Fatty acid 9- and 13-hydroperoxide lyases from cucumber[1]

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Abstract Fatty acid hydroperoxide lyase (HPL) is a novel *P*-450 enzyme that cleaves fatty acid hydroperoxides to form short-chain aldehydes and oxo-acids. In cucumber seedlings, the activities of both fatty acid 9HPL and 13HPL could be detected. High 9HPL activity was especially evident in hypocotyls. Using a polymerase chain reaction-based cloning strategy, we isolated two HPL-related cDNAs from cucumber hypocotyls. One of them, C17, had a frameshift and it was apparently expressed from a pseudogene. After repairing the frameshift, the cDNA was successfully expressed in *Escherichia coli* as an active HPL with specificity for 13-hydroperoxides. The other clone, C15, showed higher sequence similarity to allene oxide synthase (AOS). This cDNA was also expressed in *E. coli*, and the recombinant enzyme was shown to act both on 9- and 13-hydroperoxides, with a preference for the former. By extensive product analyses, it was determined that the recombinant C15 enzyme has only HPL activity and no AOS activity, in spite of its higher sequence similarity to AOS.

Key words: Fatty acid hydroperoxide lyase; Allene oxide synthase; Volatile aldehyde; *Cucumis sativus*

1. Introduction

Oxylipins are compounds derived from oxygenated fatty acids formed via the action of lipoxygenases (LOXs), cytochrome *P*-450 monooxygenases, or cyclooxygenase-like oxygenases [1]. The LOX-derived compounds in plants, include jasmonates, epoxy- and hydroxy-fatty acids, divinyl ether fatty acids, and short-chain aldehydes. Evidence for diverse roles of oxylipins in plants has been accumulating [1]. For example, jasmonates are established as modulators of fruit ripening, production of viable pollen, root growth, tendril coiling and plant resistance to insects and pathogens [2]. Jasmonates are synthesized in several enzymatic steps from α-linolenic acid, its (13*S*)-hydroperoxide and through the formation of an unstable intermediate, allene oxide, which is formed by 13-allene oxide synthase (13AOS) (Fig. 1) [2]. C_6- or C_9-aldehydes, which are formed by hydroperoxide lyase (HPL) action on 13- or 9-hydroperoxides of linoleic or linolenic acids (Fig. 1), are reported to be involved in the hypersensitive resistance response of plants infected by pathogens [3]. Furthermore, it has been reported that these aldehydes can induce expression of a subset of genes involved in disease resistance [4] and that they are involved in a defense response against insect herbivores [5]. Recently, both AOS and HPL have been purified and cloned from plants. It was found that they are heme enzymes grouped into novel families of the cytochrome *P*-450s, CYP74A and 74B, respectively [6–15]. Both AOS and HPL need no cofactor, such as molecular oxygen or reducing equivalents, which are generally essential for most *P*-450 enzymes. It has been assumed that HPL and AOS share at least some common features of their catalyses.

HPLs isolated from tea leaves [6], bell pepper fruits [9], *Arabidopsis* leaves [12,13], and tomato fruits [15] all have high substrate specificity for the 13-hydroperoxide of α-linolenic acid, and nearly no activity can be found with the 9-hydroperoxide. Nevertheless, it has been known that some plants, especially the Cucurbit family have the ability to form C_9-aldehydes such as (3*Z*)-nonenal, or (3*Z*,6*Z*)-nonadienal from the 9-hydroperoxides of linoleic acid or linolenic acid, respectively [1,16]. To date, there has been no report on the molecular structure of a HPL acting on 9-hydroperoxides, and it has not been known whether 9HPL is structurally related to 13HPL or AOS. The objective of this study was to isolate a gene encoding a HPL which can act on 9-hydroperoxides to form C_9-aldehydes, and to determine the properties of this HPL. Upon cloning the cucumber hypocotyl 9HPL, we found that this HPL has a higher structural similarity to AOS than to the other 13HPLs. Nonetheless, the 9HPL showed no AOS activity.

