Biosynthesis of New Epoxy Fatty Acids from C18 Polyunsaturated Fatty Acids by Linoleate 9-Lipoxygenase from *Sphingopyxis macrogoltabida*

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Abstract

Epoxy fatty acids (EFAs), which exist in the human body, are signaling molecules that maintain homeostasis. They are involved in anti-inflammation and are precursors of dihydroxy fatty acids. EFAs derived from the C20 polyunsaturated fatty acid (PUFA) arachidonic acid by lipoxygenases, such as 5,6-epoxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid and 8,9-epoxy-5*Z*,10*E*,12*E*,14*Z*-eicosatetraenoic acid, are found in humans and mice, respectively. However, EFAs derived from C18 PUFAs by lipoxygenases have not been identified to date. In this study, the putative lipoxygenase gene of *Spingopyxis macrogoltabida* was cloned and expressed in *Escherichia coli*. The activity and catalytic efficiency ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) of the recombinant enzyme were the highest for linoleic acid among the C18 PUFAs, including also α-linolenic acid and γ-linolenic acid. The product obtained from the conversion of linoleic acid by the putative lipoxygenase was identified as 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE) by high-performance liquid chromatography using 9-HODE and 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE) standards. These results indicate that the enzyme is a linoleate 9-lipoxygenase. The enzyme converted linoleic acid, α-linolenic acid, and γ-linolenic acid into 9-HODE, 13-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid, and 9-hydroxy-6*Z*,10*E*,12*Z*-octadecatrienoic acid, respectively. Moreover, the enzyme also converted the three C18 PUFAs into 9,10-epoxy-11*E*,13*E*-octadecadienoic acid, 12,13-epoxy-8*E*,10*E*,15*Z*-octadecatrienoic acid, and 9,10-epoxy-6*Z*,11*E*,13*E*-octadecatrienoic acid, respectively, which were identified as new EFAs by liquid chromatography-mass spectrometry/mass spectrometry. To our knowledge, this is the first report on the biosynthesis of EFAs from C18 PUFAs via a lipoxygenase.

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Running title: Lipoxygenase-Derived Epoxy Fatty Acids

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Abstract Epoxy fatty acids (EFAs), which exist in the human body, are signaling molecules that maintain homeostasis. They are involved in anti-inflammation and are precursors of dihydroxy fatty acids. EFAs derived from the C20 polyunsaturated fatty acid (PUFA) arachidonic acid by lipoxygenases, such as 5,6-epoxy-7E ,9E ,11Z ,14Z -eicosatetraenoic acid and 8,9-epoxy-5Z ,10E ,12E ,14Z -eicosatetraenoic acid, are found in humans and mice, respectively. However, EFAs derived from C18 PUFAs by lipoxygenases have not been identified to date. In this study, the putative lipoxygenase gene of *Spingopyxis macrogoltabida* was cloned and expressed in *Escherichia coli* . The activity and catalytic efficiency ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) of the recombinant enzyme were the highest for linoleic acid among the C18 PUFAs, including also α-linolenic acid and γ-linolenic acid. The product obtained from the conversion of linoleic acid by the putative lipoxygenase was identified as 9-hydroxy-10E ,12Z -octadecadienoic acid (9-HODE) by high-performance liquid chromatography using 9-HODE and 13-hydroxy-9Z ,11E -octadecadienoic acid (13-HODE) standards. These results indicate that the enzyme is a linoleate 9-lipoxygenase. The enzyme converted linoleic acid, α-linolenic acid, and γ-linolenic acid into 9-HODE, 13-hydroxy-9Z ,11E ,15Z -octadecatrienoic acid, and 9-hydroxy-6Z ,10E ,12Z -octadecatrienoic acid, respectively. Moreover, the enzyme also converted the three C18 PUFAs into 9,10-epoxy-11E ,13E -octadecadienoic acid, 12,13-epoxy-8E ,10E ,15Z -octadecatrienoic acid, and 9,10-epoxy-6Z ,11E ,13E -octadecatrienoic acid, respectively, which were identified as new EFAs by liquid chromatography-mass spectrometry/mass spectrometry. To our knowledge, this is the first report on the

biosynthesis of EFAs from C18 PUFAs via a lipoxygenase.

