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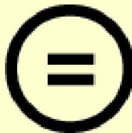

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Abstract

Synthesis of Milk Protein-derived Oligopeptides and Their Effect on Melanogenesis

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Milk is generally considered to make skin milky-white because of its unique color. Milk protein such as κ-casein and β-lactoglobulin has been reported to suppress melanogenesis in cultured human melanocytes, and peptides derived from the aforementioned proteins were recently isolated and their antioxidant activity was studied. For these reasons, we expected that milk protein-derived peptides could have both antioxidant and tyrosinase inhibitory acitivity.

We selected four kinds peptides derived from milk protein (Tyr-Phe-Tyr-Pro-Glu-Leu, Met-His-Ile-Arg-Leu, Tyr-Val-Glu-Glu-Leu, Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala) and screened peptide fragments containing four amino acids to find the active part of the peptide or shorter peptide with strong activity. All of the peptides were prepared by solid-phase peptide synthesis using an Fmoc strategy, and characterized by RP-HPLC and ESI-MS. Rink amide AM resin was chosen as a solid support.

Tyrosinase inhibitory activities of milk protein-derived peptides were evaluated by mushroom tyrosinase inhibition assay. We found that some of

the tetrapeptides showed superior activities compared to the original peptide, though others had reduced tyrosinase inhibitory activity. The IC_{50} values of the peptides were also calculated by measuring tyrosinase inhibitory activities at different concentrations. In the mushroom tyrosinase inhibition assay system, MHIRL and its fragments sufficiently inhibited tyrosinase, and exhibited the lower IC_{50} values. Therefore, we designed other derivatives by replacing an amino acid from their sequences. The tyrosinase inhibitory activities of these derivatives were also measured. In addition, we found that the positive charge of arginine was crucial for their tyrosinase inhibitory activity. MHIRL derivatives were tested for cytotoxicty and anti-melanogenesis activity in B16F10 cells and Mel-Ab cells. All of the peptides did not affect cell viability, and suppressed melanogenesis sufficiently in B16F10 cells. From these studies, we found that MHIRL derivatives could act as good tyrosinase inhibitors as well as antioxidants. We believe that these peptides could be applied in the fields of cosmetics, medicine, and in the agriculture industry.

Keywords: melanin, melanogenesis, tyrosinase inhibitor, peptides, milk protein, hypo-pigmenting activity, tyrosianse inhibitory acitivity, antioxidant activity

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List of Abbreviations

BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophos-phate
DHI	5,6-Dehydroxyindole
DHICA	5, 6-Dihydroxyindole-2-carboxylic acid
DIPEA	*N, N*-Diisopropylethylamine
DODT	3,6-Dioxa-1,8-octanedithiol
L-DOPA	L-3,4-Dihydroxyphenylalanine
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
KA	Kojic acid
NMP	*N*-Methyl-2-pyrrolidone
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

Chapter1. Introduction

1.1 Melanogenesis in Human

Melanin is a complex polyphenol-like biopolymer and its chemical structure and physiological roles are not fully understood. Melanin has excellent photoprotectant properties by transforming the harmful ultraviolet radiation into harmless heat, (McGinness J *et al.*, 1973; Proctor PH *et al.*, 1986; Hill HZ *et al.*, 1992) and plays an important role in determination of the actual color of skin, hair and eye, etc. In addition, melanin is mainly associated with various dermatological disorders, such as melasma, age spots, sites of actinic damage and even including malignant melanoma. There are two types of melanin. Eumelanin is brown-black biological melanin and the most common form. Phenomelanin is another form of yellow-red polymer. Melanin is synthesized in dendrite-shaped cell, Melanocyte, located in the dermis of the skin (Figure 1.1), and this process called Melanogenesis. Melanogenesis contains a series of oxidative reactions in the presence of tyrosinase as a key enzyme.

Upon exposure of the skin to ultraviolet radiation, the first step of Melanogenesis is initiated. Tyrosinase converts tyrosine to L-3,4-dihydroxy-phenylalanine (L-DOPA) and then to dopaquinone. After this step, the oxidative reaction from L-DOPA to dopachrome is divided into two different pathways depending on pH, intramolecular 1,4-addition reaction to the benzene ring or a water addition reaction (García-Cánovas F. *et al.*, 1982). Eumelanogenesis after dopachrom formation is also divided into two reactions involving 5,6-dehydroxyindole (DHI) or 5, 6-dihydroxyindole-2-carboxylic acid (DHICA). The former reaction includes slow chemical decarboxylation of dopachrome to DHI and the latter reaction takes place by

dopachrome tautomerase. Although two different reactions produce the same product, eumelanin, the properties differ. DHI-derived melanin is black while DHICA-derived melanin is yellow-brown. If cysteine or glutathione is present, the reaction leads to pheomelanogenesis. The thiol group of cystein or glutathione attacks dopaquinone to produce cysteinyldopa or glutathionyldopa. It can be added to 5-position. After this uncharacterized series of reaction take place that leads to the production of phenomelanin, red, and yellowish melanin (Figure 1.2 (a)) (Kim *et al.*, 2005). As shown in Figure 1.2, tyrosinase catalyzes three reactions in the synthesis of melanin in melanocyte: the hydroxylation of tyrosine to L-DOPA, the oxidation of L-DOPA to dopaquinone, and the oxidation of 5,6-dehydroxyindole (DHI) to indole-5,6-quinone. During these reactions, tyrosinase participates in monophenolase and diphenolase reactions in three forms, Oxy, Met and Deoxy form (Rescigno *et al.*, 2002) (Figure 1.2 (b)).

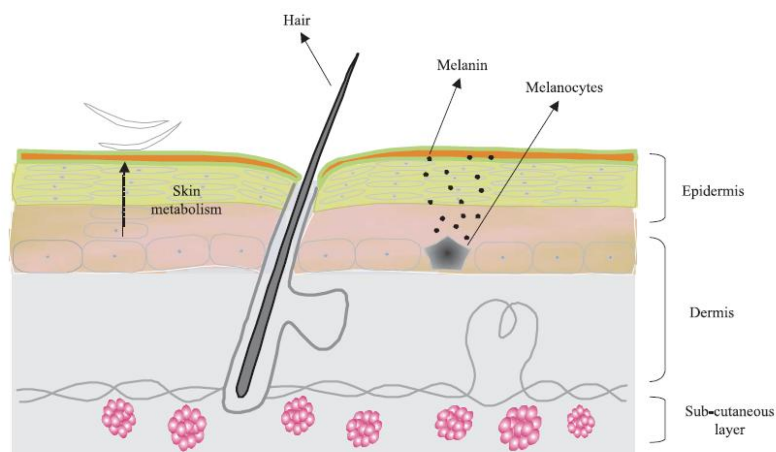
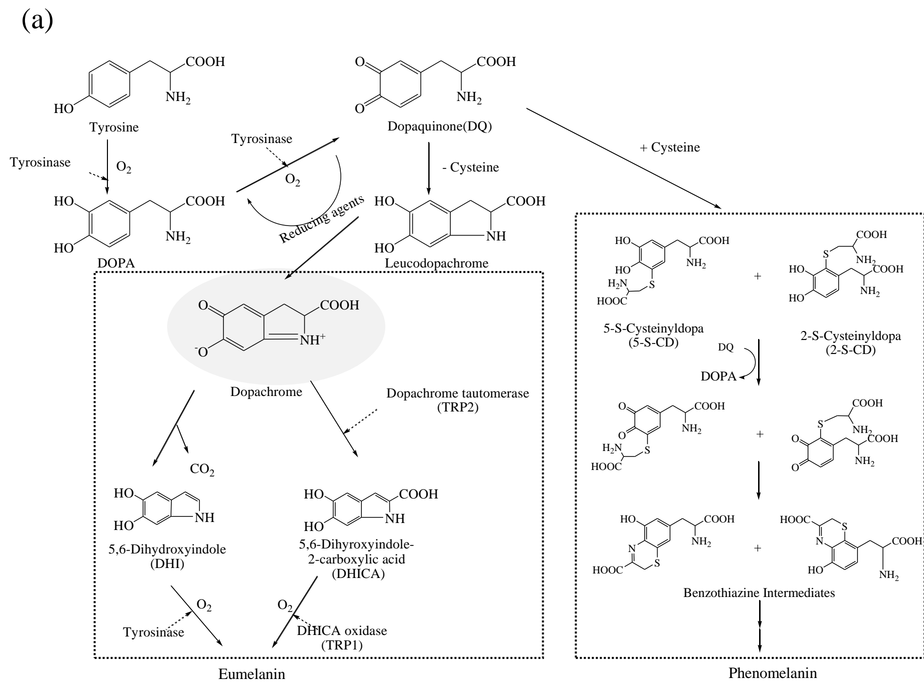


Figure 1.1 Structure of mammalian skin (Parvez e*t al.,* 2006).



