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# Lipase-catalyzed synthesis of lauroyl tripeptide-KHA with multi-functionalities: Its surface-active, antibacterial, and antioxidant properties

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ABSTRACT

Lipase-catalyzed acylation of a hydrophilic tripeptide-KHA (TP-KHA; amino acid sequence Lys-His-Ala) with a lipophilic lauric acid was performed to produce a multi-functional compound, lauroyl tripeptide-KHA (TPL-KHA), with surface, antibacterial, and antioxidant activities. The significant acylation reaction parameters were optimized as follows: organic solvent of 2-methyl-2-butanol, reaction temperature at 55 °C, substrate molar ratio (lauric acid:TP-KHA) of 4.0, and reaction time for 72 h. Structural analyses by LC-ESI-MS and $^{1}$H NMR identified that $N^{\varepsilon}$-lauroyl tripeptide-KHA was chemo-selectively synthesized by the acylation reaction under the optimum conditions. TPL-KHA showed the surface activity at the air–water interface with critical micelle concentration (CMC) of 2.71 mM and $\gamma_{CMC}$ of 30.44 mN/m. TPL-KHA exhibited bacteriostatic and bactericidal effects on Gram-positive and Gram-negative foodborne pathogens (minimum inhibitory concentrations: 2.83–4.00 mM, minimum bactericidal concentrations: 3.17–5.83 mM). Moreover, it was demonstrated that TPL-KHA had the ability to scavenge $ABTS^{+}$ radicals and inhibit the lipid oxidation.

## 1. Introduction

In emulsion-based foods, lipid oxidation and contamination by foodborne pathogens diminish the quality and the safety from manufacture to consumption. Under the strategy to control the risks with a single food additive, erythorbyl laurate (6-*O*-lauroyl-erythorbic acid) which has surface, antioxidant, and antibacterial activities was suggested as a promising multi-functional food emulsifier (Park, Lee, Sung, Lee, & Chang, 2011). Erythorbyl laurate, which is amphiphilic, is produced by lipase-catalyzed esterification between hydrophilic erythorbic acid and lipophilic lauric acid, which show antioxidant and antibacterial activity, respectively (Yu, Lee, Shin, Park, & Chang, 2019). In an oil-in-water emulsion system, erythorbyl laurate exhibits antioxidant activity against thermal and photo-oxidation of lipids, which is higher than that of erythorbic acid because the antioxidant moieties are located at the surface of lipid droplets (Park et al., 2017). In addition, erythorbyl laurate has bacteriostatic and bactericidal effects on Gram-positive bacteria by disrupting the cell membrane (Park et al., 2018).

Antioxidant peptides, typically containing 2–20 amino acids, have been identified from natural sources including milk, egg, and soy which have no adverse or safety concerns (Sarmadi & Ismail, 2010). Some antioxidant peptides inhibit lipid oxidation in emulsion model systems and food matrices such as fish and meat products (Cheng, Xiong, & Chen, 2010; Sakanaka & Tachibana, 2006). Therefore, the natural antioxidant peptides can be used in lipid-based foods as alternatives to synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), use of which is restricted due to potential health risks.

Lipopeptides, composed of a peptide with a covalently attached fatty acid moiety, have been attracted as a potent antimicrobial agent due to broad antimicrobial spectrum (Vila, Badosa, Montesinos, Feliu, & Planas, 2013). The cationic and amphiphilic nature of lipopeptides disrupts the bacterial cell membrane by mediating electrostatic and hydrophobic interactions (Pinazo et al., 2016). Their antibacterial activity can be enhanced by replacing hydrophobic amino acids with fatty acids or by modifying the amino acid sequence to promote hydrophobic interactions with the cell membrane (Schmidtchen, Pasupuleti, & Malmsten, 2014). Short lipopeptides and lipoamino acids, produced by acylation of cationic peptides or basic amino acids (*e.g.*, lysine, arginine, and histidine), have been suggested as novel antibacterial agents due to their simple chemical structures and biocompatibility (Makovitzki, Avrahami, & Shai, 2006). Recently, ethyl lauroyl arginate,

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prepared by acylation of arginine with lauric acid, was approved as a food preservative to control microbial contamination and a generally recognized as safe (GRAS) substance for food applications (Nerin, Becerril, Manso, & Silva, 2016).

Acylation of amino acids or peptides with fatty acids has been used to produce biocompatible surfactants. The acylation reaction is generally performed under highly alkaline conditions with chemical catalysts, resulting in the generation of many byproducts because the reaction is not selective. By contrast, enzymatic synthesis of acylated amino acids or peptides has selectivity and requires mild conditions, in accordance with the green chemistry principle (Dettori, Jelsch et al., 2018, Dettori, Vibert et al., 2018). Enzymatic methodologies for the acylation reactions using immobilized lipase with high stability and broad substrate specificity have been proposed (Dettori, Jelsch et al., 2018, Dettori, Vibert et al., 2018; Husson et al., 2011).

The overall aim of the present study was to prepare a novel multifunctional compound with surface, antibacterial, and antioxidant activities via lipase-catalyzed acylation of a peptide with a fatty acid. Lauric acid showing antibacterial activity against a variety of foodborne pathogens was employed as a lipophilic substrate (Lieberman, Enig, & Preuss, 2006). Tripeptide-KHA (TP-KHA; amino acid sequence Lys-His-Ala) was selected as a hydrophilic substrate among antioxidant peptides (Saito et al., 2003; Samaranayaka & Li-Chan, 2011) by considering hydrophilicity and simplicity of chemical structure. In this study, the enzymatic reaction conditions were optimized and the structure of lauroyl tripeptide-KHA (TPL-KHA), a product from the reaction, was identified. The multi-functionalities of TPL-KHA including surface active, antibacterial, and antioxidant properties were evaluated.

