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A multifunctional lipoxygenase from *Pyropia haitanensis*— The cloned and functioned complex eukaryotic algae oxylipin pathway enzyme

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ABSTRACT

Lipoxygenases (LOXs) are critical starting biocatalysts for the synthesis of signaling compounds derived from lipid peroxidation. However, LOXs from prokaryotes or lower eukaryotes always show nonspecific and multifunctional properties. Here, an oxylipin pathway enzyme gene of eukaryotic algae, *Pyropia haitanensis* of Bangiaceae (Rhodophyta), named PhLOX was cloned and characterized; a phylogenetic analysis was also performed. This unique LOX isoform is a multifunctional enzyme, combined unusually high hydroperoxidelyase (HPL), lipoxygenase, and allene oxide synthase (AOS) three catalytic activities within one catalytic domain of the protein, which may explain the observed diversity of *P. haitanensis* oxylipins. Phylogenetic analysis indicated that red algae LOXs separated from the LOX clades of the ancestor of higher plant and animal in the early stages of evolution. The substrate flexibility, oxygenation position flexibility, and functional versatility of PhLOX represented typical properties of lower organisms. These results indicated that the origin of the oxylipin biosynthetic gene and the oxylipin biosynthetic pathway of some red algae are unique, which may shed light on the specific defense strategies of these red algae and the evolution of the oxylipin pathway as well as the compact genome of red algae.

1. Introduction

Lipid peroxidation is an oxidative process common to all biological systems. Oxylipins derived from the oxidative metabolism of polyunsaturated fatty acids (PUFAs) are known to appear in developmentally regulated processes and in response to environmental changes in both terrestrial plants and animals [1]. Marine algae live in a complex seawater environment, with sessile and intertidal properties of many marine macroalgae continuously challenged by a variety of potentially pathogenic organisms and multivariate environmental changes [2]. These algae obviously had to evolve defensive strategies to survive in such a hostile environment [3]. As a defense against both abiotic and biotic stresses, evolved oxylipin mechanisms have been found to be ubiquitous among eukaryotic algae [4]. In metabolic studies of red and brown algae, a diversity of hydroperoxides, prostaglandins, short chain aldehydes and alcohols has been found [1,5]. However, until recently, very limited information has been available regarding the enzymes responsible for the biosynthesis of these compounds, with many important questions as yet unaddressed. First, what enzymes produce these complex algal oxylipins? The biosynthesis of oxylipins involves lipoxygenases (LOXs) and several enzyme members of the cytochrome P450 family, designated CYP74, which includes allene oxide synthase (AOS), hydroperoxide lyase (HPL), and divinyl ether synthase [6]. In most plant species, LOXs are encoded by gene families, comprising a number of isozymes, which differ in the substrate oxygenation position and substrate specificity [7]. However, in the lower organism, the oxylipin pathway is strikingly simple and its multifunctional properties have been increasingly reported [8–12]. In previous research, we have found the oxylipins in *Pyropia haitanensis* were different from that of other algae, with no hydroperoxides detected but an abundance of short chain volatiles found. There is a possibility that the LOX of *P. haitanensis* and those reported in lower organisms possess common properties, including substrate flexibility and function versatility. Second, what was the role of algae in the evolution of the oxylipin defense pathway? Flowering plants appear to use octadecanoic PUFAs, such as linoleic (18:2, LA) and α-linolenic acid (α-18:3, ALA) as the main precursors for jasmonic acid or other oxylipins. In invertebrate animals and mammals, oxylipins and particularly prostaglandins derive predominantly from eicosanoic PUFAs [13]. Researchers have found that both octadecanoid and eicosanoid metabolism occurred in ancient algal stages, suggesting that they have both animal as well as plant

Abbreviations: LOX, lipoxygenases; AOS, allene oxide synthase; HPL, hydroperoxide lyase; PUFA, polyunsaturated fatty acid; LA, linoleic; ALA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EST, expressed sequence tag; HETE, hydroxyl eicosatetraenoic acid; HEPE, hydroxyl eicosapentaenoic acid; HpODE, hydro(pero)xy octadecadienoic acid; HpOTE, hydro(pero)xy octadecatrienoic acid; HpETE, hydro(pero)xy eicosatetraenoic acid; HpEPE, hydro(pero)xy eicosapentaenoic acid; HpDHE, hydro-(pero)xy docosahexaenoic acid; EETE, epoxy eicosatrienoic acid; GSH-GPx, glutathione–glutathione peroxidase; SPME, solid phase micro-extraction; SRPBCC, START/RHOalphaC/PITP/Bet v1/CoxG/CalC.

