Selective Production of 9R-Hydroxy-10E,12Z,15Z-Octadecatrienoic Acid from α-Linolenic Acid in Perilla Seed Oil Hydrolyzate by a Lipoxygenase from Nostoc Sp. SAG 25.82

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Abstract

Hydroxy fatty acids (HFAs) derived from omega-3 polyunsaturated fatty acids have been known as versatile bioactive molecules. However, its practical production from omega-3 or omega-3 rich oil has not been well established. In the present study, the stereo-selective enzymatic production of 9R-hydroxy-10E,12Z,15Z-octadecatrienoic acid (9R-HOTE) from α-linolenic acid (ALA) in perilla seed oil (PO) hydrolyzate was achieved using purified recombinant 9R-lipoxygenase (9R-LOX) from Nostoc sp. SAG 25.82. The specific activity of the enzyme followed the order linoleic acid (LA) > ALA > γ-linolenic acid (GLA). A total of 75% fatty acids (ALA and LA) were used as a substrate for 9R-LOX from commercial PO by hydrolysis of Candida rugosa lipase. The optimal reaction conditions for the production of 9R-HOTE from ALA using 9R-LOX were pH 8.5, 15°C, 5% (v/v) acetone, 0.2% (w/v) Tween 80, 40 g/L ALA, and 1 g/L enzyme. Under these conditions, 9R-LOX produced 37.6 g/L 9R-HOTE from 40 g/L ALA for 1 h, with a conversion yield of 94% and a productivity of 37.6 g/L/h; and the enzyme produced 34 g/L 9R-HOTE from 40 g/L ALA in PO hydrolyzate for 1 h, with a conversion yields of 85% and a productivity of 34 g/L/h. The enzyme also converted 9R-hydroxy-10E,12Z-octadecadienoic acid (9R-HODE) from 40 g/L LA for 1.0 h, with a conversion yield of 95% and a productivity of 38.4 g/L. This is the highest productivity of HFA from both ALA and ALA-rich vegetable oil using LOX ever reported. Therefore, our result suggests an efficient method for the production of 9R-HFAs from LA and ALA in vegetable oil using recombinant LOX in biotechnology.

Introduction

Lipoxygenase (LOX) catalyzes the regio- and stereo-selective hydroperoxidation of free polyunsaturated fatty acids (PUFAs) possessing one or more (1Z,4Z)-pentadienes to form
hydroperoxy fatty acids (HPFAs) that reduced to hydroxy fatty acids (HFAs) [1]. These hydroxyperoxy- and hydroxy-fatty acids act as precursors for the biosynthesis of signaling molecules by further enzymatic cascade reactions and have a variety of functions [2, 3]. Extensive research has been performed using plant and animal LOX systems to study the physiological functions [4]. However, recent reports have described the different substrate specificity and stereo-selectivity of bacterial and fungal LOXs for omega-3 PUFAs compared to those of plant and animal LOXs [5–7].

To date, LOX has mainly been applied to the production of HPFAs, which are utilized to produce green note volatiles combined with hydroperoxy lyase [8]. Due to its highly reactive hydroperoxy forming activity, LOX is also used to modify the food polymers including wheat proteins [9, 10]. Application of LOX for pulp processing from lignocellulogic agro-materials has shown a variety of peroxy products [11]. Interestingly, new emerging applications of LOX include the production of HFAs, because they are widely used as starting materials for the synthesis of a variety of polymers and as additives in the manufacturing of lubricants, emulsifiers, and stabilizers in the chemical, food, cosmetic, and pharmaceutical industries, owing to their higher reactivity, solvent miscibility, and stability compared to non-hydroxylated fatty acids [12, 13]. HFAs are also notable intermediates for bio-grade flavor lactone production [14]. Therefore, the biotechnological production of HFAs from PUFAs is valuable.

α-Linolenic acid (ALA; 18:3n-3) is an essential fatty acid that requires dietary uptake as it is not synthesized by the human body due to the absence of endogenous desaturases such as Δ5- and Δ6-desaturases. Perilla (Perilla frutescens) seed oil (PO) is known to contain approximately 60% ALA [15, 16]. Therefore, PO is one of the most important and rich sources of ALA in East Asian countries, including Korea, China, and Japan, and is widely used as a vegetable oil in traditional foods. Increasing ALA uptake shows remarkable pharmacological effects in the improvement of inflammatory and immune disorders, as well as preventing in cardiovascular disorders [17]. Furthermore, LOX-derived bioactive HFAs from ALA, including 9-hydroxy-10E,12Z,15Z- or 13-hydroxy-9Z,11E,15Z-octadecatrienoic acid (9- or 13-HOTE), have been known as antifungal [18], anti-inflammatory [19], and signal modulation compounds [20]. Although these compounds have been isolated and identified from a wide range of taxonomic origins [18, 19, 21, 22], their specific production, especially using omega-3 rich oil, is scarce. Recently, 9R-LOX from Nostoc sp. showed a wide range of substrate specificity for PUFAs that form HPFAs [6]. Therefore, Nostoc 9R-LOX can be applied to produce HFA from ALA in omega-3 rich vegetable oil.

In the present study, the stereo-selective biocatalyst 9R-LOX from Nostoc sp. was expressed in Escherichia coli and purified. The purified enzyme was applied to the production of 9R-HOTE. The reaction conditions, including pH, temperature, organic solvent, detergent, and the concentrations of enzyme and substrate, were optimized. Under the optimized conditions, the stereo-specific production of 9R-HOTE was attempted using ALA and ALA-rich PO.

