that mRNA expression for Slc45a2, which plays a role in H^+ extrusion, was reduced in a concentration-dependent manner. Expression of Slc24a5, which is an Na^+/Ca^{2+} exchanger, was also reduced. Based on these results, we determined a mechanism for the anti-

melanogenic action of propylparaben, as follows: H^+ influx is reduced with decreased expression of the Slc45a2 gene, and intramelanosomal pH becomes acidic. Based on our findings, we consider that propylparaben-induced melanosomal acidification and the resulting tyrosinase degradation represents a promising new cosmetic mechanism.

Pmel17 exists in immature melanosome (stage II, III), and Trp-1 exists mainly in mature melanosome (stage IV) [61,62]. Dct exists mainly in stage I melanosome/coated endosome [63]. B16 melanoma cells treated with propylparaben were thought to consist of many stage II and III melanosomes. The Pmel17 level was thought to be increased by propylparaben because the maturity of melanosome was controlled by promoting the acidification of the pH of melanosome. Moreover, as Mitf participates in the survival of pigment cells, Mitf level was increased not only by propylparaben, but also by 4-n-butylresorcinol, which inhibits melanin production at non-cytotoxic concentrations in cultured B16 melanoma cells (data not shown). Increased melanin inhibition showed a relationship with increased Mitf level in B16 melanoma cells treated with propylparaben, and further research is required on this.

2.5. Conclusions

Many active cosmetic ingredients formulated as medicated brightening products (quasi-drugs) achieve their effect through inhibition of tyrosinase activity, but no products can achieve this effect through degradation of intramelanosomal tyrosinase. Here, we report on the results of our study, in which tyrosinase degradation was enabled through intramelanosomal pH reduction. It is thought that aspartic protease and cysteine protease are participating in the degradation of tyrosinase in acidic melanosomes. Based on our findings, we found that propylparaben, which is an agent of melanosomal acidification (pH-decreasing potential), can reduce melanogenesis in melanocytes in skin.

Chapter 3 *Angelica acutiloba* Root Extract Improves Hyperpigmentation via the Mechanism of Digestion of Melanosome Complexes

3.1. Introduction

Melanin is synthesized within unique membrane-bound organellas termed melanosomes in melanocytes. Melanosomes are transferred to surrounding keratinocytes to form melanin caps above their nuclei, where this process affects skin color. In normal keratinocytes, melanosomes incorporated into lysosomes are degradated by lysosomal enzymes when keratinocytes move to the skin surface by turnover, which leads to lighten skin tone. On the other hand, it has been reported that abnormal accumulation of large melanosome complexes in basal keratinocytes is observed in senile lentigo [64]. In studies on racial or skin color differences, it has been reported that melanosomes in keratinocytes from light skin are more rapidly degradated than those from dark skin [30], and that the activity of cathepsin L2, one of the lysosomal enzymes, is higher in keratinocytes from lightly pigmented, relative to darkly pigmented skins [31]. It has also reported that the average number of dividing cells in solar lentigo is significantly lower than in the adjacent normal skin [65].

Based on these reports, we hypothesized that enhancement of lysosomes function as the digestive system in highly pigmented keratinocytes could promote the digestion of melanosome complexes and recover decreased cellular proliferation, which leads to improve hyperpigmentation. The object of our study is to find active ingredients from plant extracts for enhancing proliferation of keratinocytes with melanins and for enhancing lysosome function in the digestion of melanosome complexes and to apply their activity to improve hyperpigmentation. In this study, we focused on the improvement of pigmentation by activating the lysosomal enzyme cathepsin to digest melanosome complexes, and reported the effects of *Angelica acutiloba* Root extract.

3.2. Materials and Methods

3.2.1. Materials

Angelica acutiloba Root extract BG(AARE) (manufactured by Maruzen Pharmaceuticals Co., Ltd.). Anti-tyrosinase-related protein-1 (TRP-1) antibody, TMH-2, was used [66]. Alexa Fluor 488 goat anti-rabbit IgG antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The other reagents used in the experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan). HaCaT cells and B16 melanoma cells were purchased from Cell Lines Service GmbH (Eppelheim, Germany).

3.2.2. Effect of AARE on the Proliferation of B16 Melanoma Cells

AARE solution of 10 mg/mL was prepared by adding 1 mL of dimethyl sulfoxide (DMSO) to 10 mg of AARE and diluted to 5 mg/mL with DMSO. Cells cultured in 10% FBS-DMEM (Fetal Bovine Serum-Dulbecco's Modified Eagle Medium) at 200,000 cells/mL, seeded in 96-well plates (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at 99 μL/well, and cultured for 1 day. Then, 1 μL of the control (DMSO) and AARE were added and cultured in 2% FBS-DMEM medium for 2 days.

Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added at 10 μL/well and cultured for 2 h, and the absorbance at 450 nm was measured with a microplate reader (Power Scan HT, BioTek, Winooski, VT, USA) and used as a measure of the cell count. In this method, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt)], which produces highly sensitive water-soluble formazan, was used. The number of living cells was determined by measuring the absorbance at 450 nm of the water-soluble formazan produced after reduction by intracellular dehydrogenase. There is a linear proportional relationship between the number of cells and the amount of formazan produced. The experiment was repeated three times (n = 3).

3.2.3. Evaluation of cathepsin activity promoting action

3.2.3.1. Evaluation using HaCaT Cells

HaCaT Cells (1×10^4 cells / well) were inoculated on a 96-well plate, cultured for 1 day, replaced with a test sample dissolved in DMEM containing 2% FBS, and cultured for 3 days. After freezing and thawing the plate washed with PBS, Synthetic substrate for total cathepsin measurement [31,65] Z-Phe-Arg-AMC / HCl (BACHEM) dissolved in 20 mmol/L acetate buffer (pH 5.0) or synthetic substrate for cathepsin K measurement [67]Z-Gly-Pro-Arg-4Mb 0.1 mmol/L of NA acetate salt (BACHEM) was added to each well, and the fluorescence intensity (Ex. 360 nm, Em. 460 nm) was measured immediately after the addition and 60 minutes or 180 minutes after the addition, respectively. At the same time, the amount of protein in each well after culturing was measured using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific), and the enzyme activity was evaluated based on the increase in fluorescence intensity per protein (RF/Protein).

3.2.3.2. Evaluation using HaCaT Cells containing melanosomes

HaCaT Cells (6×10^4 cells/well) were seeded on a 24-well plate and cultured for 1 day. Separately, B16 melanoma cells cultured in a 75 cm^2 flask were peeled off with 0.05% trypsin-EDTA solution (Thermo Fish-er Scientific), centrifuged, and collected, sterilized PBS was added and homogenized under ice-cooling using a sterilized homogenizer. The supernatant was discarded by centrifugation at 12000 rpm, and homogenized under ice-cooling using a sterilized homogenizer. 1 mL of sterile PBS was added and centrifuged at 500 rpm to make the turbid part at the boundary a melanosome fraction. HaCaT Cells were cultured overnight in a medium containing this melanosome fraction to phagocytose the melanosomes. The test sample dissolved in DMEM containing 2% FBS was cultured for 4 days and then washed with PBS. After thawing the plate, 0.1 mmol/L Z-Gly-Pro-Arg-4Mb NA acetate salt dissolved in 20 mmol/L acetate buffer (pH 5.0) was added to each well. The fluorescence intensity (Ex. 360 nm, Em. 460 nm) was measured immediately after the addition and 60 minutes after the addition, and the enzyme activity was similarly evaluated based

on the increase in the fluorescence intensity per protein (RF/Protein). In addition, in order to confirm the effect of the test sample on the cells, the number of viable HaCaT cells cultured in the same medium containing the test sample on a 24-well plate was measured using the Cell Counting Kit-8 (Dojin Chemical Research Institute).

3.2.3.3. Evaluation of cathepsin gene expression promoting action

HaCaT cells were seeded in 35 mm dishes at 0.999 mL/dish (25,000 cells/mL) and cultured for 1 day. AARE was dissolved in DMSO to prepare solutions of 12.5 mg/mL, 25 mg/mL and 50 mg/mL, and 1 μL of extract was added to each dishes and cultured for 1 day (n = 3). RNA was extracted from the cultured cells using an RNA extraction kit (RNeasy Mini Kit). A real-time PCR system was used to measure the relative mRNA expression levels of CTSK, CTSL2, and GAPDH. Primers were obtained from Qiagen N.V. (Venlo, The Netherlands).

3.2.4. Evaluation of melanosome digestion promoting action

3.2.4.1. Evaluation of lightening effect on melanosomes-containing keratinocytes (cell observation)

HaCaT cells cultured in a 100 mm petri dish were fed with melanosomes prepared in the same way as in 3.2.3.2, cultured for 1 day. The test sample dissolved in DMEM containing 2% FBS was added and incubated for 3 days. The cultured cells were peeled off, centrifuged, and then photographed.

3.2.4.2. Evaluation of melanosome digestive action on melanosome-containing keratinocytes (immunohistochemical staining)

HaCaT cells cultured in chamber slide were fed with melanosomes prepared in the same way as in 3.2.3.2, cultured for 1 day. The AARE was dissolved in DMSO to prepare solutions of 25 and 50 mg/mL, 1 μL was added per chamber and cultured for 5 days.

The chamber slides were washed with PBS, 500 μL of 4% PFA was added for 10 min at 4°C to fix the cells. After fixation, the cells were washed with PBS and incubated with 100 μL of 10% goat serum-PBS solution for 60 min. The

cells were washed five times with PBS and then permeabilized with 0.1% Triton X-100 for 30 min. The cells were washed four times with PBS solution. The primary antibody (anti-TRP-1 antibody) was added and the cells were incubated for 2 h at room temperature. The cells were washed three times with PBS, and then 2000-fold diluted Alexa Fluor 488 goat anti-rabbit IgG antibody was added for 1 h at room temperature. The nuclei of the cells were stained and observed with the confocal laser scanning microscope.

3.2.4.3. Evaluation of melanosome digestive action on melanosome-containing keratinocytes (Transmission Electron Microscopy)

HaCaT cells cultured in dish were fed with melanosomes prepared in the same way as in 3.2.3.2, cultured for 1 day. The AARE was added and cultured for 5 days. We asked the Bozo Research Center for post-fixation, section preparation, and observation with a transmission electron microscope (TEM) of the cultured cells. The cultured cells were fixed with 1.5% paraformaldehyde / 0.5% glutaraldehyde / PBS at 4°C for 1 hour, washed with stirring with phosphate buffer, and centrifuged to collect the cells. Then, it was post-fixed with 1% osmium tetroxide, and similarly centrifuged, cells were collected, solidified with agar, and then chopped. After dehydration and substitution with the ascending sequence of alcohol and embedding in Epon resin to prepare an electron microscope block, a toluidine blue-stained specimen was prepared. After preparing ultrathin sections from the electron microscope block, electron microscopy was performed mainly on lysosomes by TEM (JEM-1400, JEOL).

3.2.5. Statistical Analysis

Measured values were analyzed for significant differences using an unpaired t-test function (two-tailed) in Microsoft Excel. $p < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Effect of AARE on the Proliferation of B16 Melanoma Cells

After 2 days of culture, the fennel extract had no statistically significant effect on the proliferation of B16 melanoma cells at any concentration added (25, 50, and 100 μg/mL) and had no effect on cell proliferation at concentrations below 100 μg/mL (**Figure 3-1**).