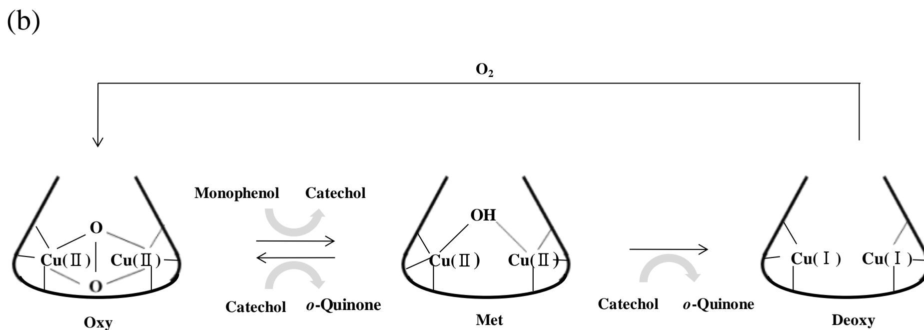


Figure 1.2 Biosynthetic pathway of melanin (Kim *et al.* 2005); (a) melanogenesis in melanocyte, (b) three oxidation states of tyrosinase.

1.2 Tyrosinase Inhibition

1.2.1 Tyrosinase Inhibitor

The production of abnormal melanin causes serious problems. As mentioned above, it leads to various pigmentation disorders in humans. In addition, it gives rise to unfavorable enzymatic browning of plant-derived food decreasing the nutritional and economic quality. Thus, controlling the tyrosinase activities has become important in various fields such as cosmetic, agriculture, medicinal products. A number of inhibitors from both natural and synthetic sources have been reported and their inhibitory mechanisms have been studied. For instance, one of the most intensively studied tyrosinase inhibitor is kojic acid (KA), derived from various fungal species, and currently applied for skin lightening agent and food additives (Ebanks *et al.*, 2009). Kojic acid inhibits tyrosinase by chelating copper atom at the active site of tyrosinase (Battaini *et al,*. 2000). It also acts as a free radical scavenger (Parves *et al.*, 2006) and chemical antioxidant by reducing the quinone. Although kojic acid has high inhibitory activities to melanin synthesis, it has once been banned because of mutagenicity concerns. Accordingly, some studies have been reported to improve the properties by modifying the structure (Noh *et al.,* 2006).

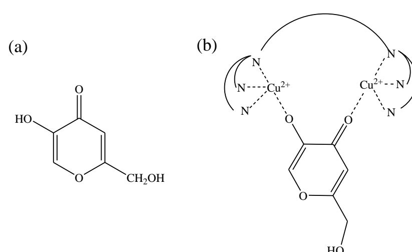


Figure 1.3 The structure of (a) kojic acid, and (b) its binding mode to the dinuclear coppers in tyrosinase.

1.2.2 Peptides as Melanogenesis Inhibitor

A number of protein or peptides having tyrosinase inhibitory activity have been studied actively since they can affect tyrosinase activity by both direct inhibition or reaction with the quinone products. There are several factors for tyrosinase inhibition such as amino acid sequence, conformation, and the peptide chain length (Schurink *et al.* 2007).

The effect of individual amino acid on tyrosinase inhibition was studied (Kahn *et al*, 1985). From this, it was found that some individual amino acids had no effect on tyrosinase inhibition; such as alanine, proline, serine, isoleucine, leucine, asparagine, valine, aspartic acid, glutamic acid, and tryptophan. Other amino acids such as lysine, glycine, histidine, and phenlyalanine inhibited the formation of dopachrome. In addition, cystein showed tyrosinase inhibitory activity since cystein plays an important role in changing the course of melanogenesis toward the formation of phenomelanin. Besides, it inhibits tyrosinase activity by reducing dopachrome. Histidine also acts as a copper chelating residue. Addtionally, the hydrophobic residues of phenylalanine can interact with the active site of tyrosinase.

The combination of amino acids is a critical factor in tyrosinase inhibition as well (Schurink *et al.*, 2007). From SPOT synthesis study, several combinations showed that they were not only binding tyrosinase but also inhibiting tyrosinase. Arginine or phenylalanine residue in combination with hydrophobic, aliphatic residues of valine, alanine or leucine leads to strong tyrosinase binding. On the other hands, peptides containing aspartic acid or glutamic acid did not bind well to tyrosinase.

There are some peptides reported for tyrosinase inhibitory activity. Milk proteins such as κ-casein (Nakajuma *et al.*, 1996), lactoferrin hydrolyzate

(Tomita *et al.*, 1995), kefir whey and kefir whey protein (Chen *et al.*, 2006) were studied for tyrosinase inhibition. Lysozyme from egg white (Li, Huang & Paskewitz *et al.*, 2006) was also claimed as a tyrosinase inhibitor. Some peptides in the human body were also reported as tyrosinase inhibitory activity; insulin (Babu *et al.*, 1998; Benathan & Labidi *et al.*, 1997), 66 kD protein from human cytosol (Vijayan *et al.*, 1982). A small peptide from honey (Ates *et al.*, 2001, Oszmianski *et al.*, 1990) and Metallothioneins (Goetghebeur & Kermasha *et al.,* 1996) from *Aspergillus niger*, which is cysteine-rich peptides of low molecular weight, are known to be tyrosinase inhibitor. Ubeid *et al*. reported on the internal library of oligopeptides showing both mushroom and human tyrosinase inhibitory activity (Ubied *et al.,* 2009). In their study, Arg-Ala-Asp-Ser-Arg-Ala-Asp-Cys and Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr were found to exhibit strong inhibitory activity against tyrosinase without cytotoxicity. Cyclic peptides showed tyrosinase inhibitory activity as well; cyclo(Pro-Tyr-Pro-Val)(Friedman *et al.*, 1995), cyclo(Gly-Thr-Leu-Pro-Ser-Pro-Phe-Leu), cyclo(Pro-Phe-Ser-Phe-Gly-Pro-Leu-Ala), cyclo(Gly-Gly-Tyr-Leu-Pro-Pro-Leu-Ser) and cyclo(Gly-Gly-Tyr-Pro-Leu-Ile-Leu) (Morita *et al.*, 1994).

1.3 Research Objectives

In previous studies, milk proteins such as κ- casein (Nakajima *et al.*, 1996) and β-lactoglobulin (Nakajima *et al.*, 1997) were reported for suppressing melanogenesis in cultured human melanocyte. Peptides derived from these proteins were recently isolated and identified as antioxidants. (Li *et al.*, 2010; Suetsuna *et al.,* 2000; Blanca *et al.* 2005) However, tyrosinase inhibitory activity has yet to be investigated. Hence, we expected that some milk protein-derived peptides might have both antioxidant and tyrosinase inhibitory activity. We chose four kinds of milk protein-derived peptides which have antioxidant activity and screened their fragments containing four amino acids to find better activities for tyrosinase inhibition. (Table1.1)

We prepared those milk protein-derived peptides reported as antioxidants and their fragments by solid-phase peptide synthesis method, and evaluated tyrosinase inhibitory activity by mushroom tyrosinase inhibition assay. To demonstrate their tyrosinase inhibitory activity effectively, kojic acid was selected as a reference compound. From this assay, we found that some peptides inhibited tyrosinase effectively compared to the original peptides.

We also prepared an oligopeptide previously known as tyrosinase inhibitor to compare its tyrosinase inhibitory activity with those of milk protein-derived peptide. Then, we focused on the peptide with higher tyrosinase inhibitory activity and various types of amino acid were replaced to find a key factor in enzyme inhibition process. The antioxidant activity of the peptides was also confirmed by lipid peroxidation test.

The milk protein-derived peptides with strong tyrosinase inhibitory activity were treated in Mel-Ab and B16F10 cells, and anti-melanogenesis test was also carried out.

Table 1.1 Milk Protein-derived Peptides and Their Tetrapeptide Fragments

Source	Peptide Sequence	Fragments (tetramer)		
Casein	YFYPEL	YFYP	FYPE	YPEL
β-Lactoglobulin	YVEEL	YVEE	VEEL	YVEL
	MHIRL	MHIR	HIRL	
	WYSLAMAA	WYSL	YSLA	SLAM
		LAMA	AMAA	

Chapter 2. Experiments

2.1 General

2.1.1 Chemicals

Fmoc-Rink amide linker coupled aminomethyl polystyrene (Rink amide AM) resin (0.82 mmol/g), 4-hydroxymethylfuran-2(5H)-one, fritted polypropylene tube reactors (5 mL, 15 mL Libra tube RT-20M), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), hydroxylbenzo-triazole (HOBt), Fmoc-protected amino acids, and the peptides such as WYSLAMAA and YRSRKYSSWY were obtained from BeadTech (Seoul, Korea). *N,N*-Diisopropylethylamine (DIPEA) was bought from Alfa Aesar (Massachusetts, USA). Ninhydrin, mushroom tyrosinase, 3,4-dihydroxy-phenylalanine (L-DOPA), ammonium thiocyanate (NH_4SCN), ferrous chloride ($FeCl_2$) and polyoxyethylenesorbitan monolaurate (Tween 20), anisole, triisopropylsilane (TIPS) and 3,6-dioxa-1,8-octanedithiol (DODT) were bought from Aldrich (St. Louis, MO, USA). *N*-methyl-2-pyrrolidone (NMP), piperidine, dichloromethane (DCM), dimethylformamide (DMF), diethyl ether, methanol were bought from Dae-Jung Chemicals (Korea). Trifluoroacetic acid (TFA) was bought from Acros Organics (Morris Plains, NJ, USA). All other solvents were used without further purification.