2. Materials and methods

Cucumber (*Cucumis sativus* L. cv. Suyo) seeds were soaked in water for 12 h and sown on moistened vermiculite. Seedlings were grown in darkness or under a 16 h light/8 h dark cycle at 25°C. For preparation of crude homogenates, cucumber organs were homogenized with four volumes of 0.1 M potassium phosphate buffer, pH 6.5, containing 0.2% Tween 20. The homogenate was centrifuged at 1000×*g* for 10 min at 4°C, and the resulting supernatant was used as crude homogenate. HPL activity was determined by high performance liquid chromatography (HPLC) quantification of the aldehydes as described [16]. With the purified enzyme, HPL activity was determined by following the decrease of the absorption at 234 nm, originating from the conjugated diene chromophore of the substrates [16]. (3*Z*)-Nonenal was formed through Jones oxidation of (3*Z*)-nonen-1-ol, and (3*Z*,6*Z*)-nonadienal was synthesized as described elsewhere [17].

Total RNA was isolated from 3-day-old cucumber hypocotyls by TRIzol reagent (BRL), and poly(A)$^+$ RNA was isolated using Oligotex mRNA kit (Qiagen, Valencia, CA, USA). Double-stranded cDNAs having adapters at both ends were synthesized with a Mar-

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[1] The nucleotide sequences reported here have been submitted to the GenBank/EMBL data bank with accession numbers AF229811 (C15) and AF229812 (C17).

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athon cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). In order to amplify the internal sequences of HPLs, the forward primers HPOL3S (5′-GGNTTYAAYGCNTWYGGNGG-3′, where N = AGCT, R = AG, Y = CT, W = AT) and HPOL4S (5′-GTNTTYGAYGANCCNGA-3′), and the reverse primers HPOL7AS (5′-CYTTNGCNGCRCAYTGYTTRTT-3′) were used. Polymerase chain reaction (PCR) was carried out with AmpliTaq GOLD (PE Biosystems, Chiba, Japan) using a touch-down program: 95°C for 10 min, 15 cycles of 94°C for 30 s, 60–51°C (decreased by 0.6°C in every cycle) for 1 min, 72°C for 2 min, then 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. With the combination of HPOL3S and HPOL7AS, amplification products of about 470 bp were obtained, while with HPOL4S and HPOL7AS, products of about 170 bp were seen. These cDNA fragments were sequenced and found to be related to HPLs, and were subsequently referred to as C15 and C17, respectively. For the isolation of the corresponding full-length cDNAs, rapid amplification of cDNA ends (RACE)-PCR was carried out as described in the manual supplied with the Marathon kit. For C15, 5′-CCCAGCCGTGGCCACCGCTCCTAGAGGTGGC-3′ (for 5′-RACE), and 5′-CTGGCGGAGGAAGTGAGGACAACCGTGAA-3′ (for 3′-RACE) were used, and for C17, 5′-CACTGTTTGTTCTTCTCGCTCGGTGTCCCCG-3′ (for 5′-RACE) and 5′-CCGGGGAGAGAAAGGGGCAGCGCTGCTGG-3′ (for 3′-RACE) were used as gene-specific primers. The amplified fragments were combined to form the corresponding full-length cDNAs by using internal restriction enzyme recognition sites. Sequence data analyses were performed using the ClustalW algorithm in the DNA data bank of Japan (http://www.ddbj.nig.ac.jp).

For heterologous expression of the two cDNAs in *Escherichia coli*, *Bam*HI sites were incorporated into the predicted N-terminal start codons using PCR, and then the cDNAs were ligated into a pQE vector. For mutagenesis of the internal stop codon in C17, a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used with 5′-GCCAGAGCCAGAAAAGATTTCAAGCTTAC-3′ and 5′-GTAAGCTTGAAATCTTTTCTGGCTCTGGC-3′ as primers. *E. coli* M15 [pREP4] was transformed with the respective constructs. Expression and purification of the recombinant HPLs was accomplished as described [15].