## 2. Materials and methods

### 2.1. Materials

Amino acid derivatives (9-fluorenylmethyoxycarbonyl [Fmoc]-His-triphenylmethyl [Trt]-OH, Fmoc-Lys-butyloxycarbonyl [BOC]-OH), resin (Fmoc-Ala-Wang resin), and a coupling reagent (benzotriazol-1-yl-oxytripyrrolidino-phosphoniumhexafluorophopsphate [PyBOP]) for synthesis of the peptides were purchased from Merck Millipore (Darmstadt, Germany). Lauric acid, triethylamine, *N*,*N*-diisopropylethylamine (DIPEA), piperidine, and triisopropylsilane (TIS) with at least 98.0% purity and molecular sieves 4 Å (8–12 mesh) were purchased from Sigma-Aldrich (St. Louis, MO). The organic solvents *N*,*N*-dimethylformamide (DMF), dichloromethane, methanol, *tert*-butylmethylether (MTBE), acetonitrile, 2-methyl 2-butanol, *tert*-butanol, and *n*-hexane (≥99.0% purity) were purchased from Daejung Chemicals (Siheung, Republic of Korea). High-performance liquid chromatography (HPLC)-grade acetonitrile, water, and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Phillipsburg, NJ). Immobilized lipase from *Candida antarctica* (Novozym 435) with a catalytic activity of 7,000 PLU/g (PLU refers to millimoles of propyl laurate synthesized per min at 60 °C) was provided by Novozymes (Bagsvaerd, Denmark). All other reagents were of analytical grade and were used without further purification.

### 2.2. Peptide synthesis

TP-KHA was produced by the solid-phase peptide synthesis procedure with the Fmoc-strategy. Briefly, Fmoc-Ala-Wang resins were washed by DMF, dichloromethane, and methanol. Deprotection to remove Fmoc-protection group was conducted by adding piperidine/DMF (1:4, v/v) and mixing for 10 min. Coupling of Fmoc-protected amino acid units was accomplished by activation with PyBOP using DIPEA for 30 min. The Fmoc-amino acids (5.0 equivalents), PyBOP (5.0 equivalents), and DIPEA (10.0 equivalents) were dissolved in DMF and subsequently mixed with the resin. The deprotection and coupling were repeated until a peptide with desired amino acid sequences was synthesized. Upon completion of synthesis, the peptide was cleaved from the resin using cleavage cocktail (TFA:TIS:water, 95:2.5:2.5, v/v/v) for 2 h. The cocktail solution with cleaved peptide was mixed with MTBE, and centrifuged for 10 min at 5,000 × *g* to precipitate peptides. The supernatant was discarded, and pellet was washed three times in MTBE to remove trace of undesired substances. The peptide was purified by preparative HPLC instrument (LC-918; JAI, Tokyo, Japan) with a polyvinyl alcohol polymer packed column (JAIGEL-GS510; JAI) and an ultraviolet detector (UV-3702; JAI) at a wavelength of 214 nm. The mobile phase was water/acetonitrile/TFA (90:10:0.1, v/v/v) at 5.0 mL/min flow rate. The purified peptide was identified by LC-ESI-MS (Thermo-Finnigan, Thermo Scientific, San Jose, CA) and lyophilized (FD85112; Ilshin Biobase, Seoul, Republic of Korea), and stored at −20 °C until use.

### 2.3. Lipase-catalyzed synthesis of lauroyl tripeptide-KHA

Lipase-catalyzed synthesis of TPL-KHA was performed in 20 mL glass vial sealed with silicone rubber caps, in a water bath at the desired reaction temperature with magnetic stirring at 500 rpm. TP-KHA (0.05 mmol) and lauric acid at the desired molar ratios were added to 10 mL organic solvents. Triethylamine (1.00 mmol), 20 molar equivalents of TP-KHA, was added to the reaction to produce neutral form of amino groups ($-NH_2$) on the peptide by eliminating salt derivatives deposited by solid-phase peptide synthesis (Reyes-Duarte, Castillo, Martínez, & López-Munguía, 2002). TPL-KHA was not produced in the absence of triethylamine (data not shown), likely because lauric acid was not accessible to the ionized amino group ($-NH_3^+$), which interacted with trifluoroacetate ($CF_3COO^-$). Molecular sieves of 100 mg were added to remove water generated during the reaction. After pre-incubation of the mixture at 500 rpm for 12 h at the desired temperature, the reaction was initiated by adding 50 mg (350 PLU) of the immobilized lipase. The conversion yield was determined using the following equation:

$$\textit{Conversion yield}\ (\%) = \frac{[\textit{TPL-KHA}]_t}{[\textit{TP-KHA}]_t + [\textit{TPL-KHA}]_t} \times 100$$

where $[\textit{TP-KHA}]_t$ and $[\textit{TPL-KHA}]_t$ are the molar concentrations of TP-KHA and TPL-KHA, respectively, in the reaction mixture at defined reaction time ($t$).

### 2.4. Determination of influence of reaction parameters on conversion yield

Influence of organic solvents including acetonitrile, *tert*-butanol, 2-methyl-2-butanol, and *n*-hexane with different log *P* values, an indicator of hydrophobicity (Sangster, 1989), on the conversion yield was investigated. In order to determine solubility of TP-KHA in the solvents, each solvent (1.0 mL) was saturated with 10.0 mM TP-KHA by incubation for 12 h with magnetic stirring at 500 rpm at 55 °C. Then, the supernatant was obtained by centrifugation for 10 min at 12,000 × *g* at 25 °C. The solubility was determined by measuring the concentration of TP-KHA in the supernatant with HPLC. The influence of reaction temperature on the conversion yield was investigated in the range of 35–75 °C at interval of 10 °C, controlled with the water bath. Moreover, the conversion yield was evaluated at the different substrate molar ratio (lauric acid:TP-KHA) from 1.0 to 5.0 while holding the concentration of TP-KHA constant at 0.05 mmol.

### 2.5. Quantitative analyses by HPLC

Quantitative analyses of TP-KHA and TPL-KHA were carried out by a HPLC instrument (Waters 600, Waters Corp., Milford, MA) with an ultraviolet detector (Waters 486, Waters Corp.) and a silica-based column (5 μm, 4.6 × 250 mm, Luna C18-AR-II, Cosmosil, Kyoto, Japan). The mobile phase was acetonitrile/water/TFA (90:10:0.1, v/v/v) at a flow rate of 1.0 mL/min for 20 min. The reaction mixture was

sampled after reaction and filtered through a membrane filter (0.45 μm); then 20 μL was injected into the HPLC instrument. Substances in the reaction mixture were identified by their retention times at wavelength of 220 nm and a calibration curve was plotted using the purified substrates (TP-KHA and lauric acid) and product (TPL-KHA).