2.1.2 Apparatus

The purity of peptides were determined by high performance liquid chromatography (HPLC, Thermo Scientific Spectra System AS3000; Thermo-Fisher, Waltham, MA, USA), using an AAPPTec Spirit Peptide C18 reverse phase column (4.6 × 250 mm; AAPPTec, Louisville, KY, USA). Two types of Mass Spectroscopy (Electrospray Ionization Mass Spectrometry, ESI-MS, LCQ; Thermo Finnigan, Waltham, MA, USA / Matrix-Assisted Laser Desorption Ionization Mass Spectrometry, MALDI-TOF, Voyager-DETM STR Biospectrometry Workstation; Applied Biosystems Inc, Carlsbad, CA, USA) were used to determine the mass of peptides. The color reactions for linoleic acid peroxidation and enzyme inhibition tests were followed by UV/Visible spectrophotometry (Optizen 2120 UV, Mecasys Co. Ltd., Daejeon, Korea). Cytotoxicity assay and anti-melanogenesis assay were followed by an ELISA reader (TECAN, Salzburg, Austria), SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), and Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA, USA).

2.1.3 Analysis Methods

Fmoc Quantitation

Thirty milligrams of dry resin containing Fmoc-groups was reacted with 20% piperidine/DMF (v/v) (3 mL) in a shaking incubator at 25 ℃ for 50 min. Then, the resin was filtered and the filterate (0.1 mL) was diluted to 10 mL with DMF. The absorbance of the diluted solution was measured at 290 nm. Loading level of amino group of the resin was determined by the following equation:

$$\text{Loading level (mmol/g)} = (55.206 \times \text{Abs}_{290\text{nm}} - 1.0223) / 30$$

Kaiser's Ninhydrin Color Test

To determine the completion of amino acid coupling and Fmoc deprotection, potassium cyanide solution (1 mL of 0.01 M KCN aqueous solution diluted in 100 mL of pyridine), ninhydrin solution (0.5 g of ninhydrin solved in 10 mL of ethanol), and phenol (80 g of phenol dissolved in 20 mL of ethanol) were prepared. After each amino acid coupling reaction and Fmoc deprotection, one or two drop of the each stock solution was added to 1~2 mg of the resin within a test tube, and the color reaction was performed at 100 ℃ for 5 min. The reaction mixture turns dark blue when amino groups remain uncoupled.

2.2 Peptide Synthesis

Milk protein-derived peptides were synthesized on Rink Amide AM resin (0.82 mmol/g) by solid-phase peptide synthesis method. To deprotect Fmoc group on Rink Amide AM resin (350 mg), 4 mL of 20% piperidine/NMP was added to the resin in a libra tube, and then, the libra tube was shaken in a shaking incubator at RT for 50 min. For amino acid coupling, Fmoc-amino acid (2 equiv), BOP (2 equiv) and HOBt (2 equiv) were dissolved in NMP, and this mixture was added to the resin with DIPEA (4 equiv). The coupling reaction was performed at RT for 2 h, and the Fmoc deprotection was carried out using 20% piperidine/NMP at RT for 30 min. Each coupling and Fmoc deprotection reaction step was monitored by Kaiser's ninhydrin color test. After repeating these steps, the final peptides were cleaved from the resin by cleavage cocktail (TFA/Anisole/TIPS/DODT = 9.3/0.3/0.2/0.2) at RT for 1.5 h. The peptide solution was filtered, concentrated in high vacuum, and precipitated with cold diethyl ether. The resulting powder from precipitation was washed with cold diethyl ether 5X and dried with nitrogen gas. The purity of peptides was checked by high performance liquid chromatography (HPLC) and the peptide products were identified by electrospray ionization mass spectrometry (ESI-MS).

2. 3 Determination of Biological Activity

2.3.1 In Vitro Assay

Mushroom Tyrosinsae Inhibition Assay

To evaluate tyrosinase inhibitory activity of milk protein-derived peptides and their fragments, mushroom tyrosinase inhibition assay was carried out. Phosphate buffer (250 μL, 0.1 M, pH 6.8), L-DOPA (250 μL, 2.5 mM), distilled water (200 μL), and 25 μL of inhibitor dissolved in DMSO were mixed together in an Eppendorf tube (1.5mL). The reaction mixture was incubated at 25℃ for 10 min after treating with 25 μL of aqueous mushroom tyrosinase solution (428 U/mL). The tyrosinase inhibitory activity was determined by measuring the decrease UV absorbance of dopachrome, an immediate precursor of eumelanin, detected at 475 nm (Figure 1.2.a), and calculated by the following equation:

$$\%\text{Inhibition} = (1\text{-Abs}_{475nm}\text{ of test sample}/\text{Abs}_{475nm}\text{ of control}) \times 100$$

Control was the reaction mixture containing 25 μL of DMSO instead of inhibitor. Each experiment was performed in triplicate at 25 ℃ for 10 min.

Lipid Peroxidation Inhibitory Activity Test

The lipid peroxidation inhibitory activity test was performed to compare the antioxidant activity of peptides fragments.

This test is based on a general method to determine the antioxidant activity indirectly by using Tween 20 emulsified linoleic acid. The alkylperoxy radicals induced by air spontaneously lead to lipid peroxidation during the experimental period.

Linoleic acid emulsion (50 mM) was prepared by mixing 0.284 g of linoleic acid, 0.284 g of Tween 20 in 50 mL of 0.1 M sodium phosphate buffer (pH 7.0). Reaction mixture contained 2.5 mL of linoleic acid emulsion, 2.0 mL of 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mL of water and 0.5 mL of test sample dissolved in methanol. The total reaction volume was 5.5 mL. The final concentration of test sample was 250 μM. The transparent glass vials (10 mL) containing the reaction mixture were capped with rubber septum, and kept at 50℃ under dark conditions for 30 h. methanol was added instead of antioxidant as a control.

The modified ferric thiocyanate (FTC) method was carried out to evaluate the amount of peroxides as follows: The reaction mixture (25 μL) was added into the solution contained 1.175 mL of 75% ethanol, 25 μL of 30% NH_4SCN, and 25 μL of 20 mM $FeCl_2$ in 3.5% HCl in an Eppendorf tube (1.5 mL). After exact 3 min, the UV absorbance of colored solution was measured at 500 nm.

The percentage of lipid peroxidation inhibitory activity (%Pi) was calculated at 24h, and the relative antioxidant activity (RAA) was also calculated based on %Pi of original peptide.

$$\%Pi = (1 - Abs_{500nm} \text{ of test sample} / Abs_{500nm} \text{ of control}) \times 100$$

2.3.2 Cellular Assay

Measurement of Cytotoxicity

Cell Counting Kit-8 (CCK-8; CK04, Dojindo, Kumamoto, Japan) was used to measure the cytotoxicity in murine melanoma cell line, B16F10 and melanocyte cell line, Mel-Ab. Mel-Ab cells (2×10^3 per well) were seeded into 96-well plates. Culture media were replaced with serum-free DMEM after 24 h, and incubated for another 24 h. Then, testpeptides were treated in cell containing new serum-free media and incubated for 24 h. After CCK-8 solution was added, the cells were incubated for another 2.5 h at 37 ℃. The amount of water-soluble formazan generated by the activity of dehydrogenase in cells was measured by optical density at 450 nm using Spectra Max Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Anti-melanogenesis Assay