Materials and Methods

Chemicals and reagents

High-quality (>99%) ALA and linoleic acid (LA; 18:2n-6) were purchased from Nu-Check Prep (Elysian, MN) and Sigma (St. Louis, MO, USA), respectively. 9-Hydroperoxy-trioctadecatrienoic acid (9-HPOTE) and 9S-HOTE standards were purchased from Cayman Chemicals (Ann Arbor, MI). 9R-HOTE standard was prepared by the previously reported procedure [23]. Solvents, including 2-propanol, acetonitrile, n-hexane, and methanol, were high-performance liquid chromatography (HPLC) grade and ethanol and ethyl acetate for extraction were reagent grade. These solvents were supplied from Duksan Chemical (Ansan, Korea). Commercial PO
was obtained from a local market. Luria-Bertani (LB) medium was purchased from Fermentas (Vilnius, Lithuania). Ampicillin, boric acid, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from USB (Cleveland, OH, USA). Lipases from recombinant Aspergillus oryzae (AOL), Candida rugosa (CRL), Pseudomonas cepacia (PCL), Pseudomonas fluorescense (PFL), Rhizomucor meihei (RML), and Thermomyces lanoginosus (TLL) were obtained from Sigma.

**Gene expression**

The constructed plasmid harboring *Nostoc* sp. 9R-lipoxygenase gene (*lox*) in pET-15b (9R-lox/pET-15b) was used for expression in *E. coli* [23]. This plasmid was transformed into *E. coli* ER2566 cells by electroporation and selected on LB agar containing 50 μg/ml ampicillin. A single colony of *E. coli* cells containing the 9R-lox/pET-15b plasmid were inoculated into 3 ml of liquid LB medium and cultivated at 37°C with shaking at 220 rpm overnight. This culture was transferred into a 2,000 ml flask containing 500 ml of LB medium and 50 μg/ml ampicillin at 37°C with shaking at 200 rpm. When the optical density of the culture reached 0.6 at 600 nm, expression of the 9R-lox gene was induced by adding isopropyl-β-D-thiogalactopyranoside with a final concentration of 0.1 mM, and the culture was further incubated for 16 h at 16°C with shaking at 150 rpm.

**Enzyme preparation**

The cultured cells were harvested by centrifugation at 6000×g for 20 min at 4°C and washed twice with 0.85% (w/v) NaCl. The cells were disrupted by sonication on ice for 2 min in 50 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl and 1 mg/ml lysozyme. The unbroken cells and debris were removed by centrifugation at 13000×g for 20 min at 4°C, and the obtained supernatant was used as a crude extract. Purification was carried out using a fast protein liquid chromatography system (Bio-Rad) in a cold room at 4°C. The crude extract was applied to a His-Trap affinity chromatography column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The bound enzyme was then eluted at 4°C with the same buffer containing 250 mM imidazole at a flow rate of 1 ml/min. The active fractions were collected and dialyzed at 4°C for 16 h against 50 mM Tris-HCl buffer (pH 8.5). The resulting solution was used as the purified enzyme.

**Determination of LOX activity and enzyme assay**

LOX activity was determined by measuring conjugated diene formation at 234 nm using a Beckman Coulter DU-700 spectrophotometer (Brea, CA, USA). An extinction coefficient of 25,000 M⁻¹ cm⁻¹ was used to calculate the activity of the enzyme for 9-HPODE or 9-HPOTE [24, 25]. Unless otherwise stated, the reaction was performed in 0.45 ml of 50 mM Tris-HCl buffer (pH 8.5) containing 50 μL substrate (10 mM ALA or LA), 0.05% (w/v) Tween 80, and 0.2–10 μg enzyme at 25°C for 5 min. The range was adjusted and monitored to give a slope within A₂₃₄ < 1.0. One unit (U) of LOX activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min. The rate of the reaction was compared to that without the addition of enzyme as a control.

**Effects of pH and temperature**

To investigate the effects of pH and temperature on 9R-LOX activity for the production of 9R-HOTE from ALA, the pH values were varied from 7.0 to 10.0 using 50 mM Tris-HCl buffer (pH 7.0–9.0) and 0.2 M sodium borate buffer (pH 9.0–10.0) at a constant temperature of 25°C, while the temperatures were varied from 10 to 50°C at a constant pH of 8.0. The effect of
temperature on enzyme stability was monitored for 4 h by incubating the enzyme solution at different temperatures from 5 to 50°C in 50 mM Tris-HCl buffer (pH 8.5). Samples were withdrawn at time intervals and then assayed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA and 0.05 U ml⁻¹ enzyme at 15°C for 5 min. The half-life of the enzyme was calculated using Sigma Plot 10.0 software (Systat Software, San Jose, CA, USA).

**Effects of solvents and detergents**

Several organic solvents at concentrations of 4% and 6% (v/v), including methanol, ethanol, butanol, isopropanol, heptanol, octanol, acetone, ethyl acetate, dichloromethane, hexane, and dimethyl sulfoxide (DMSO) were tested for selecting the optimum solvent. The effect of solvent concentration on LOX activity was evaluated by varying the concentration of acetone from 1% to 10% (v/v). The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA, 0.1 U/ml enzyme, and acetone at 15°C for 10 min. Several detergents, including Tween 20, Tween 40, Tween 80, Span 20, Span 60, Span 80, Triton-X 100, Brij 58, and sodium dodecyl sulfate (SDS), were tested to find the suitable detergent for the maximum production of 9R-HOTE from ALA. Tween 80 concentration was varied from 0.005% to 1% (w/v) to determine the optimum concentration of the detergent. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA, 0.1 U/ml enzyme, 5% (v/v) acetone, and Tween 80 at 15°C for 10 min.

