A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Chemo-enzymatic Synthesis of Lauroyl Peptides with Antibacterial and Antioxidative Activities: Influence of Hydrophilicity and Charge of Non-fatty Acid Moiety on Antibacterial Activity

항균 및 항산화성 lauroyl peptide의
화학효소적 합성:
비지방산 잔기의 친수성 및 전하가
항균성에 미치는 영향

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석사학위논문

Chemo-enzymatic Synthesis of Lauroyl Peptides with Antibacterial and Antioxidative Activities: Influence of Hydrophilicity and Charge of Non-fatty Acid Moiety on Antibacterial Activity

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이 논문을 석사학위 논문으로 제출함

2018년 2월

서울대학교 대학원

농 생 명 공 학 부

이 수 지

이수지의 석사 학위논문을 인준함

2018년 2월

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Abstract

Deterioration of food quality in terms of safety and nutritional value caused by lipid oxidation and microbial contamination is a critical problem in the food industry. In order to control lipid oxidation and microbial contamination simultaneously, multifunctional lauric acid derivatives were synthesized enzymatically in the previous study. Even though erythorbyl laurate, one of the lauric acid derivatives, retained antibacterial activity after enzymatic synthesis, most of them such as sesamol laurate, methyl laurate, and isoamyl laurate, lost antibacterial activities. Therefore, in order to study the influence of non-fatty acid moiety on antibacterial activities of lauric acid derivatives, lauroyl peptide model was designed due to the simplicity to control physicochemical properties of non-fatty acid moiety. In addition, a multi-functional lauric acid derivative having antioxidative and antibacterial activities, was lipase-catalytically synthesized using an antioxidative peptide.

Lipase-catalyzed synthesis was conducted to produce six lauroyl peptides (RHK-L, AHK-L, NHK-L, AHK-L, LK-L, and LWK-L). As comparing antibacterial activities of lauroyl peptides, NHK-L caused 3.5 log reduction after treatment for 12 h, and RHK-L caused 5.6 log reduction after treatment for 3 h, representing RHK-L had the strongest antibacterial activity

among lauroyl peptides. In order to study influence of non-fatty acid moiety on the antibacterial activities of lauroyl peptides, hydrophilicity and charge of non-fatty acid moiety were compared. As a result, hydrophilicity of non-fatty acid moiety of RHK-L and NHK-L was higher than those of the other lauroyl peptides. In addition, it was revealed that non-fatty acid moiety of RHK-L showing the strongest antibacterial activity was more positively charged than that of NHK-L. Therefore, it was implied that the hydrophilicity of non-fatty acid moiety is considered as a major influence on antibacterial activities of lauric acid derivatives, and charge also affects the antibacterial activities of the derivatives.

Evaluation of the antibacterial activity of RHK-L in O/W emulsion indicated that RHK-L had antibacterial activity against gram-positive bacteria. In addition, by fluorescence microscopy, it was revealed that RHK-L caused cell membrane rupture, which implied the antibacterial activity was derived from lauric acid. The evaluation of antioxidative activity of RHK-L identified that antioxidative activity of RHK was retained after enzymatic synthesis.

In this study, it was investigated that the antibacterial activity of lauric acid derivatives is affected by the hydrophilicity and charge of non-fatty acid moiety. In addition, RHK-L was suggested as a multi-functional lauric acid

derivative possessing both antibacterial and antioxidative activities.

***Keywords*: lauroyl peptide, multi-functionality, antibacterial activity, non-fatty acid moiety, hydrophilicity, charge**

***Student number*: 2016-21737**

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1. Introduction

Prevention of degradation in foods by lipid oxidation and microbial contamination has been an important issue (Viuda-Martos, Mohamady, Fernández-López, Abd ElRazik, Omer, Pérez-Alvarez, et al., 2011). Lipid oxidation and contamination of food pathogens reduce shelf life of lipid-based foods and lead to illness such as food poisoning (Maijala, Lyytikäinen, Johansson, Autio, Aalto, Haavisto, et al., 2001; Mason, Williams, Salmon, Lewis, Price, Johnston, & Trott, R., 2001). The aforementioned problems in terms of safety can be controlled simultaneously by using multi-functional additives with antioxidative and antibacterial activities (Food & Administration, 2001).

In the previous study, erythorbyl laurate with multi-functionality was synthesized by lipase-catalyzed esterification between erythorbic acid having antioxidative activity (hydrophilic head group) and lauric acid having antibacterial activity (hydrophobic tail group) (Park, Jo, Yu, Park, Choi, Lee, & Chang, 2017; Park, Lee, Sung, Lee, & Chang, 2011; Park, Lee, Jo, Choi, Lee, & Chang, 2017).

Lauric acid is a medium-chain fatty acid with high antibacterial activity against a range of food-borne pathogens and it can inhibit the growth of a

wide variety of microorganisms (Dayrit, 2015; Fischer, Drake, Dawson, Blanchette, Brogden, & Wertz, 2012; Kabara, Swieczkowski, Conley, & Truant, 1972). Therefore, lauric acid can be used as a substrate for synthesis of multi-functional substances with antibacterial activity.

In previous study, it was investigated that erythorbyl laurate, one of the lauric acid derivatives, has high antibacterial activity, however, most lauric acid derivatives such as sesamol laurate, methyl laurate, and isoamyl laurate lost their antibacterial activity after combining with non-fatty acid moiety.

In order to study the influence of properties of non-fatty acid moiety on antibacterial activity of the lauric acid derivatives, lauroyl peptide model was constructed. In the model, peptide was used as non-fatty acid moiety due to simplicity to change the physicochemical properties. The influence of non-fatty acid moiety on the antibacterial activity of lauric acid derivatives was investigated by the model. Moreover, in order to suggest the multi-functional lauric acid derivatives, antioxidative peptides were used as substrates for synthesis of lauroyl peptides.

In this study, various lauroyl peptides were prepared by chemo-enzymatic synthesis using antioxidative peptides and antibacterial lauric acid as substrates. The influence of non-fatty acid moiety on antibacterial activity of lauric acid derivatives was investigated by comparing the antibacterial activity of lauroyl peptides. Additionally, a novel multi-functional lauric acid

derivative, which simultaneously controls lipid oxidation and microbial contamination, was synthesized.

2. Materials and Methods

2.1. Materials

9-Fluorenylmethoxycarnobyl (Fmoc)-Lys-butyloxycarbonyl(Boc)-Wang resin, Fmoc-Ile-Wang resin, Fmoc-Arg-2,2,4,6,7-pentamethyldihydro-benzofurane-5-sulfonyl(Pbf)-OH, Fmoc-Asn-triphenylmethyl(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Glu-*tert*-butylester(OtBu)-OH, Fmoc-Ala-OH, and benzotriazol-1-yl-oxytripyrrolidino-phosphoniumhexa-fluorophosphate (PyBOP) (≥99.0%), were purchased for Fmoc solid phase peptide synthesis from Merck Millipore (Darmstadt, Germany). Immobilized lipase, from *Candida antarctica* (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435), was purchased from Novozymes (Bagsvaerd, Denmark) with a catalytic activity of 7,000 PLU/g (the activity of PLU refers to the millimoles of propyl laurate synthesized per min at 60℃). Lauric acid (≥99.0%), piperidine (≥99.0%), and dichloromethane (DCM) were purchased from Daejung Chemicals & Metals Co. (Siheung, Korea). *N*,*N*-Dimethylformaide (≥99.0%), *tert*-butyl methyl ether (≥99.0%), and *tert*-

butanol (≥99.0%) were purchased from Samchun Chemical (Pyeongtaek, Korea). *N*,*N*-Diisopropylethylamine (DIPEA) and triethylamine (≥99.0%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were of extra pure grade and were used without further purification.

2.2. Chemo-enzymatic synthesis of lauroyl peptides

2.2.1. Chemical synthesis of peptides

The solid phase peptide synthesis of peptides was accomplished using Fmoc-Lys(Boc)-Wang resin and the resin was covered with DCM for swelling. Fmoc was removed using a solution of 20% (v/v) piperidine in DMF for 10 min. Coupling of Fmoc protected amino acid units was accomplished by activation with PyBOP using DIPEA in DMF for 30 min. The Fmoc amino acids (5.0 equivalent), PyBOP (5.0 equivalent), and DIPEA (10.0 equivalent) were dissolved in DMF and subsequently mixed with the resin. After coupling the amino acid, deprotecting step was conducted and the resin was dried under vacuum. Steps of washing the resin were included between each step with DMF and DCM. Upon completion of synthesis, the peptide resin was subjected to a cleavage cocktail (trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water, 95:2.5:2.5, v/v/v) for 2 h. The resin was filtered and the combined filtrates were air-dried for 5 min. Peptides

were washed with 40 mL of *tert*-buthylmethylether for three times and the supernatants were discarded after centrifuging at 3,000xg for 10 min. Consequently, the peptides were dissolved with distilled water and lyophilized (Stawikowski & Fields, 2001).