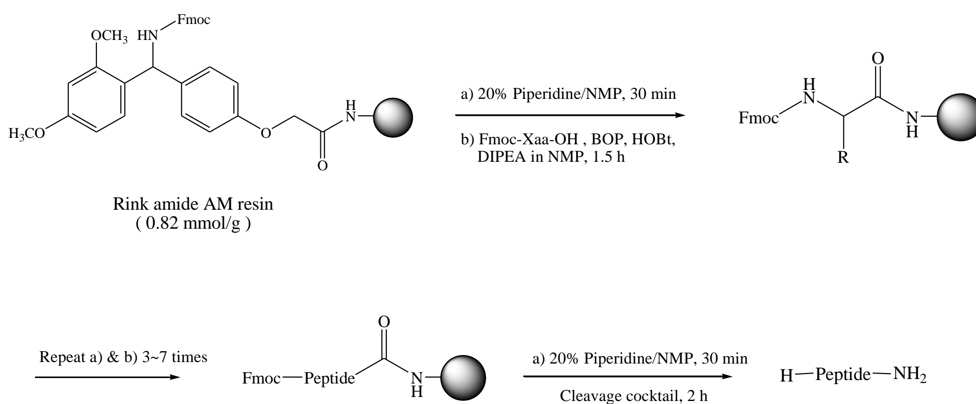
For anti-melanogenesis assay, Mel-Ab cells were cultured in DMEM with 10% FBS, 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 nM cholera toxin (CT), 50 µg/mL of streptomycin, and 50 U/mL penicillin at 37 ℃ in 5% CO_2. When the peptides were added into the Mel-Ab cell line up to 100 µM, and incubated for four days, the peptide-treated cells produced less amount of melanin than the cells without it. Then, the cells were dissolved in 1 mL of 1*N* NaOH at 100 ℃ for 30 min, and centrifuged for 20 min at 16,000 G, after which the optical densities of the supernatants were measured at 400 nm. Each experiment was performed in triplicate and averaged.

Chapter3. Results & Discussion

3.1 Synthesis and Characterization of Peptides

All the peptides were synthesized by solid-phase peptide synthesis method on Rink amide AM resin with Fmoc strategy (Scheme 3.1). We chose four kinds of milk protein-derived peptides having antioxidant activity and synthesized their fragments (tetrapeptides). Furthermore, several peptides derived from HIRL were synthesized by the same method to study the roles of peptide structure.

The purity of peptides was determined by HPLC (Table 3.1), and most of peptides were obtained with high purity (>90%). ESI-MS also used to identify the peptides.



Scheme 3.1 Synthesis of oligopeptides.

Table 3.1 The Purity and Characterization of Oligopeptides

Compounds	Purity (%)	Mass ($[M+H]^+$)	
		Calculated	Found
YFYPEL	90	830.4	830.3
YFYP	>99	588.67	588.3
FYPE	>99	554.61	554.2
YPEL	>99	519.59	520.3
YVEEL	90	651.3	651.3.
YVEE	99	538.56	538.1
VEEL	86	488.55	488.2
YVEL	>99	522.61	522.2
MHIRL	85	668.4	668.5
MHIR	95	555.71	555.3
HIRL	93	537.67	537.4
WYSLAMAA	88	911.4	911.3
WYSL	91	567.65	567.2
YSLA	98	452.52	452.2
SLAM	87	420.54	420.1
LAMA	97	403.54	404.1
AMAA	92	362.46	362.1

Table 3.1 The Purity and Characterization of Oligopeptides (continued)

Compounds	Purity (%)	Mass ($[M+H]^+$)	
		Calculated	Found
FIRL	77	588.67	588.3
AIRL	77	554.61	554.2
HILL	93	519.59	520.3
HIEL	95	651.3	651.3
HIKL	>99	538.56	538.1
YRSRKYSSWY	94	1394.54	1394.67

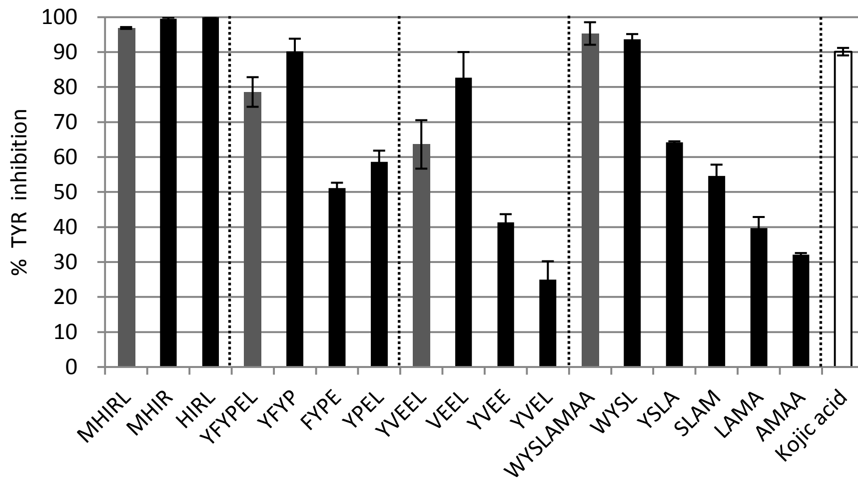
3.2 Evaluation of Tyrosinase Inhibitory Activity

3.2.1 Milk Protein-derived Oligopeptides

To determine tyrosinase inhibitory activity of milk protein-derived peptides, we performed mushroom tyrosinase inhibition assay. To compare tyrosinase inhibitory activity of milk protein-derived peptides, kojic acid (KA) was used as a reference compound.

We measured the tyrosinase inhibitory activity at 250 μM (Figure 3.1.a), and 100 μM (Figure 3.1.b). As predicted, all of the peptide fragments originated from milk protein-derived peptides showed different tyrosinase inhibitory activity with the original ones. When the concentration of peptide was 250 μM, MHIR, HIRL, YFYP, and VEEL enhanced tyrosianse inhibitory activity comparing to the original peptides, while, the other fragments showed lower tyrosinase inhibitory activity. According to this result, we assumed that some amino acid sequence played important roles in tyrosinase inhibition. For instance, the peptides related to YFYPEL and WYSLAMAA have a tendency to inecrease tyrosinase inhibitory activity as the ratio of aromatic group to the peptide was increased. They have amino acid such as phenylalanine (F) and tryptophan (W), which are similar to tyrosine (Y), the natural substrate, and they could inhibit tyrosinase by binding to the hydrophobic pocket of the enzyme. Similarly, the peptides related to YVEEL also have a tendency to enhance tyrosinase inhibitory activity when tyrosine (Y) was removed. When the concentration of peptide decreased to 100 μM, the same tendency was observed, too. MHIR and HIRL sufficiently inhibited tyrosinase (>60%), and MHIRL, YFYPEL, YFYP, WYSLAMAA and WYSL showed moderated tyrosinase inhibitory activity. The results of these assays were dependent on concentration, and thus, we assume that this result is reliable.

(a)



(b)

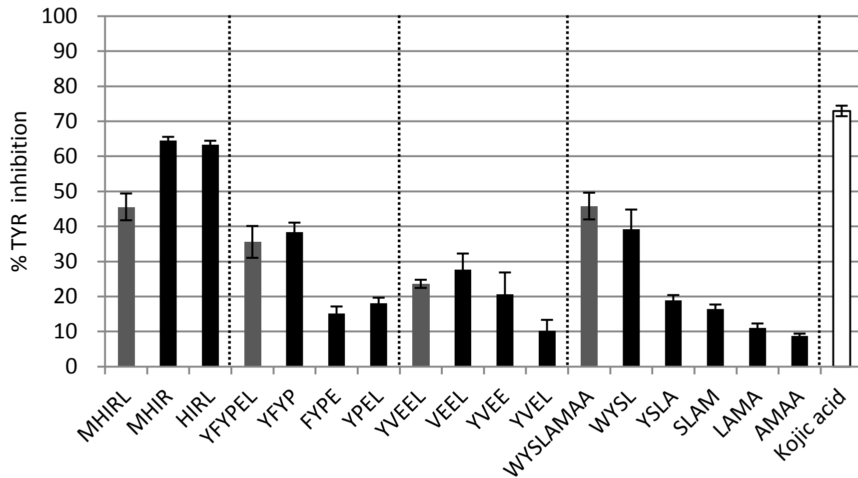


Figure 3.1 Tyrosinase inhibitory activities of milk protein-derived peptides. Concentration of inhibitor was (a) 250 μM, (b) 100 μM. Conditions: Mushroom tyrosinase inhibition assay was performed for 10 min at 25 ℃ and, the UV absorbance was measured at 475 nm. The values are given as the mean ± standard error.

To compare the tyrosinase inhibitory activity of peptides efficiently, IC_{50} of each peptide were calculated (Table 3.2). IC_{50} values were determined by measuring the tyrosinase inhibitory activity at different peptide concentrations (Figure 3.2). Peptides related to MHIRL showed low IC_{50} values. Especially, IC_{50} values of MHIR and HIRL were the lowest among milk protein-derived peptides. As assumed, WYSLAMAAA, YFYP and VEEL gave the lowest IC_{50} value among their families.