**Determination of kinetic parameters**

Determination of all kinetic measurements was obtained by monitoring the time-dependent consumption of O₂ using a Clark-type electrode (oxygen probe, YSI Model 5331, Yellow Springs, OH, USA) with a high sensitivity membrane and oxygen monitor (YSI Model 5300A) in a thermostatic oxygen cell with a cell volume of 1.5 ml at 25°C [5, 24, 26]. The cell was filled with air-saturated 50 mM Tris-HCl buffer (pH 8.5) containing substrate (0.1 mM), and was then allowed to equilibrate for approximately 5 min under a controlled O₂/N₂ atmosphere. The reactions were initiated by the addition of 1–5 μL concentrated enzyme, corresponding to 0.1–2 U/mg specific activity, via a gastight syringe. Nitrogen was used to adjust to different O₂ concentrations. Enzyme activity was determined from the initial rate of oxygen uptake and consumption, as reported previously [24, 26]. LA, ALA, and γ-linolenic acid (GLA) at 100 μM were used as substrates to determine kinetic parameters. The kinetic parameters were calculated by nonlinear regression fitting of the experimental points to the Michaelis–Menten equation.

**Effects of enzyme and substrate concentrations on 9R-HOTE production**

The optimal concentrations of enzyme and substrate for the increased production of 9R-HOTE from ALA by recombinant 9R-LOX were determined by varying the enzyme concentration from 0.05 to 2 mg/ml at a constant ALA concentration of 10 g/L and varying the substrate concentration from 5 to 50 g/L at a constant enzyme concentration of 0.5 mg/ml. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing different concentration of ALA and enzyme at 15°C for 20 min.

**Production of 9R-HOTE and 9R-HODE from ALA and LA**

The production of 9R-HOTE from ALA by recombinant 9R-LOX was performed in 50 mM Tris-HCl buffer (pH 8.5) containing 40 g/L ALA, 5% (v/v) acetone, and 0.2% (w/v) Tween 80
at 15°C for 80 min. 9R-HODE production from LA was carried out under the same conditions as the production of 9R-HOTE from ALA.

Production of 9R-HOTE and 9R-HODE from PO hydrolyzate

PO with a high content of ALA was hydrolyzed using AOL, CRL, PFL, RML, and TLL. The reaction was carried out in 9 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 10 g/L PO, 1 g/L CRL (700 U/mg), and 0.02% (w/v) Tween 80 at 37°C with shaking at 250 rpm for 2 h. The reaction was stopped by boiling the reaction mixture at 100°C for 50 min. The sample was kept at −20°C for analyzing the concentrations of fatty acids. The samples were extracted twice using ethyl acetate and dried under reduced pressure. The dried samples were collected and used for the production of 9R-HOTE. The time course reactions for the production of 9R-HOTE from ALA by 9R-LOX were carried out in a 500 ml baffled flask in 80 ml of 50 mM Tris-HCl buffer (pH 8.5) consisting of PO hydrolyzate, which contained 40 g/L ALA, 10 g/L LA, 15.3 g/L of other fatty acids, 5% (v/v) acetone, 0.2% (v/v) Tween 80, and 1 g/L purified 9R-LOX at 15°C for 100 min, with agitation at 250 rpm and oxygen supplementation for efficient oxygenation. After the reactions, 9R-HOTE and 9R-HODE were obtained from 9R-HPODE and 9R-HPOTE by the addition of 8 ml of 1 M NaBH₄, and an equal reaction volume of ethyl acetate. The ethyl acetate in the reaction mixture was removed using a rotary evaporator under reduced pressure, and the concentrations of 9R-HODE and 9R-HOTE in the reaction solutions were determined.

Preparation of 9R-HOTE and 9R-HODE

9R-HOTE and 9R-HODE standards were prepared by a preparative HPLC after solvent extraction, as described previously [23]. The purified products were identified by chiral phase HPLC and liquid chromatography-mass spectrometry (LC-MS).

Fatty acid analysis of PO hydrolyzate

The composition of total fatty acids from PO hydrolyzate was determined by fatty acid methyl ester (FAME) analysis, as described previously [27]. Briefly, 0.1 g of PO was mixed with 2 ml of 0.5M NaOCH₃ and 0.05 ml of tridecanoic acid (C13:0, 0.5 mg/ml in methanol) as an internal standard in a screw-cap Pyrex tube (16×150 mm). The mixture was incubated at 55°C in a water bath for 1 h. The reactant was cooled and then 2 ml of saturated NaOCH₃ solution and 3 ml of hexane were added in the same reaction tube for extraction. The hexane layer containing the FAME was taken into a gas chromatography (GC) vial. The vial was capped and placed at −20°C until GC analysis. A GC-flame ionization detector (FID) (Agilent 6890N) equipped with a DB-23 column (0.25 mm×30 m, 0.25 μm, J&W Scientific, Santa Clara, CA) was used for analysis. The chromatographic conditions were as follows: injector temperature, 250°C; oven temperature, started at 80°C, increased to 180°C at 25°C min⁻¹, increased to 250°C at 4°C/min, and held for 5 min; column temperature, maintained at 160°C for 1 min, and then raised to 250°C at a rate of 4°C/min. Identification of compounds was confirmed by comparison with authenticated reference standards for PO (GLC-16, Nu-chek Prep). The concentrations of fatty acids were determined using the peak of the internal standard as a reference value and the standard curves of authentic compounds.

Analysis for Substrates and Products of 9R-LOX

Substrates, ALA and LA were analyzed by non-specific measurement at 202 nm, while 9R-LOX products, including 9-HPOTE, 9-HOTE, 9-HPODE, 9-HODE, 13-HOTE, and 13-HODE,
were analyzed by specific measurement at 234 nm using an reversed-phase HPLC system (Agilent 1260, Palo Alto, CA) equipped with a UV detector and a Nucleosil C18 column (150 × 2.1 mm, 5 μm particle size; Phenomenex, Torrance, CA) with a guard cartridge. The column was run and eluted with a gradient of solvent A (acetonitrile/water/acetic acid; 50:50:0.1, v/v/v) and solvent B (acetonitrile/acetic acid; 100:0.1, v/v) as described [6, 23]. The chiral phase HPLC was run with a Chiralcel OD-H column (150 × 2.1 mm, 5 μm particle size; Daicel, Tokyo, Japan) and a solvent system of n-hexane/2-propanol/acetic acid (100:5:0.1, v/v/v) [6]. The amounts of HFAs using 9R-LOX were determined using linear calibration curves and correlating the peak areas to the concentrations of standards.

