Stabilization and improved activity of arachidonate 11S-lipoxygenase from proteobacterium Myxococcus xanthus

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Abstract  Lipoxigenases (LOXs) catalyze the dioxygenation of PUFAs to produce regio- and stereospecific oxygenated fatty acids. The identification of regio- and stereospecific LOXs is important because their specific products are involved in different physiological activities in various organisms. Bacterial LOXs are found only in some proteobacteria and cyanobacteria, and they are not stable in vitro. Here, we used C20 and C22 PUFAs such as arachidonic acid (ARA), eicosapentaenoic acid, and docosahexaenoic acid to identify an 11S-specific LOX from the proteobacterium Myxococcus xanthus and explore its in vitro stability and activity. The activity and stability of M. xanthus ARA 11S-LOX as well as the production of 11S-hydroxyeicosatetraenoic acid from ARA were significantly increased by the addition of phosphatidylycholine, Ca²⁺, and coactosin-like protein (newly identified in the yeast Rhodosporidium toruloides) as stimulatory factors; in fact, LOX activity in the presence of all three factors increased approximately 3-fold. Our results indicate that these stimulatory factors can be used to increase the activity and stability of bacterial LOX and the production of bioactive hydroxy fatty acids, which can contribute to new academic research.—An, J-U., and D-K. Oh. Stabilization and improved activity of arachidonate 11S-lipoxygenase from proteobacterium Myxococcus xanthus. J. Lipid Res. 2018. 59: 2153–2163.

Supplementary key words  11S-hydroxyeicosatetraenoic acid • stimulatory factors • coactosin-like protein

The substrates for lipoxigenases (LOXs) are PUFAs, including octadecanoids derived from linoleic acid [18:2(n-6)], α-linolenic acid [18:3(n-3)], and γ-linolenic acid [18:3(n-6)], and eicosanoids derived from arachidonic acid [ARA; 20:4(n-6)], EPA [20:5(n-3)], and DHA [22:6(n-3)]. LOXs catalyze regio- and stereospecific dioxygenation of PUFAs containing 1,4-cis,cis-pentadiene units into hydroperoxy fatty acids (HPFAs), a type of oxylipin that contains cis-trans conjugated dienes that are reduced to hydroxy fatty acids (HFAs). LOXs have been mainly reported in eukaryotes, and their biological roles have been mostly revealed (1). LOXs have also recently been reported in other organisms such as corals (2), fungi (3), and bacteria (4–7). It has been suggested that bacterial LOXs originate from higher eukaryotes by horizontal gene transfer (8), and LOXs are rarely found in bacteria (9). Bacterial LOXs have been reported only in some cyanobacteria and proteobacteria of gram-negative bacteria to date. In proteobacteria, LOXs were reported first in Pseudomonas aeruginosa and second in Burkholderia thailandensis. Several proteobacteria have LOX genes (10), and some Myxococcus species, including M. fulvus, M. virescense, and M. xanthus, are thought to have LOX isoforms.

The different regio- and stereospecific LOX-derived products are important signaling molecules with different physiological activities in various organisms (1, 11). In particular, HFAs in animals, which are converted from C20 and C22 PUFAs, act as lipid mediators. HETEs, one type of lipid mediator, are involved in eosinophil chemotaxis (12), neutrophil degradation (13), cancer cell proliferation (14), platelet aggregation (15), vasodilation (16), vascular smooth muscle cell proliferation (20), and 11-hydroperoxyeicosatetraenoic acid (HPETE) is a precursor of prostaglandin H₂ (21). 11-HETE stimulates chemotaxis of human eosinophils and neutrophils (19) and inhibits human vascular smooth muscle cell proliferation (20), and 11-hydroperoxyeicosatetraenoic acid (HPETE) is a precursor of prostaglandin H₂ (21). 11-HETE is present in marine organisms and mammals (22, 23).

Abbreviations:  ARA, arachidonic acid; CLP, coactosin-like protein; CP, chiral phase; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propane sulfonic acid; HDOHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HFA, hydroxy fatty acid; HPETE, hydroperoxyeicosatetraenoic acid; HPFA, hydroperoxy fatty acid; LOX, lipoxigenase; PL, phosphatidylycholine; PE, phosphatidylethanolamine; PLAT, polycystin-1, lipoxigenase, and α-toxin.
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enzymes that use ARA are 11-LOXs in cyanobacteria (4), corals (24), algae (25), cyclooxygenases, and cytochrome P450s (26). However, all of the reported enzymes produce 11R-HETE. The existence of ARA 11S-LOX in whole organisms and the production of 11S-HETE have not been reported to date.