### 2.6. Purification of lauroyl tripeptide-KHA

TPL-KHA was purified from the reaction mixture by the preparative-HPLC and solvent-separation (Park et al., 2011). The reaction mixture was filtered through 0.45 μm membrane filter to separate the immobilized lipase and molecular sieves, and the solvent was removed by lyophilization at −76 °C. The concentrate was washed with 5 mL *n*-hexane and centrifuged at 7,000 × g for 10 min; then the supernatant was discarded to remove residual lauric acid. The retentate was dissolved in 3 mL distilled water and filter through the membrane filter. TPL-KHA was purified from the solution by removing TP-KHA by the preparative HPLC. Purified TPL-KHA was lyophilized and stored at −20 °C until use.

### 2.7. Structural analyses of lauroyl tripeptide-KHA by LC-ESI-MS and $^1$H NMR

Purified TPL-KHA was identified by ion-trap mass spectrometry (Thermo-Finnigan LCQ Deca XP plus, Thermo Scientific). Analytes were subjected to electrospray ionization (ESI) at a capillary temperature of 275 °C. The ion source voltage and sheath and auxiliary gas were set at 5 kV, 30, and 5 units, respectively. The capillary voltage was set at 45 V in positive-ionization mode and −15 V in negative-ionization mode.

TPL-KHA was prepared in dimethyl sulfoxide-$d_6$ at 10 mg/mL for $^1$H-nuclear magnetic resonance (NMR) analyses. $^1$H NMR spectra, including two-dimensional $^1$H–$^1$H homonuclear correlated spectroscopy (COSY) analyses, were recorded using a Bruker Avance 600 spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts (δ in ppm) were referenced to the resonance of tetramethylsilane (TMS; $\delta = 0$) for dimethyl sulfoxide-$d_6$.

### 2.8. Measurement of surface tension

Surface tension was measured at 25 ± 0.2 °C by the Wilhelmy plate method using a platinum plate and a tensiometer (K100SF, Krüss, Hamburg, Germany). TPL-KHA and lauric acid based-surfactants including Tween 20, sodium dodecyl sulfate (SDS), and dodecyltrimethylammonium bromide (DTAB) were prepared in distilled water (pH of 5.6 ± 0.2) at 24 h before the measurement. The plate was cleaned with distilled water and dried by flaming after each measurement. Each measurement was performed for 5 min in which the change in surface tension was < 0.1 mN/m. The CMC was obtained by plotting the surface tension as a function of the logarithm of concentration and determined at the intersection point of the two regression lines that best fit through pre- and post CMC. All the measurements were done in triplicate.

### 2.9. Evaluation of antibacterial activity

The antibacterial activity of TPL-KHA was evaluated against the following two Gram-positive and two Gram-negative foodborne pathogens: *Staphylococcus aureus* ATCC 12692, *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 35150, and *Salmonella* Typhimurium ATCC 43971. The bacteria were cultured in tryptic soy broth (TSB) for 12 h at 37 °C, and stock cultures were maintained in 50% glycerol (w/v) at −80 °C until use.

Minimum inhibitory concentration (MIC) was determined by the broth micro-dilution method with slight modification (Wiegand, Hilpert, & Hancock, 2008). The bacteria were aerobically propagated in TSB for 12 h, and bacterial suspensions were prepared in Mueller-Hinton broth (MHB) to a final concentration of 5.0 × $10^5$ colony-forming units (CFU)/mL based on turbidity to a McFarland standard 0.5. TPL-KHA was dissolved in and serially diluted with sterile distilled water to the desired concentrations. TPL-KHA (100 μL) was added to each well of a 96-well polypropylene microplate (Costar 3790, Corning Inc., Corning, NY), and the bacterial suspension of 100 μL was inoculated to the each well. The pH of the solution in the each well was 7.2 ± 0.1. The plate was incubated at 37 °C for 18 h. The lowest concentration of TPL-KHA which completely inhibited the growth of bacteria by measurement of absorbance at 600 nm was determined as the MIC. The minimum bactericidal concentration (MBC) was defined as the lowest concentration that induced a > 3.0 log reduction in the number of viable cells in the subcultured well contents relative to the initial inoculum.

### 2.10. Evaluation of antioxidant activity

#### 2.10.1. $ABTS^+$ radical-scavenging activity

The $ABTS^+$ radical-scavenging assay was performed as described previously with slight modification (Zheng, Zhao, Xiao, Zhao, & Su, 2016). A mixture of 7.0 mM ABTS diammonium and 2.45 mM potassium persulfate in distilled water was stored in the dark for 12 h to produce $ABTS^{\cdot+}$. The solution was diluted with 50 mM sodium phosphate buffer (pH 7.0) to an absorbance of 0.70 ± 0.02 at 734 nm. A sample (40 μL) was added to 160 μL of diluted $ABTS^{\cdot+}$ solution in the wells of a 96-well microplate. The reaction mixture was stored in the dark for 60 min at room temperature and the absorbance was measured at 734 nm. The $ABTS^+$ radical-scavenging activity was determined as the percentage of inhibition of $ABTS^{\cdot+}$ and was expressed as Trolox equivalent antioxidant capacity (TEAC) values as μmol Trolox equivalent (TE)/μmol sample using a calibration curve of a linear regression of Trolox.

#### 2.10.2. Lipid peroxidation inhibitory activity

The antioxidant activity of TPL-KHA was evaluated using a linoleic acid model system with slight modification (Esteve, Marina, & Garcia, 2015). Briefly, 2.0 mL 50 mM sodium phosphate buffer (pH 7.0), 1.0 mL 83.5 mM linoleic acid in 99.5% ethanol, 1.0 mL 100 μM sample, and 1.0 mL distilled water or ethanol were mixed in glass vials sealed with silicone rubber caps. The mixture was incubated for 10 days at 40 °C in the dark and the degree of oxidation was evaluated at 24 h intervals by the ferric thiocyanate method. The reaction mixture (50 μL) was mixed with 2.35 mL 75% (v/v) ethanol, 50 μL 30% (w/v) ammonium thiocyanate, and 50 μL 20 mM ferrous chloride in 3.5% (v/v) HCl. After allowing to stand for 10 min, the absorbance at 500 nm of the solution was measured. Moreover, the lipid peroxidation inhibitory activity was calculated using the following equation:

$$\text{Lipid peroxidation inhibitory activity (\%)} = \left(1 - \frac{Absorbance_{sample}}{Absorbance_{blank}}\right) \times 100$$

### 2.11. Statistical analyses

All experiments were performed in triplicate, unless stated otherwise, and the results are presented as means and standard deviations. Analysis of variance was performed using SPSS version 25.0 (IBM Corp., Armonk, NY) and the significance of differences was determined using a Duncan's test with a 95.0% confidence interval ($p < 0.05$).