An affinity-purified recombinant barley AOS-1 (2 μg) [14] or the recombinant C15 enzyme diluted in 0.9 ml 100 mM potassium phosphate buffer, pH 7.0, was incubated with either 100 nmol of [1-^{14}C]13HPOD or 100 nmol of [1-^{14}C]9HPOD for 20 min at room temperature. The reaction was stopped by adding 100 μl of glacial acetic acid and extracted twice with diethyl ether. The combined organic phases were evaporated under a stream of nitrogen and lipids were reconstituted in HPLC solvent and HPLC analysis was performed essentially as described previously [14,18].

3. Results

3.1. High 9HPL activity was found in cucumber hypocotyls

Although molecular characterization of 13HPLs from various plants has been reported, that of 9HPL has not. To isolate a cDNA clone encoding a HPL having the capacity to form C_9-aldehydes, we selected cucumber seedlings as the enzyme and mRNA source because it is known that cucumber seedlings have both 9- and 13HPL activities [16]. As shown in Fig. 2A, both of these activities could be found in each organ of cucumber seedlings grown in the dark. The relative ratios of the two HPL activities differed to each other, i.e. 9HPL activity was higher than 13HPL activity in hypocotyls, while cotyledons and roots showed higher 13HPL activity. This suggested that cucumber seedlings have at least two HPL enzymes differing in their substrate/product specificities and their expression characteristics. In hypocotyls, the HPL activities continuously increased during the growth of the seedlings (Fig. 2B). In the hypocotyls of 3-day-old seedlings, there was little activity of 9HPL but significant activity of 13HPL could be detected. After 5 days 9HPL activity started to increase. At 7 days post-germination, almost the same activities could be seen, and slightly higher 9HPL activity could be detected thereafter. This profile of the developmental changes of HPL activities could also be observed even if the activities were expressed per g fresh weight. When the etiolated seedlings were transferred into the light, elongation of the hypocotyls stopped. However, both 9HPL and 13HPL activities continued to increase and the developmental time-course of the activities was scarcely changed (not shown). This developmental time-course of 9HPL activity is totally different from that observed for alfalfa seedlings, where the activity decreased gradually after germination [19].

3.2. Molecular cloning of two HPL cDNAs from cucumber hypocotyls

We extracted total RNA from 3-day-old cucumber hypocotyls and constructed a double-stranded cDNA pool derived from the organ. By aligning HPL sequences from bell pepper fruits [10], *Arabidopsis* leaves [12,13], and banana leaves [20], we identified seven highly-conserved regions suitable for the design of degenerate primers. Among these primers, the combination of HPOL3S and HPOL7AS, and that of HPOL4S and HPOL7AS, resulted in the amplification of DNA fragments having expected lengths; i.e. 470 bp for C15 and 170 bp for C17, respectively. Sequencing revealed that they related to HPL and AOS genes. Thus, RACE-PCR was performed to obtain corresponding full-length cDNAs. The C15 cDNA is 1813 bp in length and contains a 67 bp 5′-untranslated region, an open reading frame of 1434 bp, and 280 bp prior to the poly(A)$^+$ sequence. The 5′-untranslated region contains a single stop codon 45 bp upstream of the first deduced methio-

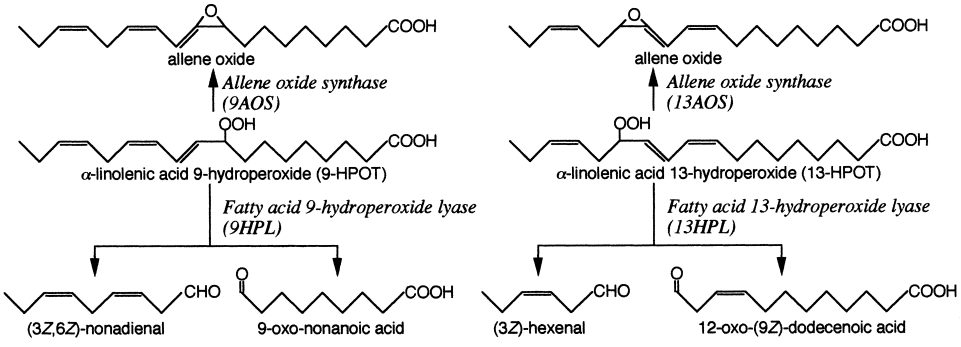

Fig. 1. The reactions catalyzed by HPL and AOS in plants. The fatty acid hydroperoxides are formed via the action of LOXs. Linoleic acid is also incorporated into this system, but (3*Z*)-nonenal and *n*-hexanal are formed by HPL instead of those in this figure. Allene oxides are unstable and are spontaneously converted into α- and γ-ketols in aqueous media.