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of HFAs was carried out using a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer (Thermo Scientific, Pittsburg, PA) with a Synergi fusion-RP 80Å column (100 × 30mm, 4 μm particle size; Phenomenex) using an electrospray ionization (ESI) interface. Two hundred microliters obtained from the HPLC fraction was concentrated under N2 stream to complete dryness and dissolved in 20 μl ethanol containing 0.1% acetic acid. The flow rate was 250 μl min⁻¹ and a gradient of 0.1% formic acid in acetonitrile was used. The operation parameters were as follows: capillary temperature, 275°C; ion source voltage, 5 kV; nebulizer gas, 40 psi nitrogen; capillary voltage, 46 V in positive ionization mode and 15 V in negative ionization mode; average scan time, 0.01 min; average time to change polarity, 0.02 min; and collision energy (CE), approximately 35% abundance of the precursor ion.

Statistical Analysis

The means and standard errors for all experiments, including determination of the concentrations of PUFAs and HFAs, evaluation of reaction conditions, and HPLC analysis, were calculated from triplicates. One-way analysis of variance (ANOVA) was carried out using Tukey’s method with a significance level of p < 0.05 using SigmaPlot 10.0 (Systat Software, Chicago, IL). Data were reported as mean ± standard deviation (SD) for triplicate treatments.

Results

Expression and purification of 9R-LOX from Nostoc sp.

The recombinant 9R-LOX from Nostoc sp. SAG 25.82 was purified as a soluble protein from crude extract by His-Trap affinity chromatography. The molecular mass of the purified enzyme analyzed by SDS-PAGE was approximately 52 kDa (Fig 1A), which was consistent with the calculated value of 52.3 kDa from the 456 amino acid residues and 6 histidine residues, as determined using the Compute pI/Mw tool (web.expasy.org/compute_pi/). Based on the molecular masses of the reference proteins, the native enzyme may exist as a trimer with a molecular mass of 156 kDa, as determined by gel filtration chromatography (Fig 1B).

Identification and analysis of products obtained from ALA and LA by 9R-LOX from Nostoc sp.

Purified recombinant 9R-LOX converted ALA to 9-HPOTE and 9-HOTE, and the formed 9-HPOTE subsequently reduced 9-HOTE by treatment with the reducing agent NaBH₄, based on reversed-phase HPLC at 234 nm (Fig 2A). After treatment with reducing agent during the same reaction, 9-HOTE (retention time: 13.1 min) and substrate ALA reduction were detected by non-specific measurement at 202 nm (Fig 2B). In the control reaction without the addition of enzyme, no HPFAs or HFAs were formed, indicating that LOX catalyzed the conversion of ALA to 9-HOTE. When the same reaction was carried out with LA, 9-HPODE and 9-HODE
were also formed. After treatment with reducing agent, the formed 9-HPODE was reduced to 9-HODE. The retention time of the 9-HODE was 14.3 min, which was different with that of 9-HOTE.

LC-MS/MS analysis was performed to identify the peak obtained from ALA by 9R-LOX reaction. The total molecular mass of the product was examined as a peak at \( m/z \) 294, corresponding to the molecular mass of 9-HOTE in full-scan mode of LC-MS/MS. [19, 22] The peaks at \( m/z \) 277 and \( m/z \) 276 were formed by the loss of OH and H2O from the total molecular mass (Fig 2C). Therefore, the peak was identified as 9-HOTE. The product formed with LA was also identified by LC-MS/MS as a total molecular mass peak at \( m/z \) 295 and \( m/z \) 171. These peaks were the same as those with 9-HODE from LA and coincide with reported data (S1 Fig) [28]. Since the total mass of 9S-HOTE is the same as that of 9R-HOTE, stereochemistry of the product was confirmed by HPLC using chiral-phase column chromatography with 9R- and 9S-HOTE standards. The retention time of the product was the same as that of the 9R-HOTE standard (Fig 2D). The 9-HODE product obtained from LA by 9R-LOX also showed...
Fig 2. Identification of reaction product of 9R-LOX by HPLC and LC-MS/MS. The reaction was performed in 50 mM Tris- HCl buffer (pH 8.5) containing 10 mM ALA, 0.05% Tween 80, and 0.25 U ml\(^{-1}\) enzyme at 20°C for 10 min. 

a) Reversed-phase HPLC for 9R-HPOTE and reduced 9R-HOTE formation from ALA.

b) Reversed-phase HPLC for 9R-HOTE formation and substrate (ALA) reduction. The control reaction showed no change in ALA concentration.

c) LC-MS/MS analysis of the reaction mixture showing the masses of 277.0 and 295.2. Peaks at 277.0 correspond to 9R-HPOTE, and peaks at 295.2 correspond to 9R-HOTE.

d) Standards for 9S-HOTE and 9R-HOTE were run alongside the reaction mixture to confirm the identity of the products.

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the same retention time as the 9R-ODE standard. Therefore, 9R-LOX catalyzed the conversion of 9R-HOTE and 9R-HODE from ALA and LA, respectively.

Effects of pH and temperature on the activity of 9R-LOX from Nostoc sp.