Table 3.2 IC_{50} of Milk Protein-derived Peptides

Compound	**IC_{50} (μM)**
MHIRL	115
MHIR	83
HRIL	70

Compound	**IC_{50} (μM)**
YFYPEL	155
YFYP	120
FYPE	225
YPEL	220

Compound	**IC_{50} (μM)**
YVEEL	200
YVEE	280
VEEL	185
YVEL	430

Compound	**IC_{50} (μM)**
WYSLAMAA	120
WYSL	135
YSLA	205
SLAM	235
LAMA	300
AMAA	310

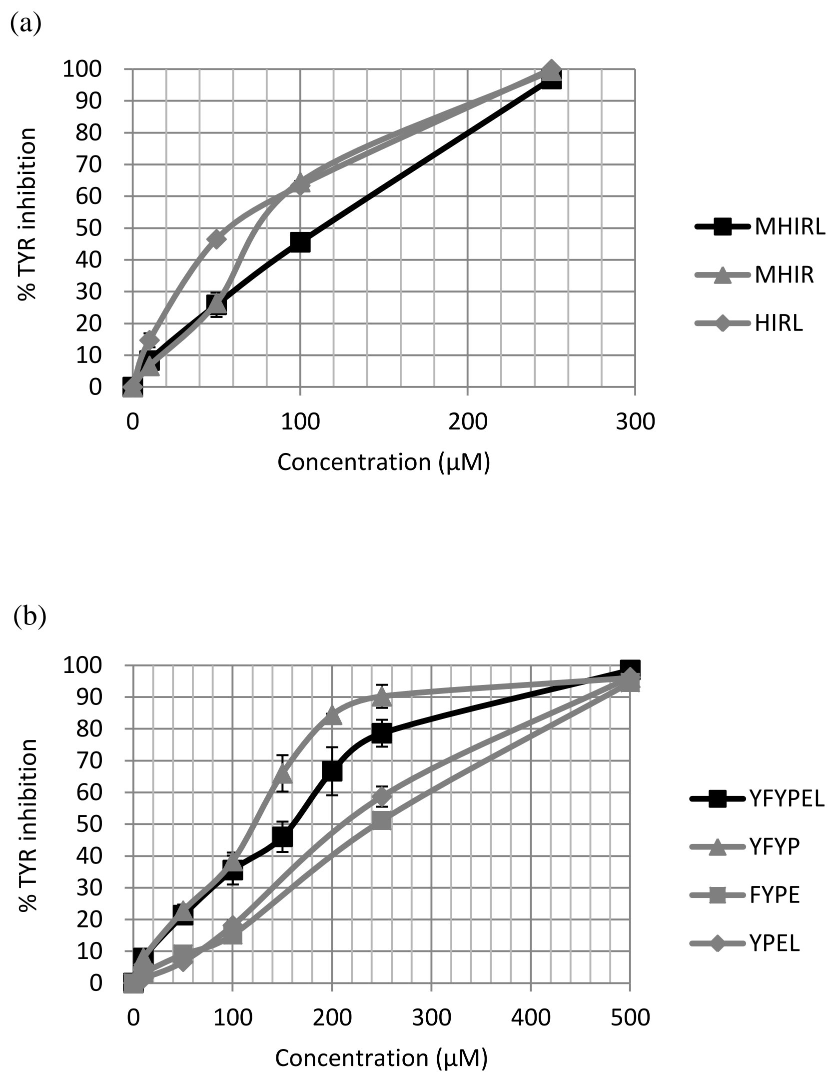
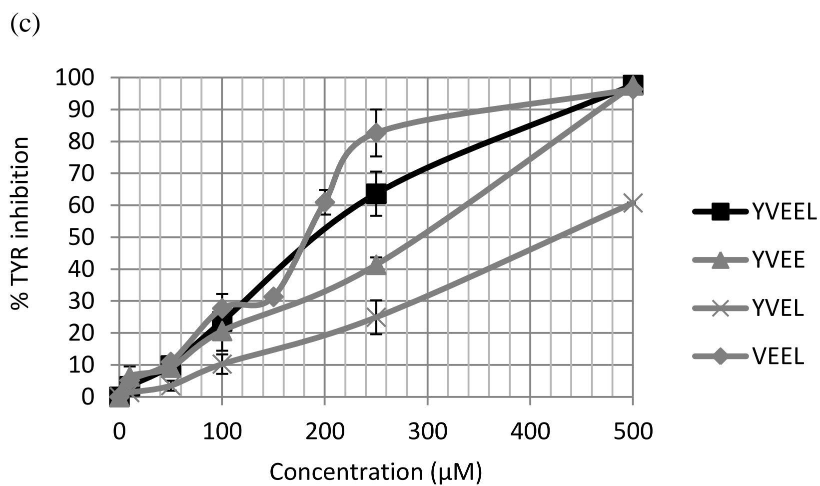


Figure 3.2 Tyrosinase inhibitory activities of milk protein-derived peptides at different concentrations. (a)MHIRL, (b)YFYPEL, (c)YVEEL, (d)WYSLAM-AA family.

(c)



(d)

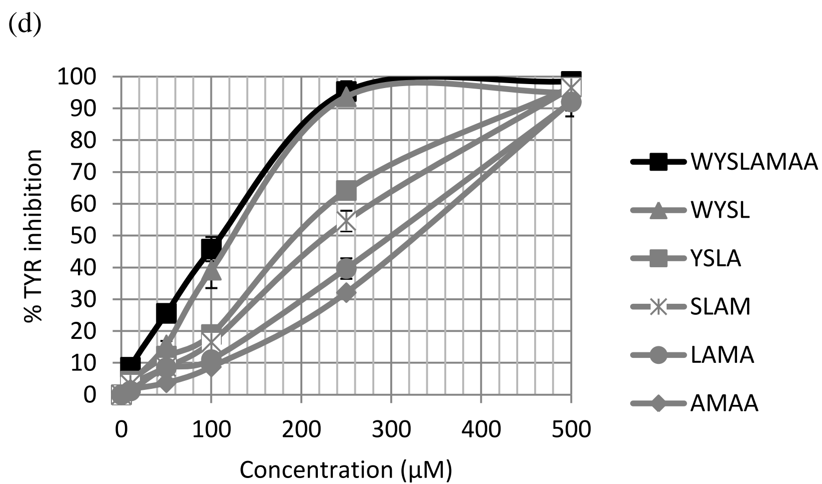


Figure 3.2 Tyrosinase inhibitory activities of milk protein-derived peptides at different concentrations (continued). (a)MHIRL, (b)YFYPEL, (c)YVEEL, (d) WYSLAMAA family.

UV-Vis spectra were measured to demonstrate that the peptides reduced dopachrom fomation. The sample was prepared as the case of mushroom tyrosinase inhibition assay. We chose MHIR and HIRL as inhibitors because they showed higher activity than other peptides. When we did not add peptides, dopachrome was clearly detected at 475 nm. Dopachrome formation was decrease the concentration of the peptide was increased. We assume that milk protein-derived peptides and their fragments inhibit the melanogenesis by suppressing dopachrome formation. A peak at 282 nm indicates the presence of L-DOPA

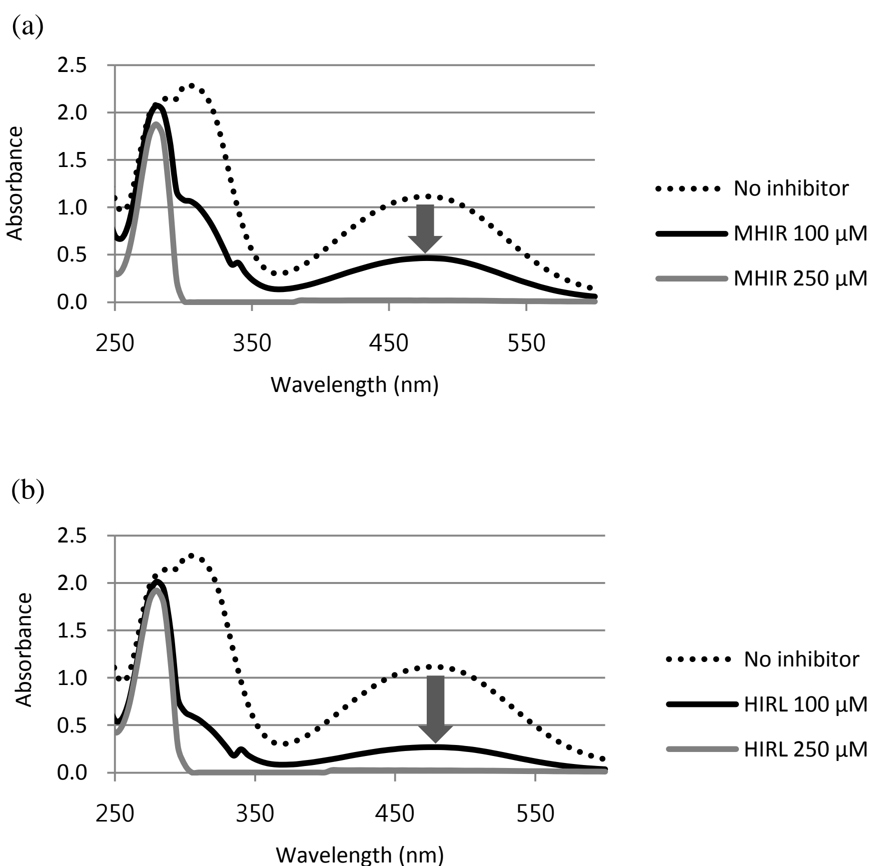


Figure 3.3 The effects of (a) MHIR and (b) HIRL on dopachrome formation.