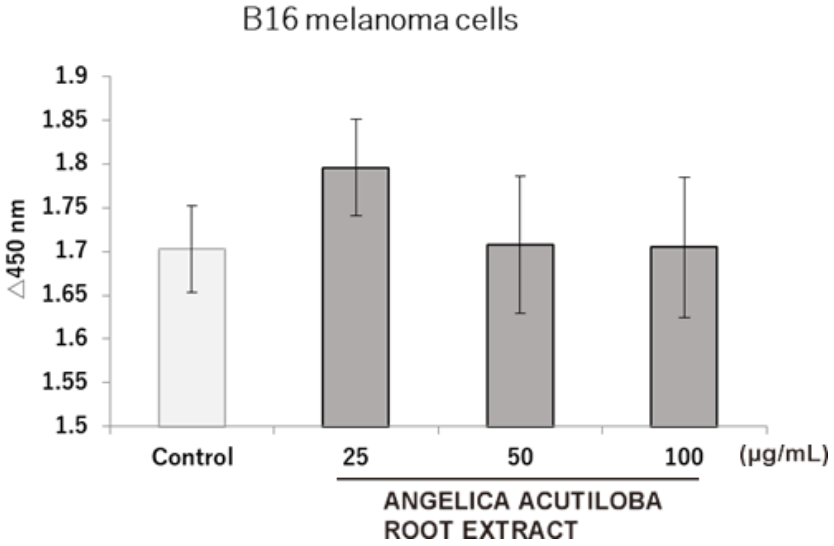


Figure 3-1. Effect of AARE on the number of cultured B16 melanoma cells. The results are expressed as the mean ± standard deviation of three experiments.

3.3.2. Evaluation of cathepsin activity promoting action

Cathepsin, which is localized in lysosomes and is known as a proteolytic enzyme, is considered to be involved in the digestion of the membrane structure of melanosomes incorporated into keratinocytes. First, the effect of AARE on the total cathepsin activity was examined. As a result, the concentration-dependent effect of promoting the activity of total cathepsin was observed in AARE (**Figure 3-2 a**). Next, as a result of focusing on cathepsin K, a concentration-dependent activity-promoting effect of cathepsin K was also observed (**Figure 3-2 b**). Since the cathepsin K activity promotion effect was observed in AARE, in order to confirm whether it has the same effect on the keratinocytes containing melanosomes in the actual pigmentation site, it was conducted using cells containing melanosomes. As a result, even in cells containing melanosomes,

cathepsin K activity promoting action was observed at a concentration that does not show cytotoxicity in AARE (**Figure 3-3**).

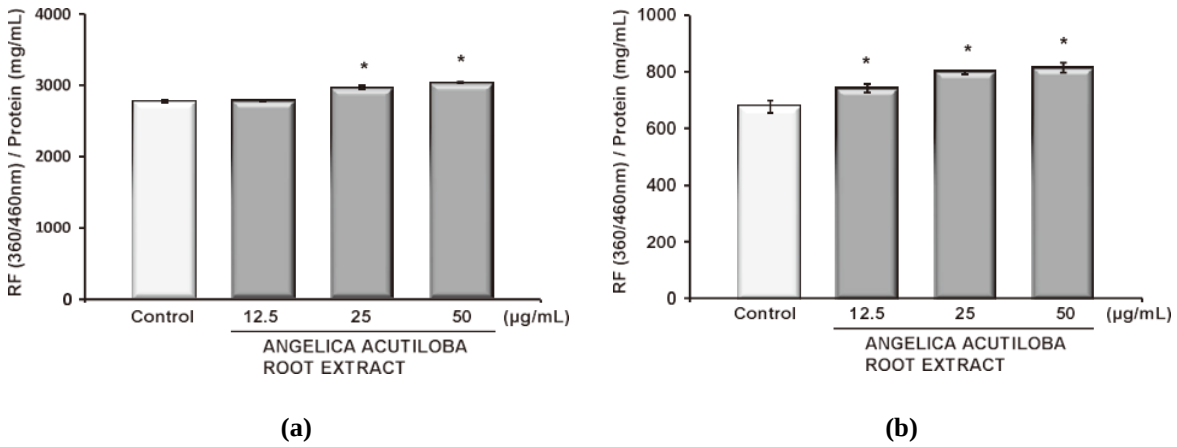


Figure 3-2. Effect of *Angelica acutiloba* root extract on cathepsin activities. HaCaT cells were treated with *Angelica acutiloba* root extract for 3 days. Enzymatic activities of total cathepsins(a) and cathepsin K(b) were measured. The results are expressed as the mean ± standard deviation of six experiments. * $p < 0.05$ vs. control.

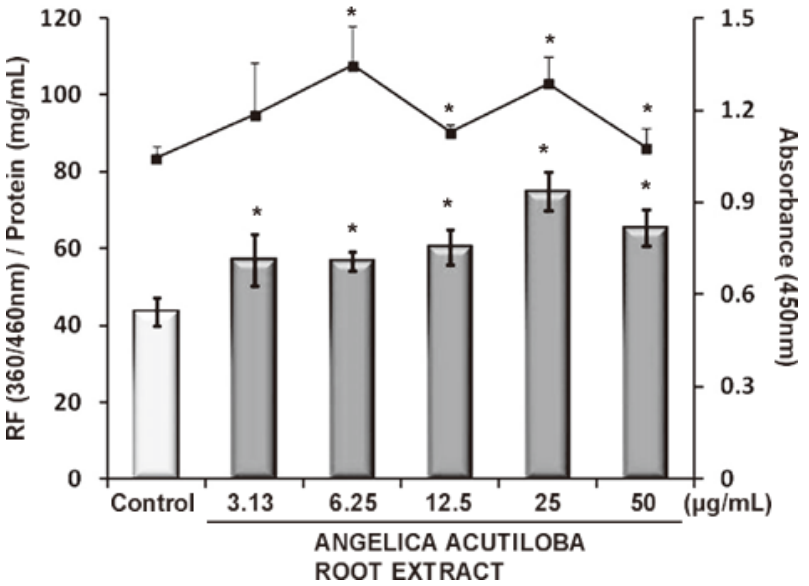


Figure 3-3. Effect of *Angelica acutiloba* root extract on cathepsin K activity in keratinocytes containing melanosomes. HaCaT cells containing melanosomes were treated with *Angelica acutiloba* root extract for 4 days. The cathepsin K activity and viable cells were measured. The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control.

3.3.3. Evaluation of cathepsin gene expression promoting action

In order to investigate the mechanism of cathepsin activity promoting action of *Angelica acutiloba* root extract, the mRNA expression levels of cathepsin K and cathepsin L2 were investigated. As a result, *Angelica acutiloba* root extract significantly increased the mRNA expression levels of cathepsin K and cathepsin L2(**Figure 3-4**).

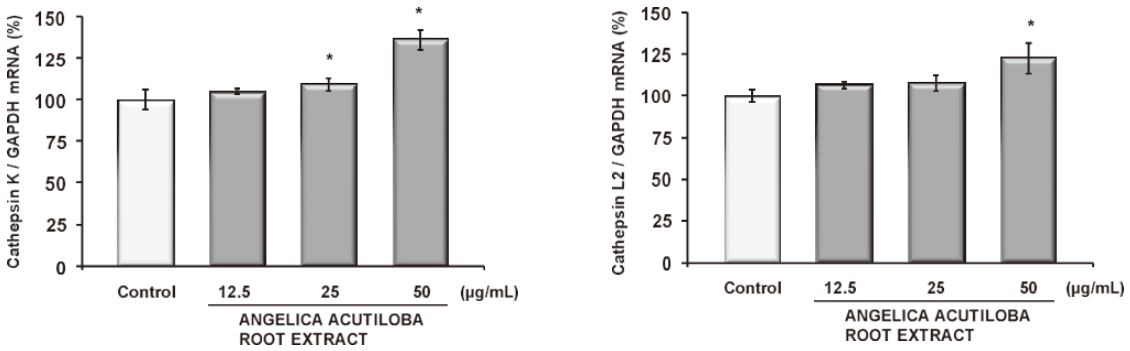


Figure 3-4. Effect of *Angelica acutiloba* root extract on cathepsin mRNA expressions. HaCaT cells were treated with *Angelica acutiloba* root extract for 24 h. Cathepsin K（a） and cathepsin L2（b） mRNA expressions were determined by real-time RT-PCR. The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control.

3.3.4. Evaluation of melanosome digestion promoting action

By adding *Angelica acutiloba* root extract, which has a cathepsin activity-promoting effect, to a keratinocyte culture system containing melanosomes, it was investigated whether the pigment actually appeared thin. As a result, it was observed that the melanosomes-containing keratinocytes were lightened by the *Angelica acutiloba* root extract (**Figure 3-5**). In addition, the results of immunostaining analysis using the expression of TRP-1 [68], which is a component of the melanosome membrane, as an index also showed that the staining intensity of TRP-1 decreased with the digestion of melanosomes (**Figure 3-6**). Furthermore, regarding the action of *Angelica acutiloba* root extract on melanosome-containing keratinocytes, the internal structure of the cell was

observed by TEM centering on lysosomes. Compared to untreated keratinocytes, keratinocytes incorporating melanosomes often contain irregularly shaped lysosomes, which are larger and have a granular component that is thought to be melanin granules inside. There were many aggregates and a part with a considerably high electron density was observed. On the other hand, in the melanosomes-containing keratinocytes treated with *Angelica acutiloba* root extract, lysosomes in which many granular components considered to be melanin granules were aggregated were not observed. Some cells had large lysosomes with agglutination of fairly small vesicles, and the electron density of the internal vesicles varied from low to high. This was considered to be an image of the digestion or digestion process of the granular component considered to be melanin granules (**Figure 3-7**).

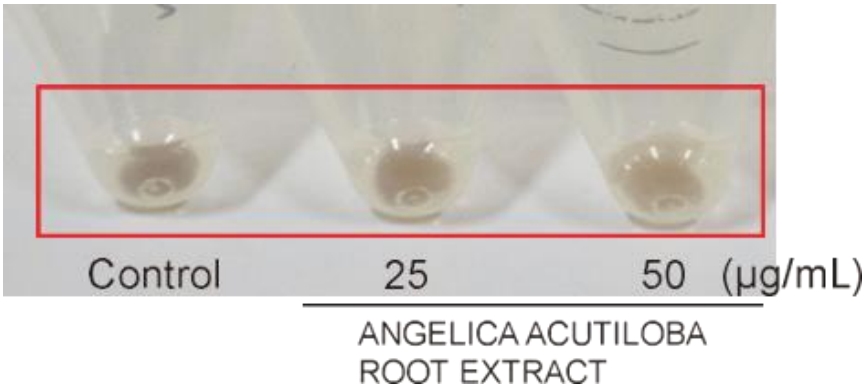


Figure 3-5. Lightening effect of *Angelica acutiloba* root extract in keratinocytes containing melanosomes. HaCaT cells containing melanosomes were treated with *Angelica acutiloba* root extract for 3 days. Lightening effect was evaluated by comparison with the control.

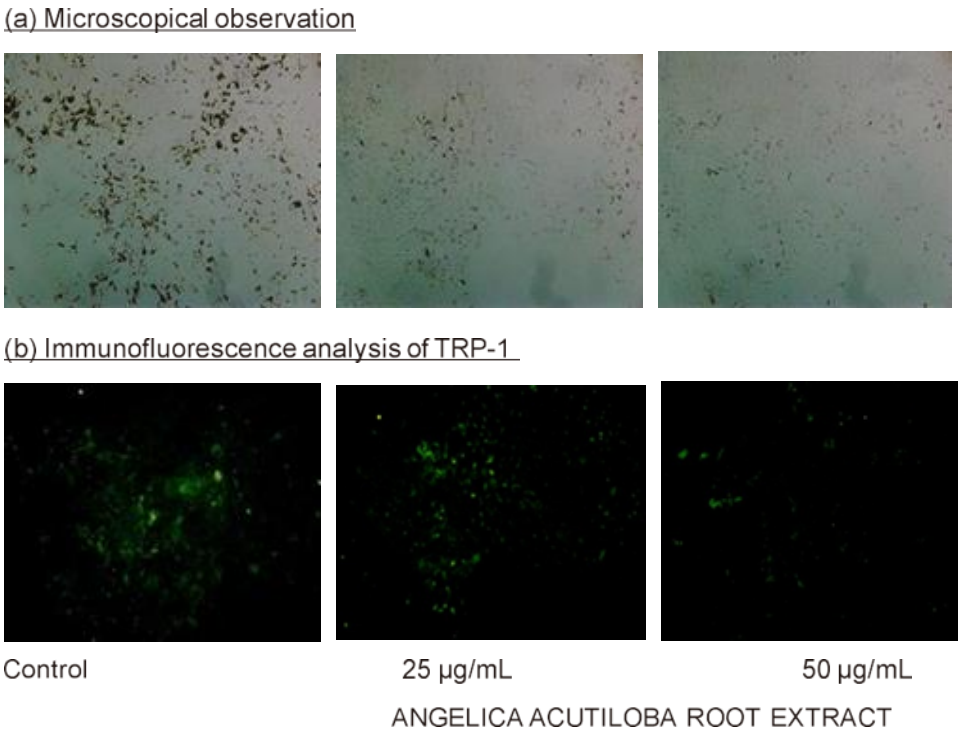


Figure 3-6. Effect of *Angelica acutiloba* root extract on melanosome digestive action in keratinocytes containing melanosomes (immunohistochemical staining). HaCaT cells containing melanosomes were incubated with *Angelica acutiloba* root extract for 5 days. The degree of melanosome digestion in each cell was observe (a). TRP-1 protein level was evaluated by immunofluorescence (b).