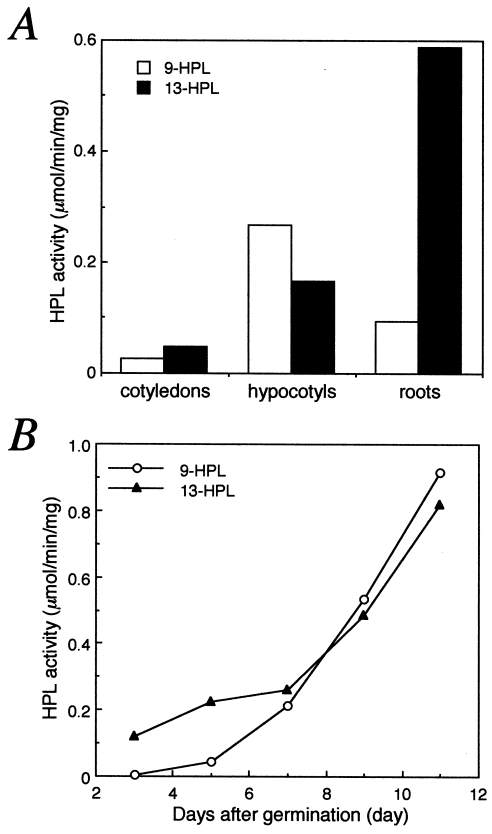

Fig. 2. Distribution of 9HPL and 13HPL activities in cucumber seedlings (A). Cucumber seedlings grown in the dark for 5 days were separated into each organ and both HPL activities were determined. Developmental time course of the activities in etiolated cucumber hypocotyls (B) are shown.

nine. The open reading frame encodes a protein of 478 amino acids with a calculated molecular mass of 53 886 Da. The deduced amino acid sequence has high sequence similarity to *Arabidopsis* AOS (51.4%), flaxseed AOS (51.2%) and rubber particle protein (51.6%), while with HPLs it shows a lower similarity of about 40%. The C17 cDNA is 1712 bp in length and contains a 81 bp 5′-untranslated region, an open reading frame of 1137 bp, and 460 bp prior to the poly(A)$^+$ sequence. Alignment of the C17 nucleotide sequence with those of other HPL and AOS sequences showed that the similarity extended throughout the sequence, even in the sequence downstream of the stop codon at position 1219. In fact, the degenerate primers used for the amplification of C17 hybridize to the 3′-untranslated region of the C17 sequence. By translating the nucleotide sequence into the corresponding amino acid sequences in all three reading frames, we realized that high similarity to the other CYP74s could be found in the region downstream of the stop codon. We found that incorporation of two adenines into a position 31 bp upstream of the stop codon made the deduced amino acid sequence have highest similarity to CYP74s. The modified cDNA sequence contains an open reading frame of 1443 bp, and could encode a hypothetical protein of 482 amino acids with a calculated molecular mass of 54321 Da. The hypothetical amino acid sequence shows high similarity (53.6–61.4%) to HPLs, while the similarity to AOSs was only 41–43%. Although some AOS and HPL proteins are reported to have a putative chloroplast transit peptide [8,11], and it has also been reported that AOS and HPL activities localize to the chloroplasts [18], both of the deduced amino acid sequences apparently lack the typical features of a transit peptide. When the sequence around the original stop codon was determined after PCR amplification of the corresponding genomic region, it was revealed that the stop codon of the C17 cDNA exists in the genomic DNA sequence as well. This indicates that the stop codon is not an artifact during reverse transcription-PCR. Existence of a poly (A)$^+$ tail in C17 also indicates that the cDNA was derived from the corresponding mRNA and not from genomic DNA contamination in the RNA preparation. Thus, it can be concluded that C17 is a pseudogene, and it is transcribed and processed to form mRNA. Southern blot analysis under high stringency conditions indicates that both C15 and C17 are present as single-copy genes (data not shown).