Most LOXs have specific conserved sequences, HxxxxH—HxxxN—IV, at the C terminus that interact with active metal ions such as iron or manganese, which are closely related to catalytic activity. However, the N-terminal sequences are different among species. Based on a comparison of the crystal structures of LOXs from different species, the active-site structures at the C terminus are similar, whereas the structures at the N terminus are different (27). At the N terminus in eukaryotic LOXs, there is a C2-like β-barrel structure termed the polycystin-1, lipoxygenase, and α-toxin (PLAT) domain that binds to the nuclear membrane in vivo, resulting in the stabilization of LOX. Thus, in vitro, most LOXs show low stability due to the absence of a nuclear membrane to bind. This problem has been solved for human ARA 5-LOX by the binding of the PLAT domain to a lipid bilayer of phosphatidylcholine (PC) as an artificial membrane and using Ca$^{2+}$ and coacase-in-like protein (CLP) as stimulatory factors (28). However, LOX from the proteobacterium P. aeruginosa has no PLAT domain activity (29). Thus, there is no evidence that the activity of proteobacterial LOX is increased by adding these stimulatory factors.

In this study, we first discovered a regio- and stereospecific ARA 11S-LOX from the proteobacterium M. xanthus by identifying the LOX products using C20 and C22 PUFAs as substrates. Moreover, the effects of the stimulatory factors PC, Ca$^{2+}$, and microbial CLP on the activity and stability of ARA 11S-LOX from M. xanthus and the biotransformation of ARA to 11S-HETE were evaluated.

**MATERIALS AND METHODS**

**Materials**

PUFA standards, including ARA, EPA, DHA, and phospholipid standards, including PC and phosphatidylethanolamine (PE), were purchased from Sigma-Aldrich. HFA standards, including 11S-HETE, 11R-HETE, 15S-HETE, 15R-HETE, 11-hydroxyicosatetraenoic acid (HEPE), and 14-hydroxyicosahexaenoic acid (HDOHE), were purchased from Cayman Chemical. M. xanthus DK1622 (KCCM 44251, Korea Culture Center of Microorganisms), Commensalibacter intestine A911 (KCTC 22117, Korea Collection for Type Cultures), and Rhodosporidium toruloides NP11 (KCTC 7134) were used as the sources of DNA templates for the cloning of one LOX and two CLP genes, respectively. Escherichia coli ER2566 and pET-28a plasmid were used as the host cells and expression vector, respectively.

**Gene cloning and site-directed mutagenesis**

The genes encoding putative LOX and CLPs were amplified by PCR using genomic DNA from the above strains as templates. The primers used for gene cloning were designed on the basis of the DNA sequences of microbial enzymes (supplemental Table S1). The DNA fragments obtained by PCR amplification using Taq polymerase (Solgent) were cloned into pET-28a. The plasmids were transformed into E. coli ER2566, which was plated on Luria-Bertani agar containing 0.1 mM kanamycin. For each construct, a kanamycin-resistant colony was selected, and plasmid DNA was isolated using a purification kit (Intron). DNA sequencing was performed at Macrogen. Site-directed mutagenesis was performed using the Quick-Change kit (Stratagene) according to the manufacturer’s instructions.

**Enzyme purification and molecular mass determination**

LOX and CLPs were expressed and purified as described previously (30). The subunit molecular masses of the purified enzymes were examined using SDS-PAGE. The molecular mass of the native LOX from M. xanthus was determined using Sephacryl S-300 HR 16/60 gel filtration chromatography (GE Healthcare). The enzyme solution was applied to the column and eluted at a flow rate of 0.6 ml/min with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with ferritin (440 kDa), catalase (292 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa) as reference proteins (GE Healthcare).

**Determination of metal ions in enzyme**

The contents of metal ions in the purified putative LOX from M. xanthus were measured by an inductively coupled plasma MS. The purified LOX with a final concentration of 0.9 mg/ml (11.84 μM) was prepared. The analysis was performed using NexOn 350D (PerkinElmer) at the National Center for Inter-university Research Facilities (Seoul National University). The instrument was calibrated by standard iron and manganese concentrations.

**Enzyme assay**

Unless otherwise stated, enzyme reactions were carried out at 35°C in 50 mM 3-[2-(2-hydroxyethyl)-1-piperazinyl]propane sulfonic acid (EPPS) buffer (pH 8.5) containing 0.1 mM PUFAs or phospholipid and 3 μg/ml enzyme for 2 min. One unit of LOX activity was defined as the amount of enzyme required to produce 1 μmol HPFA per minute at 35°C and pH 8.5. The effects of pH and temperature on the activity of the putative LOX from M. xanthus were investigated by varying the pH from 3.0 to 9.5 using 50 mM citric acid-phosphate buffer (pH 3.0–6.0), 50 mM sodium phosphate buffer (pH 6.0–8.0), 50 mM EPPS buffer (pH 8.0–9.0), and 50 mM 2-(cyclohexylamino)ethanesulfonic acid buffer (pH 8.5–9.5) at 35°C and by varying the temperature from 20 to 40°C at pH 8.5. The effect of temperature on enzyme stability was investigated by varying the temperature from 25 to 45°C at pH 8.5.

