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Regiospecificity of a novel bacterial lipoxygenase from *Myxococcus xanthus* for polyunsaturated fatty acids

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ABSTRACT

Lipoxygenase (LOX) is the key enzyme involved in the synthesis of oxylipins as signaling compounds that are important for cell growth and development, inflammation, and pathogenesis in various organisms. The regiospecificity of LOX from *Myxococcus xanthus*, a gram-negative bacterium, was investigated. The enzyme catalyzed oxygenation at the n-9 position in C20 and C22 polyunsaturated fatty acids (PUFAs) to form 12*S*- and 14*S*-hydroxy fatty acids (HFAs), respectively, and oxygenation at the n-6 position in C18 PUFAs to form 13-HFAs. The 12*S*-form products of C20 and C22 PUFAs by *M. xanthus* LOX is the first report of bacterial LOXs. The residues involved in regiospecificity were determined to be Thr397, Ala461, and Ile664 by analyzing amino acid alignment and a homology model based on human arachidonate 15-LOX with a sequence identity of 25%. Among these variants, the regiospecificity of the T397Y variant for C20 and C22 PUFAs was changed. This may be because of the reduced size of the substrate-binding pocket by substitution of the smaller Thr to the larger Tyr residue. The T397Y variant catalyzed oxygenation at the n-6 position in C20 and C22 PUFAs to form 15- and 17-hydroperoxy fatty acids, respectively. However, the oxygenation position of T397Y for C18 PUFAs was not changed. The discovery of bacterial LOX with novel regiospecificity will facilitate the biosynthesis of regiospecific-oxygenated signaling compounds.

1. Introduction

Lipoxygenases (LOXs) are non-heme iron enzymes that belong to the family of lipid peroxidation enzymes. LOXs catalyze regiospecific dioxygenation of only polyunsaturated fatty acids (PUFAs) containing one or several *cis*,*cis*-1,4-pentadiene units into specific hydroperoxy fatty acids (HpFAs) with *cis-trans* conjugated dienes, which are readily reduced to hydroxy fatty acids (HFAs) under physiological conditions. They are widely distributed in eukaryotes kingdoms, including animals [1] and plants [2]. LOXs are also found in other organisms such as fungi [3], coral [4], and bacteria [5]. The products of LOXs are involved in homeostatic biological functions, inflammation, and cell communication [6–8]. Animal-derived LOXs have molecular masses of 75–80 kDa, mainly use C20 and C22 PUFAs such as arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) as substrates, and are classified as 5-, 8-, 12-, and 15-LOXs based on ARA. In contrast, the molecular masses of LOXs originating from plants and fungi are 94–104 kDa. They catalyze oxygenation at C9 or C13 in C18 PUFAs such as linoleic acid (LA), α-linolenic acid (ALA), and γ-linolenic acid (GLA) and are classified as 9- and 13-LOXs based on LA. In addition, several fungal manganese LOXs catalyze oxygenation at C11 in C18 PUFAs [9–11].

Bacterial LOXs have been found in only Gram-negative bacteria [5], including the cyanobacteria *Anabaena* sp. [12], *Cyanothece* sp. [13], *Nostoc* sp. [14], and *Acaryochloris marina* [15] and the proteobacteria *Pseudomonas aeruginosa* [16], *Burkholderia thailandensis* [17], and *Myxococcus xanthus* [18]. These LOXs have molecular masses of 49–75 kDa and are completely conserved in key residues associated with their activities, like other reported LOXs. Among bacterial LOXs, the crystal structures have been determined for only LOXs from *Cyanothece* sp. [19] and *P. aeruginosa* [16], which differ from eukaryotic LOXs mainly at the *N*-terminal domain. Bacterial LOXs may be involved in the infection of hosts, including animals, plants, and pathogenic fungi, and the origin of bacterial LOX genes has been suggested as

Abbreviations: LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; HpFA, hydroperoxy fatty acid; HFA, hydroxy fatty acid; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxy-9,11,15(*Z*,*E*,*Z*)-octadecatrienoic acid; HOTrEγ, hydroxy-6,9,11(*Z*,*Z*,*E*)-octadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HDOHE, hydroxydocosahexaenoic acid; LB, Luria-Bertani; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]-propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; PDB, protein data bank

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horizontal gene transfer because of the symbiotic relationship of these bacteria with higher organisms [20].

The *N*-terminal domain of LOXs is associated with stabilization, while the *C*-terminal domain is associated with catalysis, which is performed by catalytic residues and iron or manganese ion [21–23]. Generally, all LOXs have a common sequence for the catalytic metal-interacting residues in the *C*-terminal domain, which are strictly conserved with HHHNI. The regiospecificity of LOXs is related to the type of substrate and size of the hydrophobic active-site pocket. A few amino acids at the bottom of the substrate-binding site are known to be regiospecific according to the structures of LOXs. For example, the regiospecificity of human ARA 5-LOX and rabbit ARA 15-LOX is associated with Phe359, Ala424, Asn425, and Ala603 [24] and Phe353, Ile418, M419, and Ile593, respectively [25]. The regiospecificity of the bacterial LOX from *P. aeruginosa* is thought to involve Glu369, Met434, Phe435, and Leu612 based on sequence alignment of the regiospecific residues of rabbit ARA 15-LOX [26]. The regiospecificity of mini-LOX from *Anabaena* sp. involves Ala215 [12].