Although HPL and AOS are grouped in the same CYP74 family, they can be distinguished from each other at the sequence level. Therefore, in more detailed classification schemes they are grouped into different subfamilies, namely, CYP74A (AOS) and CYP74B (HPL). A phylogenetic tree was constructed to determine the evolutionary placement of the C15 and modified C17 protein sequences in relation to the other CYP74 sequences (Fig. 3). From this tree it is apparent how HPLs and AOSs can be placed in distinctive groups from each other. Within the tree, C15 grouped closely with AOSs, while the modified C17 grouped with HPLs. Because C15 and the modified C17 were placed into different groups, it can be assumed that these two genes diverged from each other in the early phase of the evolution of cucumber.

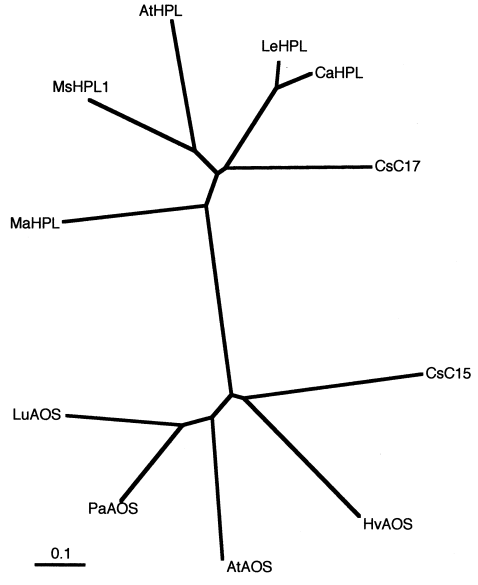

Fig. 3. The unrooted phylogenetic tree of HPLs and AOSs from several plant species. Cucumber C15 (CsC15), cucumber C17 (CsC17), *Arabidopsis* HPL (AtHPL; acc. no. AAC69871), bell pepper HPL (CaHPL; acc. no. AAA97465), tomato HPL (LeHPL; acc. no. CAB43002), alfalfa HPL (MsHPL; acc. no. CAB54849), banana HPL (MaHPL; [21]), barley AOS (HvAOS; acc. no. CAB86384), flaxseed AOS (LuAOS; acc. no. P48417), *Arabidopsis* AOS (AtAOS; acc. no. Q96242), and gyuayule rubber particle protein (PaAOS; acc. no. CAA55025).

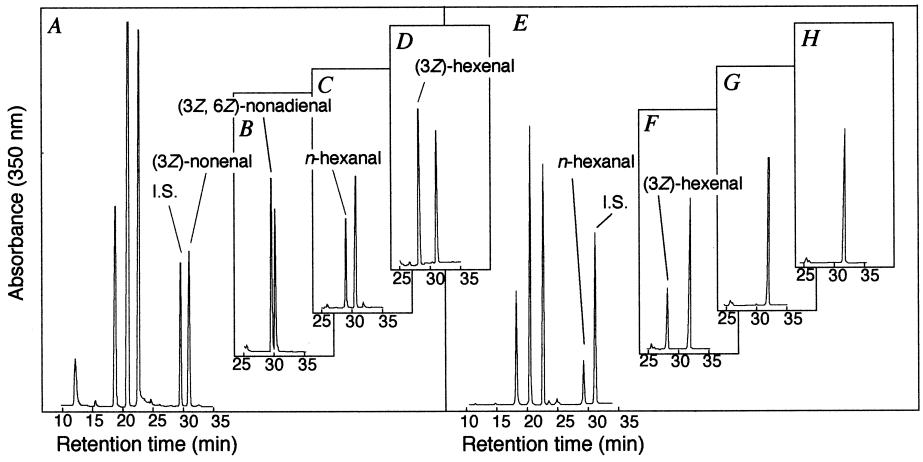

Fig. 4. HPLC detection of C_6 and C_9 aldehydes formed after the reaction of the recombinant C15 (A to D) or of the recombinant modified C17 (E to H) enzymes with 9HPOD (A, G), 9HPOT (B, H), 13HPOD (C, E) or 13HPOT (D, F).

