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Characterization of three cloned and expressed 13-hydroperoxide lyase isoenzymes from alfalfa with unusual N-terminal sequences and different enzyme kinetics

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Three full-length cDNAs from alfalfa seedlings coding for hydroperoxide lyases were cloned and expressed in *Escherichia coli* and characterized as cytochrome P450 enzymes. The isoenzymes were specific for 13-hydroperoxy linoleic and linolenic acids and did not use the 9-hydroperoxy isomers as substrates. Because alfalfa contains both specificities, this indicates the presence of two different types of hydroperoxide lyases, each specific for one kind of substrate. The enzymes contain 480 amino acids (54 kDa) and contain an unusual, nonplastidic N-terminal sequence of 22 amino acids, which strongly reduces the enzyme activity. The only known presequence of a hydroperoxide lyase (from *Arabidopsis thaliana*) was considered to be a transit sequence. The reduced enzyme activity, however, indicates that the hydroperoxide lyases with N-terminal extensions could be pro-enzymes. This hypothesis is supported by the fast release of hydroperoxide lyase products by plants upon wounding. One of the isoenzymes showed a strongly decreased V_{max} and K_m compared to the other two. Because this is probably due to the substitution of Ser377 by Phe; the residue at position 377 seems to be important. This is the first time that sufficient quantities of hydroperoxide lyase have been obtained for characterization studies, by circumventing difficult purification procedures and degradation of the enzyme. The high expression level, easy purification, good stability and high specificity make these cloned hydroperoxide lyases excellent tools to study the reaction mechanism and structure. We postulate an integrated reaction mechanism, based on the known chemistry of cytochrome P450 enzymes. This is the first mechanism that unifies all observed features of hydroperoxide lyases.

Keywords: cytochrome P450; hydroperoxide lyase; lipoxygenase pathway; *Medicago sativa*; natural flavors.

Volatile C6- and C9-aldehydes and their corresponding alcohols are important constituents of the characteristic flavors of fruits, vegetables and green leaves and are widely used as food additives. They are produced by higher plants and derived from linoleic and α-linolenic acids by a wound-inducible catalytic route involving four enzymes (Fig. 1). Fatty acids are peroxidized by lipoxygenase and subsequently cleaved by hydroperoxide lyase (HPO lyase), which leads to the formation of short-chain aldehydes and ω-oxo-acids. The products can be enzymatically isomerized from the 3*Z*- to the 2*E*-enal form, and the short-chain aldehydes can concomitantly be reduced to alcohols by alcohol dehydrogenase. The short-chain aldehydes might be involved in wound healing and pest resistance [1,2]. The ω-oxo acid 12-oxo-(9*Z*)-dodecenoic acid can be converted into the wound hormones traumatin [12-oxo-(10*E*)-dodecenoic acid] and traumatic acid [3].

HPO lyase has been purified from different sources and two genes coding for HPO lyases have been published [4,5]. Currently, little is known about its structure and reaction mechanism, due to its instability and difficult purification. Hatanaka *et al*. [6] proposed a heterolytic mechanism similar to the acid-catalyzed cleavage of linoleate hydroperoxides in an aprotic solvent [7]. However, the recently found homology of HPO lyase with cytochrome P450 enzymes suggests a homolytic reaction mechanism [4].

Two different types of substrates for plant HPO lyases are known: 13- and 9-hydroperoxy derivatives of C18 polyunsaturated fatty acids. In watermelon seedlings, tea leaves, tomato fruit and leaves, apples, green bell peppers and soybeans, only 13-HPO lyase activity was found [8,13], whereas pears showed only 9-HPO lyase activity [14]. Soybean seeds/seedlings, cucumber fruit and seedlings and alfalfa seedlings contain both HPO lyase activities [15–17]. Matsui *et al*. [18] have succeeded in separating the 13- and 9-HPO lyase activities from cucumber seedlings.

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Abbreviations: AOS, allene oxide synthase; FID, flame ionization detection; HPO lyase, hydroperoxide lyase; 13-HPOD, 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 13-HPOT, 13(*S*)-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid; 9-HPOD, 9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; 9-HPOT, 9(*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid; IMAC, immobilized metal affinity chromatography; nitriloacetic acid, nitrilotriacetic acid; SPME, solid phase micro extraction; TMBZ, 3,3′,5,5′-tetramethylbenzidine; U, unit.

Note: the DNA sequences of the *CYP74B4v1*, *CYP74B4v2* and *CYP74B4v3* genes have been deposited in the EMBL database under the accession numbers AJ249245, AJ249246and AJ249247, respectively.