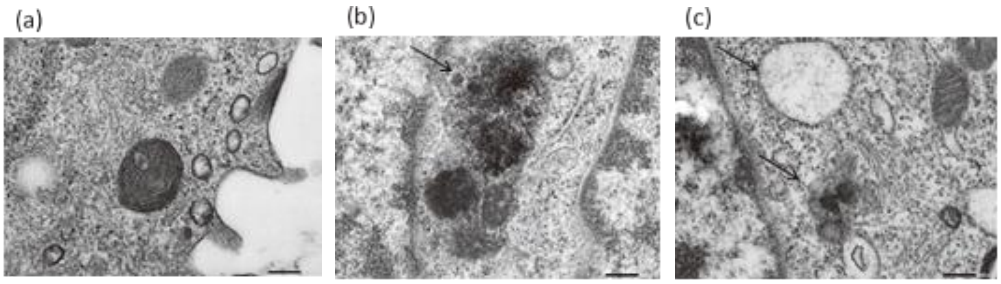


Figure 3-7. Effect of *Angelica acutiloba* root extract on melanosome digestion in lysosomes by Transmission Electron Microscopy (TEM). HaCaT cells containing melanosomes were incubated with *Angelica acutiloba* root extract for 5 days. The cells were stained with toluidine blue-stain and semi-thin sections were observed by TEM. (a)HaCaT cells without melanosomes. (b)HaCaT cells with melanosomes. (c)the extract treated HaCaT cells with melanosomes.

3.4. Discussion

"Stains," which are melanin pigmentation phenomena that occur on the skin, increase with aging and ultraviolet rays, and the main causes are increased production of various cytokines, hormones, chemical mediators, and other melanocyte-stimulating substances in keratinocytes, and melanocytes. This is due to an excessive increase in the amount of melanin, such as an increase in melanin production due to activation of tyrosinase in the skin. On the other hand, long-term retention of melanin, such as excessive accumulation of melanin in keratinocytes and delayed epidermal turnover, can also contribute to pigmentation. Known coping strategies for these factors include control of melanocyte stimulating substances, inhibition of melanin synthesis, inhibition of melanin delivery to keratinized cells, and promotion of epidermal turnover to promote melanin excretion. From the report that a large melanosome complex is observed in the keratinocytes of the basal layer in senile pigment spots [64], we disrupted the membrane structure of melanosomes and dispersed melanin in the cells by enhancing the function of lysosomes responsible for the digestive function of melanosomes, we thought that pigmentation could be improved without reducing the amount of melanin.

Many hydrolases are contained in the lumen of lysosomes, and the action of these enzymes decomposes unnecessary biopolymers inside and outside the cell. Of the degradation products, useful substances are absorbed into the cytoplasm and then reused, and unnecessary substances are either excreted extracellularly by exocytosis or remain inside the cell as a residual body [69]. More than 20 types of proteolytic enzymes "cathepsin" are localized in lysosomes, and melanosomes taken up in keratinized cells are digested by cathepsin after fusion with lysosomes. We focused on cathepsin activity as an index.

Cathepsins are classified into cysteine proteases (Cathepsin B, C, F, H, L, K, O, S, V, W, X), aspartic proteases (Cathepsin D, E) and serine proteases (Cathepsin A, G) due to differences in amino acid residues in the active center. Among the cysteine proteases to which many cathepsins belong, we focus on cathepsin K. Cathepsin K is produced and secreted by osteoclasts and decomposes type I collagen as the main bone matrix [70,71], causing

osteoporosis and rheumatoid arthritis, and its inhibitors are well-known therapeutic agents. In recent years, as a side effect of osteoporosis drugs (cathepsin K inhibitors), it has been observed that patients with osteoporosis have skin stiffness [72]. The activity of cathepsin K expressed in the lysosomes of skin fibroblasts decreases with age, and the degradation of intracellular elastin activity decreases, leading to abnormal stagnation of extracellular elastin metabolism and promoting the formation of actinic elastosis [73]. In addition, it is reported that cathepsin K remodels extracellular matrix such as type I and type IV collagen through endocytosis [74,75], so the activity of cathepsin K in fibroblasts is thought to play an important role in the homeostasis of the extracellular matrix. On the other hand, it is reported that in the epidermis from the elderly, the activity of cysteine cathepsin is reduced, and the amount of cathepsin K, S, and V in the cysteine cathepsin is reduced compared to the epidermis from young people. This is one of the reasons leading to the reduction and accumulation of unnecessary substances, which is considered to be related to the aging process. It has also been reported that the cell division ability of epidermal basal cells containing a large amount of melanin observed in senile pigment spots has decreased [76]. In other words, by activating cathepsin to improve the digestive function of lysosomes, reduce the excessive accumulation of melanosomes taken up by epidermal basal cells in the spots, so as to normalize epidermal metabolism and improve pigmentation, which is considered to be possible.

Therefore, when keratinocytes were used to study cathepsin activity, it was found that *Angelica Acutiloba* Root extract promoted the activity of total cathepsin and cathepsin K. In addition, in the study of gene level, it was found that *Angelica Acutiloba* Root extract significantly increased the expression level of cathepsin K mRNA. In summary, the *Angelica Acutiloba* Root extract increases the expression of cathepsin K at the gene level, and the activity of cathepsin K may be promoted by increasing the amount of cathepsin K. In addition, because the expression level of cathepsin L2/V mRNA was also significantly increased, the promotion of total cathepsin activity may be contributed by other cathepsins than cathepsin K, L2/V, which is a subject of future research.Next, the effects of *Angelica acutiloba* root extract on melanosome digestion were investigated from macro and micro

perspectives using melanosome-containing keratinocytes. As a result, remarkable digestive action and lightening of melanosomes by *Angelica acutiloba* root extract were confirmed.

Angelica is a perennial plant in the Umbelliferae family. In order to improve women's symptoms such as body cold, anemia, and irregular menstruation, the root is used as a medicinal ingredient and is used as the main medicine in many Chinese herbal medicines for the treatment of gynecological diseases. In addition, in an ancient Chinese story, a wife who had been suffering from gynecological diseases for a long time recovered through a herbal medicine (Angelica sinensis). Because she missed her husband who had been away for a long time, she stood at the door of the house and shouted expectantly: "Husband, you Time to go home." Therefore, this herb was named Angelica sinensis [37–39].

Based on the results herein, we believe that the *Angelica acutiloba* root extract, which is called "return to ideal state", can improve the digestive function of lysosomes by promoting the activation of cathepsin K, thereby destroying the structure of melanosomes and dispersing melanin, improving pigmentation without inhibiting melanin synthesis.

3.5. Conclusions

In this study, we focused on the large melanosome complexes in the epidermal basal cells of the spotted skin, and promoted their digestion by promoting the activity of cathepsins located in the lysosomes, thereby promoting the dispersion of melanin. We think this may dilute the spots. The results confirmed that the *Angelica acutiloba* root extract promoted the activity of total cathepsin, increased the activity of cathepsin K in cathepsin and its gene expression level, and significantly digested melanosomes in keratinocytes containing melanosomes. From the above results, it can be considered that *Angelica acutiloba* root extract is a useful substance, which can disperse melanin and improve pigmentation by promoting cathepsin activity in keratinocytes containing melanosomes in spots.

Chapter 4 Involvement of Cathepsin K and Cathepsin L2 in the Digestion of Melanosome Complexes in Epidermal Keratinocytes by Fennel Extract

4.1. Introduction

Epidermal melanin is important in the regulation of skin color and determines the wide variety of skin colors associated with ethnic diversity [7]. In healthy skin, melanosome complexes in the epidermis are digested along with keratinization. However, in senile lentigo, they accumulate as large melanosome complexes in the basal and spinous layers of the epidermis. Thus, the size, number, and distribution of melanosome complexes are the main determinants of skin color. Melanin is synthesized in melanosomes, which are the organelles found in the pigment cells (melanocytes) in the basal layer of the epidermis. In the melanosomes, tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA), and DOPA to dopaquinone, by the action of the enzyme tyrosinase. After dopaquinone formation, the eumelanin pathway proceeds as follows: first, dopachrome is converted to 5,6-dihydroxy-1H-indole (DHI) by spontaneous decarboxylation or to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) catalyzed by dopachrome tautomerase (DCT) [8]. Melanogenesis can culminate in eumelanin by oxidative polymerization reactions following the formation of DHI and DHICA [8]. Tyrosinase, a melanin synthase, is synthesized separately from melanosomes and transported by vesicles to immature melanosomes. Tyrosinase-related protein-1 (TRP-1) is present in mature melanosomes and has been used as a marker for melanosomes [10,11]. The melanin synthesized near the nucleus of melanocytes accumulates in melanosomes, and the mature melanosomes are transported to the vicinity of the cell membrane via long-range bidirectional microtubules and directly under the cell membrane via short-range unidirectional actin fibers. Subsequently, it combines with the cell membrane, is transferred to the adjacent keratinocytes, and accumulates on the nucleus of the keratinocytes to form a nuclear cap (melanin cap) [4,5]. We also reported in an earlier study that large melanosome complexes accumulate in the keratinocytes of the basal and

spinous layers [5], resulting in senile pigmented lesions.

Melanosome complexes are known to be degraded by lysosomes in normal human epidermal keratinocytes [28]. It has been reported that aspartic proteases and cysteine proteases are involved in the promotion of tyrosinase degradation in acidic melanosomes [29]. Cysteine proteases are a type of acidic protease, and cathepsin L2 is considered to act as a cysteine protease in the degradation of tyrosinase in melanosomes. Studies on racial and skin color differences have reported that melanosomes of light-skinned keratinocytes are degraded faster than those of dark-skinned keratinocytes [30] and the activity of the enzyme cathepsin L2 is reported to be higher in light-skinned keratinocytes than in dark-skinned keratinocytes [31]. Because cathepsin K is also a cysteine protease, it is thought to have an effect of degrading tyrosinase in melanosomes.

In Japan, medicated brightening cosmetics (“quasi-drugs”) that inhibit tyrosinase activity, such as kojic acid, arbutin [48], and rucinol, promote the degradation of tyrosinase, such as linoleic acid [49]. In addition, stable vitamin C derivatives, vitamin C ethyl with antioxidant properties [50], and anti-inflammatory properties, such as that in tranexamic acid [51], are being researched and developed [52]. However, agents such as rhododendrol and magnolignan are currently not used because they release hydroxyl radicals from tyrosinase, are highly cytotoxic to melanocytes, and may induce leukoderma [53]. The use of such substances, wherein hydroxyl radicals are generated by tyrosinase to regulate melanin production, poses a risk of causing vitiligo. Therefore, there is a growing need for developing skin-brightening ingredients with a mechanism that promotes the degradation of melanosome complexes accumulated in keratinocytes.