**Table 1**
Solubility of tripeptide-KHA and conversion yield in organic solvents. Values in the same columns with different letters are significantly different ($p < 0.05$).

| Organic solvent | Log *P* | Solubility of tripeptide-KHA (mM) | Conversion yield (%)* |
|---|---|---|---|
| Acetonitrile | −0.34 | 7.98 ± 0.48$^{c}$ | 56.90 ± 1.34$^{c}$ |
| *tert*-Butanol | 0.35 | 5.87 ± 0.37$^{b}$ | 38.12 ± 3.22$^{b}$ |
| 2-Methyl-2-butanol | 0.89 | 7.42 ± 0.27$^{c}$ | 63.30 ± 3.08$^{d}$ |
| *n*-Hexane | 4.00 | 0.01 ± 0.01$^{a}$ | 4.32 ± 4.75$^{a}$ |

* Reaction conditions: tripeptide-KHA; 10.0 mM, lauric acid; 10.0 mM, reaction temperature; 55 °C, reaction time; 72 h.

## 3. Results and discussion

### 3.1. Influence of reaction parameters

In enzymatic synthesis reactions, the organic solvent is a determinant of the conversion yield. The chemical properties of organic solvent influence on enzyme operational stability, which is directly associated with activity. Lipases are generally more stable in hydrophobic than hydrophilic organic solvents, because the hydrophilic solvents denature enzymes by stripping the water from enzyme surface, required to maintain an active conformation (José et al., 2011). In addition, in cases of lipase-catalyzed acylation reactions between hydrophilic and hydrophobic substrates, substrate solubility is a critical determinant of the conversion yield. Hydrophobic substrates (*e.g.*, fatty acids) typically have adequate solubility in most organic solvents, however, hydrophilic substrates (*e.g.*, hydrophilic peptide) often have low solubility, limiting their utility (Husson et al., 2011). The influence of organic solvents on the reaction was presented in Table 1. The minimum solubility of lauric acid was 75.4 mM in 2-methyl-2-butanol, which was higher than maximum concentration of lauric acid (50.0 mM) applied in this study. Therefore, the solubility of lauric acid does not limit the conversion yield of the reaction. Conversely, TP-KHA was scarcely dissolved in *n*-hexane, the most hydrophobic solvent among the tested solvents. However, the solubility was not exactly correlated with hydrophobicity of organic solvent. The solubility (7.42 mM) in 2-methyl-2-butanol with log *P* value of 0.89 was significantly higher ($p < 0.05$) than in *tert*-butanol (5.87 mM) with log *P* value of 0.35. That is, a decrease in the log *P* value (*i.e.*, an increase in hydrophilicity) does not increase the solubility of TP-KHA; thus, the solubility of TP-KHA may be related to other parameters of the organic solvent, such as the dielectric constant or dipole moment (Stergiou et al., 2013). Compared to the conversion yields in *n*-hexane (4.32%), 2-methyl-2-butanol (63.30%), and *tert*-butanol (38.12%), the solubility of TP-KHA was the factor limiting the conversion yield. Meanwhile, the conversion yield in 2-methyl-2-butanol was significantly higher ($p < 0.05$) than that in acetonitrile (56.90%) despite the fact that solubility of TP-KHA in 2-methyl-2-butanol was similar ($p > 0.05$) to that in acetonitrile (7.98 mM). This may be attributed to the effects of the different hydrophilicities of 2-methyl-2-butanol and acetonitrile on enzyme operational stability. Acetonitrile may distort the water bound on the surface of the lipase more severely than 2-methyl-2-butanol due to its higher hydrophilicity (Stergiou et al., 2013). As a result, 2-methyl-2-butanol was selected as the most suitable organic solvent for the synthesis of TPL-KHA.

The reaction temperature influences the conversion yield by affecting enzyme activity and the molecular dynamics of the substrates. A decrease in temperature may increase the viscosity of the reaction mixture and so reduce diffusion of the substrates, possibly decreasing the probability of collision between the enzyme and substrates and formation of enzyme-substrate complexes, decreasing conversion yield (Yu et al., 2010). However, a high temperature may reduce enzyme activity by denaturing the enzyme during the reaction. It was found that an increase in reaction temperature in the range of 35–55 °C