It is important to obtain a LOX with distinct regiospecificity because the regiospecific-products of LOX are important oxylipins that regulate physiological activity *in vivo* in various organisms. Bacterial LOXs are more active and stable *in vitro* and expressed more easily and rapidly than eukaryotic LOXs. Thus, identifying a new regiospecific LOX from bacterial source is valuable for the biosynthesis of oxylipins. Here, a LOX with unique regiospecificity was found in the proteobacterium *M. xanthus*, which was different with the previously reported LOX from the same strain [18]. The residues involved in the regiospecificity of *M. xanthus* LOX were identified, and a novel variant with altered regiospecificity was identified by investigating the regiospecificity of the variants for these residues based on structural analysis of the homology model.

2. Materials and methods

2.1. Materials

PUFA standards, including LA, ALA, GLA, ARA, EPA, and DHA, were purchased from Sigma (St. Louis, MO, USA) and HFA standards, including 13-hydroxy-9,11(*Z*,*E*)-octadecadienoic acid (13-HODE), 13-hydroxy-9,11,15(*Z*,*E*,*Z*)-octadecatrienoic acid (13-HOTrE), 13-hydroxy-6,9,11(*Z*,*Z*,*E*)-octadecatrienoic acid (13-HOTrEγ), 12-hydroxyeicosatetraenoic acid (12-HETE), 15-HETE, 12-hydroxyeicosapentaenoic acid (12-HEPE), 15-HEPE, 14-hydroxydocosahexaenoic acid (14-HDOHE), and 17-HDOHE, were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Bacterial strains, plasmid, gene cloning, and site-directed mutagenesis

M. xanthus DK 1622 (KCCM, Seoul, Republic of Korea), *Escherichia coli* ER2566, and pET-28a were used as the source of genomic DNA, host cells, and expression vector, respectively. Cloning and site-direct mutagenesis were carried out using primers synthesized by Macrogen (Seoul, Republic of Korea) (Supplementary Table 1). Primer sequences with *Eco*RI and *Not*I restriction sites designed based on the DNA sequence of a putative LOX from *M. xanthus* (GenBank accession number, WP_011551854.1) were used for gene cloning. The gene encoding the putative LOX was amplified by PCR using *M. xanthus* genomic DNA as the template and *Taq* polymerase (Solgent, Daejon, Korea). The DNA fragment was ligated with the pET-28a vector and transformed into *E. coli* ER2566. Recombinant *E. coli* was plated on Luria-Bertani (LB) agar containing 0.1 mM kanamycin, an antibiotic resistant colony was selected, and the plasmid DNA sequence was checked by Macrogen. Site-directed mutagenesis was carried out using the Quick-Change kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions.

2.3. Cellular fatty acid composition

Cellular fatty acid composition was measured by the Microbial Identification Incorporation (MIDI) Sherlock system. The harvested *M. xanthus* cells (40 mg) were resuspended in 1 mL of reagent A (50% methanol containing 150 mg sodium hydroxide), and the mixture was heated in boiling water on 30 min for saponification. After cooling, 2 mL of reagent B (methanol/hydrochloric acid, 1:1.2 *v/v*) was added to the mixture and heated for 10 min at 80 °C for methylation. Fatty acid methyl esters were extracted with 1.25 mL of reagent C (hexane/methyl *tert*-butyl ester, 1:1 *v/v*) and the aqueous phase was removed. The extracted fatty acid methyl esters in the solvent phase were analyzed by GC (Agilent 6890N, Santa Clara, CA, USA) with a flame ionization detector.

2.4. Culture conditions and enzyme expression

Recombinant *E. coli* was cultivated at 37 °C in a 2-L flask containing 450 mL of LB medium supplemented with 0.1 mM kanamycin with shaking at 200 rpm. When the optical density of the bacterial culture at 600 nm reached 0.6, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce enzyme expression, and the culture was further incubated at 16 °C with shaking at 150 rpm for 18 h. Recombinant cells expressing LOX were harvested by centrifugation at 13,000 × *g* for 20 min at 4 °C and stored at −80 °C.

2.5. Enzyme purification

The harvested cells were used for enzyme purification as described previously [17]. The cells were disrupted using a sonicator on ice for 20 min. Unbroken cells and cell debris were removed by centrifugation at 13,000 × *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45-μm-pore-size filter. The filtered solution 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 300 mM NaCl. Bound protein in the cartridge was eluted with a linear gradient of 10–250 mM imidazole at a flow rate of 1 mL min^{-1}. Active fractions were collected and loaded onto a Bio-Gel P-6 desalting cartridge (Bio-Rad) equilibrated with 50 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl]-propanesulfonic acid (EPPS) buffer (pH 8.5). The loaded protein was eluted using the same buffer at a flow rate of 1 mL min^{-1}, and the eluted protein was used as the purified enzyme.

2.6. Determination of molecular mass and metal ion in enzyme

The subunit molecular mass of the putative LOX from *M. xanthus* was examined by SDS-PAGE under denaturing conditions using molecular mass marker proteins as reference proteins. The total molecular mass of the 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 150 mM NaCl at a flow rate of 1 mL min^{-1}. The retention time of the putative LOX from *M. xanthus* was measured during elution. The column was calibrated with catalase (206 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) as a gel filtration calibration kit (Amersham Pharmacia Biotech, now changed to GE healthcare) [27,28]. The total molecular mass of the enzyme was calculated by comparison with the retention times of the reference proteins. The contents of metal ions in the purified putative LOX from *M. xanthus* were measured by ICP-MS. The purified LOX with a final concentration of 370 μg mL^{-1} (4.62 μM) was prepared. The analysis was performed using NexOn 350D (PerkinElmer SCIEX, Wellesley, MA, USA) at the NCIRF facility (Seoul National University, Seoul, Republic of Korea). The instrument was calibrated by