**Determination of specific activity and kinetic parameters**

The specific activity of the putative LOX from M. xanthus was determined by measuring the increase in absorbance at 234 nm using a Beckman Coulter DU-800 spectrophotometer after incubation at 35°C in 50 mM EPPS (pH 8.5) buffer containing 0.1 mM substrate and 3.0–6.0 μg/ml enzyme in the presence of 250 μg/ml PC and 1 mM CaCl$_2$ for 2 min. Only the part of each reaction showing a linear correlation between product concentration and time and the extinction coefficient of 25,000 M/cm for conjugated products was used to calculate enzyme activity. To determine kinetic parameters, the reactions were conducted at 35°C in 50 mM EPPS (pH 8.5) buffer by varying the amounts of PUFAs from 10 to 900 μM for 1 min, and enzyme activity was determined by measuring the absorbance. $K_m$ (mM) and $k_{cat}$ (min$^{-1}$) were determined on the basis of a Hanes-Woolf plot derived from the Michaelis-Menten equation. To calculate $k_{cat}$, the amount of protein was divided by the total molecular mass.
Effects of stimulatory factors
The effects of PG (0–1,000 μg/ml) and Ca2+ (0–10 mM) concentrations on the activity of LOX from *M. xanthus* were investigated using 0.1 mM ARA at 35°C for 2 min. Microbial CLPs, which were selected on the basis of a comparison with human CLP (GenBank accession number: AAB88822.1), were cloned and expressed in *E. coli*. CLP from *R. toruloides* was used at the same molar concentration as that of LOX because the binding molar ratio of CLP to human ARA 5-LOX is 1:1 (31). The effects of combinations of stimulatory factors were evaluated using 1 mM CaCl2, 250 μg/ml PC, and/or 0.65 μg/ml CLP at 35°C for 2 min. Enzyme stability was investigated at 25 and 45°C with and without stimulatory factors.

Effects of enzyme and substrate concentrations
The effects of enzyme and substrate concentrations were investigated with 5 mM ARA by varying the enzyme concentration from 1.5 to 9 mg/ml and with 6 mg/ml enzyme by varying the substrate concentration from 1 to 7 mM, respectively. The reactions were conducted in 50 mM EPPS (pH 8.5) buffer containing ARA, enzyme, 1 mM CaCl2, 250 μg/ml PC, and the same molar concentration of CLP from *R. toruloides* as that of LOX (1.3 mg/ml) at 35°C for 30 min with shaking at 200 rpm in a 100 ml baffled flask containing 10 ml reaction solution.

Biotransformation of ARA to 11S-HETE
The conversion of ARA to 11S-HETE by *M. xanthus* ARA 11S-LOX was conducted in 50 mM EPPS (pH 8.5) buffer containing 5 mM ARA, 6 mg/ml enzyme, 1 mM CaCl2, 250 μg/ml PC, and 1.5 mg/ml CLP from *R. toruloides* at 35°C for 90 min. 11SHPETE in the reaction solutions was reduced to 11S-HETE by adding 10 mM cysteine.

Chemotaxis assay
TPM agar plates containing 10 mM Tris-HCl (pH 7.6), 1 mM potassium phosphate (K2HPO4), 8 mM MgSO4, and 1.5% agar were allowed to dry at 25°C for 18 h. After drying, 5 μl spots of 5 or 25 mg/ml PE, PUFAs, and HFAs were placed on the surface of each agar plate and incubated at 30°C for 24 h to establish the lipid gradient. When the optical density at 600 nm of wild-type *M. xanthus* reached 1.2, the bacterial broth was concentrated by centrifugation to 9 × 10^10 cells/ml in 10 mM MOPS buffer (pH 7.6) containing 8 mM MgSO4 and India ink. To determine twitching motility, 1 μl of cell suspension was placed at an approximate distance of 5 mm from the center of the lipid drop on the TPM agar and allowed to incubate at 30°C for 18 h. The twitching phenotypes of *M. xanthus* were observed under an electron microscope, and the distribution area was quantified using Image J (https://imagej.nih.gov/ij/).

Analytical methods
The reaction products were extracted using an equal volume of ethyl acetate. The solvent was removed with a rotary evaporator, and methanol was added to the dried extracts. All fatty acids were quantitatively analyzed using an HPLC system (Agilent 1100) with a UV detector at a wavelength of 202 nm and a reversed-phase Nucleosil C8 column (3.2 × 150 mm; 5 μm particle size; Phenomenex). Phospholipids were analyzed using a normal-phase Zorbax RX-SIL column (2.13 × 150 mm; 5 μm particle size; Agilent) with an evaporative light scattering detector. Chiral-phase (CP)-HPLC was run using a Chiralcel OD-H column (2.1 × 150 mm; 5 μm particle size; Daicel). Elution was carried out as described previously (30). The HFAs were subjected to LC/MS/MS analysis using a Thermo-Finnigan LCQ Deca XP Plus ion trap mass spectrometer (Thermo Fisher Scientific). Ionization of the samples was carried out using ESI. The operation parameters were the same as described previously (30).