3.2.2 The Tyrosinase Inhibitory Activities of Peptides

To compare the tyrosinase inhibitory acitivity of milk protein-derived peptides, the same assay method for tyrosinase inhibitor was performed as with an oligopeptide, YRSRKYSSWY (Ubied *et al.,* 2009). The peptide was also prepared by solid-phase peptide synthesis method as an amide form.

As shown figure 3.4, HIRL exhibited higher tyrosinase inhibitory activity than the known tyrosinase inhibitor peptide, YRSRKYSSWY, even though it contained few amino acids. The tyrosinase inhibitory activities (%) at 100 μM were as follows: Kojic acid (74.46 ± 1.34) > HIRL (63.35 ± 1.0) > YRSRKYSSWY (43.10 ± 10.21)

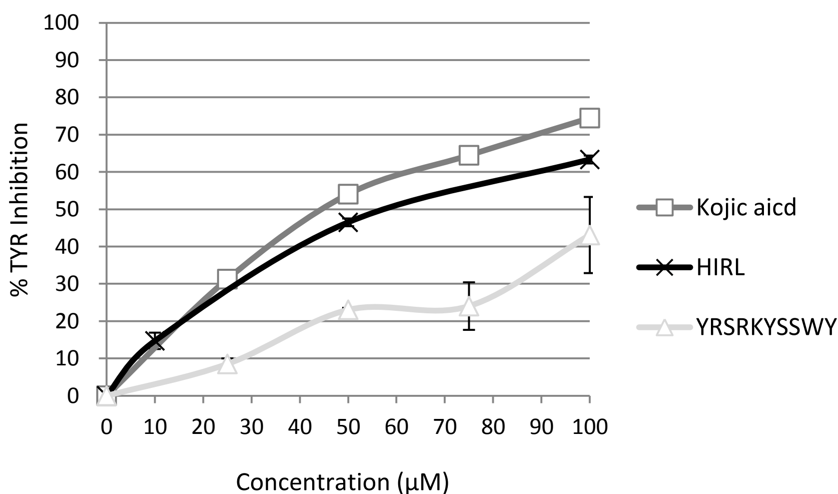
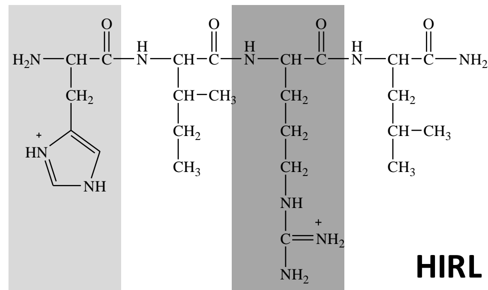


Figure 3.4 Tyrosinase inhibitory activities of kojic acid, HIRL, and YRSRKYSSWY at difference concentrations.

3.2.3 Tyrosinase Inhibitory Activities of HIRL Derivatives

In previous study, HIRL showed the lowest IC_{50} value. Thus, we designed various HIRL derivatives to figure out which amino acid is important for HIRL to inhibit tyrosinase. Although MHIR also exhibited higher tyrosinase inhibitory activity, it was excluded from this study since it contained methionine, which could change the melanogenesis pathway to phenomelanogenesis.

We prepared HIRL derivatives in two ways (Figure 3.5). First, FIRL and AIRL were synthesized to figure out the roles of histidine. L-Phenylalanine (F) was chosen instead of histidine to figure out the roles of aromatic ring on tyrosinase inhibition, and L-alanine (A) was chosen instead of histidine to compare the tyrosinase inhibitory activities between FIRL and AIRL, which contains aromatic ring and aliphatic residue, respectively. In addition, three kinds of peptides were synthesized to figure out the roles of arginine (R). Thus, we introduced negative charge or no charge into peptides by replacing arginine to glutamic acid (E) and leucine (L), respectively. Lysine, which contains different type of positive charge with arginine, was also chosen instead of arginine to determine the roles of arginine.



The roles of His	The roles of Arg
FIRL	HIEL
	HILL
AIRL	HIKL

Figure 3.5 Structures of HIRL derivatives.

From this assay, we found that the order of tyrosinase inhibitory acitivity was as follows: HIRL (63.35 %) > FIRL (34.94%) > AIRL (25.09%) > HIEL (2.04%), HILL (1.54%), HIKL (1.79%).

As shown in Figure 3.6, FIRL and AIRL exhibited lower tyrosinase inhibitory activity than HIRL, and the tyrosinase activity was more inhibited by FIRL than AIRL. From this result, we assumed that the aromatic ring might enhance tyrosinase inhibitory activity. Additionally, the peptide which contains different charge or no charge such as HIEL and HILL did not show any tyrosinase inhibitory activity. Although HIKL contained positive charge, it did not show tyrosinase inhibitory activity, either. From these results, we could conclude that the positive charge of arginine plays a key core role in tyrosinase inhibition.

In summary, positive charge of arginine is very important and, aromatic ring of histidine plays additional roles on tyrosinase inhibition.

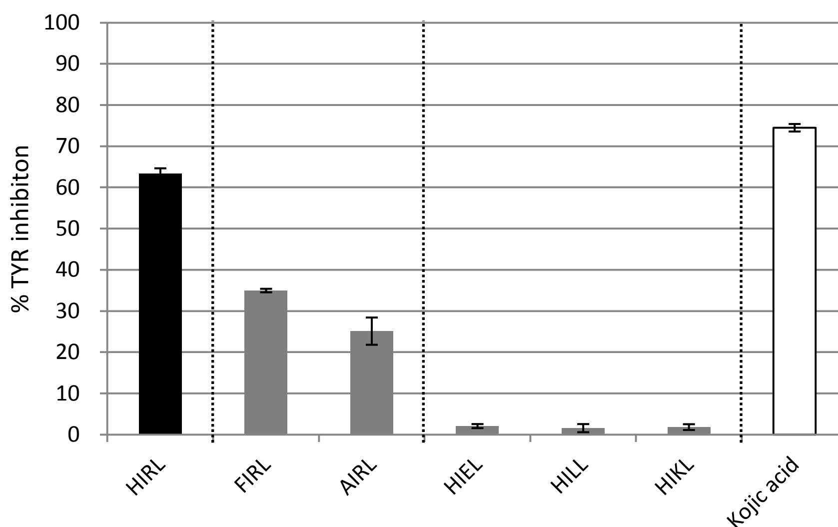


Figure 3.6 Tyrosinase inhibitory activities of HIRL derivatives.

3.3 Evaluation of Antioxidant Activity

We checked whether antioxidant activity of nine antioxidant peptide derived tetrapeptides maintained their original activity or not. The test was performed using Tween 20 emulsified linoleic acid. The alkylperoxy radicals induced by air spontaneously cause lipid peroxidation during the experimental period. We selected nine kinds of milk protein-derived peptides including four kinds of aforementioned peptides, MHIRL, YFYPEL, YVEEL, WYSLAMAA and five fragments, MHIR, HIRL, YFYP, VEEL, WYSL, having high tyrosinase inhibitory activity. The reaction was performed at 50 ℃ under dark condition and the UV absorbance of colored solution was measured by UV spectra at 500 nm.

The relative antioxidant activity was calculated and summarized in Figure 3.7. The Pi (%) value of each original peptide was set to 1.0. As shown in Figure 3.7, some tetrapeptides such as MHIR, YFYP and WYSL showed enhanced antioxidant activity when the peptide size were reduced while keeping methionine, tyrosine, phenylalanine, and tryptophan in the sequence. Methionine (M) has been reported to possess antioxidant activity, because it contained sulfide group, which is easy to form sulfoxide. Thus, the antioxidant activity of HIRL was decreased because methionine was omitted from MHIRL. The other amino acids, tyrosine, phenylalanine, tryptophan are already known as antioxidant. Therefore, the antioxidant activity of VEEL was decreased by omitting Tyr from YVEEL.