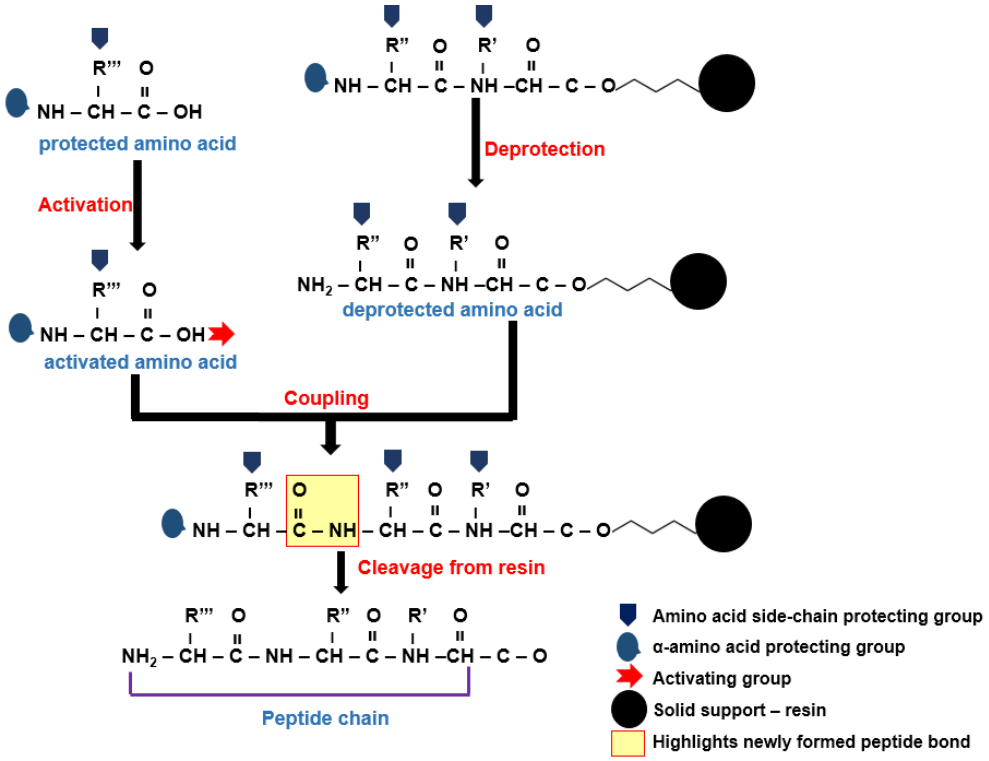


Fig. 1. Schematic procedure for the Fmoc solid phase peptide synthesis (SPSS).

2.2.2. Identification of peptide synthesis

Between deprotecting steps and coupling steps, it is needed to confirm whether the amino acid is synthesized or not. 2,4,6-trinitrobenzenesulfonic acid (TNBS) test was conducted for checking primary amines. 10 μL of 1% (w/v) TNBS in DMF and 10 μL of 10% (v/v) DIPEA solution in DMF were added to resin, and the color of beads was checked under the microscope after 10 min. If the beads are orange-red, there are free amino groups and after deprotecting step is corresponded to this case. If the beads are colorless, it means that the protecting group exists after coupling step is conducted.

2.2.3. Enzymatic synthesis of lauroyl peptides

Peptides (10 mmol) and lauric acid (40 mmol) were placed in a crimp top glass vial with 15 mL of *tert*-butanol. Triethylamine (200 mmol) was added to the reaction media to favor the neutral form of amino groups. Peptides exhibit low solubility in organic medium; consequently few hours are necessary to allow the formation of complex between the amino group of the lysine side chain of peptide and hydroxyl group of lauric acid. This complex favored the solubilization of the peptide (Husson, Humeau, Paris, Vanderesse, Framboisier, Marc, et al., 2009). After solubilization of the substrates for 12 h at 55℃, the reaction was initiated by adding 150 mg of the immobilized lipase (840 PLU/mL) into the mixture. Reaction media were

kept constant at 55±1℃ and stirred at 200 rpm. The immobilized lipase catalyzes amidation of the amino group of the lysine side chain with hydroxyl group of lauric acid according to the regio- and chemo-selectivity (Ferrari, Paris, Maigret, Bidouil, Delaunay, Humeau, et al., 2014; Husson, et al., 2009).

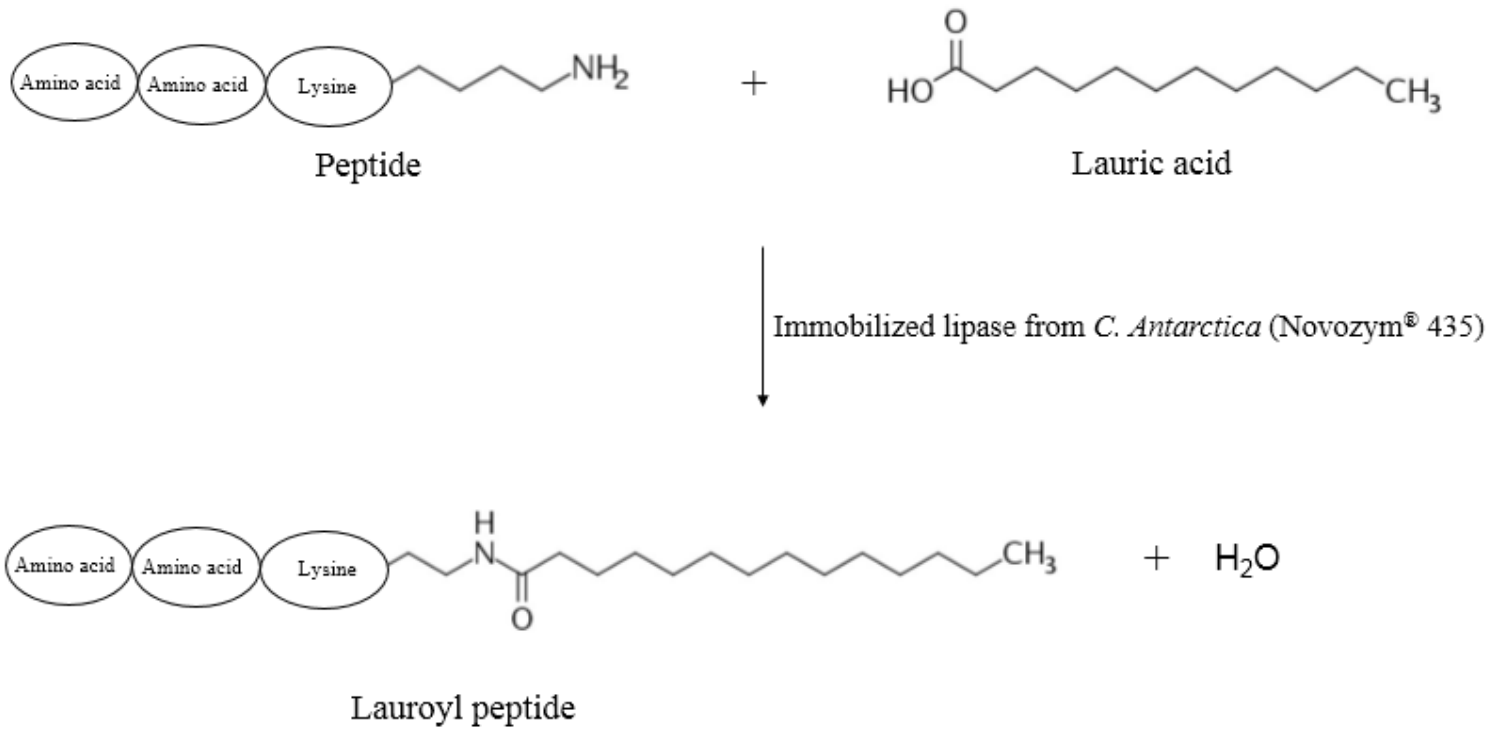


Fig. 2. Schematic representation of lipase-catalyzed synthesis for the preparation of lauroyl peptides.

2.3. HPLC analysis of lauroyl peptides

Analysis of synthesized lauroyl peptides in this study, was accomplished using an HPLC instrument (Waters 717, Waters 600, and Waters 486) equipped with a silica-based column (5 μm, I.D. 4.6 mm x 250 mm: Luna C_{18}-AR-Ⅱ, Cosmosil, Kyoto, Japan). The mobile phase was acetonitrile/water/TFA (90:10:0.1, v/v/v) at 0.5 mL/min flow rate for 15 min. The reaction mixture was sampled after reaction and filtered through a membrane filter (0.45 μm); then 15 μL of each aliquot was injected into the HPLC. Peptides with triethylamine and lauroyl peptides and lauric acid were detected, respectively, using a UV detector at 220 nm.

2.4. Purification and identification of lauroyl peptides

After enzymatic synthesis, lauroyl peptides were isolated from the reaction mixture by a slight modification of some methods (Park, Lee, Sung, Lee, & Chang, 2011; Yan, Bornscheuer, & Schmid, 1999). Briefly, the reaction mixture was filtered through a membrane (0.45 μm) to separate the immobilized lipase, and solvent was removed by a FD8512 freeze-dryer (Ilshin Lab Co., Ltd., Seoul, Korea) at -76℃. The concentrate was washed three times with distilled water (10 mL) and the supernatant was discarded to remove the residual peptide after centrifugation at 12,000xg, and remained solvent was removed. Then, *n*-hexane (10 mL) was added to the precipitate

and mixed vigorously for 30 s to make a suspension. Consequently, the lauroyl peptides were dissolved in acetonitrile/water/ethanol (1:2:1, v/v/v), and lyophilized. After freeze-drying, lauroyl peptides were stored at -20℃ until further analysis.