Fennel (scientific name: *Foeniculum vulgare* Mill.) is a perennial herb belonging to the Apiaceae (Umbelliferae) family. The young leaves and seeds (fennel seeds) have a sweet aroma and bitter taste, and effectively promote digestion and deodorizing. Since ancient times, fennel has been used as a spice and herb for food and medicinal purposes. The constituents of fennel seed oil are (E)-anethole, limonene, methyl chavicol, fenchone, α-pinene, and (Z)-β-ocimene [41,42]. Fennel seed oil reportedly exhibits anti-inflammatory properties [41]. In

addition, fennel seed oil and seed extract in methanol or ethanol reportedly display antioxidant properties (DPPH scavenging activity, ferric reducing activity, and inhibition of peroxidation of linoleic acid) [43–45]. The composition of fennel seed methanolic extracts was characterized by its richness in quinic acid, 4-O-caffeoylquinic acid, p-coumaric acid, and 4-O-caffeoylquinic acid [44]. The water extract of fennel fruits also reportedly exhibits strong antioxidant activity (inhibition of peroxidation of linoleic acid) [45–47], and 4-O-β-D-glucosyl sinapyl alcohol, 4, 9-di-O-β -glucosyl alcohol, and 4-β-glucosyloxy benzoic acid were identified as its active components [47].

Based on this background, we investigated the degradation of melanosome complexes in cultured human keratinocytes by fennel extract, a natural component with reportedly strong antioxidant properties and analyzed the mechanism by focusing on lysosomes effects.

4.2. Materials and Methods

4.2.1. Materials

Dried fennel fruit (*Foeniculum vulgare* Miller fruits; Place of origin: China), sold as a crude drug, was purchased from Nakaya Hikojuro Pharmacy Co. Ltd. (Ishikawa, Japan). Anti-tyrosinase-related protein-1 (TRP-1) antibody, TMH-2 [77], was used. Anti-cathepsin K and anti-cathepsin L2 antibodies were purchased from BioVendor Laboratory Medicine, Inc. (Brno, Czech Republic) and Abcam plc. (Cambridge, United Kingdom). Alexa Fluor 488 goat anti-mouse IgG antibody, Alexa Fluor 488 goat anti-rabbit IgG antibody, Alexa Fluor 546 goat anti-rabbit IgG antibody, Alexa Fluor 546 goat anti-rat IgG antibody, Alexa Fluor 647 goat anti-rat IgG antibody, Lyso Tracker Green DND-26, Lyso Tracker RED DND-99, and Hoechst 33342 Nuclear Stain were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The other reagents used in the experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan). HaCaT cells and B16 melanoma cells were purchased from Cell Lines Service GmbH (Eppelheim, Germany). Human epidermal 3D models were purchased from Japan Tissue Engineering Co., Ltd (Aichi, Japan).

4.2.2. Fennel extract preparation

Thirty grams of fennel was soaked in 300 mL of 0%, 25%, 50%, 75%, and 99.5% ethanol solution for one week. The solvent was removed using a centrifugal concentrator (Savant SpeedVac Concentrators, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Bio Freeze Dryer (BFD-6F2, Nihon Freezer Co., Ltd., Tokyo, Japan); then, the yield was determined. The inhibition of L-DOPA autoxidation and the promotion of melanosome degradation in keratinocytes were measured as follows: First, L-DOPA autoxidation was measured by adding 90 μL of a PBS solution (pH 7.4) of 10 mmol/L L-DOPA to the wells of a 96-well microplate. Then, 10 μL of fennel extract was added with concentrations of 0 (control), 1.25, 2.5, and 5 mg/mL and incubated at 37°C for 2 days. The change in absorption at 550 nm was measured and used to quantify the L-DOPA autoxidation relative to the control. Second, for measuring the melanosome degradation-promoting effect, 20,000 cells/99 μL of melanosome-containing and melanosome-free HaCaT cells were seeded in the wells of a 96-well microplate. Then, 1 μL of fennel extract was added with concentrations of 0 (control), 1.25, 2.5, and 5 mg/mL and incubated at 37°C for 4 days. The absorption at 550 nm was measured and used to calculate the ratio of melanosome-containing to melanosome-free HaCaT cells. The absorption value for each fennel extract trial relative to the control was calculated; this value represents the extent of degradation in melanosome-containing keratinocytes. Both experiments were conducted with n = 3.

4.2.3. Effect of Fennel Extract on the Proliferation of HaCaT Cells and B16 Melanoma Cells

A fennel extract solution of 10 mg/mL was prepared by adding 1 mL of dimethyl sulfoxide (DMSO) to 10 mg of fennel extract and diluted to 5 mg/mL with DMSO. Cells cultured in 10% FBS-DMEM (Fetal Bovine Serum-Dulbecco's Modified Eagle Medium) at 200,000 cells/mL, seeded in 96-well plates (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at 99 μL/well, and cultured for 1 day. Then, 1 μL of the control (DMSO) and fennel extract were added and cultured in

2% FBS-DMEM medium for 2 days.

Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added at 10 μL/well and cultured for 2 h, and the absorbance at 450 nm was measured with a microplate reader (Power Scan HT, BioTek, Winooski, VT, USA) and used as a measure of the cell count. In this method, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt)], which produces highly sensitive water-soluble formazan, was used. The number of living cells was determined by measuring the absorbance at 450 nm of the water-soluble formazan produced after reduction by intracellular dehydrogenase. There is a linear proportional relationship between the number of cells and the amount of formazan produced. The experiment was repeated three times (n = 3).

4.2.4. Melanosomal Lysis and Absorbance Measurement

The medium in the 100 mm dish to which control, 15 μg/mL and, 30 μg/mL of fennel extract and 15 μmol/L of chloroquine had been added was removed. Trypsin-EDTA (10 mL) was added and left in a 5% CO_2 incubator for 30 min, and the cells were collected in a 15 mL centrifuge tube and counted. The medium in the centrifuge tube was removed, the cell count was measured, and 1 mol/L NaOH was added according to the number of cells. In a 96-well plate, 100 μL of each sample was added and the absorption spectra from 400 nm to 700 nm and the value at 550 nm were measured.

4.2.5. Effect of Fennel Extract on Cathepsin K and Cathepsin L2 in Keratinocytes

A solution of 4 mmol/L acetate buffer (pH 5.6), phosphate-buffered saline (PBS; pH7.4), 1 mmol/L cathepsin K substrate, 0.1 mmol/L cathepsin L substrate, and cathepsin B inhibitor solution was prepared. Cathepsin L2 specifically degrades the substrate Z-Phe-Arg-AMC and releases the fluorescent substrate 4-methyl-cumaryl-7-amide (AMC) [66,78]. The reaction equation is shown in Equation (1).

Z-Phe-Arg-AMC → Z-Phe-Arg + AMC (1)

The fluorescence intensity of AMC was detected using a fluorescence microplate reader (Power Scan HT) at 37°C. As Z-Phe-Arg-AMC is also a substrate of cathepsin B, cathepsin L2 activity was specifically measured by mixing the reaction solution with [L-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline (CA-074), a specific inhibitor of cathepsin B [79].

The cells were prepared at 200,000 cells/mL, and seeded at 99 μL/well in 96-well plates, and cultured for 1 day. After 3 days of culture, the medium on the 96-well plate was removed and discarded. The cell plates were washed twice with PBS, and frozen. After thawing, (1) 10 μL of 1 mmol/L cathepsin K substrate and 90 μL of PBS buffer were added to each well, or (2) 10 μL of 0.1 mmol/L cathepsin L2 substrate, 10 μL of cathepsin B inhibitor solution, and 80 μL of 4 mmol/L acetate buffer (pH 5.6) were added to each well.

The fluorescence intensity (excitation, 360 nm, emission, 460 nm) was measured with a microplate reader, and the plate was placed in an incubator at 37°C for 4 h. The activities of cathepsin K and cathepsin L2 were measured over 4 h. The experiment was repeated three times (n = 3).

4.2.6. Effect of Fennel Extract on mRNA Expressions of Cathepsin K and Cathepsin L2

HaCaT cells were seeded in 35 mm dishes at 0.999 mL/dish (30,000 cells/mL) and cultured for 1 day. Fennel extract was dissolved in DMSO to prepare solutions of 25 mg/mL and 50 mg/mL, and 1 μL of extract was added to each dishes and cultured for 3 days (n = 3). RNA was extracted from the cultured cells using an RNA extraction kit (RNeasy Mini Kit). A real-time PCR system was used to measure the relative mRNA expression levels of CTSK, CTSL2, and GAPDH. Primers were obtained from Qiagen N.V. (Venlo, The Netherlands).

4.2.7. Confocal Laser Scanning Microscopy Analysis of the Effects of Fennel Extract on Lysosomes and TRP-1 in Melanosome-Containing Keratinocytes

HaCaT cell suspensions (70,000 cells/mL) containing melanosomes were

prepared, and 1 mL of the suspension was added to each chamber slide and cultured for 1 day. The fennel extract was dissolved in DMSO to prepare solutions of 6.25, 12.5, and 25 mg/mL, 1 μL was added per chamber and cultured for 3 days.

The chamber slides were washed with serum-free DMEM, and fluorescence reagent (Lyso Tracker Green DND-26 diluted 60,000-fold in 10% FBS-DMEM medium) was added (500 μL per slide). After collecting the fluorescence reagent and washing with PBS, 500 μL of 4% PFA was added for 10 min at 4°C to fix the cells. After fixation, the cells were washed with PBS and incubated with 100 μL of 10% goat serum-PBS solution for 60 min. The cells were washed five times with PBS and then permeabilized with 0.1% Triton X-100 for 30 min. The cells were washed four times with PBS solution. The primary antibody (anti-TRP-1 antibody) was added and the cells were incubated for 2 h at room temperature. The cells were washed three times with PBS, and then 2000-fold diluted Alexa Fluor 546 goat anti-rat IgG antibody was added for 1 h at room temperature. The nuclei of the cells were stained and observed with the confocal laser scanning microscope.

4.2.8. Confocal Laser Scanning Microscopy Analysis of the Effects of Fennel Extract on Lysosomes and Cathepsin L2 in Melanosome-Containing Keratinocytes

Melanosome-incorporated cells were added to chamber slides at 500 μL/well (100,000 cells/mL) and cultured for 2 days. Then, 5 μL of solution was added so that the final concentrations of fennel extract and chloroquine were 15 μg/mL, 30 μg/mL, and 15 μmol/L, and the cells were cultured for 2 days. As a control, 5 μL of DMSO was added.

The chamber slides were washed with serum-free DMEM, and the fluorescence reagent (LysoTracker Green DND-99 diluted 60,000 times in 10% FBS-DMEM medium) was prepared and added to the slides (500 μL per slide), and cultured for 60 min. After removing the fluorescence reagent, the slides were washed with PBS solution, and 500 μL of 4% paraformaldehyde (PFA) in phosphate buffer was added for 10 min at 4°C to fix the cells. After fixation, the

cells were washed with PBS and incubated with 100 μL of 10% goat serum-PBS solution for 60 min. The cells were washed five times with PBS solution and permeabilized with 0.1% Triton X-100 for 5 min. The cells were washed four times with PBS solution. The primary antibody (anti-cathepsin L2 antibody) was then added, and the cells were incubated for 2 h at room temperature. The cells were washed with PBS solution three times, then Alexa Fluor 546 goat anti-rabbit IgG antibody (1:2000 dilution) was added for 1 h at room temperature. The stained cells were examined under a fluorescence microscope (FV3000, Olympus, Tokyo, Japan).

