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Epoxyalcohol synthase of *Ectocarpus siliculosus*. First CYP74-related enzyme of oxylipin biosynthesis in brown algae☆

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ABSTRACT

Enzymes of CYP74 family play the central role in the biosynthesis of physiologically important oxylipins in land plants. Although a broad diversity of oxylipins is known in the algae, no CYP74s or related enzymes have been detected in brown algae yet. Cloning of the first CYP74-related gene *CYP5164B1* of brown alga *Ectocarpus siliculosus* is reported in present work. The recombinant protein was incubated with several fatty acid hydroperoxides. Linoleic acid 9-hydroperoxide (9-HPOD) was the preferred substrate, while linoleate 13-hydroperoxide (13-HPOD) was less efficient. α-Linolenic acid 9- and 13-hydroperoxides, as well as eicosapentaenoic acid 15-hydroperoxide were inefficient substrates. Both 9-HPOD and 13-HPOD were converted into epoxyalcohols. For instance, 9-HPOD was turned primarily into (9*S*,10*S*,11*S*,12*Z*)-9,10-epoxy-11-hydroxy-12-octadecenoic acid. Both epoxide and hydroxyl oxygen atoms of the epoxyalcohol were incorporated mostly from [$^{18}O_2$]9-HPOD. Thus, the enzyme exhibits the activity of epoxyalcohol synthase (EsEAS). The results show that the EsEAS isomerizes the hydroperoxides into epoxyalcohols via epoxyallylic radical, a common intermediate of different CYP74s and related enzymes. EsEAS can be considered as an archaic prototype of CYP74 family enzymes.

1. Introduction

Enzymes of CYP74 family play a central role in the plant lipoxygenase pathway [1–3]. They control the conversions (dehydration or isomerization) of fatty acid hydroperoxides to numerous oxylipins, including the bioactive compounds like jasmonates, divinyl ethers, aldehydes and others [1–3]. The diversity of CYP74-related enzymes has recently been extended to some proteobacteria, rhizobacteria and Metazoa [4]. These new members expanded the CYP74 diversity from family to the clan (for definitions of P450 family and clan see [5]). Most of these new clan members are waiting for their cloning and studies of specificity of their action. Besides, typical CYP74 products like divinyl ethers have been detected in some living organisms where CYP74-related genes and enzymes have not been encountered yet. Marine algae possess a broad diversity of oxylipins [6–9]. For instance, the brown alga *Laminaria sinclairii* [10], and red alga *Polyneura latissima* [11] contain the divinyl ethers. Identical or analogous oxylipins are well known as the products of divinyl ether synthases (CYP74 family), which are widespread in higher plants [12]. The biosynthetic origin of algal divinyl ethers has not been revealed yet. Involvement of CYP74-related enzymes in the biosynthesis of algal oxylipins seems to be very likely.

Although the CYP74 family enzymes are widespread in land plants, none of CYP74 clan members has been detected in algae until recently. The freshly discovered allene oxide synthase (AOS) of green alga *Klebsormidium flaccidum* [13] presents the only exception. An unusual gene *CYP5164B1* homologous to the CYP74s has been detected recently as a result of the annotation of brown alga *Ectocarpus siliculosus* (Ectocarpaceae, Phaeophyceae) genome [14]. These considerations prompted us to clone the *CYP5164B1* gene and test putative fatty acid hydroperoxide metabolizing activity of the recombinant protein. The results are reported in the present paper.

Abbreviations: EAS, epoxyalcohol synthase; EsEAS, *E. siliculosus* epoxyalcohol synthase; DES, divinyl ether synthase; AOS, allene oxide synthase; HPL, hydroperoxide lyase; 9-H(P)OD, (9*S*,10*E*,12*Z*)-9-hydro(pero)xy-10,12-octadecadienoic acid; 9-H(P)OT, (9*S*,10*E*,12*Z*,15*Z*)-9-hydro(pero)xy-10,12,15-octadecatrienoic acid; 13-H(P)OD, (9*Z*,11*E*,13*S*)-13-hydro(pero)xy-9,11-octadecadienoic acid; 13-H(P)OT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydro(pero)xy-9,11,15-octadecatrienoic acid; 15-H(P)EPE, (5*Z*,8*Z*,11*Z*,13*E*,15*S*,17*Z*)-13-hydro(pero)xy-5,8,11,13,17-eicosapentaenoic acid; IMAC, immobilized metal affinity chromatography; Me, methyl; TMS, trimethylsilyl; SIM, selected ion monitoring; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

☆ This paper is dedicated to the memory of Professor Victor E. Vaskovsky (1935-2016), a distinguished researcher in the fields of lipidology and marine biology, whose friendly care and encouragement were essential for this work.