Keywords C18 Polyunsaturated fatty acids [?] 9,10-Epoxy-11E ,13E -octadecadienoic acid [?] 12,13-Epoxy-8E ,10E ,15Z -octadecatrienoic acid [?] 9,10-Epoxy-6Z ,11E ,13E -octadecatrienoic acid [?] Lipoxygenase [?] Liquid chromatography-mass spectrometry/mass spectrometry

Abbreviations

PUFA	Polyunsaturated fatty acid
EFA	Epoxy fatty acid
HFA	Hydroxy fatty acid
HpFA	Hydroperoxy fatty acid
9-HODE	9-Hydroxy-10E,12Z-octadecadienoic acid
13-HODE	13-Hydroxy-9Z,11E-octadecadienoic acid
13-HOTrE	13-Hydroxy-9Z,11E,15Z-octadecatrienoic acid
9-HOTrEγ	9-Hydroxy-6Z,10E,12Z-octadecatrienoic acid
9-HpODE	9-Hydroperoxy-10E,12Z-octadecadienoic acid
13-HpOTrE	13-Hydroperoxy-9Z,11E,15Z-octadecatrienoic acid
9-HpOTrEγ	9-Hydroperoxy-6Z,10E,12Z-octadecatrienoic acid
9,10-EpODE	9,10-Epoxy-11E,13E-octadecadienoic acid
12,13-EpOTrE	12,13-Epoxy-8E,10E,15Z-octadecatrienoic acid
9,10-EpOTrEγ	9,10-Epoxy-6Z,11E,13E-octadecatrienoic acid
HEPPS	3-[4-(2-Hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
ITMS-ESI	Ion trap mass spectrometry-electrospray ionization
LIPID MAPS	Lipid metabolites and pathways strategy

Introduction

Unsaturated fatty acids are converted into valuable products such as hydroxy fatty acids (HFAs) (Kim and Oh, 2013), epoxy fatty acids (EFAs) (Zhang et al., 2014), epoxy hydroxy fatty acids (Teder et al., 2017; Yang et al., 2019), dihydroxy fatty acids (Morisseau et al., 2010), and trihydroxy fatty acids (An et al., 2019) by fatty acid oxygenating enzymes, such as cytochrome p450s, hydratases, diol synthases, and lipoxygenases, and epoxide hydrolases. Among these enzymes, cytochrome p450 epoxygenases and lipoxygenases can synthesize EFAs with an epoxy ring via different mechanisms. Cytochrome p450 epoxygenases convert a double bond in polyunsaturated fatty acids (PUFAs) into a single bond (Aliwarga et al., 2017), whereas lipoxygenases move the double bond by one carbon position (Haeggstrom and Funk, 2011). Therefore, they produce different EFAs using the same PUFAs. The newly formed EFAs are rapidly converted into dihydroxy fatty acids by soluble epoxide hydrolases*in vivo* (Inceoglu et al., 2015).

Cytochrome p450 epoxygenases convert C18 unsaturated fatty acids, such as oleic acid, linoleic acid, and α-linolenic acid, into 9,10-epoxyoctadecanoic acid (Tsikas et al., 2003); 9,10-epoxy-12Z -octadecenoic acid (Spector and Kim, 2015) and 12,13-epoxy-9Z -octadecenoic acid (Vatanparast et al., 2020); 9,10-epoxy-12Z ,15Z -octadecadienoic acid (Wang et al., 2019), 12,13-epoxy-9Z ,15Z -octadecadienoic acid (Walker et al., 2021), and 15,16-epoxy-9Z ,12Z -octadecadienoic acid (Holt et al., 2015), respectively. Cytochrome p450 epoxygenases convert C20 and C22 PUFAs, such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, into C20 and C22 EFAs, such as 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids, 5,6-, 8,9-, 11,12-, 14,15-, and 17,18-epoxyeicosatetraenoic acid, 7,8-, 10,11-, 13,14-, 16,17-, and 19,20-epoxydocosapentaenoic acids, respectively (Spector and Kim, 2015). These cytochrome p450 epoxygenase-derived EFAs decrease hypertension and pain perception and are involved in homeostasis maintenance and

anti-inflammation (McReynolds et al., 2020). Lipoxygenases convert the C20 PUFA arachidonic acid into C20 EFAs, such as 5,6-epoxy-7E ,9E ,11Z ,14Z -eicosatetraenoic acid, which is a pro-inflammatory compound (Jafaru and Justin, 2000), and 8,9-epoxy-5Z ,10E ,12E ,14Z -eicosatetraenoic acid (Kawajiri et al., 2005). However, EFAs derived from C18 PUFAs by lipoxygenases have not been identified to date.