The obtained lauroyl peptides were identified by LC-ESI-MS (Thermo Fisher Scientific, Waltham, MA, USA) equipped with PDA-UV detector.

2.5. Evaluation of antibacterial activity of lauroyl peptides in O/W emulsion

2.5.1. Cell culture

The gram-positive strains were *Staphylococcus aureus* ATCC 49444 and *Listeria monocytogenes* ATCC 7644. Gram-negative strains were *Escherichia coli* ATCC 35150 and *Salmonella* Typhimurium ATCC 43971. Stock cultures were stored in tryptic soy broth (TSB) supplemented with 50% glycerol at -80℃. All microorganisms were cultured for 12-18 h at 37℃ in TSB.

2.5.2. Preparation of O/W emulsion

The emulsions were prepared to test the antibacterial activity of lauroyl peptides in oil in water emulsion (O/W emulsion). The O/W emulsion was composed of soybean oil (5%, w/w), polyoxyethylene sorbitan monolaurate

(Tween 20) (2%, w/w), various concentrations of lauroyl peptides, and sterilized water (93%, w/w). Concentrations of soybean oil (5%, w/w) and Tween 20 (2.0%, w/w) were fixed for all emulsion formulations. Emulsions were prepared by mixing the oil and Tween 20 with various concentrations of lauroyl peptides, followed by the addition of sterilized water. The emulsion were then sonicated with 20 KHz ultrasonicator (ULH-700S, Jeiotech, Korea) for 10 min at 210 W and each cycle consisted of 1 s pulse on and 4 s pulses off at 4℃ (Ghosh, Mukherjee, & Chandrasekaran, 2013; Moghimi, Aliahmadi, McClements, & Rafati, 2016).

2.5.3. Time-killing assay

Time-killing assay was accomplished to study the concentration and time-dependent killing effect (Zhao, Zhang, Hao, & Li, 2015). Overnight cultures were centrifuged for 10 min at 4,000xg at 4℃. The supernatant was discarded and the cells were washed twice with 0.1 M phosphate buffer (pH 7.4, PBS) and resuspended in the same buffer. The suspension was adjusted to achieve a bacterial concentration of $1.0x10^8$ colony-forming units (CFU)/mL based on 0.5 McFarland standard for bactericidal testing.

In case of O/W emulsion containing lauroyl peptides, 100 μL inoculum was added to 900 μL O/W emulsion containing lauroyl peptides. After inoculation, all the samples were incubated at 37℃ under shaking conditions

(220 rpm). After incubation for 0, 3, 6, 9, 12h, an aliquot (100 μL) was taken out and serially diluted. And the diluted samples were inoculated on tryptic soy agar (TSA) plate and incubated for 24 h at 37℃. The number of survivors (CFU/mL) was determined by counting the colonies and time-killing curves were constructed by plotting the log CFU/mL versus time. The limit of detection in the assay was 1.0 log CFU/mL. And the experiments were conducted in triplicate.

2.6. Fluorescence microscopy

The Live/Dead BacLight viability kit (Molecular Probes, Inc., Eugene, OR, USA) was used for assessment of cell rupture according to the manufacturer instructions. In this assay, the SYTO 9 and propidium iodide stains compete for binding to the bacterial nucleic acid. SYTO 9 labels cells with both damaged and intact membranes, whereas propidium iodide penetrates only cells with damaged membranes. A culture of *S. aureus* ATCC 49444 as a model organism was grown for 18-24 h in TSB. The bacterial culture was harvested by centrifugation at 10,000xg for 10 min at 4℃. The supernatant was removed and the pellet was washed once with PBS (pH 7.4) and resuspended in 0.85% NaCl solution. Bacterial suspension adjusted to $1.0x10^5$ CFU/mL were treated with RHK-L and then, all the suspensions were incubated at 37℃ for 30 min. At the end of the incubation period, the

dye mixture (3 μL) was added to bacterial suspensions (1.0 mL) including untreated bacterial suspensions. After another incubation in the dark at 25℃ for 15 min, an aliquot (5 μL) of the stained bacterial suspension was applied to a microscope slide with a coverslip, and examined using a DE/Axio imager A1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with fluorescence filters for SYTO 9 (filter set 38 HE, Carl Zeiss) and propidium iodide (filter set 43 HE, Carl Zeiss).

2.7. Evaluation of antioxidative activity

Linoleic acid was oxidized in a linoleic acid model system to measure the antioxidative activity of peptides or lauroyl peptides by a slight moidification of methods in the several studies (Rajapakse, Mendis, Byun, & Kim, 2005). Briefly, 50 mM sodium phosphate buffer (2.0 mL, pH 7.0), 83.5 mM linoleic acid in 99.5% ethanol (1.0 mL), 100 μM samples (peptides or lauroyl peptides) in 99.5% ethanol (1.0 mL), distilled water (1.0 mL) were mixed in a glass vial. Reactors were sealed tightly with silicon rubber caps and kept at 40℃ in the dark. Aliquots of the reaction mixtures were withdrawn at 24 h intervals for the measurement of antioxidative activity by the ferric thiocyanate method (Mendis, Rajapakse, & Kim, 2005; Rajapakse, Mendis, Byun, & Kim, 2005; Wang, Li, Chi, Ma, Luo, & Xu, 2013). The ferric thiocyanate analysis was performed by mixing the reaction mixture (50

μL) with 75% (v/v) ethanol (2.35 mL), 30% (w/v) ammonium thiocyanate (50 μL), and 0.02 M ferrous chloride in 3.5% hydrochloric acid (50 μL). After 3 min, the absorbance of the colored solution was measured at 500 nm every 24 h with UV/VIS spectrophotometer (Optizen POP BIO, Mecasys Co., Ltd, Korea).

3. Results and discussion

3.1. Chemo-enzymatic synthesis of lauroyl peptides

3.1.1. Chemical synthesis of peptides

Peptides can be synthesized stepwise from C to N terminus using N^{α}-protected amino acids by solid phase peptide synthesis. In order to synthesize peptide composed of specific amino acid sequences, 9-fluorenylmethoxycarbonyl (Fmoc) group was used for N^{α} protection. The deprotecting step to remove protecting group and the coupling step were proceeded to form a peptide bond between N^{α}-protected amino acid and C-terminus of amino acid corresponding to the next sequence. To monitor the synthesis of peptides between each step, TNBS test was performed under a microscope (Fig. 3). TNBS reagent serves as a color reagent by detecting the amine group. After the deprotecting step, the protecting group was removed and the beads turned orange-red since the amine group was detected. After the coupling step, the color of beads was colorless since the amine group was not exposed due to the protection by Fmoc group. TNBS test was conducted at each step of the synthesis, and it was confirmed that all of the peptides were synthesized with the correct sequence.

(a) (b)

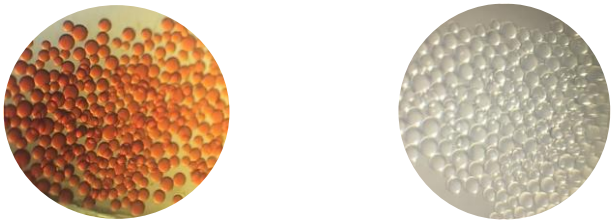


Fig. 3. Micrographs of beads by TNBS test; (a) the positive result representing first amine group is detected after the deprotecting step (orange-red) and (b) the negative result representing protecting group exists after the coupling step (colorless).

3.1.2. Antioxidative activity screening of peptides

Analysis of antioxidative activity of peptides were synthesized by SPSS, was performed (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005; Liu, Zheng, Li, Wang, Wu, Wang, et al., 2015; Saito, Jin, Ogawa, Muramoto, Hatakeyama, Yasuhara, et al., 2003; Suetsuna, 1999). As a result of analyzing the antioxidative activity of peptide against thermal oxidation, the lipid peroxide of the control group reached the maximum after incubation for 9 days and then it gradually decreased. This result could be explained by the production of secondary hydrogen peroxide which was not detected by FTC method in this study.

As a result, it was revealed that Lys-Val-Ile (KVI), Pro-Pro-Lys (PPK), and Glu-Ala-Lys (EAK) as well as lysine which was a non-fatty acid moiety of lauroyl lysine (LL) had no antioxidative activity. In contrast, Ala-His-Lys (AHK), Pro-His-Lys (PHK), Arg-His-Lys (RHK), Asn-His-Lys (NHK), Leu-Trp-Lys (LWK), and Leu-Lys (LK) were revealed to have antioxidative activity similar to butylated hydroxyanisole (BHA) and α-tocopherol used as positive controls. As a result, six antioxidative peptides (AHK, PHK, RHK, NHK, LWK, and LK), had relatively significant antioxidative activity, were selected as substrates for enzymatic synthesis of lauroyl peptides, and used for further study.