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2. Materials and methods

2.1. Materials

[^{18}O]Water (95% ^{18}O) and $^{18}O_2$ gas (88% ^{18}O) were purchased from ZAO Neogaz (Moscow, Russia). [$^2H_6$]Benzene (99.5% ^{2}H) was acquired from FSUE RSC "Applied Chemistry" (St. Petersburg, Russia). Linoleic, α-linolenic, and eicosapentaenoic acids, as well as the soybean lipoxygenase type V, were purchased from Sigma. [1-^{14}C]Linoleic acid (2.072 MBq/μmol) was purchased from Perkin Elmer (former New England Nuclear). $NaBH_4$ and silylating reagents were purchased from Fluka (Buchs, Switzerland). (9*S*,10*E*,12*Z*)-9-Hydroperoxy-10,12-octadecadienoic (9-HPOD), and (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic (9-HPOT) acids, as well as the [1-^{14}C]9-HPOD (5.78 kBq/μmol), were prepared by incubation of linoleic, α-linolenic, and [1-^{14}C]linoleic acids, respectively, with the recombinant maize 9-lipoxygenase (GeneBank: AAG61118.1) [15] at 0 °C, Na-phosphate buffer (100 mM, pH 6.0), under continuous oxygen bubbling. (9*Z*,11*E*,13*S*)-13-Hydroperoxy-9,11-octadecadienoic (13-HPOD), (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic (13-HPOT), and (5*Z*,8*Z*,11*Z*,13*E*,15*S*,17*Z*)-13-hydroperoxy-5,8,11,13,17-eicosapentaenoic (15-HPEPE) acids were obtained by incubation of linoleic, α-linolenic, and eicosapentaenoic acids, respectively, with the soybean lipoxygenase type V at 23 °C, Tris-HCl buffer (50 mM, pH 9.0), under continuous oxygen bubbling. The extracted hydroperoxides (as free carboxylic acids) were purified by normal phase HPLC (NP-HPLC) on the Kromasil Si columns (7 μm; 4.0 × 250 mm; Elsico, Moscow, Russia) under the isocratic elution with the solvent mixture hexane-isopropanol-acetic acid 98.1:1.8:0.1 (*v*/v) at a flow rate of 0.4 mL/min. Hydroperoxides were chromatographically pure and at least 98% optically pure, as judged by chiral phase HPLC [16]. Labelled [$^{18}O_2$-hydroperoxy]9-HPOD was obtained similarly by incubations under $^{18}O_2$ atmosphere.

2.2. Bioinformatics methods

The search for CYP74-related proteins predicted from *E. siliculosus* genome data was performed using the theoretical BLAST analyses in NCBI and Online Resource for Community Annotation of Eukaryotes (ORCAE) databases. Primer construction and multiple sequences alignments were performed using Vector NTI program (Invitrogene, U.S.A.). The BLAST analyses of the CYP74s were performed using the protein NCBI BLAST tool. Multiple protein alignments have been made using the Clustal Omega. The phylogenetic trees of selected CYP74s and P450s were built using the ClustalW2 – Phylogeny and viewed with the TreeView software.