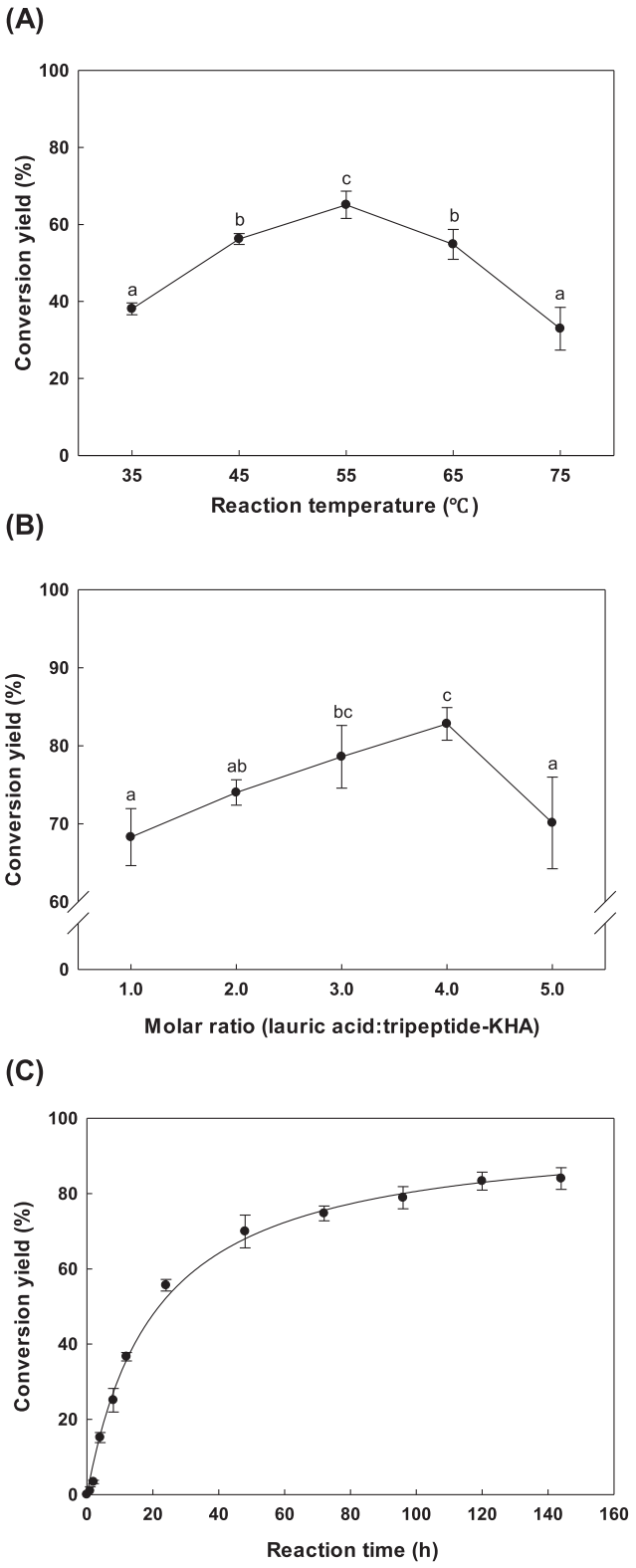


**Fig. 1.** Effects of reaction parameters on conversion yield. (A) Reaction temperature, (B) substrate molar ratio (lauric acid:tripeptide-KHA), and (C) reaction time. Reaction conditions: (A) organic solvent; 2-methyl-2-butanol, substrate molar ratio; 1.0, reaction time; 72 h, (B) organic solvent; 2-methyl-2-butanol, reaction temperature; 55 °C, reaction time; 72 h, (C) organic solvent; 2-methyl-2-butanol, reaction temperature; 55 °C, substrate molar ratio; 4.0. Different letters indicate significant differences ($p < 0.05$).

significantly enhanced ($p < 0.05$) the conversion yield (Fig. 1A), likely by decreasing the viscosity. However, the conversion yield decreased significantly ($p < 0.05$) as reaction temperature increased from 55 to 75 °C. Hence, the optimum reaction temperature was determined at 55 °C.

The conversion yield of lipase-catalyzed acylation is influenced by the substrate molar ratio due to the reversibility of the reaction. In general, the reaction equilibrium is shifted towards product synthesis in the presence of excessive acyl donor, inhibiting the inverse reaction (*i.e.*, hydrolysis) (Sen & Puskas, 2015). The conversion yield steadily increased as the molar ratio increased from 1.0 to 4.0 (Fig. 1B), indicating a shift in the reaction equilibrium towards acylation. However, the conversion yield was significantly decreased ($p < 0.05$) by further increase of the substrate molar ratio more than 4.0. This could be attributed to substrate inhibition, which is typical for lipase-catalyzed acylation and esterification (Kim & Park, 2017). In the presence of too much excess acyl donor, the acyl donor-enzyme complex sterically hinders binding of an acyl acceptor to the enzyme, causing decrease in conversion yields. The optimum substrate molar ratio of lauric acid to TP-KHA was 4.0.

We also investigated the effects of reaction time on the conversion yield (Fig. 1C). The concentration of TP-KHA decreased with increasing concentration of TPL-KHA as the reaction time increased (Fig. S1). The conversion yield gradually increased to a peak at 144 h (84.01%), indicating the reaction achieved the equilibrium. The conversion yield was 74.73% at 72 h and increased by only 9.28% from 72 to 144 h, indicating TPL-KHA was synthesized rapidly at the beginning of 72 h. Therefore, despite the gradual increase in conversion yield until 144 h, the optimum reaction time was 72 h.

### 3.2. Structural analyses of lauroyl tripeptide-KHA

TPL-KHA was identified by LC-ESI-MS (Fig. S2). TP-KHA was acylated with one equivalent of lauric acid and no TP-KHA was produced from two equivalents of lauric acid; *i.e.*, $N^{\alpha}$,$N^{\varepsilon}$-dilauroyl TP-KHA. The spectrum showed two molecular ions at *m/z* of 269.3 ($[M+2H]^{2+}$) and 537.5 ($[M+H]^{+}$), corresponding to the estimated molar mass of TPL-KHA (536.7 g/mol). The relative abundance of the double-charged molecular ion, caused by multiple isotopes, was higher than that of single-charged ion.

The molecular structure of TP-KHA exhibited two potential positions for *N*-acylation with lauric acid, the α- and ε-amino group ($-NH_2$) of lysine. To identify the acylation position, structural analyses of synthesized TPL-KHA were performed with $^1$H NMR (Fig. 2A and Table S1). The proton signals were assigned from the COSY spectra based on the cross-peaking with each vicinal proton (Fig. 2B). The key protons on the amine groups of alanine, histidine, and lysine to elucidate the acylation position were observed at downfield chemical shifts of 7.84–8.33 ppm (Fig. 2C). The acylation position was determined by analyzing the spin–spin coupling patterns and each signal at the field as described previously (Husson et al., 2009). In the spectra of the field, the protons on amine groups of alanine ($\delta = 8.16$ ppm, $J = 7.1$ Hz, 1H) and histidine ($\delta = 8.33$ ppm, $J = 7.8$ Hz, 1H) individually participated in amide bonds (*i.e.*, peptide bond; NH–C═O), as both signals were doublets, indicating that the amine groups were directly attached to the –CH. However, the protons on amine group of lysine ($\delta = 7.84$ ppm, $J = 5.6$ Hz, 1H) was observed as a triplet, demonstrating that neighboring hydrocarbon was $-CH_2$ at δ-position on lysine side chain (Fig. 2D). If the α-amino group of lysine was acylated with lauric acid, its signal should be doublet since the neighboring hydrogen carbon was –CH. Hence, only the ε-amino group on lysine side chains of TP-KHA was selectively *N*-acylated with lauric acid by the enzymatic acylation reaction.