4.2.9. Cathepsin K, Cathepsin L2, and TRP-1 Staining in Normal Human Epidermal Keratinocytes (NHEKs)

Normal human epidermal keratinocytes (NHEKs, Kurabo Industries Ltd., Osaka, Japan) were cultured in a 75 cm^2 tissue culture flask in a CO_2 incubator with HuMedia-KG2 (Kurabo), a serum-free medium for tissue culture. After the culture medium was discarded, and melanosomes suspended in the medium were added, the NHEKs were cultured for 1 day to allow melanosomes incorporation. The medium used for the melanosome-incorporated NHEKs was discarded, and the cells were incubated with trypsin/EDTA solution for 10 min in a CO_2 incubator. The cells were then seeded into 35 mm dishes with 1 mL of trypsin/EDTA solution and incubated in the CO_2 incubator for 1 day. The cells were incubated in the CO_2 incubator for 2 days, and then the final concentrations of fennel extract were 15 μg/mL and 30 μg/mL. DMSO was added as a control. Staining for cathepsin L2, cathepsin K, and TRP-1 was performed as described in previous sections. Cathepsin K was stained used by anti-cathepsin K mouse monoclonal antibody (Santa Cruz Biotechnology Inc. Dallas, TX, USA). Alexa Fluor 647 goat anti-rat IgG antibody was used as the secondary antibody for TRP-1 staining.

4.2.10. Immunohistochemical Analysis of Fennel Extract on the Expression of Cathepsin K andCathepsin L in the 3D models of Human Epidermis

The human epidermis models were cultured in a dedicated medium in the presence (12.5, 25, and 50 μg/mL) and absence of fennel extract for 5 days, then embedded in a Tissue-Tek optimum cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). The sections were incubated in 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4) for 10 min at 4°C, washed three times with PBS, then incubated with 10% goat serum in PBS for 1 h at room temperature in a humid chamber. After three washes with PBS, the sections were incubated overnight at 4°C with anti-cathepsin K and anti-cathepsin L2 antibodies diluted 500 × in PBS. The sections were washed three times with PBS supplemented with 0.05% Tween 20, then incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (1:2000 dilution) or Alexa Fluor 488 goat anti-rabbit IgG antibody (1:2000 dilution) at 37°C for 30 min in the humid chamber. Following one wash with PBS, the sections were mounted onto slides using Fluoromount (Japan Tanner Corporation, Osaka, Japan), covered with a cover glass, and observed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan). The fluorescence intensity and fluorescence area of three tissue sections were quantified using ImageJ (NIH, Bethesda, MD, USA). The fluorescent area/total area × 100 was used as the area ratio, and the results of the area ratio × fluorescence intensity calculation were compared.

4.2.11. Statistical Analysis

Measurements were analyzed for significant differences using the paired t-test function (two-sided) in Microsoft Excel. A P value of < 0.05 was considered statistically significant.

4.3. Results

4.3.1. Fennel extraction, L-DOPA autoxidation, and melanosome degradation

The results are summarized in **Table 4-1**. The highest extract yield was obtained using 99.5% ethanol and was in the form of an oil. The extract obtained from 75% ethanol was in the form of a plaque, whereas the extracts from 50%, 25%, and 0% ethanol were in the form of dry powders. The autoxidation of L-DOPA was significantly inhibited by the extracts from the 50% ethanol solution; however, it was promoted by the extracts from the 0% ethanol solution. The extracts from the 50% ethanol solution had the strongest effect on melanosome degradation in melanosome-containing keratinocytes. Subsequent experiments were carried out using fennel extracts from the 50% ethanol solution.

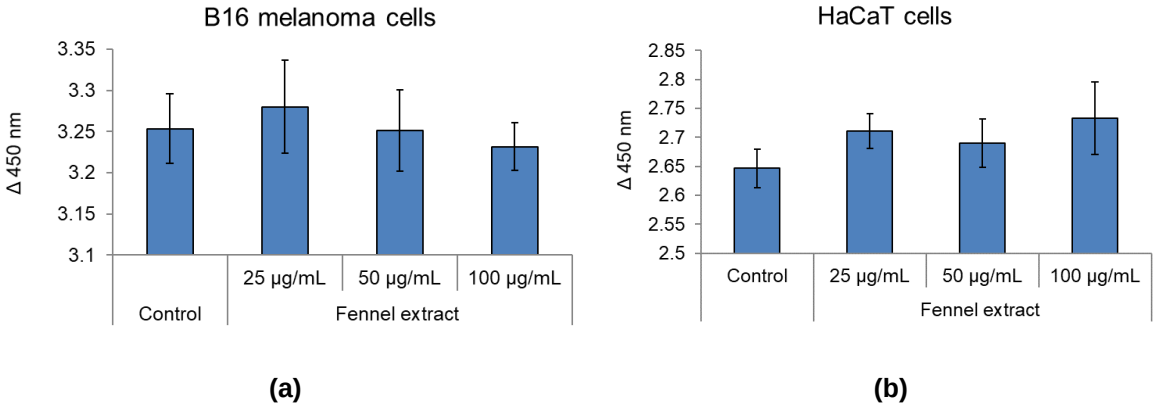
Table 4-1. Calculated yields of fennel extractions, inhibitory effect on the autoxidation of L-DOPA, and enhancement of melanosome degradation in melanosome-containing keratinocytes as a function of ethanol concentration.

	Concentration	Ethanol (%)				
Extraction condition		99.5	75	50	25	0
Yield (%)		48.5	17.1	12.4	14.4	21.4
Oxidation of L-DOPA (relative to the control at 550 nm as 100)	0.125 mg/mL	85.1	88.0	89.2 *	89.0	89.8
	0.25 mg/mL	85.7	95.2	91.3 *	95.1	85.7
	0.5 mg/mL	83.8	82.9	94.3	108.5	129.9 ***
Melanosome degradation in keratinocytes (relative to the control at 550 nm as 100)	12.5 μg/mL	83.4	85.5	76.7 *	78.4	76
	25 μg/mL	79.5	76.7	71.7 *	75.4	72.1 *
	50 μg/mL	70.3 *	80.6	62.5 **	90.1	88.7

The oxidation and degradation results are expressed as the ratio of the absorbance value at 550 nm of each solvent extract to that of the control, and averaged across three trials. The p-values are indicated as * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control.

4.3.2. Effect of Fennel Extract on the Proliferation of HaCaT Cells and B16 Melanoma Cells

After 2 days of culture, the fennel extract had no statistically significant effect on the proliferation of B16 melanoma cells or HaCaT cells at any concentration added (25, 50, and 100 μg/mL) and had no effect on cell proliferation at concentrations below 100 μg/mL (**Figure 4-1 (a)** and Figure **4-1 (b)**).



(a) **(b)**

Figure 4-1. (a) Effect of fennel extract on the number of cultured B16 melanoma cells. (b) Effect of fennel extract on the number of cultured HaCaT cells. The results are expressed as the mean ± standard deviation of three experiments.

4.3.3. Effect of Addition of Fennel Extract and Chloroquine on the Absorbance of Melanin in Cultured Melanosome-Containing Keratinocytes

The absorption spectra of the cells lysed in 1 mol/L NaOH and the values at 550 nm are shown in **Figure 4-2**. Compared with the control, the absorbance was lower in the presence of 15 μg/mL and 30 μg/mL of fennel extract, and higher in the presence of 15 μM chloroquine. Compared with the control, the absorbance at 550 nm was lower in the presence of fennel extract and higher in the presence of chloroquine (**Figure 4-2**).

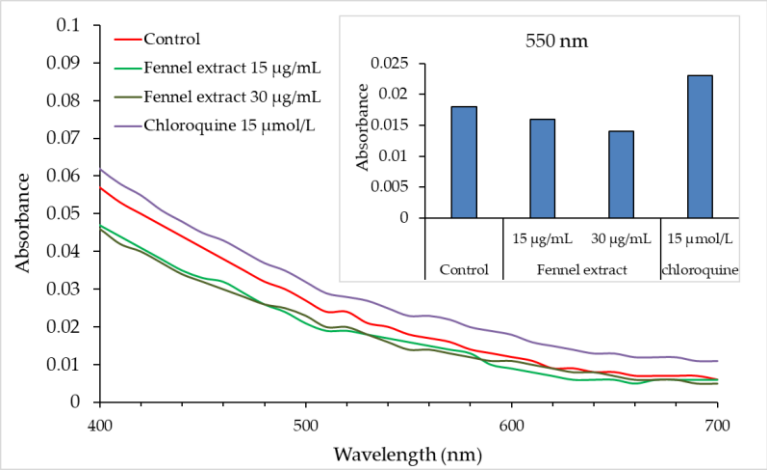


Figure 4-2. Effect of fennel extract addition and chloroquine addition on the color of melanin in cultured melanosome-containing keratinocytes.

4.3.4. Intracellular Localization of Cathepsin K and Cathepsin L

The intracellular localization of cathepsin K and cathepsin L was examined using immunofluorescent labeling. The localization of cathepsin K and lysosomes was consistent, but cathepsin L was found to exist in the cytoplasmic vesicles as well as lysosomes (**Figure 4-3**).

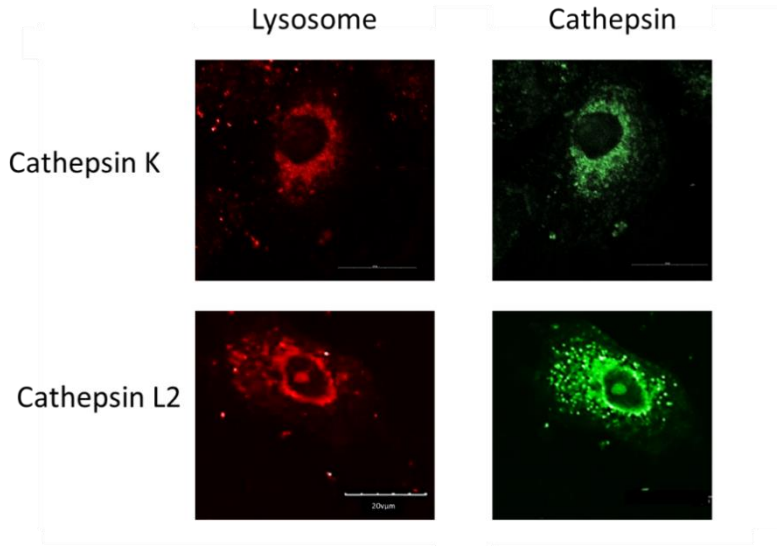


Figure 4-3. The intracellular localization of Cathepsin K and Cathepsin L observed by confocal laser scanning microscopy. The fluorescent intensity of intracellular lysosomes [LysoTracker Green DND-99 (red), a red fluorescent dye that stains acidic compartments in living cells]. Cathepsin K and Cathepsin L were labeled with antibodies of anti-cathepsin K and anti-cathepsin L2, and Alexa Fluor 488 secondary antibody. Scale bar = 20μm.

4.3.5. Effects of Fennel Extract on Cathepsin K and Cathepsin L2 in Keratinocytes

The effect of fennel extract on the activities of cathepsin K and cathepsin L2 in cultured keratinocytes was investigated at 25, 50, and 100 μg/mL, and it was found that the activities of these digestive enzymes were promoted higher than 25 μg/mL (**Figure 4-4 (a) and (b)**). We also examined the effect of fennel extract on the mRNA expression of cathepsin K, cathepsin L2 in cultured keratinocytes. At 50 μg/mL, the fennel extract increased the expression of CTSK

mRNA. The expression of CTSL2 mRNA was increased by 25 μg/mL and 50 μg/mL of fennel extract (**Figure 4-4 (c) and (d)**).