Phylogenetic analysis
ARA 11S-LOX from *M. xanthus* was used as query sequence for a blast search against the amino acid sequences of other organisms. All hits with an expected value of ≤10^-10 were compiled from the database and aligned using the MUSCLE algorithm in MEGA 7. Sequences with poor alignment and annotated as unrelated proteins were removed. Phylogenetic trees were built using the neighbor-joining method in MEGA 7 with 1,000 bootstraps.

Homology modeling and substrate docking
Homology modeling of *M. xanthus* LOX was performed using the Build Homology Models module in the MODELER application of Discovery Studio 4.1 (Accelrys Software) based on the determined structure of human ARA 5-LOX (Protein Data Bank number 3O8Y) as a template. The generated structure was improved by subsequent refinement of the loop conformations by assessing the expected value of ≤10^-10 compatibility of amino acid sequences with the known Protein Data Bank structure. The geometry of the loop region was corrected using Refine Loop/ MODELER, and the best model was chosen. Hydrogen atoms were added to the models and minimized to have stable energy conformations and to relax the conformation from close contacts.

ARA or DHA as a substrate was docked into the active-site pocket of the homology model of *M. xanthus* LOX using the CDOCKER module of Discovery Studio 4.1. Substrate poses were refined by full-potential final minimization, and candidate poses were constructed using random rigid-body rotations followed by simulated annealing. The structure of the enzyme-ligand complex was subjected to energy minimization using the CHARMM force field of Discovery Studio 4.5.

RESULTS

Biochemical properties of the putative LOX from *M. xanthus*
The gene (2,028 bp) encoding a putative LOX from *M. xanthus*, with the same sequence as that reported in GenBank (accession number ABE88826.1), was cloned and expressed in *E. coli* as a His-tagged protein that was purified by HisTrap HP affinity chromatography. The protein presented as a single band in SDS-PAGE with a molecular mass of 70–80 kDa (supplemental Fig. S1A), which is consistent with the value (76 kDa) calculated on the basis of the 675 amino acids plus hexahistidine tag. The molecular mass of the native protein was 230 kDa, as determined by gel filtration chromatography (supplemental Fig. S1B), indicating that LOX is a trimer, like human ARA 15-LOX (32). However, most LOXs are dimers (33). The amino acid sequence of the catalytic domain in *M. xanthus* LOX was aligned with those of the previously reported LOXs (Fig. 1). The metal binding residues were three His residues (His366, H371, and His549), which were completely conserved, and Val675, which was Val or Ile for LOXs. The Gly/Ala switch, which is reported to be a determinant of isomer chirality, was Ala for SLOXs and Gly for RLOXs (34). The Gly/Ala switch of *M. xanthus* LOX was Ala409, indicating that the LOX is an SLOX.
The contents of metal ions in the purified putative LOX from \textit{M. xanthus} were analyzed by inductively coupled plasma MS. The molar ratios of iron (11.43 ± 0.2 μM) and manganese (0.27 ± 0.0 μM) to protein (11.84 μM) were 96.5% and 2.2%, respectively. The results suggest that \textit{M. xanthus} LOX has iron as a metal cofactor in the active site and binds to the LOX with the approximate ratio of 1:1. The effects of pH and temperature on the activity of the putative LOX from \textit{M. xanthus} were investigated, and the activity was maximal at pH 8.5 and 35°C (supplemental Fig. S2A, B). The thermostability of the putative LOX from \textit{M. xanthus} was assessed by measuring the residual activity after incubation at temperatures between 25 and 45°C. Thermal inactivation of the enzyme followed first-order kinetics, with half-lives of 48, 15, 2.9, 1.3, and 0.2 h at 25, 30, 35, 40, and 45°C, respectively (supplemental Fig. S2C).

**Identification of the reaction products from the conversion of C20 and C22 PUFAs by the putative LOX from \textit{M. xanthus}**

The putative LOX of \textit{M. xanthus} converted C20 and C22 PUFAs to HPFAs, which were reduced to HFAs by treatment with cysteine as a reducing agent. The total molecular masses of the HFA products obtained from the conversion of ARA, EPA, and DHA were represented by peaks at m/z 319.2, 317.2, and 343.2, respectively, in the

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**Fig. 1.** Alignment of amino acid sequences of the catalytic domain in LOXs. LOXs with the GenBank accession numbers are as follows: \textit{M. xanthus} A11S-LOX, Q1DBH9; \textit{M. xanthus} A12S-LOX, Q1DBH8; \textit{B. thailandensis} A15S-LOX, Q2SW25; \textit{P. aeruginosa} A15S-LOX, Q9I4G8; \textit{Cyanothece} sp. L09R-LOX, B7K2Q5; \textit{Fusarium oxysporum} L11R-LOX, F9FRH4; \textit{soybean} L13S-LOX, P08170; human A5S-LOX, P09917; human A15S-LOX, Q12996; and mouse A15S-LOX, P39654. The metal binding residues are shown as a black box and white color. The Gly/Ala switch is shown as a white box and black color. A, arachidonate; L, lineolate.