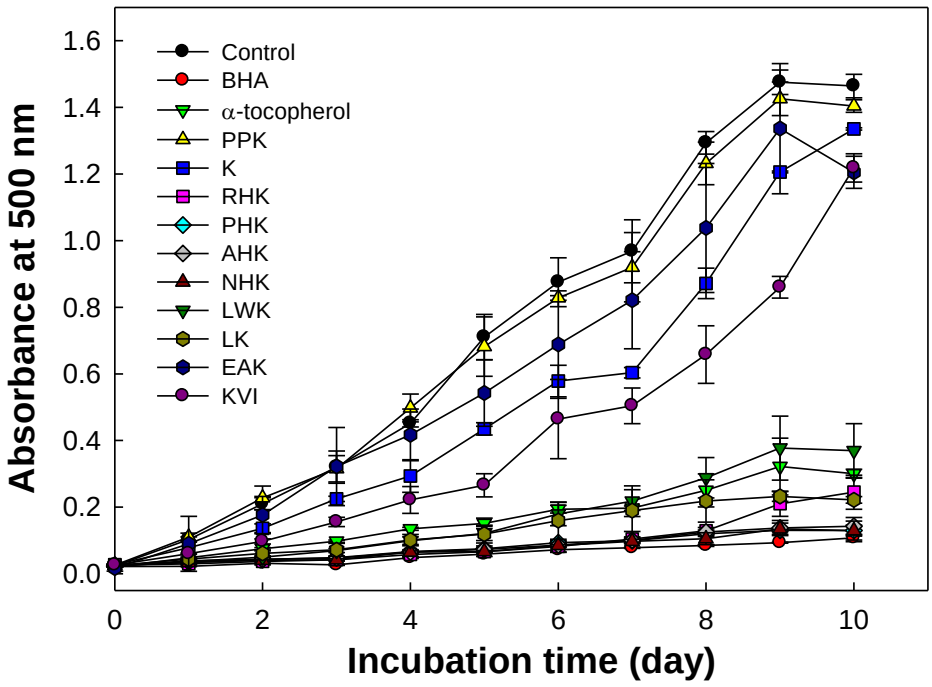


Fig. 4. Antioxidative activity screening of peptides by ferric thiocyanate method in linoleic acid peroxidation system. The degree of linoleic acid oxidation was assessed by measuring absorbance at 500 nm at every 24 h. Butylated hydroxyanisole (BHA) and α-tocopherol were used as positive controls.

3.1.3. Enzymatic synthesis of lauroyl peptides

To monitor the enzymatic synthesis between six peptides and lauric acid, products obtained from enzymatic synthesis after reaction for 72 h were analyzed by HPLC. HPLC chromatograms of each reactant obtained from enzymatic synthesis are presented in Fig. 5. The peaks of each reactant were identified by comparing retention time with peaks of purified peptides and lauric acid. Consequently, HPLC analysis showed that all six lauroyl peptides were synthesized.

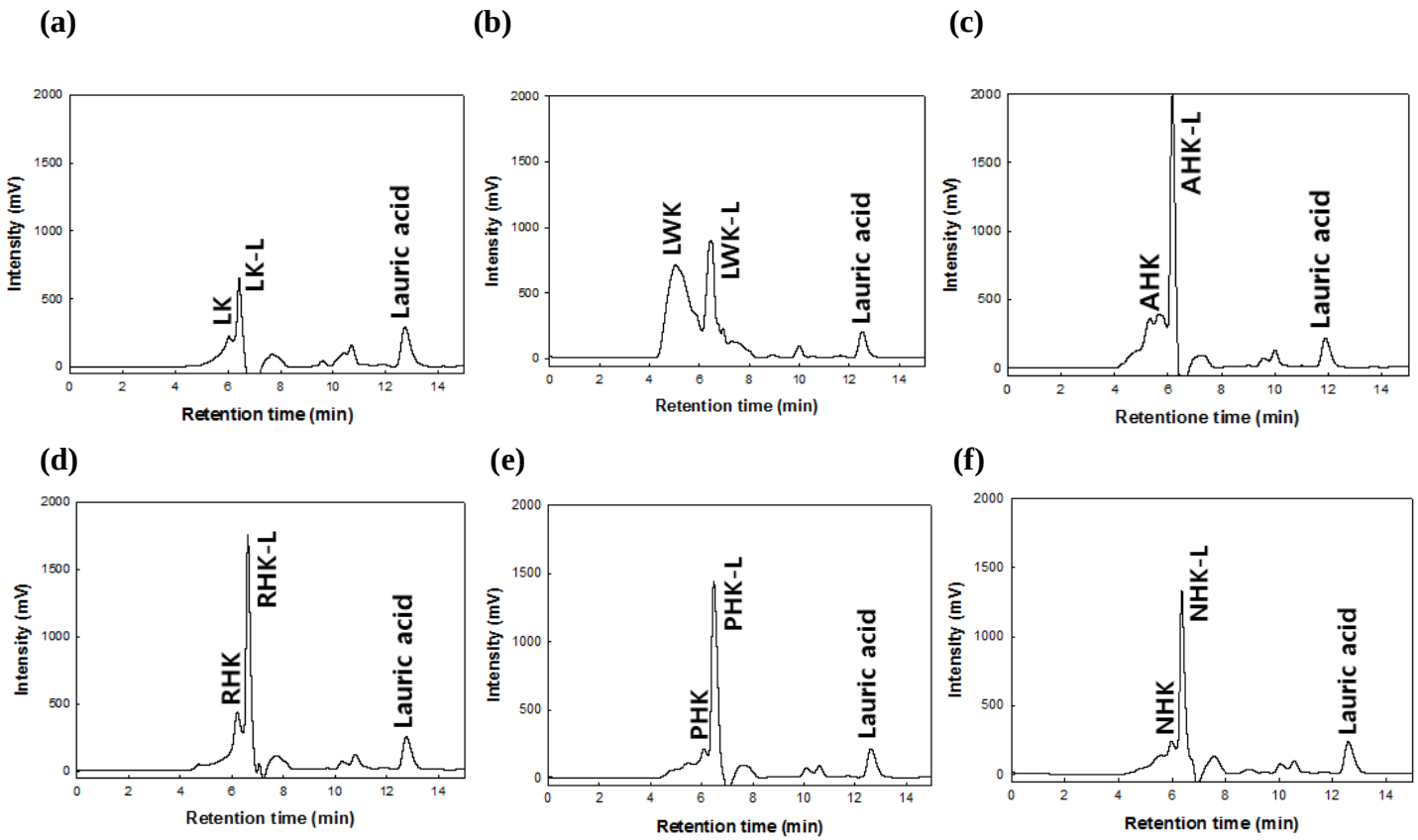
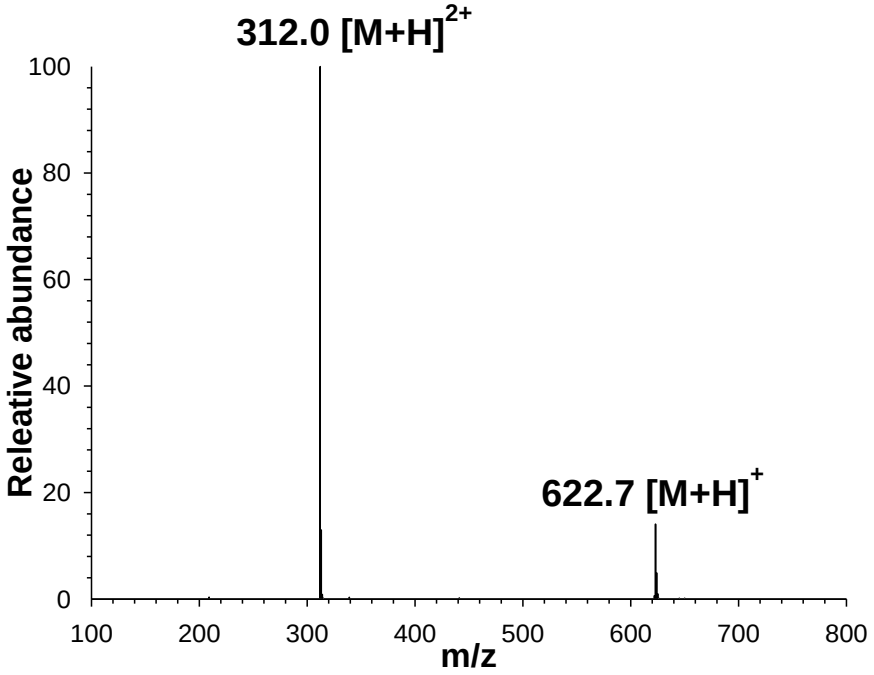


Fig. 5. HPLC chromatograms of lauroyl peptides (a) RHK-L, (b) PHK-L, (c) NHK-L, (d) LK-L, (d) LWK-L, and (f) AHK-L obtained from enzymatic synthesis between peptides and lauric acid in *tert*-butanol.