The effect of pH on the activity of 9R-LOX from Nostoc sp. for the conversion of ALA to 9R-HOTE was examined in a pH range from 7.0 to 10.5 (S2A Fig). Maximal activity was observed at pH 8.5. The activity at pH 4.5 and pH 6.5 was approximately 50% of the maximum. The effect of temperature on 9R-LOX activity was investigated by varying the temperature from 10 to 45°C at pH 8.5 (S2B Fig). Maximal activity was observed at 15°C. The thermal stability of 9R-LOX was examined at temperatures ranging from 15 to 40°C for 3 h (S2C Fig). The activity of 9R-LOX was maintained at 100% at temperatures within 15°C. However, above this temperature, the activity decreased with increasing temperature.

Effects of solvent and detergent on the activity of 9R-LOX from Nostoc sp.

The effect of organic solvent on the activity of 9R-LOX from Nostoc sp. for 9R-HOTE formation was examined at the concentrations of 4% and 6% (v/v). Among the solvents tested, acetone was the most effective solvents for LOX activity (Fig 3A). The effect of acetone concentration on the activity of 9R-LOX was evaluated by varying the concentration from 1% to 10% (v/v). The maximum enzyme activity occurred at 5% (v/v) acetone (Fig 3B). The effect of detergent on the activity of 9R-LOX for 9R-HOTE production was also investigated at concentrations of 0.02% and 0.1% (v/v). Tween 20, Tween 40, and Tween 80 at these concentrations showed 1.2- to 1.6-fold higher activity than that of control (Fig 4A). Tween 80 was the most active detergent for the production of 9R-HOTE from ALA. The effect of Tween 80 concentration on the activity of 9R-LOX was evaluated by varying the concentration from 0.1% to 0.8% (v/v). The maximum enzyme activity occurred at 0.2% (v/v) Tween 80 (Fig 4B). Solvent and detergent are important sources for the LOX reaction in mixing, dispersing reactant, and providing more chance for the substrate and catalyst to interact. The addition of 5% (v/v) acetone and 0.2% (v/v) Tween 80 was 1.9-fold higher in the conversion activity of 9R-LOX than that without the addition of solvent and detergent.

Substrate specificity of 9R-LOX from Nostoc sp.

LOX activity was optimal under the conditions of 5% (v/v) acetone and 0.2% (w/v) Tween 80 at pH 8.5 and 15°C for 5 min. Under these conditions, the specific activity and Michaelis–Menten constants (K_m), turnover numbers (k_cat), and catalytic efficiencies (k_cat/K_m) of Nostoc sp. 9R-LOX were investigated using PUFAs, including LA, ALA, and GLA (Table 1 and S3 Fig). The specific activity of the enzyme followed the order LA > ALA > GLA with 18.4, 6.3, and 5.4 U mg⁻¹, respectively. The specific activity of Nostoc sp. 9R-LOX for ALA was lower than that for LA. The catalytic efficiencies of Nostoc sp. 9R-LOX for LA, ALA and GLA were 507, 137, and 109 mM⁻¹ min⁻¹, respectively, and their order was the same as that observed for the specific activity.
Optimization of reaction conditions for the increased production of 9\textit{R}-HOTE from ALA by 9\textit{R}-LOX from \textit{Nostoc} sp.

To determine the maximal production of 9\textit{R}-HOTE from ALA using \textit{Nostoc} sp. 9\textit{R}-LOX, the enzyme concentration was investigated by varying the enzyme concentration from 0.25 to 2 g/L in the presence of 5% (v/v) acetone and 0.2% (w/v) Tween 80 at pH 8.5 and 15°C. The maximum production of 9\textit{R}-HOTE was observed at 1.0 g/L enzyme (Fig 5A). The concentration of ALA for the maximum production of 9\textit{R}-HOTE was also investigated by varying the

\textbf{Fig 3.} Effect of solvent on the activity of 9\textit{R}-LOX from \textit{Nostoc} sp. The data represent the means of three separate experiments, and error bars represent the standard deviation. \textbf{a} Effect of solvent type. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA, 0.1 U ml\textsuperscript{-1} enzyme, and 4 (black bar) or 6% (grey bar) (v/v) of solvent at 15°C for 10 min. \textbf{b} Effect of acetone concentration. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA and 0.1 U ml\textsuperscript{-1} enzyme by varying the acetone concentration from 1 to 10% (v/v).

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concentration of ALA as a substrate from 10 to 60 g/L using 1 g/L enzyme in the presence of 5% (v/v) acetone and 0.2% (w/v) Tween 80 at pH 8.5 and 15°C (Fig 5B). At concentrations below 50 g/L ALA, 9R-HOTE production was increased with increasing ALA concentration. However, the conversion yield decreased as the concentration of substrate increased. Therefore, a substrate concentration of 40 g/L ALA for 9R-HOTE production was selected as a suitable concentration for improving the performance in terms of both conversion yield and product concentration.

Fig 4. Effect of detergent on the activity of 9R-LOX from Nostoc sp. The data represent the means of three separate experiments, and error bars represent the standard deviation. a Effect of detergent type. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA, 0.1 U ml⁻¹ enzyme, and 0.02 (black bar) or 0.1% (grey bar) (w/v) of detergent at 15°C for 10 min. b Effect of Tween 80 concentration. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA and 0.1 U ml⁻¹ enzyme by varying the Tween 80 concentration from 0.005 to 1% (w/v) at 15°C for 10 min.