**Fig. 2.** LC/MS/MS and HPLC profiles of the reaction product obtained from the conversion of ARA by the wild-type and A409G variant LOXs from \textit{M. xanthus}. A: LC/MS/MS spectrum and chemical structure of the reaction product of ARA. The product was identified as 11-HETE. The asterisk indicates an MS/MS fragment resulting from the cleavage of the hydroxyl group. B: Reversed-phase HPLC profiles of the reaction products with 11-HETE and 15-HETE standards. C: CP-HPLC profiles of the reaction products with 11S-HETE, 11R-HETE, 15S-HETE, and 15R-HETE standards.
LC/MS/MS spectrum. A peak at \( m/z \) 167.2 for the products of ARA (Fig. 2A) and EPA resulted from the cleavage of the hydroxyl group at the C11 position. A peak at \( m/z \) 152.9 for the product of DHA resulted from the cleavage of the hydroxyl group at the C10 position. On the basis of the LC/MS/MS data, the reaction products of ARA, EPA, and DHA were identified as 11-HETE, 11-HEPE, and 10-HDOHE, respectively (Table 1, supplemental Fig. 2A). The chirality of 11-HETE produced by the 11-LOX from \( M. \) xanthus was identified as 11\( S \)-HETE by CP-HPLC with the separately injected 11\( S \)-HETE and 11\( R \)-HETE standards (Fig. 2C). To investigate the effect of Ala or Gly as the Gly/Ala switch in \( M. \) xanthus LOX on stereospecificity, the Gly/Ala switch residue Ala409 was substituted with Gly. The A409G variant LOX from \( M. \) xanthus converted ARA into 11\( S \)-HETE as well as 15\( R \)-HETE (Fig. 2B, C). Similar results were shown in the A420G variant of \( P. \) aeroginosa ARA 15\( S \)-LOX (35). The wild-type LOX from \( P. \) aeroginosa formed 15\( S \)-HETE, whereas its A420G variant formed not only 15\( S \)-HETE but also 11\( R \)-HETE. The substrate specificity for C20 and C22 PUFAs was investigated. The specific activity and \( k_{cat}/K_m \) were the highest for ARA (Table 1). Thus, the putative LOX from \( M. \) xanthus was an ARA 11\( S \)-LOX.

Effects of PC, \( \text{Ca}^{2+} \), and microbial CLP as stimulatory factors on the activity of the ARA 11\( S \)-LOX from \( M. \) xanthus

The effect of PC as an artificial membrane on the activity of the LOX in vitro was investigated. The LOX activity at
250 µg/ml was the highest, which was 1.8-fold higher than that in the absence of PC (supplemental Fig. S3A). In vivo, Ca$^{2+}$ is known to help the binding of LOX to the nuclear membrane (28). Thus, the effect of Ca$^{2+}$ was investigated. LOX activity at 1 mM CaCl$_2$ in the presence of PC was the highest and increased approximately 2.3-fold compared with that in the absence of Ca$^{2+}$ (supplemental Fig. S3B). CLP aids in the binding of human ARA 5-LOX to the nuclear membrane, thereby increasing LOX stability and activity (36). A gene blast search was conducted using human CLP (GenBank accession number AAA88022.1) as a template to search for microbial CLPs. CLPs with identities of 16.6% and 28.5% were obtained from the bacterium C. intes- tine (GenBank accession number EHD13870.1) and the yeast R. toruloides (GenBank accession number EMS22454.1), respectively. The Leu residues at positions 75 and 131 in human CLP are crucial for the interaction with human ARA 5-LOX (37). In microbial CLPs, Leu75 was conserved; however, Leu131 was replaced with Gln (supplemental Fig. S4A). The CLPs purified from C. intes- tine and R. toruloides were identified by SDS-PAGE as single bands of 23 and 16 kDa, respectively (supplemental Fig. S4B). When the same molar concentration CLP as that of LOX was used, CLP without PC and Ca$^{2+}$ and C. intestine CLP with PC and Ca$^{2+}$ did not have an effect; however, R. toruloides CLP with PC and Ca$^{2+}$ increased the LOX activity by approximately 3-fold (supplemental Fig. S4C).