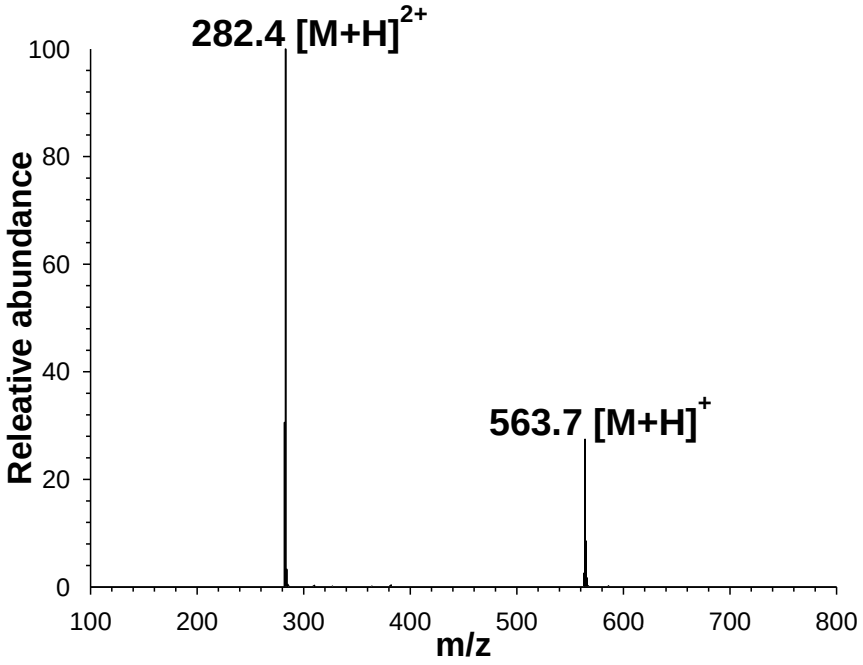
3.1.4. Identification of lauroyl peptides

After the purification procedure, lauroyl peptides were identified by LC-ESI-MS. Mass spectrometry in full mode revealed the presence of lauroyl peptides. The spectra gave a molecular ion at m/z = 442.5 $[M+H]^+$ of lauroyl peptide Leu-Lys (LK-L), 628.7 $[M+H]^+$ of lauroyl peptide Leu-Trp-Lys (LWK-L), 537.6 $[M+H]^+$ of lauroyl peptide Ala-His-Lys (AHK-L), 580.7 $[M+H]^+$ of lauroyl peptide Asn-His-Lys (NHK-L), 563.7 $[M+H]^+$ of lauroyl peptide Pro-His-Lys (PHK-L), 622.7 $[M+H]^+$ of lauroyl peptide Arg-His-Lys (RHK-L), corresponding exactly to the estimated molecular mass of each lauroyl peptides (Fig. 6). For AHK-L, NHK-L, PHK-L, and RHK-L, the relative abundance of multiple charges caused by multiple isotope was higher than single charge. Consequently, the lauroyl peptides were identified as expected products.

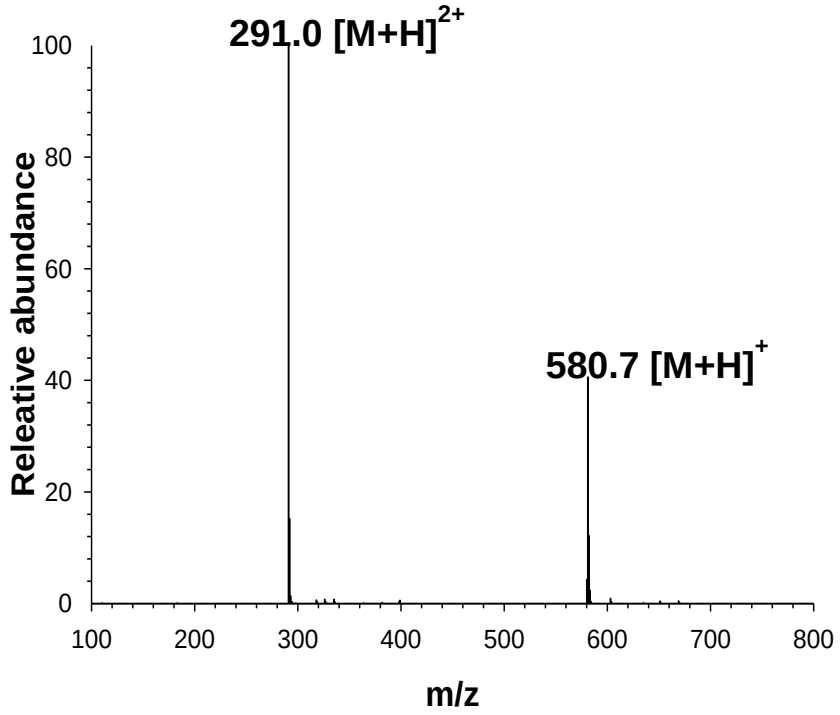
(a)


312.0 [M+H]2+
622.7 [M+H]+
Releative abundance
m/z
0
20
40
60
80
100
100
200
300
400
500
600
700
800

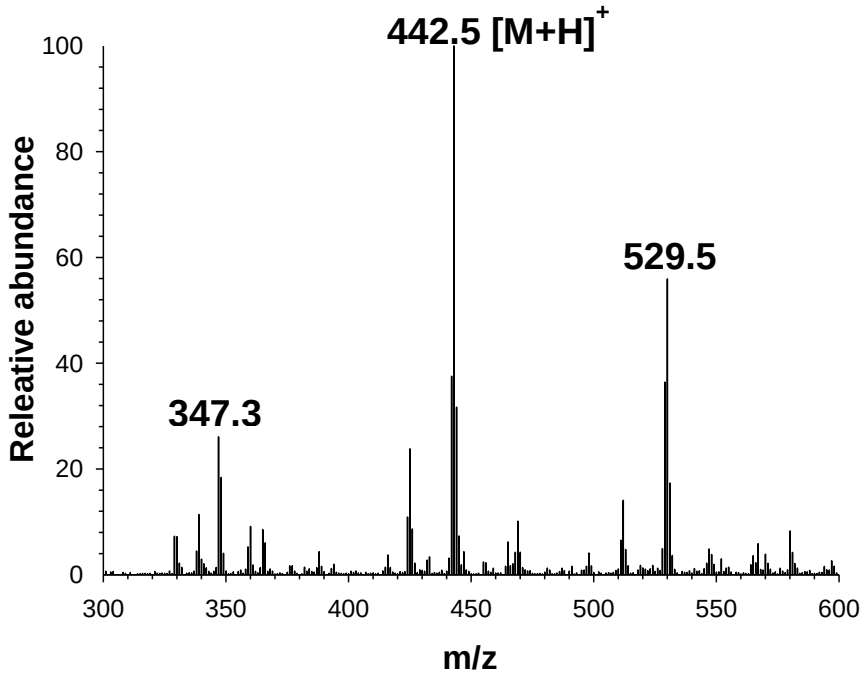
(b)


282.4 [M+H]2+
563.7 [M+H]+
Releative abundance
m/z
0
20
40
60
80
100
100
200
300
400
500
600
700
800

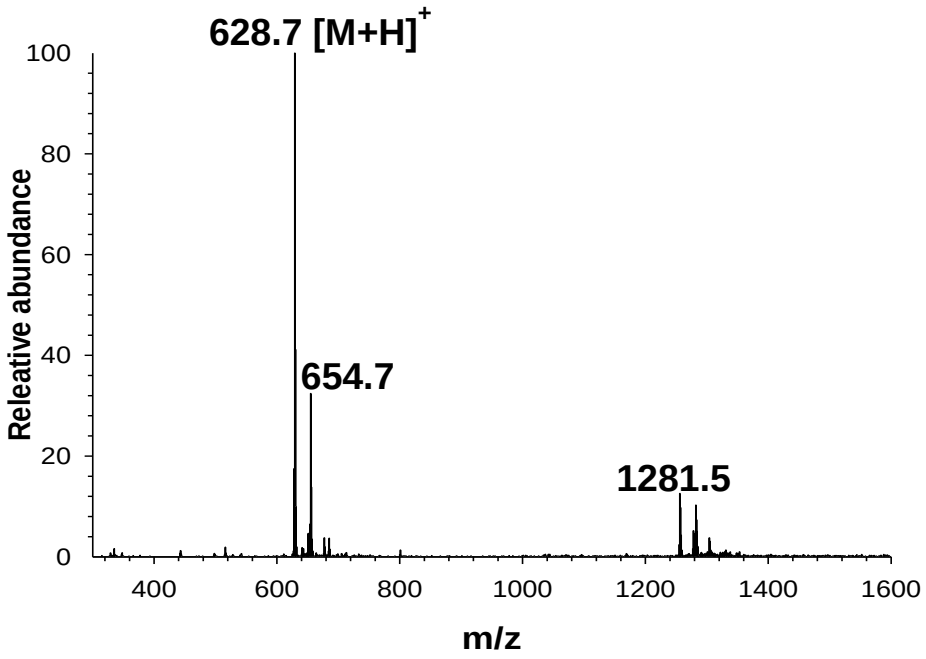
(c)


291.0 [M+H]2+
580.7 [M+H]+
Releative abundance
m/z
0
20
40
60
80
100
100
200
300
400
500
600
700
800

(d)


442.5 [M+H]+
529.5
347.3
Releative abundance
m/z
0
20
40
60
80
100
300
350
400
450
500
550
600

(e)



(f)

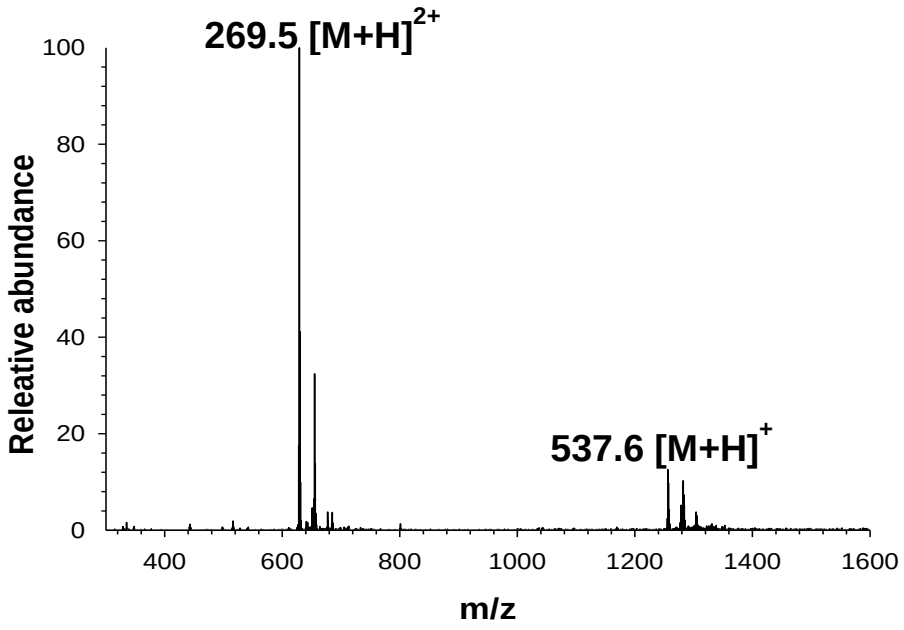


Fig. 6. LC-ESI-MS spectra of lauroyl peptides (a) RHK-L, (b) PHK-L, (c) NHK-L, (d) LK-L, (e) LWK-L, and (f) AHK-L obtained from enzymatic synthesis between peptide and lauric acid in this study (full scan mode).