3.3. Expression of recombinant proteins

In order to analyze the enzymatic activities of the two cDNA sequences, both the coding regions of C15 and modified C17 were expressed in *E. coli*. The lysate of cells harboring the C15 cDNA degraded both 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid (9HPOD) and 13-hydroperoxy-(9*Z*,12*E*)-octadecadienoic acid (13HPOD), 9HPOD being the better substrate. During the enzyme reaction, only a decrease in absorbance at the substrate λ_{max} of 234 nm could be detected, and there was no other visible change in absorbance from 200 to 340 nm. When the lysate of the cells harboring the modified C17 cDNA was incubated with 13HPOD or 9HPOD, only 13HPOD was degraded. Using the original unmodified C17 cDNA for *E. coli* expression under the same experimental conditions, no HPO-degrading activity could be detected.

In order to study properties of the recombinant C15 HPL in more detail, the enzyme was affinity-purified to an apparent homogeneous state. As shown in Fig. 4A–D, when HPLC detection was performed, formation of (3*Z*)-nonenal, (3*Z*,6*Z*)-nonadienal, *n*-hexanal, and (3*Z*)-hexenal from 9HPOD, 9-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid (9HPOT), 13HPOD, and 13-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13HPOT), respectively, was observed. This

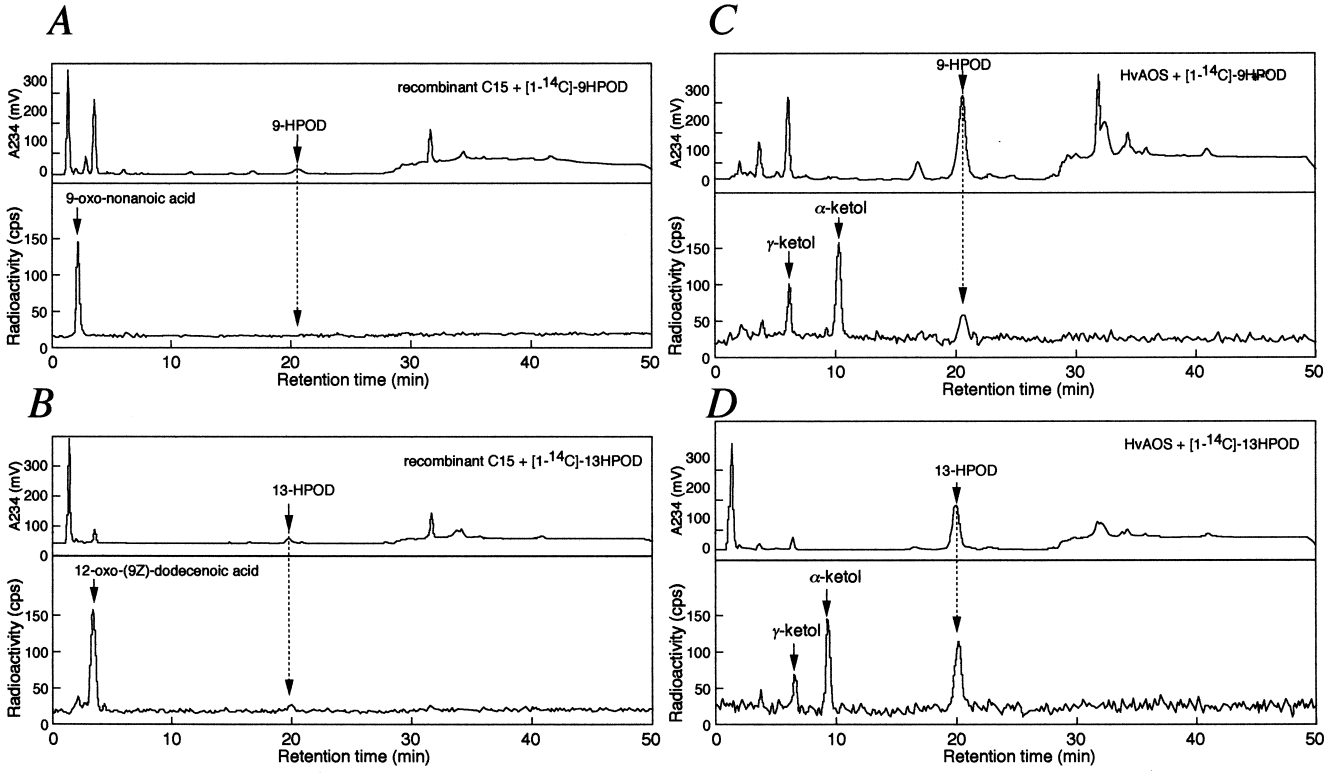

Fig. 5. Radio-HPLC detection of the products formed from [1-^{14}C]9HPOD (A, C) or [1-^{14}C]13HPOD (B, D) by the recombinant C15 enzyme (A, B) or by the recombinant barley AOS1 (C, D).

detection system was not suitable for detection of the corresponding ω-oxo-acids, and they were scarcely visible. Other than these compounds, there were no other significant peaks in the chromatogram; thus, the short-chain aldehydes seemed to be the sole aldehyde products. When the kinetics of substrate consumed and products formed were followed during the reaction, a stoichiometry of almost 1:1 could be observed, which suggests that the aldehydes were the main products formed from the hydroperoxy fatty acids. It has been reported that some plants can form divinyl ether fatty acids, such as colneleic acid and colnelenic acid, from fatty acid hydroperoxides [21,22]. These divinyl ether fatty acids are acid-labile, and upon treatment with acid, they