**Table 1. Specific activity and kinetic parameters of M. xanthus 11S-LOX for C20 and C22 PUFAs**

| Chain Length | Substrate | Product | MS/MS Fragments [M-H] | Specific Activity (U mg$^{-1}$) | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$) |
|--------------|-----------|---------|------------------------|-------------------------------|------------|---------------------|----------------------------------|
| C20          | ARA (ω6, C20:4$^{5Z,8Z,11Z,14Z}$) | 11S-HETE | 116.2, 275.3, 301.2, 319.2 | 5.2 ± 0.1 | 0.299 | 323 | 1,081 |
|              | EPA (ω3, C20:5$^{5Z,8Z,11Z,14Z,17Z}$) | 11S-HEPE | 121.1, 167.2, 195.2, 255.1, 317.2 | 2.4 ± 0.3 | 0.083 | 65.8 | 798 |
| C22          | DHA (ω3, 22:6$^{4Z,7Z,10Z,13Z,16Z,19Z}$) | 10S-HDOHE | 121.2, 152.9, 181.0, 281.2, 334.2 | 1.8 ± 0.2 | 0.100 | 69.2 | 693 |

Bold indicates an MS/MS fragment resulting from the cleavage of the hydroxyl group. Data represent the means of three experiments.
PC and Ca\(^{2+}\); PC and \(R.\) toruloides CLP; Ca\(^{2+}\) and \(R.\) toruloides CLP; and PC, Ca\(^{2+}\), and \(R.\) toruloides CLP increased LOX activity by approximately 2.3-, 2.5-, 1.7-, and 3.3-fold, respectively (Fig. 3), indicating that the addition of all stimulatory factors was the most effective for increasing the activity of \(M.\) xanthus ARA 11\(\Delta\)5-LOX. Therefore, 250 µg/ml PC, 1 mM CaCl\(_2\), and 1.3 mg/ml CLP from \(R.\) toruloides were used for the biotransformation of ARA to 11\(\Delta\)HETE. The effects of combinations of the stimulatory factors on the stability of the enzyme were studied at 25 and 45°C with or without all combined stimulatory factors by measuring the residual activity. The residual activity of \(M.\) xanthus LOX with stimulatory factors was 80% at 25°C after 360 min and 40% at 45°C after 120 min, whereas that without stimulatory factors was 58% at 25°C and 4% at 45°C (supplemental Fig. S5). These results indicated that the stimulatory factors increase the stability of ARA 11\(\Delta\)5-LOX from \(M.\) xanthus.

**Biotransformation of ARA to 11\(\Delta\)HETE by the ARA 11\(\Delta\)5-LOX from \(M.\) xanthus**

11\(\Delta\)HETE production was maximal at 6 mg/ml and 5 mM ARA (supplemental Fig. S6). The time-course reactions were conducted with 5 mM ARA and 6 mg/ml enzyme with and without 250 µg/ml PC, 1 mM CaCl\(_2\), and 1.3 mg/ml CLP from \(R.\) toruloides, at pH 8.5 and 35°C for 90 min (Fig. 4). \(M.\) xanthus ARA 11\(\Delta\)5-LOX with stimulatory factors converted 5 mM ARA to 11\(\Delta\)HETE with a molar conversion yield of 100%, whereas the enzyme without stimulatory factors converted 5 mM ARA to 2.8 mM 11\(\Delta\)HETE after 90 min, with a molar conversion yield of 56%.

**Effects of reaction products obtained from PUFAs by \(M.\) xanthus ARA 11\(\Delta\)5-LOX on the twitching motility of \(M.\) xanthus**

The effects of PUFAs and HFAs as chemotactic substances on the twitching motility of wild-type \(M.\) xanthus were investigated. PE has been involved in the chemotaxis of \(M.\) xanthus (12). In the agar plates, not only PE but also ARA (PUFA) and 11-HETE (HFA) showed the twitching motility of \(M.\) xanthus, although PE showed the highest twitching motility (supplemental Fig. S7A). The twitching motility of PE at 5 mg/ml was approximately 2.2-fold higher than that of the nontreated control. PUFAs at 5 or 25 mg/ml increased 1.1–1.2-fold, and HFAs increased 1.2–1.6-fold, in the twitching motility of \(M.\) xanthus compared with that of the nontreated control (supplemental Fig. S7B). Among PUFAs and HFAs, 11-HETE at 25 mg/ml showed the highest twitching motility with an approximately 1.6-fold increase.