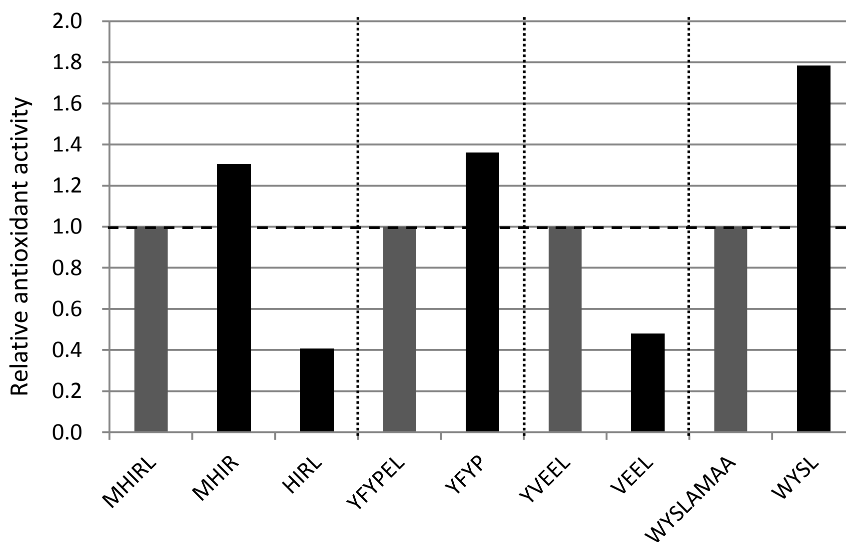


Figure 3.7 The relative antioxidant activities of peptides after 24h. The Pi (%) was measured by lipid peroxidation inhibition assay. The absorbance of colored reaction mixture was measured at 500 nm. The reaction was performed at 50℃ under dark condition. Each experiment was performed in triplicate and final concentration of antioxidant was 250 μM. The values are given as the mean ± standard error.

3.4 Melanogenesis Inhibitory Activity in Cell System

3.4.1 Cytotoxicity of Milk Protein-derived Peptides

MHIRL derivatives, MHIR and HIRL, which exhibited good tyrosinase inhibitory activity *in vitro*, were chosen, and their melanogenesis inhibitory activities in cell system were measured. Mel-Ab cell and B16F10 cell were selected for this assay, and the concentration of peptides was fixed at 100 μM. Arbutin, which is a well known skin whitening agent, was used as a reference compound. As shown figure 3.8, all the peptides did not show cytotoxicity on both cell lines.

(a)

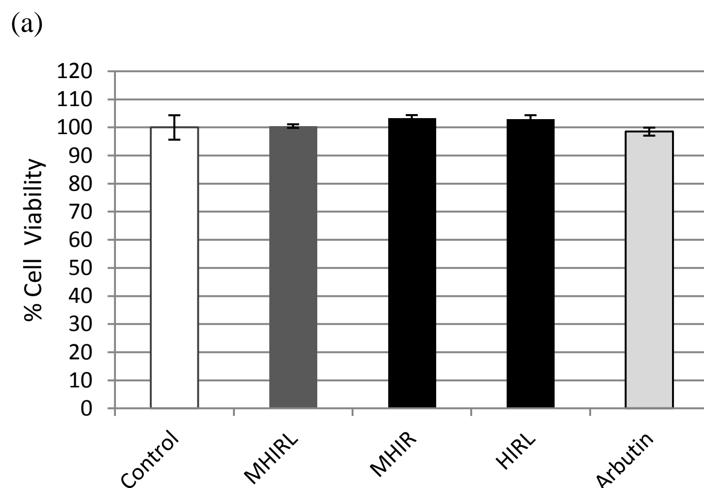


Figure 3.8 Cytotoxicity of MHIRL derivatives and arbutin on cell viability (a) in Mel-Ab cells (b) in B16F10 cells. [inhibitor] = 100 μM.

(b)

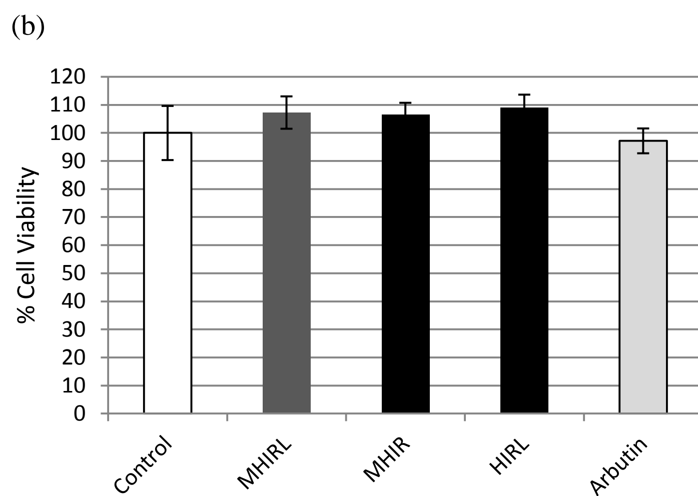


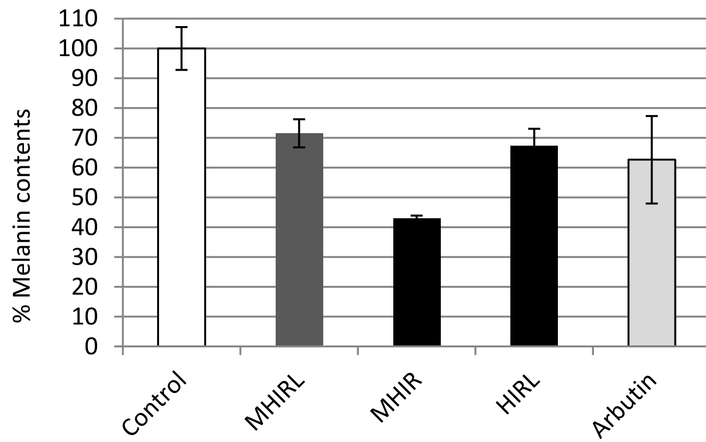
Figure 3.8 Cytotoxicity of MHIRL derivatives and arbutin on cell viability (continued) (a) in Mel-Ab cells (b) in B16F10 cells. [inhibitor] = 100 μM.

3.4.2 Melanogenesis Inhibitory Activities of MHIRL Derived Tetrapeptides

Melanogenesis inhibitory activities of MHIRL family were measured in Mel-Ab cell and B16F10 cell. The concentration of inhibitors was 100 μM, too. As shown in Figure 3.9 (a), MHIR sufficiently inhibited melanogenesis in Mel-Ab cell, and gave higher inhibitory activity than arbutin. The other peptides also exhibited slight anti-melanogenesis activity. Interestingly, all of peptides showed good melanogenesis inhibitory activity in B16F10 cells (Figure 3.9 (b)), and gave superior activity than arbutin. From these results, we found that MHIR commonly inhibited melanogenesis in both cell lines, though each cell line has different inhibitory mechanisms. We assumed that methionine of MHIR enhanced the melanogenesis inhibitory activity by similar to the cystein which can change melanogenesis pathway to phenomelanogenesis. In addition, methionine can act as a reducing agent which is able to convert o-quinones back to the catechols by forming methionine sulfoxide.

The anti-melanogenesis activity of MHIR was measured at various concentrations; 1, 5, 10, 50, and 100 μM. Figure 3.10.a shows that melanin contents were decreased depending on the concentration of MHIR in Mel-Ab cells. Arbutin did not give melanogenesis inhibitory activity under this condition. The same test was performed in B16F10 cells (Figure 3.10.b), and MHIR exhibited similar melanogenesis inhibitory activity at 50 μM concentration.

(a)



(b)

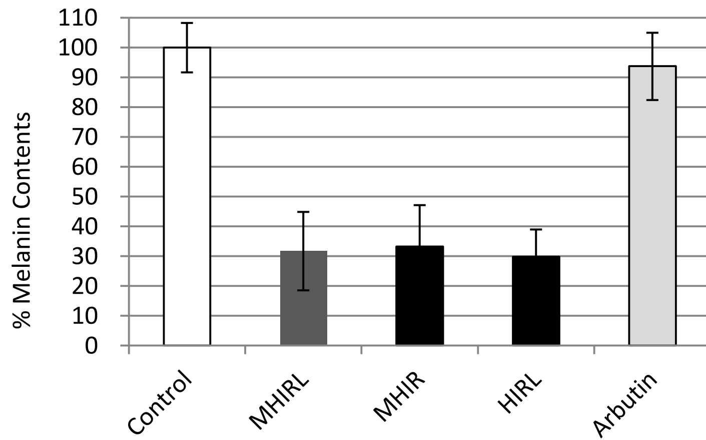


Figure 3.9 Inhibitory activities of MHIRL family on melanogenesis in (a) Mel-Ab cell, and (b) B16F10 cell.