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properties [14]. Thus, as the initial enzyme of oxylipin pathway, how have the LOXs from red algae evolved and what are the evolutionary relationships between LOXs from plants, animals, and prokaryotes?

LOXs are the initial and key enzymes in the oxylipin pathway and are ubiquitous in plants, prokaryotes, and animals [15]. Recently, data regarding LOXs from lower organisms, such as cyanobacteria, coral, and moss, have been published [1,8–10]. Nonetheless, there is little information concerning the origin and function of marine algal LOXs and of how and to what extent the oxylipin pathway is present in marine algal kingdoms. In addition, there is the question of whether these pathways evolved independently in the different kingdoms. In this study, a LOX gene from *P. haitanensis* (Bangiales, Rhodophytes), a typical warm, temperate zone species [16], was identified and its versatile functions and enzyme products described. The results raised interesting questions concerning this enzyme's evolutionary heritage and its relationship to the known diversity of oxylipin structures and functions. Finally, the catalytic character of this LOX variety was elaborated to illuminate its role as a defense strategy in red algae.

2. Materials and methods

2.1. Plant material

A gametophyte of *P. haitanensis* was collected at Hepu, Xiangshan Harbor, Zhejiang Province, China (29°09′18″N, 121°54′05″W) in 2011.

2.2. DNA and RNA isolation

P. haitanensis gametophyte genomic DNA and total RNA were isolated using the E.Z.N.A.™ HP Plant DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and Takara RNAiso Plus kit (Takara Bio Inc., Otsu, Japan), respectively. The total RNA was then retranscribed into cDNA using a Takara Primescript RT reagent kit (Takara Bio Inc.).

2.3. Isolation and sequence analysis of P. haitanensis LOX gene

Retrieval and alignment of algal LOX associated sequences in NCBI and *Pyropia yezoensis* EST database (http://est.kazusa.or.jp/en/plant/porphyra/EST/) were performed to design a pair of primers for amplification of the *P. haitanensis* LOX gene. The primers were LOXs/LOXa (5′ CTCACCCGCAAGGGAGATGG3′/5′GGACGCTGGGAAGGAGGTGA3′), and a fragment of 624 bp was obtained following a PCR procedure at 94 °C for 3 min; 94 °C for 30 s, 58 °C for 35 s, 72 °C for 40 s, 35 cycles and then 72 °C for 10 min. The technique of 3′ and 5′ RACE (Rapid Amplification of cDNA Ends) was used to obtain its complete ORF. The primers were LOX-os/LOX-is (5′GCCCTCCCGTCCACCCACGTT3′/5′TGCCCCAC TTCGCCGACACC3′) and LOX-oa/LOX-ia (5′CGAGCCCAGGAAGTCCCA CCCTT3′/5′GCCGCCGAGAAGACGTCCATCC3′) for 3′ and 5′-terminal amplification, respectively, by following the nested PCR procedure. The obtained PCR fragment was sequenced and blasted in NCBI data bank and designated as *PhLOX*. The phylogenetic analysis was monitored by aligning on http://clustalw.ddbj.nig.ac.jp/ with default parameters and then edited in TreeView. An LOX domain structural graphic was constructed according to N- and C-terminal domains, based on all the LOX gene sequences annotated in NCBI "Conserved domains". The graphic architecture was based on the diagram of Whittaker's five-kingdom system.