**DISCUSSION**

Among Myxococcus species, two LOXs from \(M.\) xanthus were already characterized. One was identified as ARA 12\(\Delta\)5-LOX (38), and the other used in this study has been characterized by Qian et al. (39). The reported LOX activity was measured at pH 2.5–7.0 using citrate-phosphate buffer and was maximal at pH 3.0, suggesting a novel acidic LOX. However, the LOX activity in this study was maximal at pH 8.5 in EPPS buffer (supplemental Fig. S2A). To carefully assess this difference, we reinvestigated the effect of pH on LOX at pH 3.0–9.0, including the same citrate-phosphate buffer (pH 3.0–6.0), by HPLC. Our results were entirely different from the previously reported results. The activity of the \(M.\) xanthus LOX in this study was nearly abolished at pH 3.0 in citrate-phosphate buffer (supplemental Fig. S8A) and was maximal at pH 8.5. The activity at various pH values was confirmed in the areas of HPLC profiles for enzymatic reaction products obtained from ARA (supplemental Fig. S8B). These results indicate that \(M.\) xanthus LOX is not an acidic LOX but prefers basic pH. Most LOXs prefer basic pH, except for those of a few plants, such as rose and the tea plant, which are acidic (40, 41). Moreover, the previous report did not identify the reaction products and the regio- and stereospecificity of the LOX from \(M.\) xanthus. Thus, the LOX is not exactly identified. In the NCBI protein database, the LOX from \(M.\) xanthus is named as ARA 5-LOX due to its high sequence identity to human ARA 5-LOX. However, in this study, the enzyme was identified as an ARA 11\(\Delta\)5-LOX through the determination of its specific activity and kinetic parameters for C20 and C22 PUFAs and the identification of the regio- and stereospecificity of the reaction products. The turnover number of ARA 11\(\Delta\)5-LOX from \(M.\) xanthus LOX was 5 s\(^{-1}\) for ARA (Table 1), which was 36- and 22-fold lower than those of the prokaryotic LOXs from \(P.\) aeruginosa

![Fig. 3. Effects of combinations of stimulatory factors on the activity of ARA 11\(\Delta\)5-LOX from \(M.\) xanthus. The reactions were conducted in 50 mM EPPS (pH 8.5) buffer containing 0.1 mM ARA and 3 µg/ml enzyme with 1 mM CaCl\(_2\), 250 µg/ml PC, and/or 0.65 µg/ml CLP from \(R.\) toruloides at 35°C for 2 min. Plus and minus indicate with and without stimulatory factor, respectively. Data represent the means of three experiments, and error bars represent the standard deviations.](image-url)
LOXs are key enzymes for the biosynthesis of oxylipins, which are involved in multiple physiological activities in various organisms; however, they are not stable in vitro. The stability in vitro is increased in human ARA 5-LOX by the addition of PC and Ca²⁺ as stimulatory factors (47). Therefore, in this study, PC and Ca²⁺ were added to M. xanthus LOX, and thereby, the activity of M. xanthus LOX was increased by 1.8- and 1.2-fold at 250 µg/ml PC and 1 mM Ca²⁺ as the optimum concentrations, respectively (supplemental Fig. S3). The simultaneous addition of both factors increased the activity by 2.3-fold. CLP, a stabilizing chaperone, belongs to the actin-depolymerizing factor/cofilin group of actin binding protein and stabilizes human ARA 5-LOX by assisting the binding of its PLAT domain to the nuclear membrane at the molar ratio of 1:1 (31). CLP can function as a scaffold for LOX in the absence of a nuclear membrane or PC, similar to the membrane (28, 37). Human ARA 5-LOX is a bifunctional enzyme that converts ARA to 5-HPETE by dioxygenation, which is subsequently converted to leukotriene A₄ by epoxidation. The LOX is structurally stabilized with help from human CLP, which increases the activity (36). Thus, the addition of CLP significantly increases the production of leukotriene A₄ (48).

Human CLP is one of the actin binding proteins, which are widely present in animals, plants, fungi, yeasts, and bacteria (49–52). However, only human CLP has been associated with LOX stabilization to date. To find an effective microbial CLP, we blast-searched using human CLP as a template and obtained a CLP from the yeast R. toruloides (supplemental Fig. S4). R. toruloides CLP alone did not increase M. xanthus LOX activity, whereas the combination of PC, Ca²⁺, and CLP increased the activity by approximately 3-fold (Fig. 3). Moreover, when combined, the three stimulatory factors increased enzyme stability (supplemental Fig. S5) and the production of 11S-HETE from ARA (Fig. 4). This is the second case of stabilization of LOX by CLP, besides human ARA 5-LOX. This finding is also meaningful in that the CLP was discovered in a nonmammalian species.

Only two crystal structures of bacterial LOXs, from P. aeruginosa (29) and Cyanothece sp. (53), have been reported. The P. aeruginosa LOX has two α-helix structures at the N terminus instead of the PLAT domain, whereas Cyanothece sp. LOX has not only a PLAT domain but also a long α-helix structure. These structures in LOXs may bind to membranes. However, there have been no studies on the membrane binding of the N-terminal structures to stabilize the structure of LOXs. A LOX from M. xanthus homologous to human ARA 5-LOX was reported to have a structure at the N terminus similar to that of the PLAT domain, suggesting that M. xanthus LOX may have a PLAT domain (supplemental Fig. S9). M. xanthus LOX showed 34% amino acid sequence identity to human ARA 5-LOX, which was the highest sequence identity, whereas LOXs from P. aeruginosa and Cyanothece sp. showed 25% and 16% identities, respectively (supplemental Table S2). A phylogenetic tree was constructed on the basis of the amino acid sequences of LOXs from M. xanthus, other bacteria, fungi, plants, and humans (Fig. 6). M. xanthus LOX was the most closely related to human LOXs compared with not only
other bacterial LOXs but also eukaryotic LOXs, which also suggests that *M. xanthus* LOX may have a PLAT domain. To confirm the exact structure of the putative PLAT domain, the crystal structure of *M. xanthus* LOX must be determined.