*Candida antarctica lipase* B (CALB) exhibits chemo-selectivity towards $N^{\varepsilon}$-acylation in the reaction between fatty acids and peptides containing lysine (Husson et al., 2009). In an acylation reaction catalyzed by immobilized CALB (Novozym 435) between oleic acid and Lys-Ser dipeptide (potential acylation positions at α- and ε-amino group of lysine and hydroxyl group of serine), neither $N^{\alpha}$- nor *O*-acylation product was detected, but $N^{\varepsilon}$-oleoyl Lys-Ser was produced. The selectivity on the amino group may be explained by the higher nucleophilicity of the primary ε-amino group than that of the α-amino group. In addition, substrate specificity of CALB, induced by its particular conformation of catalytic cavity at active site, may lead to the selectivity of the reaction (Dettori, Jelsch et al., 2018, Dettori, Vibert et al., 2018). The presence of bulky groups at the α-position could prevent the α-amino group from entering the active site of CALB. Moreover, docking simulations suggested that the tightness and the depth of the active site in CALB explain the preference on ε-amino group of lysine (Ferrari et al., 2014). Due to the conformation, only functional groups with a long enough chain (ε-amino group in this study), can reach the bottom of the catalytic cavity. The preference of CALB towards ε-amino groups of lysine suggests that enzymatic production of $N^{\varepsilon}$-acylated peptide has advantages over chemical synthesis.

### 3.3. Surface activity

The surface tension at various concentrations of TPL-KHA was measured and compared to those of lauric acid based-surfactants (Fig. 3). Tween 20, SDS, and DTAB were used as non-ionic, anionic, and cationic surfactants, respectively. The surface tension decreased as the concentration of TPL-KHA increased from 0.05 to 2.0 mM, indicating that TPL-KHA has surface activity at air–water interfaces. Compared to the surface tension of distilled water (72.57 mN/m) as a control, that of TP-KHA at 5.0 mM (72.35 mN/m) was similar (data not shown), suggesting that the surface activity of TPL-KHA is obtained by acylation with lauric acid. However, surface tension was constant at > 2.0 mM TPL-KHA, suggesting that the interface is fully occupied by TPL-KHA and thus micelles begin to form. The critical micelle concentration (CMC), the concentration at which surface tension is independent of surfactant concentration, is determined by the intersection of extrapolations above and below the break of the surface tension-concentration curve (Reis et al., 2004). The CMC and the surface tension at the CMC ($\gamma_{CMC}$) are important parameters determining efficiency of surfactants. The lower the CMC, the greater the surfactant form micelles solubilizing hydrophobic compounds in hydrophilic phase, and $\gamma_{CMC}$ indicates the ability of a surfactants to reduce surface tension (Otzen, 2017).

The CMCs of Tween 20, SDS, and DTAB were 0.053, 10.07, and 13.35 mM, respectively, as reported previously (Rosen & Kunjappu, 2012). TPL-KHA showed a CMC of 2.71 mM, which was the two orders of magnitude higher than that of Tween 20 (non-ionic surfactant) but 3.7- and 4.9-fold lower than that of SDS (anionic surfactant) and DTAB (cationic surfactant), respectively. Charge is a critical determinant of the CMC, together with size, molecular structure, and hydrophobicity. Generally, non-ionic surfactants have lower CMC than ionic surfactants with the same hydrophobic tail group, since the head group of ionic surfactants repel each other in the micelles and the repulsion prevents to form micelles (Rosen & Kunjappu, 2012). In this context, the CMC of TPL-KHA, which was two orders of magnitude higher than that of the non-ionic surfactant and lower than those of the ionic surfactants, could be explained in terms of charge. The head group of TPL-KHA (TP-KHA) has three ionizable groups. At the ionized state, the α-amino group of lysine and the imidazole group of histidine side chain serve positive charges and the carboxyl group of alanine provides a negative charge. It is thought that TPL-KHA has amphoteric properties, that is, TPL-KHA can have non-ionic, cationic, and anionic properties depending on pH. Although the exact ionized state of TPL-KHA was not determined, the lower charge density of TPL-KHA than those of ionic surfactants may result in a less-intense repulsive force among the head groups. These results are consistent with a previous report that lauroyl arginine-glycine and lauroyl arginine-phenylalanine, amphoteric compounds, have