degrade into their corresponding HPL products [21]. Thus, 9HPOD was incubated with the recombinant C15 in buffers of various pH from 4.0 to 10.0, and formation of colneleic acid was monitored spectrophotometrically as described [22]. However, there was no detectable formation of colneleic acid at any pH. Because the primary sequence of C15 has a higher resemblance to AOSs than HPLs, it was hypothesized that the recombinant C15 enzyme might have AOS activity. To test this hypothesis, radiolabeled substrates were used for the enzyme assay. When [1-^{14}C]9HPOD was incubated with the recombinant C15 enzyme, almost all of the added substrate was consumed and 9-oxo-nonanoic acid was the only product formed (Fig. 5A). Products formed by the action of a 9AOS from barley leaves [14] on 9HPOD, namely α- and γ-ketols, could not be detected (Fig. 5C). Furthermore, no formation of divinyl ether fatty acids was observed by this method as well. This was also the case with [1-^{14}C]13HPOD, and 12-oxo-(9*Z*)-dodecenoic acid was the only product formed by recombinant C15 from this substrate (Fig. 5B). For comparison, a similar reaction was followed again with 9AOS from barley leaves as shown in Fig. 5D, and α- and γ-ketols were formed from 13HPOD as primary products. It should be noticed that there was no detectable formation of ω-oxo-acids by the 9AOS. Taken together, these results indicate that the recombinant C15 enzyme is a HPL and has no detectable AOS activity. A HPL which can act on 9-hydroperoxides was recently purified from cucumber fruits [23]. The substrate/product specificity of the cucumber fruit HPL is similar to that of the recombinant C15.

The activity of the recombinant modified C17 enzyme was relatively low. In the membrane fraction prepared from the lysate of transformed *E. coli* cells, C17 showed 200 times lower activity than the corresponding preparation of the recombinant C15. Thus, no attempt at purification of the recombinant C17 enzyme was made. As expected from the high similarity of the C17 sequence with most 13HPLs, the products formed from 13-HPOD and 13-HPOT were *n*-hexanal and (3*Z*)-hexenal, respectively (Fig. 4E,D). No aldehyde products from the 9-isomers were detected with the enzyme (Fig. 4G,H). Thus, it can be concluded that the modified C17 cDNA encodes a 13HPL.