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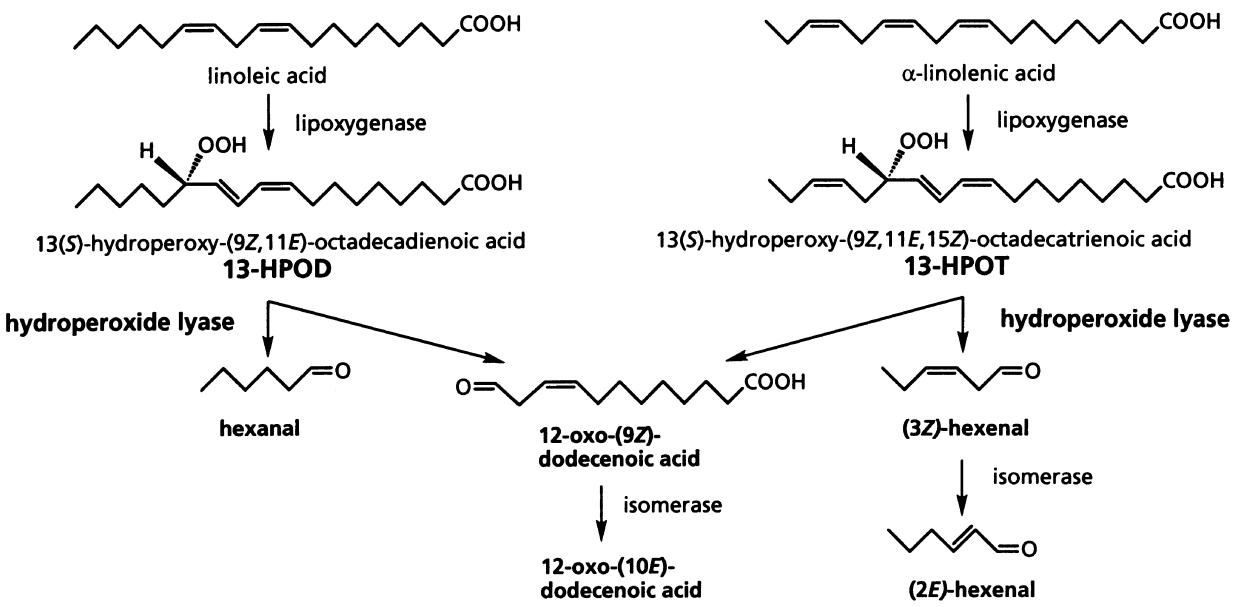

Fig. 1. Lipoxygenase pathway.

Until now, it has been unclear whether alfalfa contains one enzyme that accepts both types of substrate, or two enzymes each specific for one positional isomer [17]. In order to answer this question and to obtain more information about the enzyme characteristics, we cloned and expressed different HPO lyase genes of alfalfa (*Medicago sativa* L.) in *Escherichia coli.* A purification method was developed and the substrate and product specificities of the enzymes were determined. The enzyme kinetics were compared to get more insight into important residues for the activity. Based on the known chemistry of cytochrome P450 enzymes, we postulate a reaction mechanism for HPO lyases which unifies all aspects of these enzymes.

MATERIALS AND METHODS

Construction of the λZAP alfalfa cDNA library

Total RNA was isolated from 7-day-old alfalfa seedlings using the procedure of Logemann *et al*. [19]. The RNA was further purified by precipitations with 2 M LiCl and with 96% ethanol. mRNA was isolated using a PolyATtract® mRNA isolation system (Promega). A λZAP cDNA library was constructed of 5 μg alfalfa mRNA using a cDNA and ZAP-cDNA® Synthesis Kit and a ZAP-cDNA® Gigapack® III Gold Cloning Kit (Stratagene). The primary library yielded 10^7 recombinants. Approximately 10^6 phages of the amplified library were screened.