Vernolic acid (12,13-epoxy-9Z -octadecenoic acid), a representative chemically synthesized EFA, can be used as a plasticizer for polyvinyl chloride in the chemical industry because of the cross-linking ability of its epoxy group, and oils containing vernolic acid are useful as adhesives and coating materials in paints (Cahoon et al., 2002). The compound 9,10-epoxy-18-hydroxyoctadecanoic acid exists as a component of cutin in plants, and is an essential polymer that protects plants by minimizing the influence of pathogens by waterproofing the leaves and fruits of plants (Elizabeth, 2002). The levels of linoleic acid-derived EFAs, namely 9,10-epoxyoctadecanoic acid and 9,10-epoxy-12Z -octadecenoic acid, are found to be increased in rheumatoid arthritis (Jira et al., 1998) and in patients with severe burns (Newman et al., 2002).

In this study, the biochemical properties, substrate specificity, and the product of the putative protein of *Sphingopyxis macrogoltabida* were evaluated, and the enzyme was identified as linoleate 9-lipoxygenase. The enzyme converted C18 PUFAs, including linoleic acid, α-linolenic acid, and γ-linolenic acid into EFAs, which were identified through liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis.

Materials and Methods

Materials

Linoleic acid, α-linolenic acid, and γ-linolenic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HFA standards, 9-hydroxy-10E ,12Z -octadecadienoic acid (9-HODE) and 13-hydroxy-9Z ,11E -octadecadienoic acid (13-HODE) were purchased from Cayman Chemical (Ann Arbor, MI, USA) (>99% purity).

Methods

Microorganisms, plasmid, and culture conditions

S. macrogoltabida DSMZ 8826 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), *Escherichia coli*ER2566, and the plasmid pET-28a were used as the source of genomic DNA, host cells, and expression vector, respectively. Recombinant *E. coli* was incubated at 37 °C and 200 rpm in a 2-L flask with 450 mL of Luria-Bertani medium (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride) containing 0.048 mg/mL kanamycin. When the optical density of the bacterial culture reached 0.6 at 600 nm, 0.024 g/L isopropyl-β-d-thiogalactopyranoside was added to induce enzyme expression, and the culture was incubated at 16 °C and 150 rpm for 16 h.

Gene cloning

The gene encoding a putative lipoxygenase was amplified by polymerase chain reaction using *S. macrogoltabida* genomic DNA as a template and *Taq* polymerase (Solgent, Daejeon, Republic of Korea) with the below described primers. The primers used for gene cloning were designed based on the DNA sequence of the putative lipoxygenase from *S. macrogoltabida* (GenBank accession number, A0A0P0DSJ0). Forward (5'-GCTCGAC*CATATG* TCCTTTGTGTCGCCTTCGCTG-3') and reverse primers (5'-GCGCCC*AAGCTT* TCAGATATTGATGCTCGTCGGG-3') were designed to introduce the *Nde* I and *Hind* III restriction sites (underlined), respectively, and were synthesized by Bioneer (Daejeon, Republic of Korea). The amplified DNA fragments were ligated into the pET-28a vector and transformed into *E. coli* ER2566. Recombinant*E. coli* was plated on Luria-Bertani agar containing 0.048 mg/mL kanamycin to select antibiotic-resistant colonies, and the plasmid DNA was sequenced at the Macrogen facility (Seoul, Republic of Korea).

Enzyme purification

The harvested cells were suspended in 50 mM phosphate buffer (pH 8.0) containing 10 mM imidazole and

1.77 g/L NaCl and disrupted by sonication in an ice bath. Cell debris were removed by centrifugation at 13,000 $\times g$ for 10 min at 4 ℃, and the supernatant was applied to an immobilized metal ion affinity chromatography cartridge (Bio-Rad, Hercules, CA, USA) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 1.77 g/L NaCl. The bound protein was eluted with a linear gradient of 10-250 mM imidazole at a flow rate of 1 mL/min. The fractions containing active proteins were collected and loaded onto a Bio-Gel P-6 desalting cartridge (Bio-Rad) equilibrated with 50 mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS) buffer (pH 8.5). The loaded protein was eluted using the same buffer at a flow rate of 1 mL/min, and the eluted protein was used as the purified enzyme for the enzyme assays.