Unfortunately, the exact biological roles of bacterial LOXs have not been elucidated to date. However, the biological role of the pathogen *P. aeruginosa* LOX on infection has been suggested as biofilm formation, infectiousness and invasiveness of *P. aeruginosa*, bacterial evasion to silence the host immune response (54), and oxidation of phospholipids in the host cell membrane (55). However, *M. xanthus* LOX did not oxidize phospholipids as substrates (supplemental Fig. S10). This result may be due to the nonpathogenic property of *M. xanthus*. Several LOXs can modify PUFAs into chemotactic substances. LOX catalyzes the dioxygenation of PUFAs to HPFAs, which are reduced to HFAs, and also catalyzes the epoxidation of HPFAs to epoxy hydroxy fatty acids. *P. aeruginosa* exhibits twitching motility toward extracellularly treated modified unsaturated fatty acids, suggesting that it may be related to LOX (56). HFAs, which were converted from PUFAs by ARA 11S-LOX from *M. xanthus*, increased the twitching motility of *M. xanthus* (supplemental Fig. S7). The results suggest that *M. xanthus* LOX is involved in the synthesis of chemotactic materials from exogenous PUFAs. ARA 11S-LOX from *M. xanthus* catalyzes the dioxygenation of ARA to 11S-HPETE, which is reduced to 11S-HETE, and the epoxidation of 11S-HPETE into hepoxilin D3. 11-HETE at 25 mg/ml showed the highest twitching motility as an approximately 1.6-fold increase among the HFAs tested, and hepoxilins are known as chemotactic factors for human neutrophils. 11S-HETE and hepoxilin D3 acted as partial agonists of PPARγ similar to thiazolidinediones, a representative full agonist of PPARγ (57). PPARγ is involved in various biological roles such as hyperalgesia, glucose metabolism, lipid accumulation, neutrophil transmigration, vascular permeability, and inflammatory response in vivo.

![Fig. 5](image.png) Proposed mechanistic schemes explaining the oxygenation of C20 PUFAs at the C11 position and oxygenation of C22 PUFAs at the C10 position in the s-configuration by LOXs from *M. xanthus*.

![Fig. 6](image.png) Phylogenetic tree on the basis of amino acid sequences of LOXs from various organisms. The tree was constructed with MEGA7 using the full amino acid sequences of 12 selected proteins. The optimal tree with the sum of the branch length = 7.45162201 is shown. The evolutionary distances were computed using the Poisson correction method and are expressed as the number of amino acid substitutions per site. GenBank accession numbers are as follows: MX A11S-LOX, ABF88826.1; MX A12S-LOX, ABF86480.1; HU A5S-LOX, AAA36183.1; HU A55-LOX, AAA67866.1; PT L9S-LOX, CA64766.1; SB L13S-LOX, AAA39861.1; MO L9S-LOX, AL229899.1; GG L13R-LOX, AAK81882.2; PA A15S-LOX, AAG04558.1; BT A15S-LOX, ABC36974.1; AM A11R-LOX, ABW28888.1; and CS L9R-LOX, ACK67087.1. A, arachidonate; AM, Acaryochloris marina; BT, *B. thailandensis*; CS, Cyanobacteria; GG, Gaeumannomyces graminis; HU, human; L, linoleate; MO, *Magnaporthe oryzae*; MX, *M. xanthus*; PA, *P. aeruginosa*; PT, potato; SB, soybean.
Thus, 11-HETE and hepxoxilin D$_3$ produced by *M. xanthus* ARA 11SLOX are important substances that may regulate various biological activities in animals.

LOXs have a variety of regio- and stereospecificities for PUFAs and produce regio- and stereospecific oxygenated fatty acids that are involved in various physiological activities (11). ARA-derived regio- and stereospecific LOX products are 5R, 8S, 8R, 11R, 12S, 12R, 15S, and 15R-HETEs, which play different important roles in eukaryotes (1, 58). Of these products, 11R-HETE is present in several species (4, 24, 25, 59, 60). However, 11-HETE has never been reported. Thus, the identification of 11-HETE-producing LOX is meaningful.

**CONCLUSION**

The biochemical properties of a putative LOX from *M. xanthus* were characterized, and the enzyme was found to be a novel ARA 11SLOX by identifying its products, which were converted from C20 and C22 PUFAs. This is the first report of an ARA 11SLOX. The activity and stability of the bacterial ARA 11SLOX and the biotransformation of ARA to 11-HETE were significantly increased by adding the stimulatory factors PC, Ca$^{2+}$, and microbial CLP. These results are expected to stimulate the biosynthesis of bioactive oxylipins involved in various physiological activities in mammals and to contribute to the academic and industrial fields.

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