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Table 1. Specific activity and kinetic parameters of recombinant Nostoc sp. 9R-LOX for PUFAs.

| Substrate       | Product                  | Specific activity (U mg⁻¹) | $K_m$ (μM) | $k_{cat}$ (1/min) | $k_{cat}/K_m$ (mM⁻¹ min⁻¹) |
|-----------------|--------------------------|---------------------------|------------|------------------|----------------------------|
| Linoleic acid   | 9R-HODE                  | 18.4 ± 1.1                | 404 ± 30   | 307 ± 8          | 760 ± 8                    |
| α-Linolenic acid| 9R-HOTE (9Z,12E,15Z)     | 6.3 ± 0.4                 | 54 ± 1.3   | 7.8 ± 0.2        | 144 ± 3                    |
| γ-Linolenic acid| 9R-HOTE (6Z,10E,12Z)     | 5.4 ± 0.6                 | 54 ± 0.8   | 5.8 ± 0.4        | 109 ± 7                    |

HODE: hydroxyoctadecadienoic acid, HOTE: hydroxyoctadecatrienoic acid

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Fig 5. Effects of the concentrations of enzyme and substrate on the production of 9R-HOTE from ALA by 9R-LOX. The data represent the means of three separate experiments, and error bars represent the standard deviation. 

a Effect of enzyme concentration. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 10 g l⁻¹ ALA, 5% (v/v) acetone, and 0.2% (w/v) Tween 80 at 15°C for 20 min by varying the enzyme concentration from 0.05 to 2 g l⁻¹.

b Effect of ALA concentrations. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 g l⁻¹ enzyme, 5% (v/v) acetone, and 0.2% (w/v) Tween 80 at 15°C for 20 min by varying the ALA concentrations from 10 to 80 g l⁻¹.

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Preparation of PO hydrolyzate by CRL and its fatty acid composition

Natural PO was hydrolyzed by CRL because *Nostoc* sp. 9R-LOX cannot direct the hydroxylation of PO. ALA from PO was prepared by the hydrolysis of PO using CRL, because CRL exhibited the highest activity for releasing free ALA from PO among the lipases tested (S4 Fig). The hydrolytic reaction for the oil was performed with 10 g/L PO and 1 g/L CRL at pH 7.5, 35°C, and 250 rpm for 120 min. At 90 min, PO hydrolyzate contained 6 g/L ALA, 1.5 g/L LA, 1.7 g/L oleic acid (OA), and 0.8 g/L saturated fatty acids (Fig 6A). After 90 min, the contents of these fatty acids decreased with increasing reaction time due to reverse reaction. Therefore, the reaction was terminated at 90 min, and the resulting composition is presented in S1 Table. The obtained PO hydrolyzate contained 60% ALA and 15% LA, and its composition was similar to that obtained the previously published data [15]. Therefore, a total of 75% substrates (ALA and LA) in PO hydrolyzate can be utilized for 9R-HOTE and 9R-HODE production using 9R-LOX.

Time-course reactions for the production of 9R-HOTE and 9R-HODE from ALA and LA by 9R-LOX from *Nostoc* sp.

The optimal conditions for 9R-HOTE conversion of the recombinant purified 9R-LOX from *Nostoc* sp. were pH 8.5, 15°C, 1 g/L enzyme, 40 g/L reagent ALA, 5% (v/v) acetone, and 0.02% (w/v) Tween 80 with agitation speed of 250 rpm and oxygen at 20 ml of working volume in a 250 ml-baffled flask. Under the optimal conditions, the production of 9R-HOTE and 9R-HODE from PO hydrolyzate containing ALA and LA were performed using 9R-LOX. The enzyme produced 36.5 g/L of 9R-HOTE and 9.5 g/L of 9R-HODE from 40 g/L of ALA and 10 g/L of LA in PO hydrolyzate for 100 min. 9R-HOTE production from ALA in PO hydrolyzate was increased to 34 g/L at 60 min and plateaued at 35 g/L at 80 min. The conversion yield and volumetric productivity for 9R-HOTE from ALA in PO hydrolyzate were 85% and 34 g/L/h, respectively. 9R-HODE production from LA in PO hydrolyzate was more rapid than that from ALA and plateaued at 20 min with a conversion yield of 95% (Fig 6B). 9R-LOX produced 37.6 g/L 9R-HOTE from 40 g/L of reagent-grade ALA at 60 min, with a conversion yield of 94%, a molar yield of 89%, and a volumetric productivity of 37.6 g/L/h (Fig 6C). Since the 9R-LOX activity for LA was highest, the enzymatic conversion of 9R-HODE from reagent grade LA was also performed under the same conditions. 9R-LOX produced 38.4 g/L 9R-HODE from 40 g/L LA at 60 min, with a conversion yield of 96%, a molar yield of 91%, and a volumetric productivity of 38.4 g/L/h (Fig 6D). These results are the first data for showing the enzymatic production of 9R-HOTE and 9R-HODE from not only reagent grade ALA and LA but also ALA and LA in PO hydrolyzate using LOX (Table 2).

Discussion

The characterization and application of bacterial LOX emerged only last decades. Recent investigations revealed that bacterial LOX possessed different properties including unusual stereoselectivity as well as substrate specificity and molecular size, comparing with plant and animal LOXs. Dimeric form comprising N-terminal domain and the catalytic β-barrel domain is common in LOX but the molecular masses of the reported bacterial LOXs were differently ranged as a mainly 44–65 kDa [6, 26], comparing with a 70–80 kDa in fungi [7], 94–104 kDa dimer in plants, and 75–80 kDa monomer in animals [3, 29]. *Nostoc* sp. recombinant 9R-LOX also showed short N-terminal domain and catalytic domain with 52 kDa of molecular mass in denatured form, however, native form of associated subunit is firstly identified as a trimeric LOX in this study (Fig 1).
Fig 6. Time-course reactions for the production of fatty acids by CRL, 9R-HOTE and 9R-HODE by recombinant 9R-LOX. The data represent the means of three separate experiments, and error bars represent the standard deviation. A. Production of fatty acids from PO by CRL. Fatty acids were α-linoleic acid (filled circle), linoleic acid (empty circle), oleic acid (filled inverse triangle), and other fatty acids (empty triangle). The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing 1 g l⁻¹ CRL, 10 g l⁻¹ PO, and 0.02% (w/v) Tween 80 at 37°C with agitation at 200 rpm for 2 h. B. Production of 9R-HOTE (empty circle) and 9R-HODE (empty triangle) from ALA (filled circle) and LA (filled inverse triangle) in PO hydrolyzate. The reactions were performed in a 500 mL baffled flask in 80 mL of 50 mM Tris-HCl buffer (pH 8.5) consisting of PO hydrolyzate, which contained 40 g l⁻¹ ALA, 10 g l⁻¹ LA, 15.3 g l⁻¹ of other fatty acids, 5% (v/v) acetonitrile, 0.2% (v/v) Tween 80, and 1 g l⁻¹ purified 9R-LOX at 15°C for 100 min with agitation at 250 rpm. C. Production of 9R-HOTE (empty circle) from reagent-grade ALA (filled circle) by 9R-LOX. The reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 40 g L⁻¹ reagent-grade ALA, 1 g L⁻¹ 9R-LOX, 5% (v/v) acetonitrile, and 0.2% (w/v) Tween 80 at 15°C for 80 min. D. Production of 9R-HODE (empty inverse triangle) from reagent-grade LA (filled circle) by 9R-LOX. The reactions were performed in the same manner as those of 9R-HOTE production, except for the substrate LA.