Determination of specific activity and kinetic parameters

The specific activity of the lipoxygenase for C18 PUFAs as substrates was determined by measuring the increase in absorbance at 234 nm due to the production of HFAs using a Beckman Coulter DU-700 spectrophotometer (Brea, CA, USA) after incubation in 50 mM HEPPS buffer containing 0.05-0.1 mM substrate and 0.05-0.2 mg/mL enzyme in the presence of 200 mM cysteine at 30 degC for 5 min. Specific activity was defined as the amount of product per unit reaction time per amount of protein. To determine the kinetic parameters, the reactions were performed in the presence of 200 mM cysteine at 30 degC in 50 mM HEPPS buffer (pH 8.5) by varying the concentration of C18 PUFA from 10 μM to 100 μM for 5 min. The values of K_{m} (mM) andk_{cat} (1/min) were determined by non-linear regression using GraphPad Prism 8.0 (San Diego, CA, USA).

Determination of molecular mass

The subunit molecular mass of the putative lipoxygenase from *S. macrogoltabida* was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions using molecular mass marker proteins as standard proteins (MBI Fermentas, Glen Burnie, MD, USA). The total molecular mass of the native enzyme was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative-grade column (GE Healthcare, Little Chalfont, UK). The enzyme solution was applied to the column and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 8.77 g/L NaCl at a flow rate of 1 mL/min. The column was calibrated with ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa), used as standard proteins (GE Healthcare). The total molecular mass of the native enzyme was calculated by comparing the retention times of the standard proteins.

Analytical methods

All compounds were analyzed at 234 nm using a high-performance liquid chromatography (HPLC) system (Agilent 1260) with a reverse-phase Nucleosil C18 column (3.5×150 mm, 5-μm particle size; Phenomenx) and a normal-phase Zorbax Rx-Sil column (2.1×150 mm, 5-μm particle size; Agilent). HFAs and EFAs were monitored using the reversed-phase column at 202 nm and HFAs were also monitored at 234 nm using a normal-phase column. The reversed-phase column was eluted at 30 °C with 100% solvent A (acetonitrile/water/acetic acid, 50:50:0.1, v/v/v) at a flow rate of 0.25 mL/min for 0-5 min, solvent A and to solvent B (acetonitrile/acetic acid, 100:0.1, v/v) at 0.25 mL/min for 5-21 min, 100% solvent B at 0.4 mL/min for 21-24 min, solvent B to solvent A at 0.4 mL/min for 24-27 min, and 100% solvent A at 0.25 mL/min for 27-30 min. The normal-phase column was eluted at 30 degC with 100% solvent A (n -hexane/diethyl ether/acetic acid, 70:30:0.25, v/v/v) at a flow rate of 0.2 mL/min for 50 min.

LC-MS/MS analysis for HFAs and EFAs was performed using an LCQ Deca XP plus ion trap mass (Thermo Fisher Scientific, Pittsburgh, PA, USA) at the NICEM facility (Seoul National University, Seoul, Republic of Korea). The electrospray ionization conditions were as follows: 275 degC; 5 kV; 30 ?; 20 V in negative mode; 46 V in positive mode; average scan time, 0.01 min; average time to change polarity, 0.02 min; and abundance of precursor ions at collision energy, 35%.

Results and Discussion

Biochemical properties of the putative protein from *S. macrogoltabida*

The gene (1938 bp) encoding a putative protein from the bacterium*S. macrogoltabida* having the same sequence as that reported in GenBank (accession number: A0A0P0DSJ0) was cloned into the pET-28a vector and transformed into*E. coli* . The protein was purified from disrupted recombinant*E. coli* expressing the putative protein using immobilized metal ion affinity chromatography and Bio-Gel P-6 desalting cartridges. The expression of the putative protein in *E. coli* was confirmed as a single band by SDS-PAGE with a molecular mass of approximately 70 kDa, which is consistent with the molecular mass calculated from the 646 amino acids plus 6 histidine residues (Fig. 1a). The total protein mass of the purified putative protein was 140 kDa by gel filtration chromatography (Fig. 1b), indicating that the putative protein exists as a homodimer.