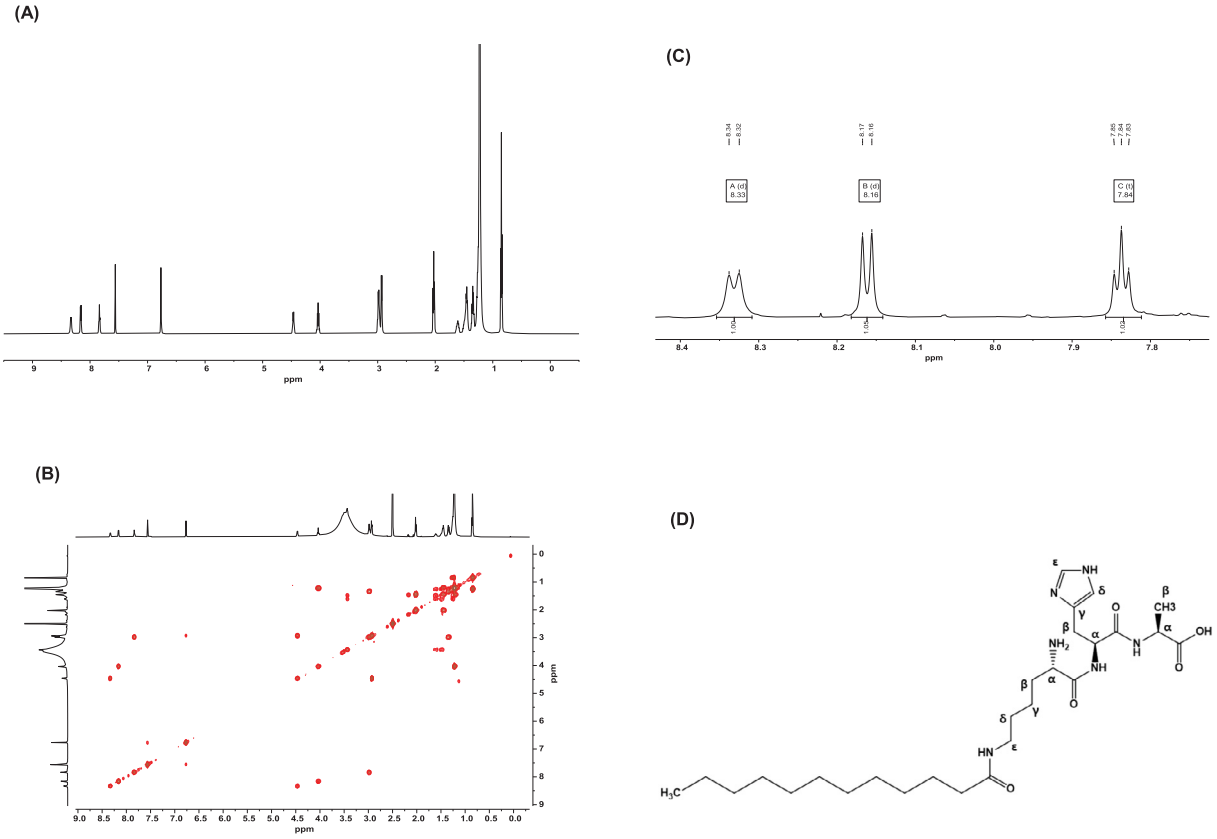


**Fig. 2.** Structural analyses of lauroyl tripeptide-KHA. (A) $^{1}$H NMR spectra ranging from 0.0 to 10.0 ppm. (B) $^{1}$H–$^{1}$H two-dimensional COSY spectra. (C) Amide region (7.7–8.4 ppm) of the $^{1}$H NMR spectra. (D) Chemical structure of lauroyl tripeptide-KHA identified by the analysis.

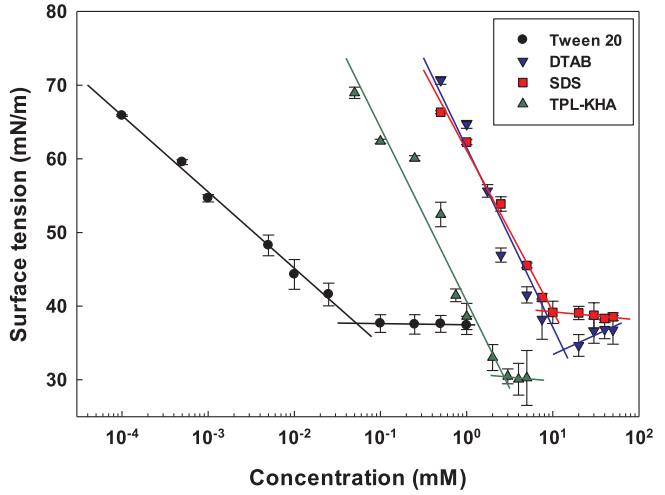


**Fig. 3.** Plots of surface tension versus concentration for lauric acid-based surfactants; Tween 20, dodecyl trimethylammonium bromide (DTAB), sodium dodecyl sulfate (SDS), and lauroyl tripeptide-KHA (TPL-KHA). The CMC was determined from the intersection point of the regression lines fitting the two part of the curve, below and above the CMC. The data were obtained from triplicate experiments and presented as mean values with standard deviations.

lower CMCs than their cationic homologues (Bustelo et al., 2017).

Despite the higher CMC of TPL-KHA than Tween 20, TPL-KHA had a lower $\gamma_{CMC}$ (30.44 mN/m) than Tween 20 (37.68 mN/m), SDS (39.23 mN/m), or DTAB (34.11 mN/m). The results showed that TPL-KHA most effectively decreased surface tension at the hydrophobic-hydrophilic interface, possibly due to its suitable amphiphilicity, which enabled it to be readily packed at the interface.

### 3.4. Antibacterial activity

The antibacterial activity of TPL-KHA against *S. aureus*, *B. cereus*, *E. coli*, *S.* Typhimurium was evaluated using the broth micro-dilution method. The growth of the four bacteria was inhibited by TPL-KHA in a concentration-dependent manner (Fig. S3). The MICs of TPL-KHA against the four bacteria were ranged from 2.83 to 4.00 mM, which were 1.06–1.46 fold lower than the MBCs (3.17–5.83 mM) (Table 2). These results indicated TPL-KHA had bacteriostatic and bactericidal effects on both Gram-positive and Gram-negative foodborne pathogens. In contrast to TPL-KHA, lauric acid at 4.00 mM significantly inhibited the growth of only *B. cereus*. TP-KHA did not show any inhibition effects on the bacterial growth at 5.0 mM (data not shown).

It has been widely accepted that free fatty acids and fatty acid derivatives, including lipoamino acids, lipopeptides, and fatty acid esters, showed antibacterial activity by damaging the cell membrane (Zhao, Zhang, Hao, & Li, 2015). However, most of the fatty acid derivatives displayed weaker activity against Gram-negative bacteria than Gram-positive bacteria, which may be due to the differences on the cell wall structures (Schmidtchen et al., 2014). While Gram-positive bacteria have a single plasma membrane surrounded by a thick peptidoglycan

**Table 2**
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of lauroyl tripeptide-KHA against Gram-positive and Gram-negative bacteria. Data are means of triplicate assays.

| Bacterial strains | | MIC (mM) | MBC (mM) |
|---|---|---|---|
| Gram-positive | *S. aureus* ATCC 12692 | 4.00 | 5.67 |
| | *B. cereus* ATCC 10876 | 3.00 | 3.17 |
| Gram-negative | *E. coli* ATCC 35150 | 2.83 | 3.83 |
| | *S.* Typhimurium ATCC 43971 | 4.00 | 5.83 |

envelope with a coarse meshwork, Gram-negative bacteria possess a negatively charged lipopolysaccharide forming a compact layer at outer membrane, which acts as a barrier to antibacterial agents (Koh et al., 2015).

Indeed, lauric acid and its esters such as sucrose monolaurate, lactose monolaurate, and glyceryl monolaurate show antibacterial activity against only Gram-positive bacteria (Dayrit, 2015; Wagh, Shen, Shen, Miller, & Walsh, 2012). In addition, it was observed that the MIC values of erythorbyl laurate against Gram-positive bacteria were 0.48–0.88 mM, but erythorbyl laurate did not exhibit antibacterial activity against Gram-negative bacteria at concentrations up to 5.0 mM (Park et al., 2018). Amphiphilicity and charge are considered as the most important factors determining the antibacterial activity of the fatty acid derivatives (Makovitzki et al., 2006). Each lauric acid ester and TPL-KHA have different amphiphilicities; however, as described in Section 3.3, TPL-KHA can have a partial positive charge, but the lauric acid esters cannot due to the absence of any ionizable moiety acting as a cation in the head groups. Hence, it is thought that positive charge is the indispensable factor for the fatty acid derivatives to possess antibacterial activity against Gram-negative bacteria with negative charged lipopolysaccharide at outer membrane. The antibacterial activity of TPL-KHA would be dependent on the pH. Since the positive charge of TPL-KHA would be higher at the acidic conditions than the alkaline, the interaction of TPL-KHA with outer membrane of the bacteria is expected to be enhanced at the acidic conditions. Therefore, the antibacterial activity might increase at the acidic conditions, especially against Gram-negative bacteria.