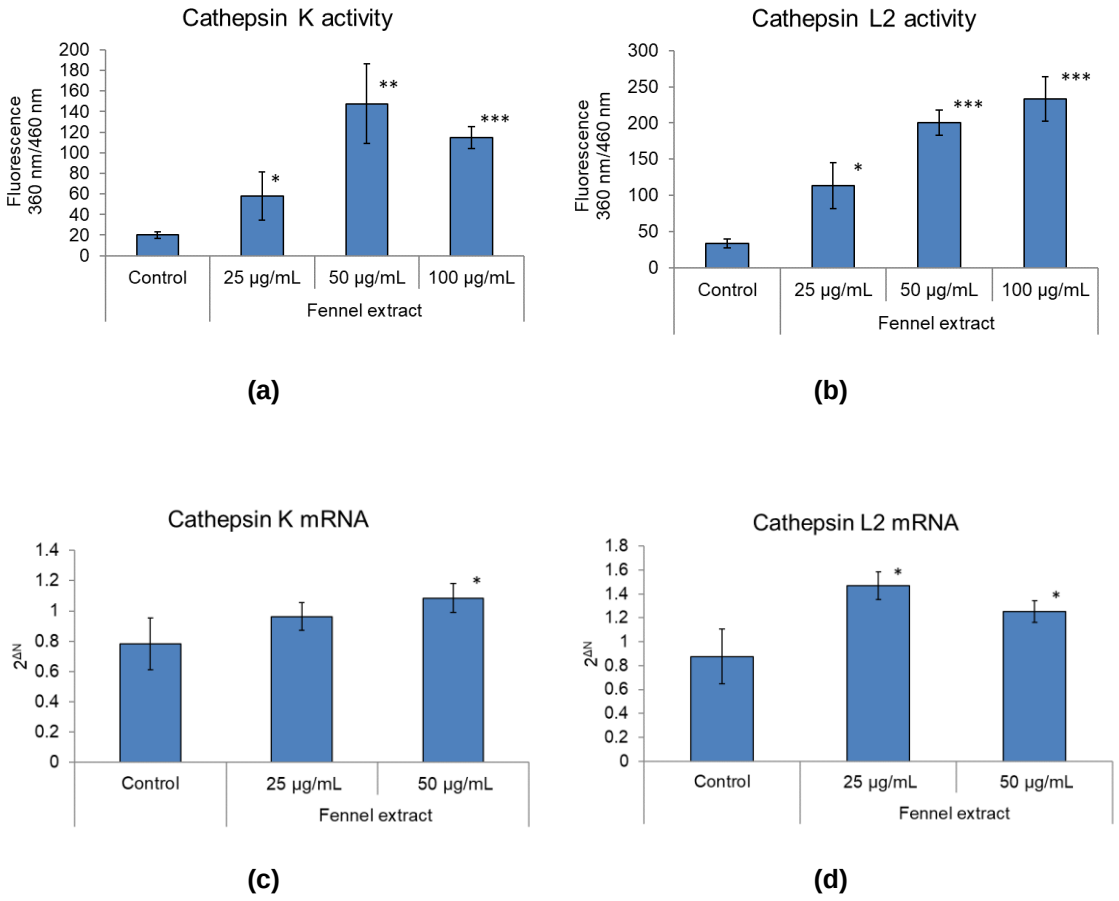


Figure 4-4. (a) Effect of fennel extract on cathepsin K in HaCaT cells. (b) Effect of fennel extract on cathepsin L2 in HaCaT cells. (c) Effects of fennel extract on relative mRNA expression level of cathepsin K(CTSK). (d) Effects of fennel extract on relative mRNA expression level of cathepsin L2 (CTSL2). The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control.

4.3.6. Effects of Fennel Extract on Lysosomes and TRP-1 of Melanosome-Containing Keratinocytes Observed by Confocal Laser Scanning Microscopy

As it was found that fennel extract promoted the activity of intracellular cathepsin K, cathepsin L2, and the relative expression levels of human CTSK mRNA and CTSL2 mRNA, the effects of fennel extract on lysosomes and TRP-1 in melanosome-containing keratinocytes were investigated using confocal laser scanning microscopy. The effects of fennel extract on lysosome and TRP-1 were visualized using immunostaining. We found that the fluorescence intensity of intracellular lysosomes (green) was stronger in the presence of fennel extract was added compared with the control solution (without fennel extract). Furthermore, the fluorescence intensity (red) of TRP-1 was weaker in cells in the presence of fennel extract (12.5, 25 μg/mL) compared with the control solution (without fennel extract). The fennel extract was found to promote the activity of lysosomes in the degradation of melanosomes, especially at concentrations of 12.5 μg/mL and 25 μg/mL (**Figure 4-5**).

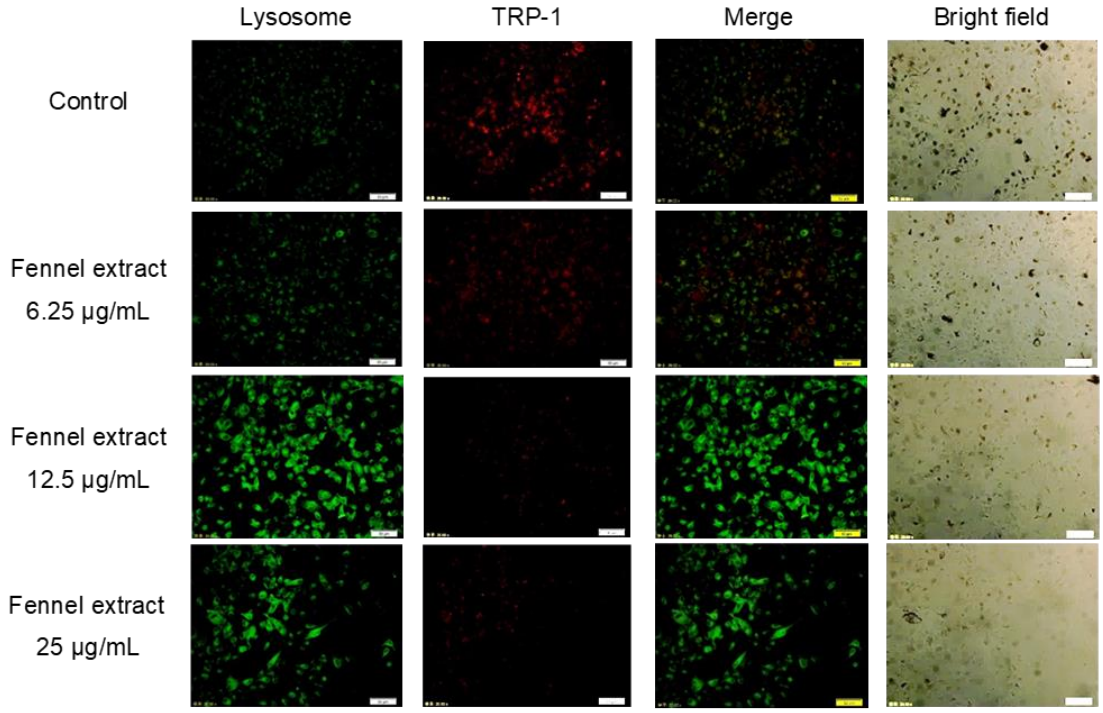


Figure 4-5. Effects of fennel extract on lysosomes and TRP-1 in melanosome-containing keratinocytes observed by confocal laser scanning microscopy. The

fluorescence intensity of intracellular lysosomes [LysoTracker Green DND-26 (green), which is a green fluorescent dye that stains acidic compartments in live cells] was stronger in the presence of fennel extract compared with the control (without fennel extract). TRP-1, a membrane protein of melanosomes, was labeled with anti-TRP-1 antibody and Alexa 546 secondary antibody. Immunoreactive TRP-1 (red) was weakly expressed in the presence of fennel extract compared with the control (without fennel extract). The areas where lysosomal localization (green) and immunoreactive TRP-1 (red) coincide are shown in yellow in the merged color image. Scale bar = 50 μm.

4.3.7. Effects of Fennel Extract and Chloroquine on Cathepsin L2 in Melanosome-Containing Keratinocytes

We observed that 15 μg/mL and 30 μg/mL of fennel extract resulted in stronger fluorescence intensity of lysosomes and cathepsin L2 compared with the control (**Figure 4-6**). The addition of 15 μmol/L of chloroquine resulted in significantly stronger fluorescence intensity of lysosomes than the addition of fennel extract (**Figure 4-6**). However, chloroquine markedly reduced the fluorescence intensity of cathepsin L2 (**Figure 4-6**).

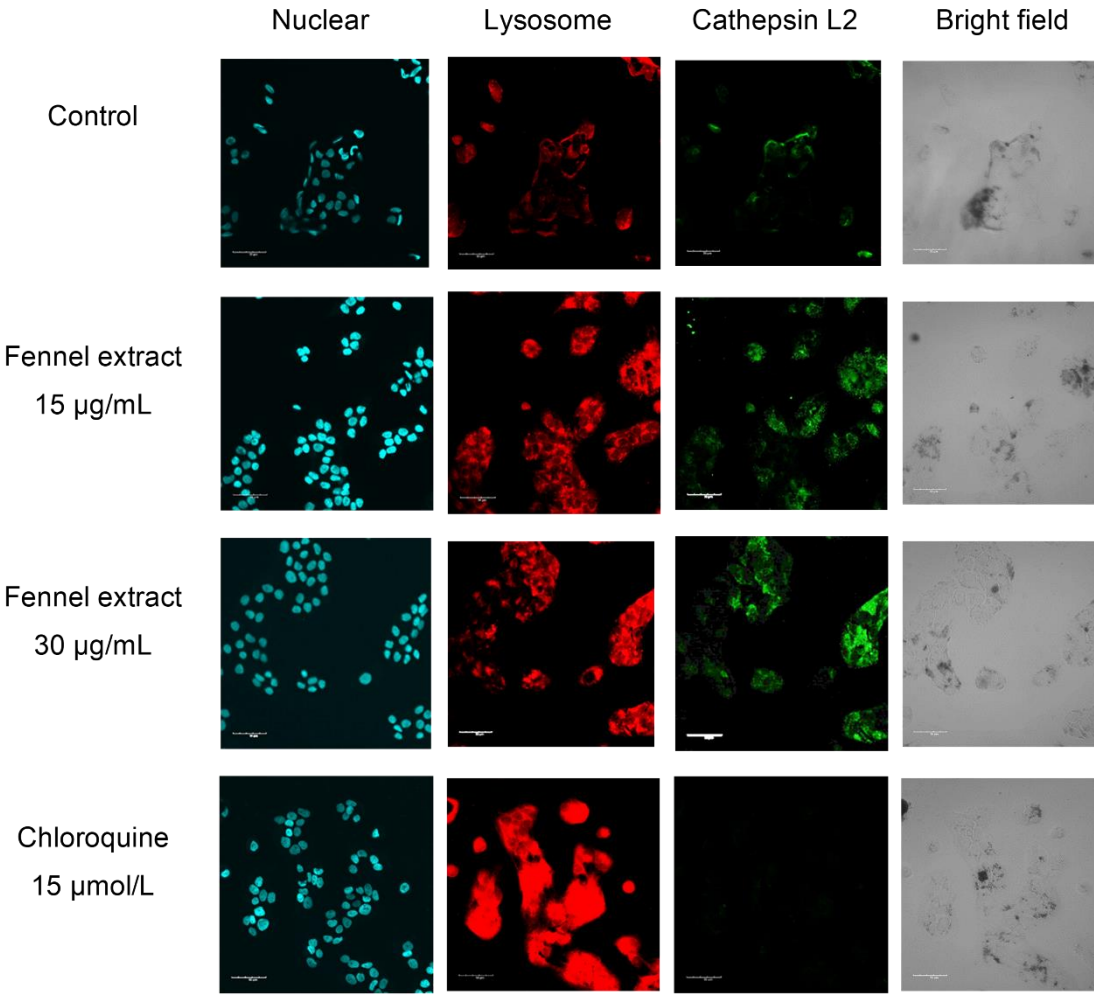


Figure 4-6. The effects of fennel extract and chloroquine on lysosomes in melanosome-containing keratinocytes were observed by confocal laser scanning microscopy. The lysosomes [LysoTracker DND-99 (red), a red fluorescent dye that stains acidic compartments in living cells] were more strongly stained in the presence of fennel extract (15 μg/mL) compared with the control (without fennel extract). With the addition of chloroquine, the fluorescence intensity of the lysosomes was stronger than in the presence of the fennel extract, Nuclei (blue), lysosomes (red). Scale bar = 50 μm.