3.2. Comparison of antibacterial activity of lauroyl peptides in O/W emulsion

Antibacterial activity of 5.0 mM lauroyl peptides were evaluated by time-killing assay in the O/W emulsion against gram-positive bacteria *S. aureus* ATCC 49444 (Fig. 7). After treatment with lauroyl lysine (LL) and LK-L for 12 h, there was no significant difference from control. In addition, LWK-L, AHK-L, and LK-L showed no bactericidal effect below 3.0 log reduction. NHK-L caused 3.5 log reduction after treatment for 12 h. Also, RHK-L caused 5.6 log reduction after treatment for 3 h, indicating it has the highest antibacterial activity.

From these results, it could be confirmed that antibacterial activity of lauric acid derivatives were displayed differently according to non-fatty acid moiety.

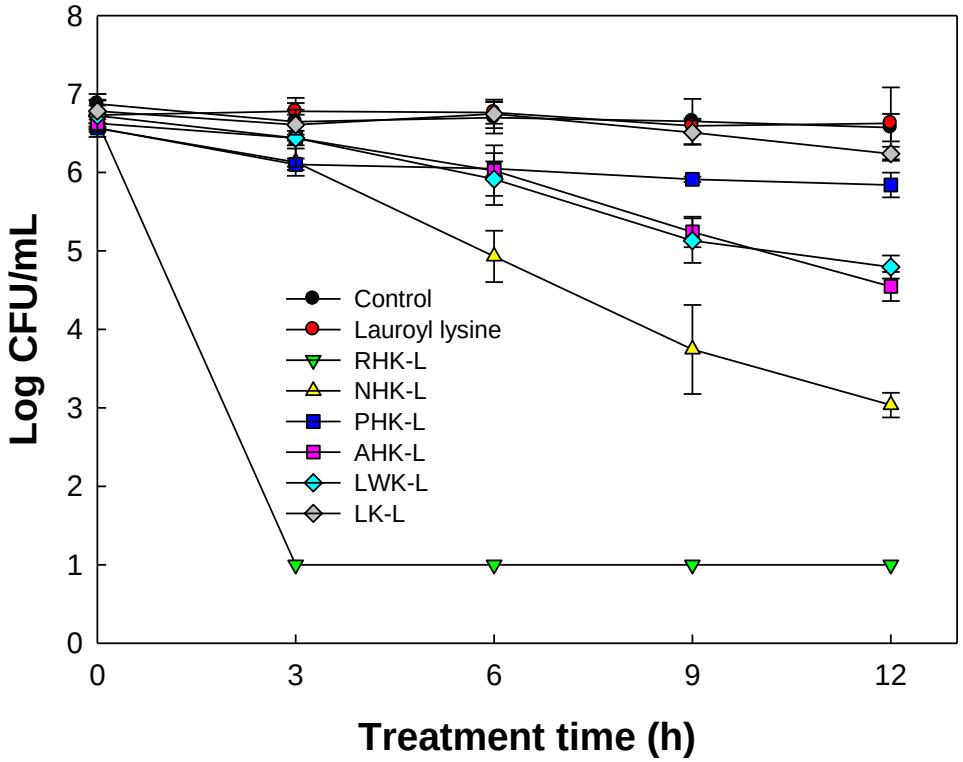


Fig. 7. Time-killing curves of lauroyl peptides of 5.0 mM in O/W emulsion against *Staphylococcus aureus* ATCC 49444.

3.3. Influence of hydrophilicity or charge of non-fatty acid moiety on antibacterial activity of lauroyl peptides

As shown in Table 1, the hydrophilicity and charge of non-fatty acid moiety of RHK-L and NHK-L which showed antibacterial activity were compared with the non-fatty acid moiety of LL, LK-L, PHK-L, AHK-L, and LWK-L having no antibacterial activity. Hydrophilicity of the non-fatty acid moiety of RHK-L and NHK-L were higher than those of the other lauroyl peptides. Comparison of charge of non-fatty acid moiety of RHK-L with those of NHK-L indicated that positive charge of non-fatty acid moiety of RHK-L was higher than that of NHK-L.

Therefore, it was inferred that the most significant property influencing the antibacterial activity of lauric acid derivatives was the hydrophilicity. Additionally, charge could affect the antibacterial activity of the derivatives. The hydrophilicity, which has a major influence on antibacterial activity of lauric acid derivatives, might be related to amphiphilicity of the derivatives. The bacterial membrane is composed of amphiphilic phospholipids. The amphiphilic lauric acid derivatives could easily interact with membrane phospholipids and exhibit an antibacterial effect. It is inferred that the antibacterial effect is exhibited by the interaction between lauric acid derivatives and the hydrophilic head of the phospholipid as the hydrophilicity of the non-fatty acid moiety increases.

Accordingly, lauric acid derivatives composed of amphiphilic structure, could exhibit antibacterial effects due to strong interaction with phospholipid of the cell membrane. Therefore, the antibacterial activity of lauric acid derivatives could be improved when hydrophilicity of non-fatty acid moiety increases (Fillion, Valois-Paillard, Lorin, Noël, Voyer, & Auger, 2015; Yeaman & Yount, 2003).

Furthermore, charge may affect the antibacterial activity of lauric acid derivatives. Gram-positive bacteria are surrounded by layers of peptidoglycan. And, teichoic acids extend from the cell surface into the peptidoglycan layer. Also, teichoic acids, which are the membrane of gram-positive bacteria are composed of negatively charged phospholipids. Substances with positive charge can affect destruction of the membrane of gram-positive bacteria due to a strong interaction with anionic lipids on the bacterial membrane. Therefore, as the positive charge of non-fatty acid moiety increases, antibacterial activity of lauric acid derivatives could be higher due to increase of the ionic bond with the membrane of gram-positive bacteria (Silhavy, Kahne, & Walker, 2010; Friedrich, Moyles, Beveridge, & Hancock, 2000; Greber, 2017; Kim & Cha, 2010; Yeaman & Yount, 2003; Zhou, Wang, Chen, Li, Qiao, Liu, et al., 2016).

Table 1. Properties (amino acid sequence, hydrophilicity, net charge) of non-fatty acid moiety and antibacterial activity of lauroyl peptides

Amino acid sequence	Hydrophilicity[a]	Charge[b]	Antibacterial activity of lauroyl peptides[c]
Arg-His-Lys (RHK)	24.1	+3	++
Asn-His-Lys (NHK)	16.6	+2	+
Pro-His-Lys (PHK)	12.0	+2	-
Ala-His-Lys (AHK)	10.2	+2	-
Lys (K)	8.8	+1	-
Leu-Lys (LK)	6.0	+1	-
Leu-Trp-Lys (LWK)	4.1	+1	-

[a] Hydrophilicity was calculated by averaging the hydrophilicity of each amino acid obtained from Engelman et al. (1986).

[b] The sum of the charge of each amino acid

[c] Antibacterial activity of lauroyl peptides were evaluated by measuring log reduction value of bacteria counts after treatment for 12 h; (++): more than 3.0 log reduction, (+): 3.0 log reduction, (-): less than 3.0 log reduction

3.4. Antibacterial activity of RHK-L in O/W emulsion

The antibacterial activity of RHK-L was analyzed against two gram-positive bacteria *S. aureus* ATCC 49444 and *L. monocytogenes* ATCC 7644, and two gram-negative bacteria *E. coli* ATCC 35150 and *S.* Typhimurium ATCC 43971. The antibacterial activity of RHK-L against *S. aureus* was proportional to the concentration when 1.0, 3.0, and 5.0 mM of RHK-L was treated in the O/W emulsion (Fig. 8a). After treatment with 5.0 mM for 3 h, no viable cells were observed below the detection limit. At a concentration of 3.0 mM, incubation for 12 h achieved 5.2 log reduction. The antibacterial activity against *L. monocytogenes* was also proportional to the concentration of RHK-L. No antibacterial activity was observed at 0.1 mM, but, 6 log reduction was observed after treatment with 0.5 and 1.0 mM for 3 h (Fig. 8b).