2.3. Expression and purification of recombinant enzyme

The CYP5164B1 coding sequence was adapted for expression in *Escherichia coli* cells (Supplementary Fig. 1) and synthesized in Evrogen Company (Moscow, Russia). The sequence was cloned into the pET-23a vector (Novagen, USA) to yield the target recombinant protein with His-tag at C-terminus. The recombinant gene was expressed in BL21(DE3)pLysS strain cells (Novagen, USA) as follows. An overnight culture (10 mL) of bacteria was inoculated into 1 L of Luria-Bertani medium supplemented with one volume of mineral medium M9. Bacteria were grown at 37 °C, 250 rpm to an OD_{600} of 0.6. Expression of the target gene was induced by addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside to the medium. Simultaneously, the medium was supplied with 5-aminolevulinic acid to facilitate the haem formation. Induction was controlled by the 12% SDS-PAGE analysis of crude cellular extracts. Bacteria were harvested by centrifugation for 15 min at 4500 rpm at 4 °C and lysed with BugBuster Protein Extraction Reagent (Novagen, USA). Purification of His-tagged recombinant protein was performed using Bio-Scale Mini Profinity IMAC cartridge in BioLogic LP chromatographic system (Bio-Rad, USA). The recombinant enzyme was eluted from the cartridge using 50 mM histidine. The recombinant protein concentration was determined using the Quant-iT™ Protein HS Assay Kit (Invitrogen, USA). The haemoprotein concentration was estimated using the pyridine haemochromogen assay [17]. The relative purity of recombinant protein was estimated by SDS-PAGE and staining of the gel with Coomassie brilliant blue R-250 (Supplementary Fig. 2). Enzymatic activity of the purified recombinant enzyme was determined by monitoring the decrease of the signal at 234 nm in a Perkin Elmer Lambda 25 UV-VIS spectrophotometer with 40 μM substrate concentration. The analyses were performed in 0.6 mL of Na-phosphate buffer (100 mM, pH 7.0) at 25 °C. The initial linear regions of the kinetic curves were used to calculate the rates. The molar extinction coefficient for 9- and 13-hydroperoxides of linoleic acid at 234 nm is 25,000 M^{-1} cm^{-1}. Five independent experiments were performed for each specified variant.

2.4. Incubations of recombinant enzyme with substrates

The recombinant enzyme (25 μg) was incubated with 100 μg of 9-HPOD, 9-HPOT, 13-HPOD, 13-HPOT, or 15-HPEPE in Na-phosphate buffer (100 mM, 10 mL), pH 7.0, 4 °C, for 15 min. The reaction mixture was acidified to pH 6.0, and the products were extracted with hexane/ethyl acetate (1:1, by volume) mixture, methylated with ethereal diazomethane and trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (1:1:1, by volume) mixture at 23 °C for 30 min. Then the silylation reagents were evaporated in vacuo. The dry residue was dissolved in 100 μL of hexane and subjected to GC-MS analyses. When specified, the products were reduced with $NaBH_4$, then methylated and trimethylsilylated. Alternatively, the products of $NaBH_4$ reduction were hydrogenated over PtO_2, then methylated and trimethylsilylated. Products (without or with the preliminary $NaBH_4$ reduction) were analyzed as Me esters/TMS derivatives (Me/TMS) by GC-MS as described before [18]. For micropreparative isolation of products, the recombinant enzyme (25 μg) was incubated with 18.5 kBq of [1-^{14}C]9-HPOD (5.78 kBq/μmol) in the same way. Products were extracted and methylated with ethereal diazomethane. The resulting methyl esters were separated and purified by NP-HPLC as described in the next section. Incubations of the recombinant enzyme (25 μg) with 100 μg of [$^{18}O_2$-hydroperoxy]9-HPOD were performed in the same way. Alternatively, the enzyme was incubated under identical conditions with the unlabelled 9-HPOD in an $^{18}O_2$ atmosphere or in the [^{18}O]water medium (1 mL). The products (Me/TMS) were analysed by the selected ion monitoring (SIM) GC-MS. The selected ions are specified in the Results. The data of the selected ion chromatograms were quantified with the Shimadzu data analysis software.

2.5. Separation and purification of individual oxylipins by normal phase HPLC

Products (Me esters) were separated by NP-HPLC on Macherey-Nagel EC 250/4.6 Nucleodur 100–3 SiOH column under elution with hexane – isopropanol 98:2 (by volume), flow rate 0.4 mL/min. Ultraviolet (UV) detection (190–370 nm) was performed with Shimadzu SPD-M20A diode array detector. Radioactivity was detected by the HPLC radiomonitor model 171 (Beckman Instruments, Fullerton, CA, USA) with a solid scintillator cell (125 μm). Separate products were collected after NP-HPLC separation, redissolved in [$^2H_6$]benzene and subjected to NMR spectral records.

2.6. Methods of instrumental analyses

The UV spectra of products were scanned during the incubations of the recombinant protein with fatty acid hydroperoxides with Varian Cary 50 spectrophotometer. The UV spectra of reaction products were recorded with the same instrument. Alternatively, the UV spectra of