The amino acid sequence of the putative protein was aligned with the amino acid sequences of other known lipoxygenases. The putative protein isolated from *S. macrogoltabida* showed 14%, 25.3%, and 13.5%; and 19.2%, 40.6%, and 38.2% amino acid sequence identity with the characterized linoleate 9-lipoxygenases of *Nostoc* sp.,*Myxococcus xanthus* , and *Acaryochloris marina* ; and linoleate 13-lipoxygenases of soybean, *Pseudomonas aeruginosa* , and *Burkholderia thailandensis* , respectively. The metal-binding residues (HHHNI) of the putative protein of *S. macrogoltabida*were the same as those of lipoxygenases (Liavonchanka and Feussner, 2006) (Table 1), suggesting that the putative protein was a lipoxygenase.

Identification of the putative lipoxygenase from *S. macrogoltabida* as linoleate 9-lipoxygenase

To identify the putative lipoxygenase, the reaction products obtained from the conversion of linoleic acid in the absence of cysteine by this enzyme were analyzed by HPLC (Fig. 2a). The three reaction products (1), (2), and (3) were identified as 9-hydroperoxy-10E ,12Z -octadecadienoic acid (9-HpODE), 9,10-epoxy-11E ,13E -octadecadienoic acid (9,10-EpODE), and 9-HODE by LC-MS/MS analysis, respectively. The reduced product (3) obtained from the conversion of linoleic acid in the presence of cysteine was analyzed by HPLC using a reverse-phase column based on the retention times using 9-HODE and 13-HODE standards. The retention time of the reaction product was the same as that of 9-HODE standard (Fig. 2b), indicating that the reaction product of the putative lipoxygenase was 9-HODE. The bacterial 9-lipoxygenases from *Nostoc* sp. (Kim et al., 2015) and *A. marina* (Gao et al., 2010) have also been shown to produce 9-HODE from linoleic acid.

The catalytic efficiency (k_{cat}/K_m) of the enzyme for C18 PUFAs was evaluated, and it was the highest for linoleic acid (1,370 min^{-1} mM^{-1}), followed by α-linolenic acid (756 min^{-1} mM^{-1}), and γ-linolenic acid (715 $min^{-1}mM^{-1}$) (Table 2). Therefore, the putative lipoxygenase from *S. macrogoltabida* is a linoleate 9-lipoxygenase. Thek_{cat}/K_m of linoleate 9-lipoxygenase from *S. macrogoltabida* for linoleic acid was 1.8-fold and 113-fold higher than those of linoleate 9-lipoxygenases from *Nostoc* sp. (Kim, et al., 2015) and *A. marina* (Gao, et al., 2010), respectively (Table 2), indicating that the enzyme is an efficient linoleate 9-lipoxygenase.

Biosynthesis of HFAs and EFAs from C18 PUFAs by linoleate 9-lipoxygenase from *S. macrogoltabida*

Linoleate 9-lipoxygenase from *S. macrogoltabida* converted linoleic acid into 9-HpODE via peroxidation. 9-HpODE was converted into 9-HODE by reduction in the presence of cysteine as a reducing agent or 9,10-EpODE by dehydration in the absence of cysteine (Fig. 3a). The enzyme also converted α-linolenic acid and γ-linolenic acid into 13-hydroperoxy-9Z ,11E ,15Z -octadecatrienoic acid (13-HpOTrE) and 9-hydroperoxy-6Z ,10E ,12Z -octadecatrienoic acid (9-HpOTrEγ), respectively, which were reduced to 13-hydroxy-9Z ,11E ,15Z -octadecatrienoic acid (13-HOTrE) and 9-hydroxy-6Z ,10E ,12Z -octadecatrienoic acid (9-HOTrEγ) in the presence of cysteine, respectively. The synthesized 13-HpOTrE and 9-HpOTrEγ were dehydrated into 12,13-epoxy-8E ,10E ,15Z -octadecatrienoic acid (12,13-EpOTrE) and 9,10-epoxy-6Z ,11E ,13E -octadecatrienoic acid (9,10-EpOTrEγ) in the absence of cysteine, respectively (Fig. 3b, c). However, even without the reducing agent, HFAs are formed simultaneously with EFAs from hydroperoxy fatty acids (HpFAs) by natural reduction.

Linoleate 9-lipoxygenases from *Nostoc* sp. (Kim, et al., 2015) and*A. marina* (Gao, et al., 2010) and linoleate 13-lipoxygenase from*Burkholderia thailandensis* (An et al., 2015) have been shown to convert C18 PUFAs

into HpFAs, which are reduced to HFAs. Linoleate 9-lipoxygenase from *M. xanthus* converts C18 PUFAs into epoxy hydroxy fatty acids via the isomerization of HpFAs (An, et al., 2019). However, EFAs have not been converted from C18 PUFAs by lipoxygenases. Therefore, to our knowledge, this is the first study reporting the conversion of C18 PUFAs into EFAs by a lipoxygenase. In this study, three new types of EFAs were obtained from the C18 PUFAs linoleic acid, α-linolenic acid, and γ-linolenic acid.