In contrast to gram-positive bacteria, no antibacterial activity was shown by treatment with 5.0 mM of RHK-L against two gram-negative bacteria *E. coli* and *S.* Typhimurium, respectively (Fig. 9).

In conclusion, RHK-L had antibacterial activity against gram-positive bacteria, but did not show the antibacterial effect against gram-negative bacteria. The selective antibacterial activity of RHK-L could be attributed to the antibacterial properties of lauric acid. It was reported that lauric acid had no antibacterial activity against gram-negative bacteria due to existence of lipopolysaccharide (LPS) layer in outer membrane of gram-negative bacteria

unlike gram-positive bacteria. Therefore, the results suggested that gram-negative bacteria were more resistant to RHK-L than gram-positive bacteria due to their outer membrane. In addition, the antibacterial activity of RHK-L might be improved by determining a proper emulsion system or by increasing the concentration (Malanovic & Lohner, 2016; Silhavy, Kahne, & Walker, 2010; Torcato, Huang, Franquelim, Gaspar, Craik, Castanho, et al., 2013; Zhao, Zhang, Hao, & Li, 2015).

(a) (b)

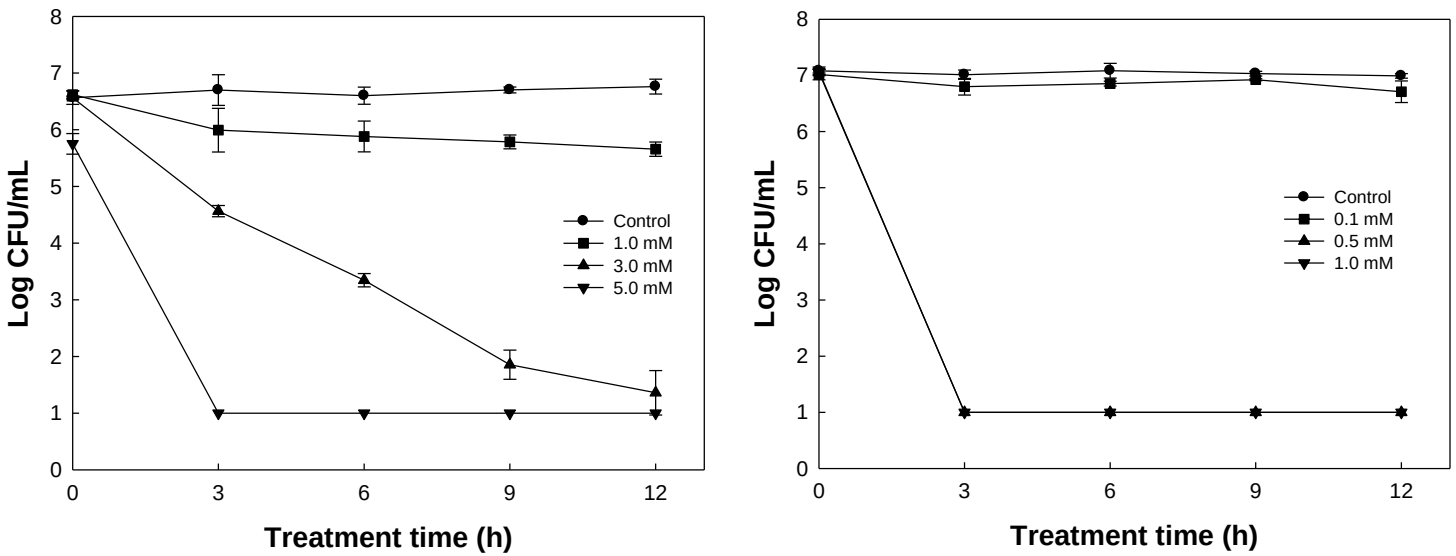


Fig. 8. Time-killing curves of RHK-L with various concentrations in O/W emulsion against (a) *Staphylococcus aureus* ATCC 49444, (b) *Listeria monocytogenes* ATCC 7644.

(a) (b)

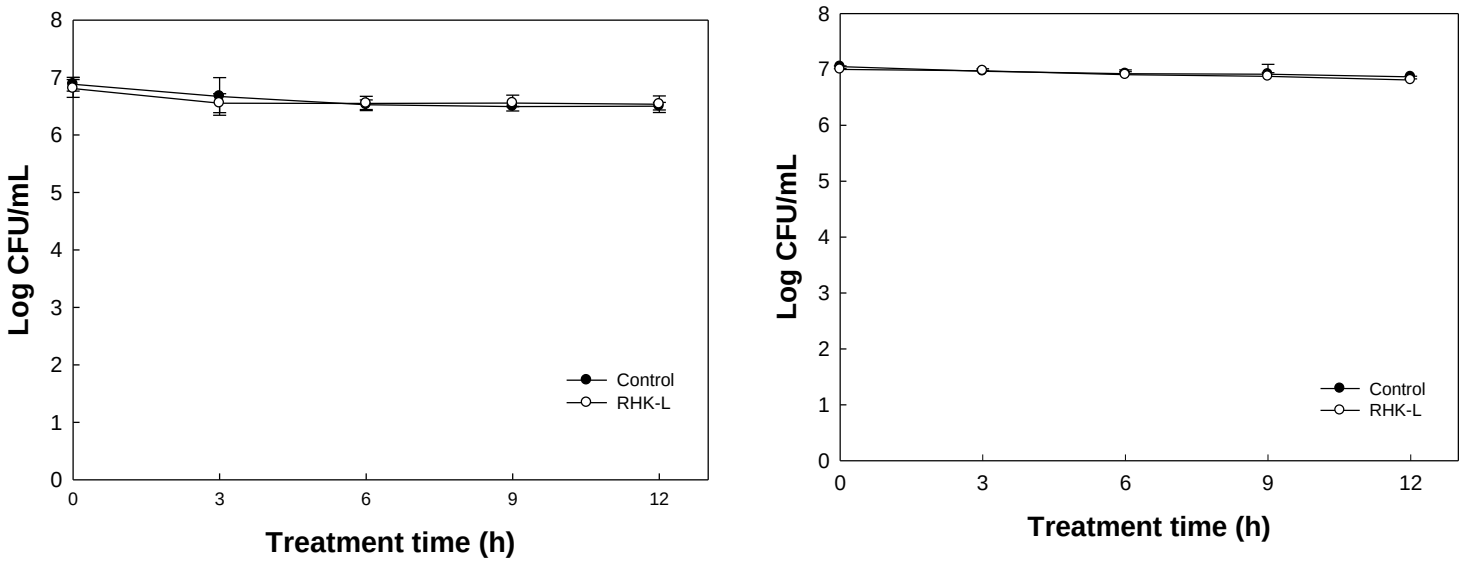


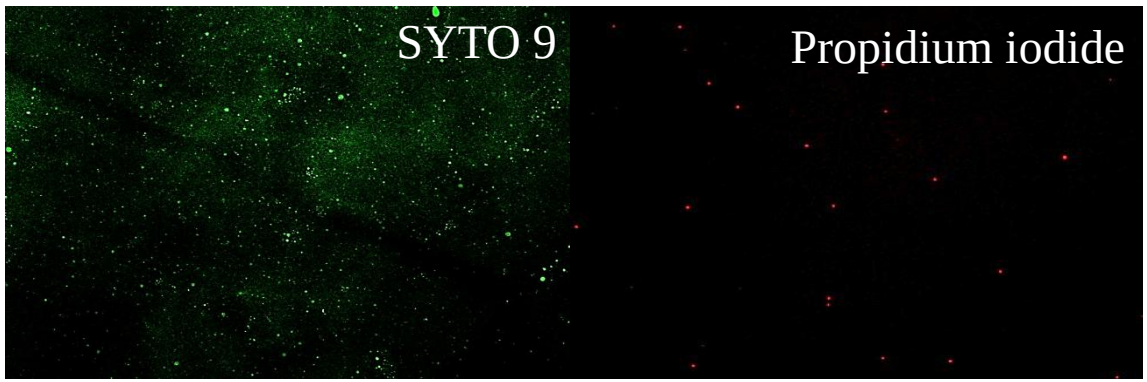
Fig. 9. Time-killing curves of 5.0 mM RHK-L in O/W emulsion against (a) *Escherichia coli* ATCC 35150, (b) *Salmonella* Typhimurium ATCC 43971.

3.5. Visualized images of cell rupture evidence by fluorescence microscopy

To monitor changes in cell membrane permeability, fluorescence microscopy was performed with Live/Dead viability kit against *S. aureus* ATCC 49444. The main target of lauric acid is cell membrane, and its influence on cell membrane interferes with the electron transport chain and oxidative phosphorylation of the microorganism, leading to bacterial growth inhibition or death (Desbois & Smith, 2010).

In fluorescence micrographs of stained bacteria suspension without RHK-L treatment, most cells showed only green light stained with SYTO 9. However, the bacteria treated with 5.0 mM RHK-L fluoresced both green and red. RHK-L induced cell membrane rupture was confirmed by staining of propidium iodide, which detects only dead cells with cell membrane rupture. As a result, it shows the number of red cells more than the untreated cells (Fig. 10). Therefore, it was confirmed that RHK-L affected the cell membrane disintegration.