3.4. Enzymatic properties of recombinant C15

The recombinant C15 exhibited a broad pH–activity profile from pH 4.5 to 7.0, with the highest activity at around pH 5.5. As described for most HPLs [6,9,15], lipophilic antioxidants were potent inhibitors of the recombinant C15 enzyme, with 0.2 mM α-tocopherol abolishing over 90% of the initial activity and 0.1 mM nordihydroguaiaretic acid over 80%. These antioxidants inhibited the activity in a reversible manner. Non-steroidal anti-inflammatory drugs such as salicylic acid, salicylhydroxamic acid, ibuprofen and mefenamic acid were also potent inhibitors of the recombinant C15 enzyme. With these drugs, the enzyme was inactivated in a time-dependent and irreversible manner as was shown for tomato 13HPL [15]. The *S–V* plots of the C15-HPL activity for the substrates listed in Table 1 yielded saturation curves and its catalysis proceeded with normal Michaelis–Menten kinetics, although there was a limit due to the low solubility of the substrate in the reaction buffer. The K_m value for 9HPOD was the lowest, followed by 9HPOT. The values for the 13-isomers were 2.5- to 3-fold higher than those for the 9-isomers. 9HPOD showed the highest V_{max} value, and a turnover rate of 700 s^{-1} with this substrate could be calculated.

4. Discussion

In this study, we isolated two cDNAs related to HPL and AOS. One of these, C15 cDNA, showed higher sequence similarity to AOSs than to HPLs, but apparently encodes a HPL. The other, C17, is a pseudogene, and unexpectedly, it is transcribed to form mature mRNA molecules. No HPL activity could be observed with the truncated peptide from the unmodified, intact C17 cDNA. This suggests that the C-terminal one-fifth of the HPL, which includes the heme-binding domain, is necessary for the described HPL activity. In the young hypocotyls 13HPL activity was much higher than 9HPL activity. Such a high ratio of 13HPL activity to 9HPL activity could be observed with mature leaves, flowers or roots. The recombinant C17 enzyme prefers 9HPOs rather than 13HPOs. Taking this specificity into consideration, this cannot be fully explained in these organs. Thus, there should be (an)other gene(s) encoding active 13HPL other than C17 in cucumber, and it may be regulated, at least in some extent, differently to that of C15 gene.

The recombinant enzyme derived from the C15 cDNA solely has HPL activity and no AOS activity nor an activity to form divinyl ether fatty acids. It has been determined that HPL and AOS are distinct types of cytochrome *P*-450s, and that they are grouped into separate subfamilies depending on their primary sequences. However, as shown in this study, some enzymatic properties, such as the effect of inhibitors or their high catalytic turnover rate, are almost similar within the recombinant C15 and other HPLs and AOSs. A HPL reaction carried out with the ^{18}O-labeled 13-hydroperoxide

Table 1
Kinetic parameters of the recombinant C15

Substrate	V_{max} (μkat/mg)	K_m (μM)
9-Hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid	9.91	3.6
9-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid	12.93	4.1
13-Hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid	5.31	12.6
13-Hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid	7.09	10.3

of linoleoyl alcohol showed that one of the hydroperoxide oxygens was incorporated solely into the oxo moiety of (3*Z*)-12-hydroxy-3-dodecenal, while with the other counterpart, *n*-hexanal, no incorporation could be detected [24]. This suggests that heterolysis of the hydroperoxide group of the substrate causes the reaction, probably followed by the formation of an epoxy-carbonium ion intermediate during HPL catalysis. Involvement of an epoxy-carbonium ion intermediate in the AOS catalysis has also been proposed [25]. Recently, Gerwick proposed that these enzymes form various structurally unrelated products through the formation of the same type of intermediate [26]. AOS and HPL are thought to share some parts of their reaction mechanism, probably the hydroperoxide activation steps, in common, but some parts should be largely different from each other. By comparing the structures of HPLs with AOSs, a residue(s) involved in exerting the difference would be identified. The finding of C15, which showed a higher structural similarity to AOS but is a HPL, would provide a good rationale of identification of the residues involved. For example, nine amino acid residues upstream of the cysteine involved in heme binding is always glutamic acid in AOSs, while a neutral amino residue is highly conserved at the corresponding position in HPLs. This residue must be the first candidate for site-directed mutagenesis study. Molecular dissection of C15 concomitant with other AOSs or HPLs will shed light on the reaction mechanism of these two interesting and related enzymes.

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