Identification of HFA products obtained from the conversion of C18 PUFAs by linoleate 9-lipoxygenase from *S. macrogoltabida*

The HFA reaction products obtained from the conversion of C18 PUFAs, including linoleic acid, α-linolenic acid, and γ-linolenic acid, by the peroxidation of 9-lipoxygenase from *S. macrogoltabida* , were analyzed using LC-MS/MS. In the full-scan mode of LC-MS, the retention times of the three reaction products obtained from the conversion of linoleic acid were 13.6 min, 14.6 min, and 15.7 min (Fig. 4a). The total molecular mass of the product with a retention time of 13.6 min was represented by a peak at *m/z* 295.4, corresponding to the molecular mass of HODE in the LC-MS. In the LC-MS/MS spectrum of the product, the peaks at *m/z* 277.2 and *m/z* 251.2 were formed by the loss of H_2O and CO_2 from the total molecular mass, respectively, and a peak at *m/z* 171.0 resulted from the cleavage of the hydroxyl group at the C9 position. These fragment peaks indicate that the reaction product was 9-HODE. This LC-MS/MS profile was confirmed by comparison with the reference compound in the Lipid Metabolites and Pathway Strategy (LIPID MAPS) database.

The retention times of the reaction products obtained from the conversion of α-linolenic acid by the linoleate 9-lipoxygenase were 12.4 min, 13.6 min, and 14.6 min. The total molecular mass of the product with a retention time of 12.4 min was represented by a peak at*m/z* 293.4, corresponding to the molecular mass of HOTrE in LC-MS (Fig. 4b). In the LC-MS/MS spectrum of the product, the peak at*m/z* 275.2, was formed by the loss of H_2O from the total molecular mass with a retention time of 12.45 min, and the peaks at *m/z* 195.1 and *m/z* 223.2 indicated a hydroxyl group at the C13 position of HOTrE. The LC-MS/MS profile of 13-HOTrE was confirmed by comparison with a reference in the LIPID MAPS database.

The retention times of the reaction products obtained from the conversion of γ-linolenic acid by linoleate 9-lipoxygenase were 13.3 min, 14.2 min, and 15.0 min. The total molecular mass of the product with a retention time of 13.3 min was represented by a peak at*m/z* 293.5, corresponding to the molecular mass of HOTrE in LC-MS. The peaks at *m/z* 275.3 and *m/z* 249.3, were formed by the loss of H_2O and CO_2 from the total molecular mass in the LC-MS/MS spectrum (Fig. 4c), and the peaks at*m/z* 141.0 and *m/z* 169.0 resulted from the cleavage of the hydroxyl group at the C9 position of HOTrE. Thus, the product was identified as 9-HOTrEγ.

The compound 9-HOTrEγ obtained from the conversion of γ-linolenic acid by 9-lipoxygenase from *M. xanthus* was identified by LC-MS/MS analysis (An, et al., 2019). In the LC-MS/MS profile, the fragment peaks at *m/z* 141.1 and *m/z* 169.0 were shown by cleavage of the hydroxyl group at 9-HOTrEγ. The same peaks were also observed in the LC-MS/MS analysis of the γ-linolenic acid-derived reaction product of linoleate 9-lipoxygenase from *S. macrogoltabida* (Fig. 4c).The LC-MS/MS profile of 13-HOTrEγ has been reported in the LIPID MAPS database. However, that of 9-HOTrEγ has not been provided. For a more accurate analysis, the fragment peaks around the hydroxyl group of the reaction product 9-HOTrEγ were compared with those of 13-HOTrEγ. The molecular mass of the fragment peak obtained from the cleavage of the hydroxyl group of 13-HOTrEγ in the LIPID MAPS database was *m/z*193.1, which was different from that of the fragment peaks at *m/z*141.1 and *m/z* 169.0 in 9-HOTrEγ (Fig. 4c). Thus, the product obtained from the conversion of γ-linolenic acid by linoleate 9-lipoxygenase was not 13-HOTrEγ, but 9-HOTrEγ.