(a)



(b)

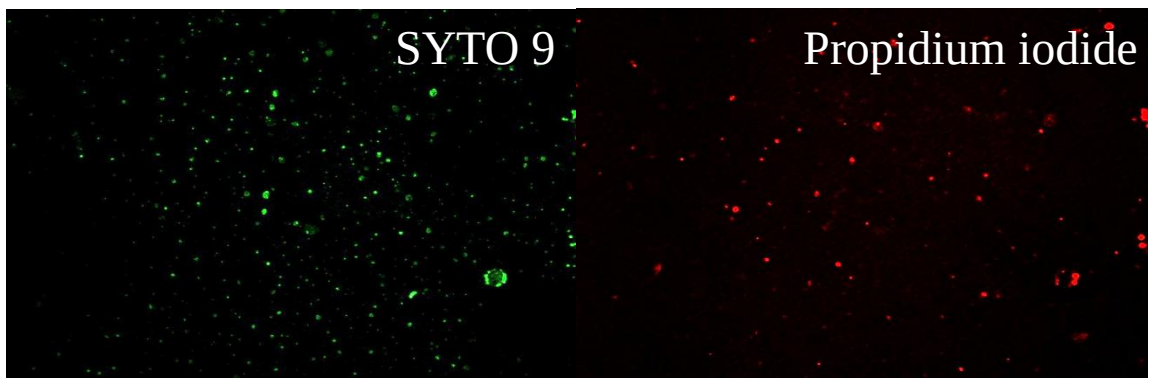


Fig. 10. SYTO 9 and propidium iodide fluorescence images of *S. aureus* ATCC 49444 (a) without treatment of RHK-L and (b) with treatment of 5.0 mM RHK-L. SYTO 9 stained both intact and damaged cells (green), and propidium iodide stained only cells with compromised membranes (red).

3.6. Verification of antioxidative activity of RHK-L

Antioxidative activity of RHK-L was evaluated against thermal oxidation at 40℃. Antioxidative activity is relatively compared to absorbance at 500 nm. After incubation for 6 days, the lipid peroxide of control was 1.3, but that of RHK-L and lauroyl lysine was 0.25 and 0.37, respectively. A significant difference of antioxidative activity between RHK-L and lauroyl lysine derived from lysine as non-fatty acid moiety was not observed ($p<0.05$).

As a result, antioxidative activity of RHK-L was identified after enzymatic synthesis.

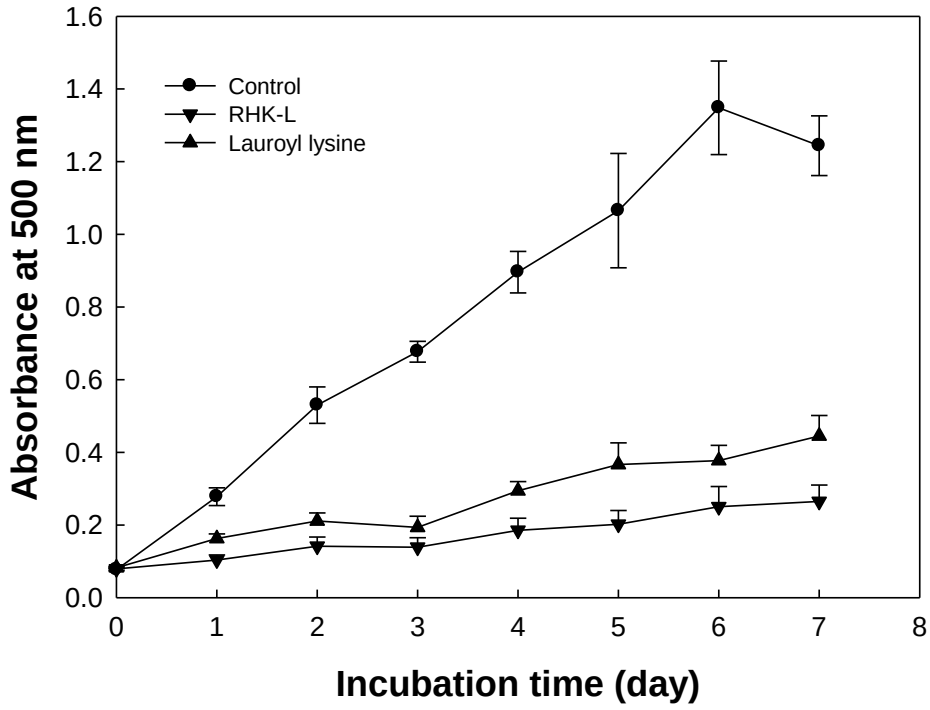


Fig. 11. Antioxidative activity of RHK-L by ferric thiocyanate in linoleic acid peroxidation system. The degree of linoleic acid oxidation was assessed by measuring absorbance at 500 nm at every 24 h. Lauroyl lysine was used as a positive control. The concentration of samples were 100 μM.

4. Conclusions

In this study, lauroyl peptide model was constructed to study the influence of properties of non-fatty acid moiety on antibacterial activity of lauric acid derivatives. As a result of comparing antibacterial activity of lauroyl peptides in O/W emulsion, it was confirmed that lauric acid derivatives had different antibacterial activity according to non-fatty acid moiety. As a result, hydrophilicity of non-fatty acid moiety was considered as the most significant property influencing the antibacterial activity of lauric acid derivatives. Furthermore, charge of non-fatty acid moiety might affect the antibacterial activity of lauric acid derivatives.

Furthermore, it was demonstrated that RHK-L had the highest antibacterial activity among lauroyl peptides. Cell rupture treated with RHK-L was observed by fluorescence microscopy, indicating the antibacterial activity of RHK-L was derived from lauric acid. In addition, the antioxidative activity of RHK-L was confirmed. Therefore, it was revealed that RHK-L had antibacterial activity and antioxidative activity, indicating the possibility of a novel multi-functional lauric acid derivative.

In this study, the lauroyl peptide model can be used as a research model to understand significant factors influencing the antibacterial activity of lauric acid derivatives. Also, it can be used as a foundation for development

of multi-functional fatty acid derivatives.

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국문초록

식품 산업에서, 지질 산화와 미생물 오염에 의한 식품 품질 저하는 매우 중요한 문제이다. 선행연구에서 지질 산화와 미생물 오염을 동시제어하기 위해 다기능성의 lauric acid 유도체를 효소적으로 합성하고자 하였다. 하지만, 효소적 합성 이후, erythorbyl laurate와 같이 항균력을 유지하는 물질이 있는 반면, sesamol laurate, methyl laurate, isoamyl laurate 등 대부분의 물질은 항균력이 소실되었다. 따라서 비지방산 잔기의 특성이 lauric acid 유도체의 항균력에 미치는 영향을 분석하기 위하여 이화학적 특성 제어가 용이한 peptide를 비지방산 잔기로 구성하여 lauroyl peptide 모델을 설계하였다. 또한 항산화력을 가지는 peptide를 활용하여 항산화력과 항균력을 동시에 갖는 다기능성 lauric acid 유도체를 lipase를 촉매로 하여 효소적으로 합성하였다.

6종의 lauroyl peptide (RHK-L, AHK-L, NHK-L, PHK-L, LK-L, LWK-L)를 생산하기 위해, lipase 촉매에 의한 효소적 합성을 수행하였다. Lauroyl peptide간의 항균력을 비교했을 때,

12시간 처리 시, NHK-L은 3.5 log reduction의 균 저감화를 보였고, RHK-L은 3시간 처리 시, 5.6 log reduction의 균 저감화를 보여 RHK-L이 lauroyl peptide 중 가장 강한 항균력을 갖는 것을 알 수 있었다. LP 간 항균력 차이의 원인을 분석하기 위하여 LP의 비지방산 잔기인 peptide의 특성을 비교해본 결과, 항균효과를 나타낸 RHK-L, NHK-L의 비지방산 잔기인 RHK, NHK는 다른 LP의 비지방산 잔기보다 더 높은 친수성을 나타냈다. 추가적으로, 가장 강한 항균효과를 나타낸 RHK-L의 비지방산 잔기는 NHK-L의 비지방산 잔기보다 더 강한 양전하를 가지고 있음을 확인하였다. 따라서 비지방산 잔기의 친수성이 lauric acid 유도체의 항균력에 주된 영향을 주며, 전하 또한 영향을 주는 것으로 추론된다.

가장 항균력이 강한 RHK-L을 대상으로 그람 양성균 2종, 그람 음성균 2종에 대한 항균력을 수중유적형 유화액상에서 평가한 결과, RHK-L은 그람 양성균에 대해 항균력을 가지고 있었다. 또한 형광 현미경을 이용한 세포막의 파괴 관찰을 통해, RHK-L의 항균효과는 lauric acid에서 기인하였음을 확인했다. RHK-L의 항산화력 분석을 통해 RHK-L의 항산화력은 효소적으로

합성된 이후에도 유지됨을 확인하였다.

결론적으로, 본 연구에서는 lauroyl peptide 모델을 통해 lauric acid 유도체의 항균력이 비지방산 잔기의 친수성에 주된 영향을 받으며, 전하에 의해서도 영향을 받을 수 있음을 확인하였다. 뿐만 아니라, 항균력과 항산화력을 동시에 보유하는 다기능성 lauric acid 유도체로서 RHK-L을 제시하였다.

주요어: 라우로일 펩타이드, 다기능성, 항균성, 비지방산 잔기, 친수성, 전하

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