TRP-1 and cathepsin K in melanosome-incorporated NHEKs were stained with anti-TRP-1 and anti-cathepsin K antibodies after culture in medium containing fennel extract for 2 days. The results of observation by confocal scanning laser microscopy are shown in **Figure 4-7**. The fluorescence intensity of TRP-1 was lower after treatment with 15 μg/mL and 30 μg/mL of fennel extract compared with the control. The fluorescence intensity of cathepsin K was increased after treatment with 15 μg/mL and 30 μg/mL of fennel extract compared

with the control. The merged images of TRP-1 and cathepsin K showed that there was stronger staining of cathepsin K in the vicinity of the TRP-1 staining after treatment with 15 μg/mL and 30 μg/mL of fennel extract compared with the control. In the bright-field images, there were fewer melanosomes compared with the control. The co-localization of TRP-1 (blue) and cathepsin K (green) is shown in yellow in the merged, enlarged color image. The localization of TRP-1 was found to be consistent with that of cathepsin K in many places.

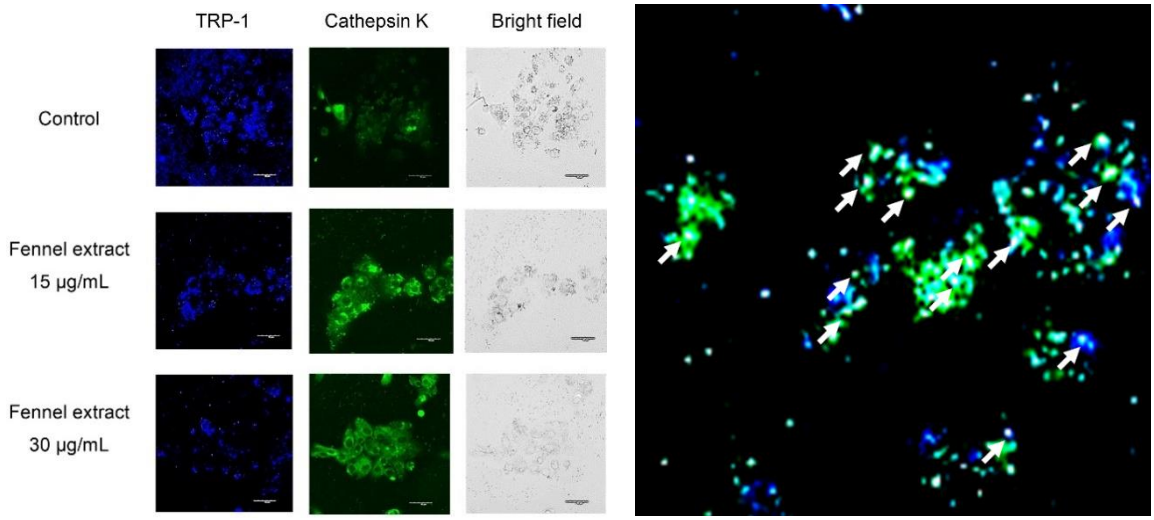


Figure 4-7. Confocal laser scanning microscopy images of TRP-1 and cathepsin K localization in melanosome-incorporated NHEKs cultured for 2 days in medium containing fennel extract. TRP-1 (blue), cathepsin K (green). Scale bar = 50 μm. The co-localization of TRP-1 (blue) and cathepsin K (green) is shown in yellow in the merged, enlarged color image. The white arrows indicate sites of co-localization.

TRP-1 and cathepsin L2 of melanosome-incorporated NHEKs were stained with anti-TRP-1 and anti-cathepsin L2 antibodies, respectively, and observed by confocal scanning laser microscopy when they were cultured in medium containing fennel extract for 2 days. The results are shown in **Figure 4-8**. In the presence of fennel extract 15 μg/mL and 30 μg/mL, the fluorescence intensity of TRP-1 was lower than that of the control. The fluorescence intensity of cathepsin L2 was higher in the presence of 15 μg/mL and 30 μg/mL of fennel extract compared with the control. The merged images of TRP-1 and cathepsin L2 showed that the staining intensity of cathepsin L2 near the TRP-1 staining was stronger in the presence of 15 μg/mL and 30 μg/mL of fennel extract compared

with the control. The bright-field images showed that there were fewer melanosomes in the presence of 15 µg/mL and 30 µg/mL fennel extract compared with the control. The co-localization of TRP-1 (blue) and cathepsin L2 (green) is shown in yellow in the merged, enlarged color image. There were also a few cases where the localization of TRP-1 coincided with that of cathepsin L2.

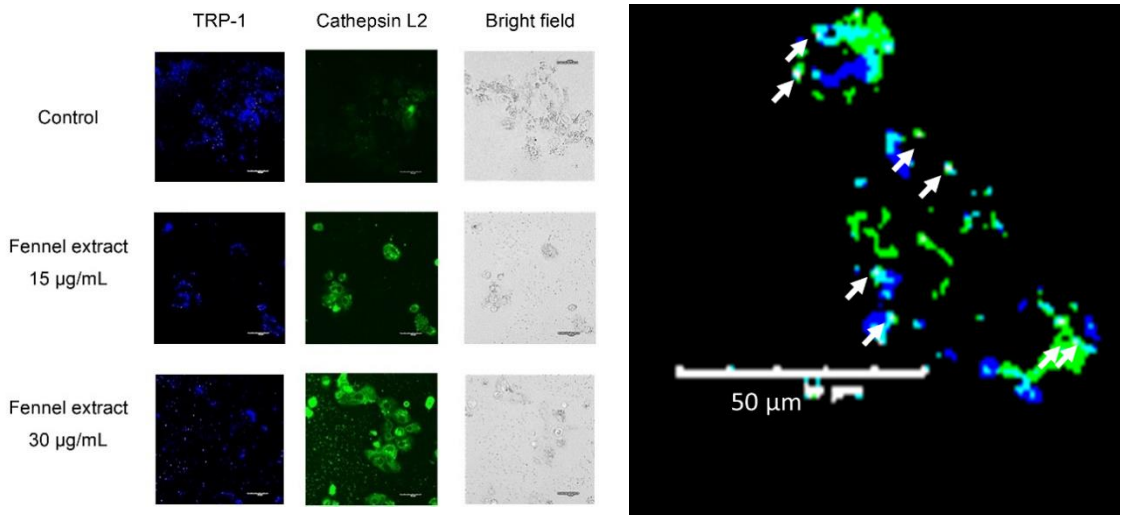


Figure 8. Confocal laser scanning microscopy images of TRP-1 and cathepsin L2 localization in melanosome-incorporated NHEKs cultured for 2 days in medium containing fennel extract. TRP-1 (blue), cathepsin L2 (green). Scale bar = 50 µm. The co-localization of TRP-1 (blue) and cathepsin L2 (green) is shown in yellow in the merged, enlarged color image. The white arrows indicate sites of co-localization.

4.3.8. Effect of Fennel Extract on Cathepsin K and Cathepsin L2 Protein Levels in Human Epidermis 3D Models

The effects of fennel extract on cathepsin K and cathepsin L2 protein levels in human epidermis 3D models were investigated through an immunohistochemical analysis. In the absence of fennel extract (control), cathepsin K and cathepsin L2 were localized predominantly in the basal and stratum corneum layers of the epidermis (**Figure 4-9 (a)**). In the 3D human epidermis models cultured in the presence of fennel extract, cathepsin K was localized in the cellular cytoplasm in the basal and spinous layers and throughout the entire stratum granulosum and stratum corneum layers of the epidermis with higher protein level than in the control (**Figure 4-9 (a)**). Cathepsin L2 was

localized in the cellular cytoplasm in the basal and the spinous layers of the epidermis in the presence of fennel extract, and the protein level was higher than that in the control (**Figure 4-9 (a)**). The imaging analysis revealed that immunoreactive cathepsin K was significantly increased in the presence of 12.5, 25, and 50 μg/mL fennel extract compared to the control (**Figure 4-9 (b)**). Immunoreactive cathepsin L2 was also significantly increased in the presence of 25 and 50 μg/mL fennel extract compared to the control (**Figure 4-9 (b)**).

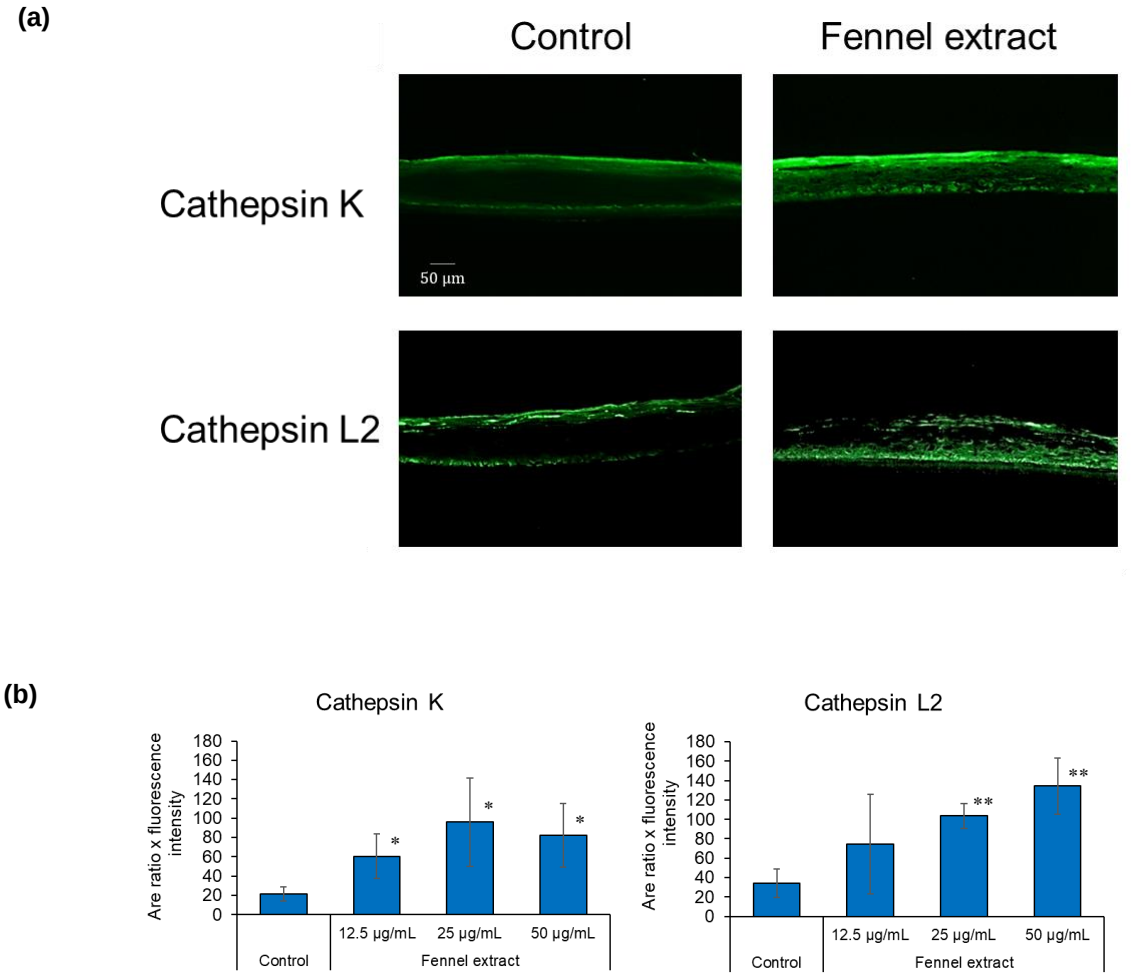


Figure 4-9. Effect of fennel extract on cathepsin K and cathepsin L2 protein levels in the models of the human epidermis. (a) The 3D models of the human epidermis were cultured with 50 μg/mL fennel extract for 5 days. Cathepsin K (green) and cathepsin L2 (green) were localized in the cellular cytoplasm in the basal and spinous layers. Scale bar = 50 μm. (b) The area ratio × fluorescence intensity of immunostained cathepsin K and cathepsin L2 in the presence and absence of the fennel extract (12.5, 25, and 50 μg/mL). The results are expressed as the mean ± standard deviation of three experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control.