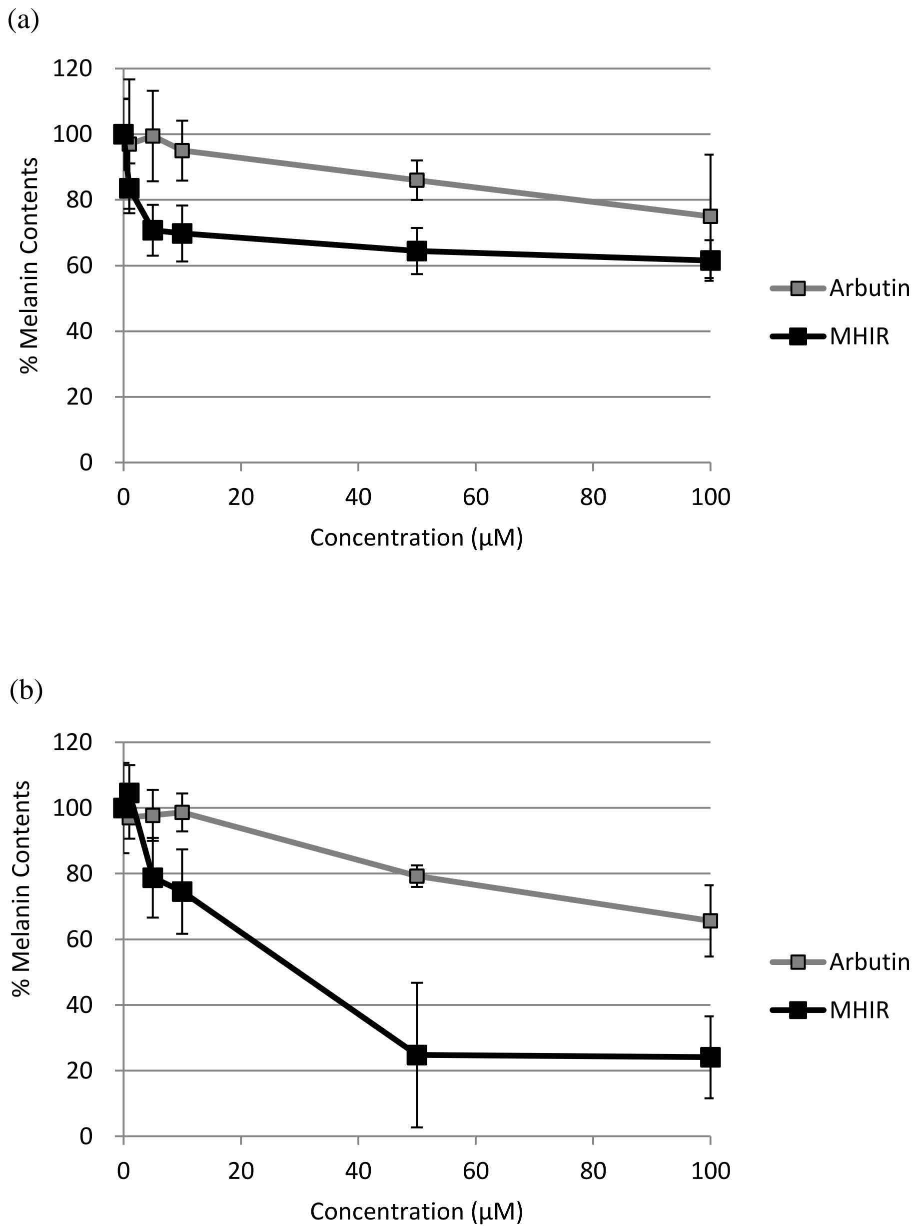


Figure 3.10 Melanogenesis inhibitory activities of MHIR at different concentrations on (a) Mel-Ab cells, and (b) B16F10 cells.

Conclusion

Four kinds of milk protein-derived peptides known as antioxidants and their fragments (tetrapeptides) were prepared by solid-phase peptide synthesis method. Mushroom tyrosinase inhibition test was performed to measure the tyrosinase inhibition activity of milk protein-derived peptides. Among four kinds of milk protein-derived peptides derivatives, MHIRL family showed higher tyrosinase inhibitory activity and HIRL exhibited the lowest IC_{50} value. From these results, we confirmed the active part of each milk protein-derived peptide and shorter peptide fragments having stronger activity.

In addition, we prepared an oligopeptide, YRSRKYSSWY, which is known as a tyroainse inhibitor to compare its tyrosinase inhibitory activity, and we found that it had lower tyrosinase inhibitory activity than HIRL.

The structure and tyrosinase inhibitory activity of relationship of HIRL was studied to find an important factor on tyrosinase inhibition. First, the roles of histidine were studied by comparing the activity of HIRL to that of FIRL and AIRL. FIRL contains phenylalanine having aromatic group. On the other hands, AIRL contains alkyl chain residue. We also synthesized HIEL, HILL and HIKL to find the role of arginine having positive charge. From these studies, we confirmed that HIRL showed the highest tyrosinase inhibitory activity than other derivatives, and the peptides without arginine such as HIEL,

HILL and HIKL did not show any tyrosinase inhibitory activity. We also found that the positive charge of arginine played a key role in tyrosinase inhibition.

The lipid peroxidation test was performed to compare the antioxidant activity of the original milk protein-derived peptides and tetrapeptide fragments. We chose five tetrapeptides, MHIR, HIRL, YFYP, VEEL, WYSL, having good tyrosianse inhibitory activity and four kinds of milk protein-derived peptides, MHIRL, YFYPEL, YVEEL, WYSLAMAA. Most of the tetrapeptides maintained their antioxidant activity of the original peptide.

The melanogenesis inhibitory activity of MHIRL family was measured in B16F10 cell. All of the peptides showed no cytotoxicity and the peptides from MHIRL family showed higher melanogenesis inhibitory activity than arbutin. The melanogenesis inhibitory activity was also measured in Mel-Ab cell. MHIR showed the highest tyrosinase inhibitory activity at 100 μM (57%) and the activity was increased in concentration dependent manner.

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초록

우유는 오래전부터 그 특유의 색깔 때문에 미백 기능을 가지는 것으로 막연히 여겨왔고, 클레오파트라를 비롯한 역사 속 미인들이 우유로 세안과 목욕을 했다고 전해지고 있다. 1990년대에 들어 유청에서 분리한 단백질 중 κ-casein과 β-lactoglobulin이 멜라닌 생합성을 효과적으로 저해한다는 논문이 발표된 바 있고, 2000년대에는 분리된 유청 단백질의 아미노산 서열을 분석하는 연구가 활발히 이루어지면서, 펩타이드 조각에 대한 연구와 동시에 이들의 항산화 기능이 밝혀졌다.

이에 본 연구에서는 미백 기능과 항산화 기능 모두를 갖는 우유 단백질 유래 펩타이드의 개발을 목적으로 하여, 기존의 항산화 기능만 보고된 우유 유래 펩타이드 중 네 가지(Tyr-Phe-Tyr-Pro-Glu-Leu, Met-His-Ile-Arg-Leu, Tyr-Val-Glu-Glu-Leu, Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala)를 선정하였다. 또한, 펩타이드의 구조 중 미백 활성을 나타내는 부분을 찾고, 짧지만 강한 활성을 가지는 펩타이드를 찾기 위해 네 가지의 아미노산을 포함하는 펩타이드 조각들을 디자인 및 합성하였다. 모든 합성은 Fmoc 화학법에 의한 고체상 펩타이드 합성을 통해 이루어졌다.

버섯의 타이로시나아제를 이용한 실험을 통해 우유 단백질 유래

펩타이드의 타이로시나아제 억제활성을 측정하였다. 그리고, 몇 가지의 펩타이드 조각이 본래 펩타이드보다 좋은 활성을 보이는 것을 확인하였다. 또한, 다양한 농도에서 활성을 측정함으로써 각 펩타이드의 IC_{50}을 계산하였다. 또한, 가장 낮은 IC_{50}을 보인 펩타이드의 유도체들을 디자인하여 펩타이드가 타이로시나아제를 억제하는 과정에 관여하는 요소에 대한 연구를 통해 아르기닌의 양전하가 중요한 역할을 하는 것을 발견 하였다.

높은 타이로시나아제 억제 활성을 보인 MHIRL과 그 유도체들 (MHIR, HIRL)을 선정하여 그 활성을 세포에서도 확인하였다. B16F10 세포와 Mel-Ab 세포에 이들 펩타이드를 처리한 결과, MHIRL 유도체가 두가지 종류의 멜라닌 세포에서 독성이 없이, 일반적인 미백제로 사용되는 알부틴보다 좋은 멜라닌 생합성 저해 활성을 보이는 것을 확인하였다.

주요 단어: 멜라닌, 타이로시나아제, 타이로시나아제 억제 활성, 펩타이드, 우유 단백질, 미백기능, 항산화 활성

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