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The substrate specificity of bacterial LOXs toward omega-3 PUFAs including ALA based on kinetic parameters is also noteworthy properties. The kinetic parameters of bacterial LOXs can be determined by not only an O₂ probe method but also a spectrophotometry method. However, it is difficult to measure the activities at the high concentrations of substrate and enzyme using the spectrophotometry method. Moreover, the spectrophotometry method is less accurate. Therefore, only O₂ probe method was used in the present study for the determination of the kinetic parameters of LOX. The kinetic parameters of the known microbial LOX enzymes for ALA have been reported only in LOXs from Acaryochloris marina, Burkholderia

Table 2. Production of HPFA and HFA from PUFA and oil hydrolyzate by LOX.

| Source of LOX | Biocatalyst | Substrate (g/L) | Product (g/L) | Conversion yield (%) | Productivity (g/L/h) | References |
|---------------|-------------|----------------|--------------|----------------------|----------------------|------------|
| potato        | immobilized | LA (0.12)      | 9-HODE (0.077) | 64.2                 | NA                   | [39]       |
| potato        | crude        | TLH (1.5)      | 9S-HPOTE (1.24) | 82.7                 | NA                   | [40]       |
| soybean       | two-phase   | LA (20)        | 13S-HPODE* (12.04) | 60.2                 | NA                   | [33]       |
| soybean       | crude        | LA (40)        | 13S-HPODE (36) | 90                    | 9                    | [34]       |
| soybean       | crude        | TLH (6**       | 13S-HPODE (5.7) | 96.1                 | NA                   | [35]       |
| soybean       | crude        | FOH (78.5**)   | 13S-HPOTE**** (62.8) | 80                  | 31.4                 | [36]       |
| soybean       | partial purified | LA (28) | 13S-HPDE (22.4) | 80                  | 22.4                 | [37]       |
| soybean       | partial purified | LA (28) | 13S-HPDE (15.1) | 54                  | NA                   | [38]       |
| G. graminis tritici LOX in T. reesei | secreted crude | LA (300) | 13S-HPODE* (120) | 40                  | 3.6                  | [42]       |

B. thailandensis LOX in E. coli
Recombinant purified enzyme

LA (20) | 13S-HODE (20.8) | 98.5 | 10.4 | [31] |

P. aeruginose LOX in T. reesei
secreted crude

LA (10) | 9S- and 13S-HPDE (7.5) | 75 | 0.16 | [41] |

Nostoc sp. LOX in E. coli
Recombinant whole cell

LA (15) | 9R-HODE (14.3) | 95 | 14.3 | [23] |

Nostoc sp. LOX in E. coli
Recombinant purified enzyme

LA(40) | 9R-HODE (38.4) | 96 | 38.4 | This study |

ALA(40) | 9R-HOTE (37.6) | 94 | 37.6 | This study |
POH (40***) | 9R-HOTE (35.2) | 85 | 34 | This study |

NA: Not available, TLH: Trilinolein hydrozate, FOH: Flax seed oil hydrozate, POH: Perilla seed oil hydrozate.

* E,Z-form only
** LA only
*** ALA only
**** E,Z,Z-form only.

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with a conversion yield of 62.8% and a productivity of 31.4 g/L/h [36]. Fungal LOX such as to 9-HODE production because of its low yield. Therefore, 9

Nostoc sp. Existing sources and methodology for the production of 9-hydroxy fatty acid relied

sion yield of 98.5% [29].

Thus, the LA-rich vegetable oil was not applied to the production of HFA by 9

as well as LA- and ALA-rich vegetable oil.

The production of HPFAs and HFAs is summarized in Table 2. Soybean LOX (SLOX) was mainly used for the enzymatic production of 13S-HPODE from LA by the crude extract or the partially purified enzyme conversion, immobilization, biphasic reaction [33–38]. Crude extract of SLOX produced 5–62 g/L with 54–96% conversion yield of 13-HPODE from LA. Alternatively, potato LOX has also been used for production of 9S-HPODE or 9S-13-HODE from LA using crude extract or by immobilization [39, 40]. A few reports showed HPODE production from vegetable oil. Trilinolein hydrolyzate (TLH) containing LA and flax seed oil hydrolyzate (FOH) containing ALA were used for the production of 13S-HPODE and 13S-13-HOPE, respectively. SLOX produced 5.7 g/L 13S-HPODE from 6 g/L LA in TLH with a conversion yield of 96% [35]. SLOX produced the 10E,12Z,15Z-form of 13S-13-HPOTE from 78.5 g/L ALA in FOH with a conversion yield of 62.8% and a productivity of 31.4 g/L/h [36]. Fungal LOX such as Gaemumownomyces graminis tritici LOX secreted in Trichoderma reesei showed a conversion yield of 40% and a productivity of 3.6 g/L/h for 10E,12Z-13S-13-HPOTE production [41]. A LOX from Pseudomonas aeruginosa that was cloned and secreted in Trichoderma reesei showed mixed production of 9S- and 13S-13-HPODE from LA with a low yield [42]. Recently, recombinant Burkholderia thailandensis LOX showed 10.4 g/L/h of productivity with a molar conversion yield of 98.5% [29].