2.4. Prokaryotic expression and purification of PhLOX in Escherichiacoli

The primers, *PhLOX*F/*PhLOX*R (5′GGAATTCCATATGATGGGGAATGCG 3′, *Nde*I-site underlined, 5′CCCAAGCTTCTAGATGTCGATGGACAG3′, *Hin*dIII-site underlined), were combined to clone the target gene from *P. haitanensis* cDNA. The amplicon was inserted into pET-28a (+) and transformed into *E. coli* BL21 cells to express a recombinant protein. The transformant protein was induced in the presence of 0.1 mM isopropyl thio-β-galactoside and the cells then harvested, lysed, and centrifuged. The resulting crude enzyme supernatant was purified using a Ni-Agarose 6×His-Tagged Protein Purification Kit (Accuprep, Bioneer Corp., Alameda, CA, USA). The PhLOX protein purity was evaluated by SDS-PAGE electrophoresis.

2.5. Sample preparation for LC–MS and GC–MS analysis

The focus here was on PhLOX catalytic activity for octadecanoids and eicosanoids (LA, α-ALA, arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), 9-hydroperoxyoctadecatrienoic acid (9-HpOTE), 12-hydroperoxyeicosatetraenoic acid (12-HpETE), and isotopically-labeled [D_8]-ARA). For LC–MS sample preparation, enzyme catalytic products were produced by incubating a 100 μM substrate in 0.5 ml 50 mM Tris buffer (pH 8.0) with 0.12 mg ml^{-1} of pure enzyme at 20 °C and the reaction terminated at defined time points by adding 1 ml of ethyl acetate and shaking under 4 °C for 1 h to extract products. In glutathione–glutathione peroxidase (GSH-GPx) system, 2 U ml^{-1} GPx and 3 mM GSH were simultaneously added in the reaction solution with substrates [17]. After centrifugation at 12,000 rpm for 10 min, the supernatant organic phase was decanted. The organic solvent was evaporated and redissolved in 500 μl of methanol for LC–MS analysis. The substrate selectivity of PhLOX was determined by adding mixed substrates, at 100 μM each, to a sample of the enzyme, as above. Substrate utilization was quantified using six PUFA standards dissolved at 100 μM in 50 mM Tris buffer (pH 8.0). Calibration curves for the standards were established by peak area integration of different fatty acid standard concentrations. Quantities from utilization analyses were determined using calibration curves. Consumption ratio = [(substrate content before reaction − substrate content after reaction) / substrate content before reaction] × 100%.

For GC–MS sample preparation, initial enzyme products were produced by incubation of a 100 μM substrate in 0.5 ml of 3.3 mg ml^{-1} of pure enzyme at 20 °C and the reaction stopped at defined time points by adjusting the pH to 12.5 with 4 M NaOH. Volatile products were extracted from the resulting mixture by incubation at 40 °C for 50 min using a solid phase micro-extraction device (Supelco Inc., Bellefonte, PA, USA).

2.6. LC–MS analysis

LC–MS analysis was carried out on a Finnigan Surveyor and TSQ Quantum Access system (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). A Hypersil Gold C18 column (2.1 × 100 mm, 3 μm; Thermo Fisher Scientific Inc.) was used at 30 °C with a solvent system of acetonitrile (A)-0.2% acetic acid (B) and a flow rate of 0.2 ml min^{-1}. The sample was eluted by a programmed solvent gradient of 30 to 55% A over 4 min, then to 80% A over 32 min, and finally to 100% A over 4 min. Absorption scanning from 234 nm to 280 nm were recorded.

High-resolution mass spectrometry was performed on a Q Exactive hybride quadrupole-Orbitrap mass spectrometer operating in the data dependent mode to automatically switch between full scan MS and MS/MS acquisition in negative ion mode. Survey full scan MS spectra with mass range scanning from 50 to 500 was acquired in the Orbitrap with 70,000 resolution (m/z 200) after accumulation of ions to a 1×10^6 target value based on predictive AGC from previous full scan. Dynamic exclusion was set to 70 s. The maximum ion time is 250 ms. The MS/MS parameters were set as follows: AGC target 2×10^5; maximum ion time 250 ms; isolation width 2 Da. Typical mass spectrometric conditions were: spray voltage, 2 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275; normalized collision energy was set to 25%, and activation time to 20 ms [18].

9-HpODE, 9-HpOTE, and 13-HpOTE were separated on a Waters Millennium HPLC system (Waters, Milford, MA) with a semi-preparative column (Nova-Pak®HR C18, 6 μm, 60 Å, 7.8 × 300 mm, PrepColumn).