Identification of new EFA products obtained from the conversion of C18 PUFAs by linoleate 9-lipoxygenase from *S. macrogoltabida*

The retention times of the three reaction products obtained from the conversion of linoleic acid were 13.6 min, 14.6 min, and 15.7 min in LC-MS (Fig. 5a). The total molecular masses of the products with the retention times of 14.6 min and 15.7 min were represented by peaks at*m/z* 311.5 (data not shown) and *m/z* 293.6, corresponding to the molecular masses of HpODE and EpODE, respectively. In the LC-MS/MS

spectrum, the peaks at *m/z* 275.2 and *m/z* 249.3 were formed by the loss of H_2O and CO_2 from the total molecular mass, respectively, and the peaks at *m/z* 141.0 and *m/z* 185.1 resulted from the cleavage of the epoxy group at the C9 and C10 positions, respectively. Based on these peaks, the product was identified as 9,10-EpODE. Therefore, the three reaction products obtained from the conversion of linoleic acid with the retention times of 13.6 min, 14.6 min, and 15.7 min in LC-MS were 9-HODE, 9-HpODE, and 9,10-EpODE, respectively.

The total molecular mass of the reaction product with the retention time of 14.6 min obtained from the conversion of α-linolenic acid by linoleate 9-lipoxygenase was represented by a peak at *m/z* 291.6 in the LC-MS (Fig. 5b), corresponding to the molecular mass of EpOTrE. In the LC-MS/MS spectrum, the peaks at *m/z* 273.2 and *m/z*247.2 were formed by the loss of H_2O and CO_2 from the total molecular mass, and the peaks at*m/z* 110.9 and *m/z* 179.1 resulted from the cleavage of the epoxy group at the C12 position. These results suggested that the product was 12,13-EpOTrE.

The total molecular mass of the reaction product with a retention time of 15.0 min obtained from the conversion of γ-linolenic acid was represented by a peak at *m/z* 291.7 in LC-MS (Fig. 5c), corresponding to the molecular mass of EpOTrE. In the LC-MS/MS spectrum, the peaks at *m/z* 273.2 and *m/z* 247.2 were formed by the loss of H_2O and CO_2 from the total molecular mass, and the peaks at *m/z* 109.1 and *m/z* 181.1 resulted from the cleavage of the epoxy group at the C10 position. These fragment peaks indicate that the reaction product was 9,10-EpOTrEγ. The molecular masses of the fragment peaks obtained from the cleavage of the epoxy group of 12,13-EpOTrEγ were *m/z* 178.0 and *m/z* 220.0. Thus, the EFA product obtained from the conversion of γ-linolenic acid by 9-lipoxygenase was 9,10-EpOTrEγ. The compounds 9,10-EpODE, 12,13-EpOTrE, and 9,10-EpOTrEγ are new EFAs because they do not match any compounds in the available databases, including LIPID MAPS.

Conclusion

In this study, the putative protein from the bacterium *S. macrogoltabida* was identified as a linoleate 9-lipoxygenase by characterizing its biochemical properties, determining substrate specificity, and identifying the reaction product of the enzyme. The linoleate 9-lipoxygenase derived from *S. macrogoltabida* showed the highest$k_{\mathrm{cat}}/K_{\mathrm{m}}$ value for linoleic acid among the 9-lipoxygenases reported in literature and is thus as an efficient linoleate 9-lipoxygenase. The enzyme converted C18 PUFAs into new EFAs, which were identified by LC-MS/MS. To the best of our knowledge, this is the first study to identify EFAs obtained from the conversion of C18 PUFAs using a lipoxygenase.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 Determination of the total and subunit molecular masses of linoleate 9-lipoxygenase from *Sphingopyxis macrogoltabida.* (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified linoleate 9-lipoxygenase from *S. macrogoltabida* stained with Coomassie blue. Lane 1, marker proteins (180, 150, 135, 100, 75, 63, 48, 35, 25, 21, 17, and 11 kDa); lane 2, pellet; lane 3, crude extract; lane 4, purified linoleate 9-lipoxygenase from *S. macrogoltabida.* (b) Determination of the molecular mass of the purified native 9-lipoxygenase from *S. macrogoltabida*using Sephacryl S-300 HR gel filtration chromatography. The reference proteins (*) were ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa). The 9-lipoxygenase (*) from *S. macrogoltabida* was eluted at a position corresponding to 140 kDa