4.4. Discussion

The addition of fennel extract decreased the amount of melanin in cultured melanosome-containing keratinocytes compared with the control conditions (the absence of fennel extract). The protein level of TRP-1 present in the mature melanosomes was also reduced, suggesting that fennel extract promoted the degradation of the melanosome complex in keratinocytes.

In the melanosome-containing keratinocytes, the lysosomal staining intensity was enhanced by the addition of fennel extract. In addition, the amount of cathepsin L2 protein was increased and the intensity of fluorescent antibody staining was also enhanced. Therefore, it is thought that the extract activates lysosomes and cathepsin L2. It has been reported that cathepsin L2 is present close to melanosomes that have transferred to keratinocytes and is involved in the degradation of the melanosome complex. In melanosome-containing keratinocytes, the addition of 15 μmol/L chloroquine resulted in stronger fluorescence intensity and a wider staining area of lysosomes compared with the control and the addition of fennel extract. However, the amount of melanin in the keratinocytes with melanosomes were incorporated was higher in the presence of chloroquine than in the control. Chloroquine is a lysosomotropic drug that raises intralysosomal pH [80] and impairs autophagic protein degradation [81]. Similarly, bafilomycin A1 has the effect of neutralizing the pH of acidic organelles, such as lysosomes, and it has been confirmed that bafilomycin A1 increases the amount of melanin [29]. The amount of melanin in melanosome-containing keratinocytes was higher than in the control in the presence of chloroquine, which may be due to the fact that chloroquine inhibited the degradation of melanosomes incorporated into keratinocytes or acidified lysosomes. Therefore, we hypothesized that melanosomes were degraded by the fusion of melanosomes and lysosomes, and further by the increased expressions of cathepsin K in lysosomes and cathepsin L2 in lysosomes and cytoplasm.

In melanosome-containing keratinocytes, as the staining intensity of the lysosomes was increased by the presence of the fennel extract. Studies on racial and skin color differences have reported that melanosomes from light-skinned keratinocytes are degraded faster than those from darker skin [30] and that the

activity of the lysosomal enzyme cathepsin L2 is higher in light-skinned keratinocytes than in dark-skinned keratinocytes [31]. The effect of fennel extract on the activity of the intracellular digestive enzyme cathepsin K and cathepsin L2 was investigated, and it was found that fennel extract promoted the activities of cathepsin K and cathepsin L2. Therefore, the fennel extract may be effective in promoting the degradation of melanosomes in epidermal keratinocytes [29].

We also observed that the fennel extract increased the expressions of cathepsin K and cathepsin L mRNAs, which are the digestive enzymes of lysosomes. The inactive cathepsin precursor is synthesized in the endoplasmic reticulum and then transported to the Golgi apparatus, where it is further glycosylated and phosphorylated to form mannose-6-phosphate protein. Finally, the modified protein is recognized by the mannose-6-phosphate receptor in the lysosome, where the proenzyme is hydrolyzed at low pH and the prodomain is removed to produce active, mature cathepsin [82]. Therefore, the degree of increase in mRNA of cathepsin K and cathepsin L2 by fennel may have been lower than the degree of increase in their activity. Furthermore, we observed the effect of fennel extract on the degradation of melanosomes in epidermal keratinocytes by confocal laser scanning microscopy, and confirmed that fennel extract reduced tyrosinase-related protein-1 (TRP-1), a melanosomal membrane protein. The localization of TRP-1 was found to be consistent with that of cathepsin K in many places. There were also a few cases where the localization of TRP-1 coincided with that of cathepsin L2. From these results, it was confirmed that fennel extract promoted the degradation of melanosomes in epidermal keratinocytes. In addition, cathepsin K and cathepsin L were thought to be involved in the degradation of melanosomes to some extent.

Melanosomes phagocytosed by keratinocytes are present in keratinocytes as melanosome complexes. However, the membrane proteins are degraded through keratinization into complexes smaller than 400 nm and dispersed to become invisible. Nevertheless, in senile pigmented spots, multiple melanosome complexes remain in the basal and spinous layers [5]. In this study, applying a human epidermal 3D model, fennel extract increased the expression of cathepsin K mainly in the stratum granulosum and stratum corneum as well as in the basal

layer and increased cathepsin L2 expression mainly in the spinous and basal layers. Therefore, these digestive enzymes might be involved in membrane protein degradation in the melanosome complexes and melanin dispersion by keratinization.

Based on the results herein, we believe that fennel extract can degrade melanosomes in epidermal keratinocytes by promoting the digestive enzymes, cathepsin K and cathepsin L2, and this is expected to be effective in helping senile pigmented spots fade.

4.5. Conclusion

Melanin amount and TRP-1 expression in mature melanosomes in melanosome-containing keratinocytes were decreased by the addition of fennel extract. Furthermore, cathepsin K and cathepsin L2 immunostaining intensity, activity, and mRNA expression increased. These results suggest that the fennel extract promoted melanosome degradation through the activation of the lysosomal digestive enzymes, cathepsin K and cathepsin L2. The intracellular co-localization of these cathepsins and TRP-1 was observed in melanosome-containing keratinocytes. In the 3D models of the human epidermis, cathepsin K expression was increased by the fennel extract, primarily in the stratum granulosum and stratum corneum but also in the basal layer, and cathepsin L2 expression was increased primarily in the spinous and basal layers. These results indicate that fennel extract can be effective in making senile pigmentation fade because it promotes the degradation of melanosomes from the basal layer to the stratum corneum of the epidermis by inducing cathepsin K and cathepsin L2 expression.

Chapter 5 Conclusions

In study focused on a new brightening mechanism related to melanosome digestion. First, we investigated the potential of pH-dependent tyrosinase degradation, focusing on the low pH of intramelanosomal environment-induced suppression of melanogenesis. We then investigated whether the mechanism of propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations (Chapter 2). Based on the results presented in Chapter 2, we focused on lysosomal cathepsins. We hypothesized that lysosomal functions in highly pigmented keratinocytes could be enhanced because the digestive system promotes the digestion of melanosome complexes and restores decreased cellular proliferation, leading to improved hyperpigmentation. The objective of Chapter 3 was to identify active ingredients from plant extracts for enhancing the proliferation of keratinocytes with melanins, enhancing lysosomal function in the digestion of melanosome complexes, and applying their activity to improve hyperpigmentation (Chapter 3). Chapter 3 found that the *Foeniculum vulgare* Miller fruit extract (fennel extract) had a more substantial effect on cathepsin promotion than the *Angelica acutiloba* root extract (touki extract). Finally, we investigated the degradation of melanosome complexes in cultured human keratinocytes using fennel extract, a natural component with potent antioxidant properties, and we analyzed the mechanism by focusing on the effects of lysosomes (Chapter 4).

The following is a description of the scientific findings of this doctoral thesis.

Chapter 2 investigated the potential for pH-dependent tyrosinase degradation, focusing on the low pH of intramelanosomal environment-induced suppression of melanogenesis. We also investigated whether the mechanism of the propylparaben is implicated in melanosomal pH regulation at non-cytotoxic concentrations. We found that tyrosinase was degraded via acidification of melanosomes, thereby decreasing its activity, and both inhibitors of aspartic protease and cysteine protease decreased tyrosinase degradation. Aspartic protease and cysteine protease were thought to be involved in the degradation

of tyrosinase in acidic melanosomes. Na^+/H^+ exchangers and V-ATPase regulate melanosomal pH. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The mRNA expression levels of tyrosinase and related proteins (TRP-1 and dopachrome tautomerase) were not affected by propylparaben; however, the protein levels in melanosomes decreased. We investigated the mechanisms underlying the inhibitory effect of propylparaben on melanin production in B16 melanoma cells. The effects of propylparaben on the mRNA expression of Na^+/H^+ exchangers, Na^+/Ca^{2+} exchangers, and melanosome pH levels were examined. The results showed that propylparaben decreased gene expression in both exchangers, and staining with an intracellular pH indicator confirmed the effect of propylparaben to decrease the pH of melanosomes.These results suggest that propylparaben downregulates melanin production via acidification of melanosomes.

Melanosomes are specialized lysosomes [12–14]. The first protein shared by lysosomes and melanosomes was the lysosomal membrane glycoprotein Lamp-1 [13,15]. Later, Lamp-1 was identified in vesicles implicated in the trafficking of the tyrosinase-related protein family to melanosomes [15,16]. Melanosomes are the principal repository in melanocytes of lysosomal hydrolytic enzymes such as β-glucuronidase, β-galactosidase, β-hexosaminidase, cathepsin B, and cathepsin L [17]. In addition, the high levels of cathepsin D were found to be present in immature melanosomes in retinal pigment epithelial cells [18]. In normal keratinocytes, melanosomes incorporated into lysosomes are degraded by lysosomal enzymes when keratinocytes move to the skin surface via turnover, which leads to a lighter skin tone. In contrast, an abnormal accumulation of large melanosome complexes in basal keratinocytes is observed in senile lentigo. Lysosomal cathepsins, enclosed in lysosomes, help maintain homeostasis by participating in the degradation of heterophagic and autophagic materials.

In Chapter 3, based on the results of Chapter 2, we focused on improving pigmentation by activating the lysosomal enzyme cathepsin to digest melanosome complexes, and we also investigated the effects of touki extract. The results confirmed that touki extract promoted the activity of total cathepsin,

increased the activity and gene expression level of cathepsin K in cathepsin, and significantly digested melanosomes in keratinocytes containing melanosomes. These results suggest that touki extract is a valuable substance that disperses melanin and improves pigmentation by promoting cathepsin activity in keratinocytes containing melanosomes in spots. We also found that fennel extract had a more substantial effect on the promotion of cathepsin than the touki extract.

Chapter 4 investigated how fennel extract affects melanosome degradation in melanosome-containing cultured epidermal keratinocytes. We also investigated the potential of fennel extract to increase intracellular lysosomal digestive enzyme activity via immunostaining and gene expression analysis. Fennel extract reduced the expression of TRP-1, a mature melanosome membrane protein, and increased cathepsin K and L2 expression. Intracellular cathepsin and TRP-1 colocalization were observed in melanosome-containing keratinocytes. Cathepsin L2 expression levels were lower in the basal layer than in the stratum corneum side of the hyperpigmented region. In addition, melanosome degradation was suppressed in cathepsin L2-knockdown cells. In a three-dimensional human epidermis model, fennel extract increased cathepsin K expression, primarily in the basal and spinous epidermal layers and in the entire stratum granulosum and stratum corneum. The expression of cathepsin L2 increased primarily in the basal and spinous epidermal layers. These results suggest that fennel extract-induced digestive enzymes are involved in melanosome complex membrane protein degradation and keratinization-related melanin dispersion. Moreover, melanosomes can be degraded in epidermal keratinocytes by promoting cathepsin K and L2 activities. Therefore, fennel may prove effective in fading senile pigmentation.

In summary, we achieved our initial research objectives. We identified a new brightening mechanism in which tyrosinase is degraded by the acidification of melanosomes, thereby decreasing its activity. Based on this result, we investigated the effect of plant extracts by degrading melanosomes in keratinocytes to lighten pigment spots and clarified its mechanism. The results indicate that plant extracts such as fennel extract decrease senile pigmentation because they promote the degradation of melanosomes from the basal layer to

the stratum corneum of the epidermis by inducing cathepsin K and L2 expression. These results are expected to be applied to medicated brightening cosmetics in the future, especially to reduce senile lentigo.

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