### 3.5. Antioxidant activity

The ability of TPL-KHA and TP-KHA to scavenge $ABTS^+$ radicals was determined using BHA and α-tocopherol as positive controls (Fig. 4A). BHA and α-tocopherol exhibited TEAC values of 1.249 and 0.960 μmol TE/μmol, respectively, higher than those of TP-KHA and TPL-KHA. The $ABTS^+$ radical scavenging activity of TP-KHA and TPL-KHA increased as concentration increased from 100 to 800 μM (data not shown). TPL-KHA showed a TEAC value of 0.206 μmol TE/μmol, significantly higher ($p < 0.05$) than that of TP-KHA (0.147 μmol TE/μmol). Although the radical scavenging activity of TPL-KHA was slightly higher than that of TP-KHA, it was identified that antioxidant activity of TP-KHA was retained after acylation with lauric acid.

The inhibitory activity of TPL-KHA against lipid oxidation was evaluated in comparison with TP-KHA, BHA, and α-tocopherol (Fig. 4B). The control group (no antioxidant) showed an increase in absorbance over 7 days, indicating formation of lipid peroxide by the thermal oxidation of linoleic acid. The absorbance peaked at 7–9 days, suggesting the oxidation of linoleic acid progressed with simultaneous conversion of lipid peroxides to secondary oxidation products such as malondialdehyde (Chen, Zhao, Zhao, Cong, & Bao, 2007). BHA at 100 μM inhibited the oxidation of linoleic acid throughout the incubation period, showing 93.93% inhibition at 9 days. TPL-KHA showed 86.28% of inhibitory activity against the oxidation at 7 days, but the activity decreased by 80.99% at 9 days. The inhibitory activity of TPL-KHA was similar ($p > 0.05$) to that of α-tocopherol at 7 days (87.79%) but slightly lower ($p < 0.05$) at 9 days (86.55%). In addition, no significant difference was observed ($p < 0.05$) between the inhibitory activity of TPL-KHA and TP-KHA at 9 days (83.60%). These results indicated TPL-KHA had ability to scavenge lipid radicals (R˙) at initiation step of linoleic acid oxidation or to react with alkoxyl (RO˙) and/or peroxyl (ROO˙) radicals at propagation step of the oxidation (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

## 4. Conclusions

In this study, lipase-catalyzed acylation of TP-KHA with lauric acid was performed to produce a novel multi-functional compound with surface, antibacterial, and antioxidant activities. The acylation reaction selectively produced $N^{\varepsilon}$-lauroyl tripeptide-KHA under the optimum conditions. It was demonstrated that TPL-KHA had surface activity at the air–water interface and showed both bacteriostatic and bactericidal effects on Gram-positive (*S. aureus*, *B. cereus*) and Gram-negative (*E. coli*, *S.* Typhimurium) foodborne pathogens. In addition, TPL-KHA exhibited the ability to scavenge $ABTS^+$ radicals and inhibit the lipid oxidation. Therefore, TPL-KHA has a potential as a multi-functional food emulsifier to simultaneously control the lipid oxidation and foodborne pathogens in emulsion-based foods.

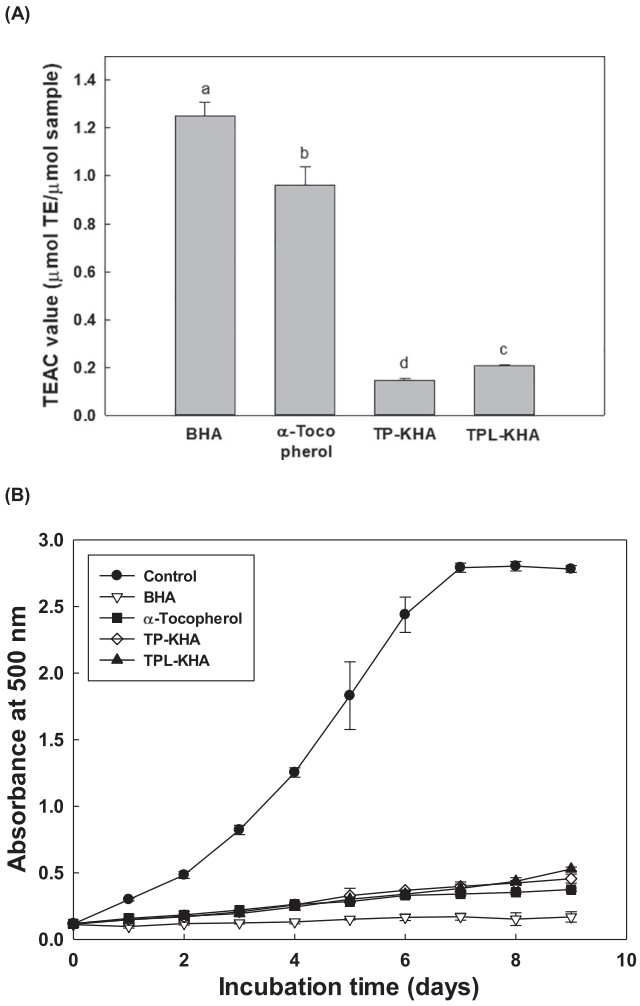


**Fig. 4.** (A) $ABTS^+$ radical scavenging activity of lauroyl tripeptide-KHA (TPL-KHA), tripeptide-KHA (TP-KHA), α-tocopherol, and BHA. Activity is expressed as the TEAC value at 60 min. Different letters indicate significant differences ($p < 0.05$). (B) Inhibition of linoleic acid oxidation by TPL-KHA, TP-KHA, α-tocopherol, and BHA at a concentration of 100 μM. The degree of oxidation was measured every 24 h for 9 days.

## CRediT authorship contribution statement

**Hyunjong Yu:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Kyung-Min Park:** Conceptualization, Formal analysis, Data curation, Visualization, Writing - review & editing. **Pahn-Shick Chang:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126533.

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