In the present study, recombinant purified 9R-LOX from Nostoc sp. produced 9R-HOTE with a higher conversion yield and productivity from ALA in PO hydrolyzate as well as reagent-grade ALA than those of other LOXs for PUFAs (Table 2, Fig 6B and 6C). 9R-LOX also converted 9R-10E-13Z-HODE from reagent grade LA with the highest yield and productivity ever reported (Table 2 and Fig 6D). As shown in Table 1, the catalytic efficiency of 9R-LOX for LA was 5.3-fold higher than that for ALA, indicating that LA is a better substrate than ALA. If LA-rich vegetable oil is used, the production of HFA by 9R-LOX from Nostoc sp. will increase. The typical LA-rich vegetable oil is safflower oil, which is consisted of 80% LA, 14% oleic acid, and 5% palmitic acid. The present study is focused on the stereo-selective enzymatic production of 9R-HOTE. However, safflower oil was not used for 9R-HOTE because it contained little ALA. Thus, the LA-rich vegetable oil was not applied to the production of HFA by 9R-LOX from Nostoc sp. Existing sources and methodology for the production of 9-hydroxy fatty acid relied on LOX from only plant source such as potato. However, plant source LOX cannot be applied to 9-HODE production because of its low yield. Therefore, 9R-LOX is an alternative and efficient biocatalyst for 9R-HOTE and 9R-13-HODE production from reagent grade ALA and LA as well as LA- and ALA-rich vegetable oil.

HFAs originating from the LOX reaction exhibited a variety of bioactivities including antifungal and immune modulation activity [19, 43]. Thus, the production and application of
HFAs from ALA using LOX is interesting for further understanding of signaling cascades. Vegetable oils are widely utilized in the human diet and in many other industrial applications [44]. Furthermore, a large volume of surplus vegetable oil is currently an important issue [45]. The PUFA component in vegetable oils provides an opportunity to manufacture value-added products such as HFAs, green notes, and lactones. ALA and LA are one of the major components in vegetable oils including PO, flax seed oil, and chia oil, which contain over 60% ALA. However, except for LA, the current LOXs are limited in their practical utilization of n-3 or n-6 PUFAs. Therefore, our study offers a new opportunity for the biotechnological production of value added product from vegetable oil using recombinant LOX, by the efficient utilization of PUFAs in vegetable oil.

Conclusion

In conclusion, the production of 9R-HOTE from ALA in PO was demonstrated through enzymatic hydrolysis and oxygenation processes. CRL-treated PO hydrolyzate containing ALA and LA was converted into 9R-HOTE and 9R-HODE by purified recombinant 9R-LOX. To the best of our knowledge, our enzymatic conversion exhibits the highest productivity and yield of 9R-HOTE and 9R-HODE from ALA and LA in natural oil by recombinant LOX, respectively, reported up to date. Thus, the enzymatic production of 9R-HOTE from PUFA of vegetable oil may be useful for efficient oxyfunctionalization and value-added product biotransformation.

Supporting Information

S1 Fig. LC-MS/MS analysis of 9R-HODE from LA by 9R-LOX. The reaction was performed in 50 mM Tris-HCl buffer (pH 8.5), 10 mM LA, and 0.25 U ml\(^{-1}\) enzyme at 25°C for 5 min. (TIF)

S2 Fig. Effects of pH, temperature, and stability on the activity of 9R-LOX from Nostoc sp. The data represent the means of three separate experiments, and error bars represent the standard deviation. a Effect of pH. The reactions were performed by varying the pH from 7.0 to 10.0 using 50 mM Tris-HCl buffer (pH 7.0–9.0) (filled circle) and 50 mM sodium borate buffer (pH 9.0–10.0) (empty circle) containing 0.25 mM ALA and 0.05 U/mL enzyme at a constant temperature of 25°C for 5 min. b Effect of temperature. The reactions were performed by varying the temperature from 10 to 50°C in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA and 0.05 U ml\(^{-1}\) enzyme for 5 min. c The effect of temperature on 9R-LOX stability. Experiments were carried out after incubation at temperatures ranging from 4 to 45°C for 4 h. Samples were withdrawn at time intervals and then assayed in 50 mM Tris-HCl buffer (pH 8.5) containing 0.25 mM ALA and 0.05 U ml\(^{-1}\) enzyme at 15°C for 5 min. (TIF)

S3 Fig. Hanes-Woolf plot (S vs S/V) from the Michaelis-Menten equation of 9R-LOX from Nostoc sp. for each unsaturated fatty acid substrate. a Reacted with LA. b Reacted with ALA. c Reacted with GLA. (TIF)

S4 Fig. Activities of lipases for releasing ALA from PO. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5), 1 U ml\(^{-1}\) lipase, and 0.1 g L\(^{-1}\) of PO at 30°C for 30 min. Recombinant AOL, CRL, TLL, RML, PCL, and PFL were used for the hydrolysis of PO. (TIF)

S1 Table. Composition of fatty acids for PO hydrolyzate. (DOCX)
Author Contributions
Conceived and designed the experiments: KRK DKO. Performed the experiments: KRK JUA SHL. Analyzed the data: KRK JUA SHL DKO. Contributed reagents/materials/analysis tools: DKO. Wrote the paper: KRK DKO.

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