Fig. 2 High-performance liquid chromatography (HPLC) chromatograms of the reaction products obtained from the conversion of linoleic acid by linoleate 9-lipoxygenase from *S. macrogoltabida* . (a) Reverse-phase HPLC chromatograms of linoleic acid standard and reaction products. The products were obtained from the conversion of linoleic acid by linoleate 9-lipoxygenase from *S. macrogoltabida*in the absence of cysteine as a reducing agent. The compounds (1), (2), and (3) were identified as 9,10-epoxy-11E ,13E -octadecadienoic acid (9,10-EpODE), 9-hydroperxy-10E ,12Z -octadecadienoic acid (9-HpODE), and 9-hydroxy-10E ,12Z -octadecadienoic acid (9-HODE), respectively, by LC-MS/MS analysis. (b) Normal-phase HPLC chromatograms of 9-HODE and 13-HODE standards and the reaction product. The reaction product was obtained from the conversion of linoleic acid by linoleate 9-lipoxygenase from *S. macrogoltabida* in the presence of cysteine. The reaction was performed in 50 mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS) buffer (pH 8.5) containing 0.28 g/L linoleic acid and 0.1 g/L enzyme in the presence of 200 mM cysteine at 30 degC for 10 min

Fig. 3 Biosynthetic pathways of hydroxy fatty acids (HFAs) and epoxy fatty acids (EFAs) obtained from the conversion of C18 polyunsaturated fatty acids (PUFAs) by linoleate 9-lipoxygenase from*S. macrogoltabida.* (a) Linoleic acid metabolism. (b) α-Linolenic acid metabolism. (c) γ-Linolenic acid metabolism. Hydroperoxy fatty acids (HpFAs) were converted into HFAs by reduction in the presence of cysteine, and they were converted into HFAs by natural reduction and into EFAs by dehydration in the absence of cysteine

Fig. 4 Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) chromatograms of the HFA products obtained from the conversion of C18 PUFAs by linoleate 9-lipoxygenase from *S. macrogoltabida.* (a) LC-MS and LC-MS/MS chromatograms of 9-HODE obtained from the conversion of linoleic acid; result of the ion trap mass spectrometry-electrospray ionization (ITMS-ESI) analysis with a full MS spectra range of $m\ /z$ 294.9-295.9 at 0-35 min and a MS/MS spectrum of $m\ /z$ 295 at 13.65-13.82 min. (b) LC-MS and LC-MS/MS chromatograms of 13-hydroxy-9Z ,11E ,15Z -octadecatrienoic acid (13-HOTrE) obtained from the conversion of α-linolenic acid; result of the ITMS-ESI analysis with a full MS spectrum range of $m\ /z$292.9-293.9 at 0-35 min and an MS/MS spectrum of $m\ /z$ 293 at 12.45 min. (c) LC-MS and LC-MS/MS chromatograms of 9-hydroxy-6Z ,10E ,12Z -octadecatrienoic acid (9-HOTrEγ) obtained from the conversion of γ-linolenic acid; result of the ITMS-ESI analysis with a full MS spectrum range of $m\ /z$292.9-293.9 at 0-35 min and a MS/MS spectrum of $m\ /z$ 293 at 13.3 min

Fig. 5 LC-MS/MS of the EFA products obtained from the conversion of C18 PUFAs by linoleate 9-lipoxygenase from *S. macrogoltabida.* (a) LC-MS and LC-MS/MS chromatograms of 9,10-EpODE obtained from the conversion of linoleic acid. ITMS-ESI analysis result with a full MS spectra range of $m\ /z$ 292.9-293.9 at 0-35 min and a MS/MS spectrum of $m\ /z$ 293 at 15.7 min. (b) LC-MS and LC-MS/MS chromatograms of 12,13-epoxy-8E ,10E ,15Z -octadecatrienoic acid (12,13-EpOTrE) obtained from the conversion of α-linolenic acid. The ITMS-ESI analysis result with a full MS spectrum range of$m\ /z$ 290.9-291.9 at 0-35 min and a MS/MS spectrum of$m\ /z$ 291.4 at 14.68 min. (c) LC-MS and LC-MS/MS chromatograms of 9,10-epoxy-6Z ,11E ,13E -octadecatrienoic acid (9,10-EpOTrEγ) obtained from the conversion of γ-linolenic acid. The ITMS-ESI analysis result with a full MS spectrum range of$m\ /z$ 290.9-291.9 at 0-35 min and a MS/MS spectrum of$m\ /z$ 291.4 at 15.0 min.

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