Probe construction

The bell pepper HPO lyase gene (a kind gift of K. Matsui, Yamaguchi University, Japan) was restricted with *Bgl*II and *Afl*III, resulting in a 482-bp 5′ fragment and a 979-bp 3′ fragment lacking the polyA tail. The fragments were separated on an agarose gel, isolated from the gel and randomly labeled with [α-^{32}P]dCTP using pd(N)$_6$ oligonucleotides (Pharmacia Biotech) and Klenow.

cDNA library screening

Plaque lifts were performed following the manufacturer's instructions (Stratagene). Filters were prehybridized with salmon sperm DNA at 55 °C for 2 h and hybridized with labeled probes at 55 °C overnight in 6 × NaCl/Cit (1 × NaCl/Cit is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's (1 × Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumine), 0.5% SDS. Filters were washed at 55 °C, with a highest wash stringency of 1 × NaCl/Cit, 0.1% SDS. The filters were exposed to X-ray films with intensifying screens at −80 °C for 2 days. Plaques giving a hybridization signal were isolated and rescreened under the same conditions until single plaques could be obtained. pBluescript® phagemids were obtained from positive plaques by *in vivo* excision as described in the manufacturer's protocol (Stratagene). Sequencing was performed with M13-20 and M13 Reverse Primers (Stratagene), on an automatic sequencing apparatus from Applied Biosystems Inc. Sequence comparisons to the genetic database were made using the BLAST algorithm.

Cloning and expression of alfalfa HPO lyase in *E. coli*

In a PCR reaction, *Bam*HI and *Sma*I restriction sites were introduced at the 5′ and 3′ gene ends, respectively. The following primers were used (Gibco-BRL Custom Primers): sense strand: 5′-GGGGATCCATGTCACTCCCACCACC-3′ (complete gene) or 5′-GGGGATCCTACCGATCCGGCAGA-TTC-3′ (gene without N-terminal sequence), and antisense strand: 5′-AGGCCCGGGACCCTAACTCTTCATTTGGC-3′. The HPO lyase genes were cloned in pQE30 or pQE32 vectors (Qiagen), containing a sequence coding for an N-terminal 6 × His-tag. Expression was performed in *E. coli* M15 as described by the manufacturer's protocol (The QIA-*expressionist*, Qiagen), except that after induction with IPTG, the cells were further grown overnight at 25–30 °C to prevent sequestering of the HPO lyase in inclusion bodies. Small cultures (up to 100 mL) were grown in Luria–Bertani medium, large cultures (up to 10 L) 1.5 × in Luria–Bertani medium

supplemented with 0.5% glucose and 1 × minimal medium buffer. Cells were harvested by centrifugation and the supernatant was discarded. The cell pellet was suspended in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5, in 5–10% of the original culture volume. The cells were sonicated on ice and recentrifuged. The supernatant contained the soluble proteins. The membrane pellet was resuspended in 5–10% of the original culture volume of 50 mM potassium phosphate buffer pH 7.5 containing 0.2% (m/v) Triton X-100 (membrane solubilization buffer) and recentrifuged. The supernatant contained solubilized membrane proteins. All centrifugation steps and potassium phosphate buffers were at 4 °C.

Enzyme purification

Purification occurred by immobilized metal affinity chromatography (IMAC). For small sample volumes (up to 10 mL), a 0.5-mL Ni-nitriloacetic acid agarose column (Qiagen) was used, which had been previously equilibrated with membrane solubilization buffer. The solubilized membrane proteins were applied and the column was washed with 4 mL of membrane solubilization buffer containing 300 mM NaCl and 20 mM imidazole. Elution was performed with 4 × 0.5 mL membrane solubilization buffer containing 300 mM NaCl and 250 mM imidazole. For larger volumes (up to 100 mL) an FPLC column containing 10 mL Ni-nitriloacetic acid superflow (Qiagen) was used, with a flow rate of 1 mL·min^{-1}. After application of the solubilized membrane proteins, the column was washed with 20 mL membrane solubilization buffer containing 300 mM NaCl, and 50 mL of the same buffer supplemented with 10 mM imidazole. Elution was performed in 100 min with a linear gradient of 10–250 mM imidazole in membrane solubilization buffer containing 300 mM NaCl. The HPO lyases eluted at an imidazole concentration of 100 mM. All steps were carried out at 4 °C.

Enzyme activity measurements

HPO lyase activity was determined in 50 mM potassium phosphate buffer, pH 6.0, containing 100 μM substrate by measuring the decrease of the A_{234} due to the cleavage of substrate. One unit of activity (U) corresponds to the amount of enzyme that converts 1 μmol of substrate per min. Protein concentrations were determined with the bicinchoninic acid method [20]. The substrates, 13-HPOD and 13-HPOT were prepared from linoleic and α-linolenic acid, respectively (≈ 99%, Fluka), with soybean lipoxygenase-1 [21]. Analogously, 9-HPOD and 9-HPOT were prepared with tomato lipoxygenase [22].

Product identification

One unit of HPO lyase (determined with 13-HPOD as substrate) was diluted to 8 mL with 50 mM potassium phosphate buffer, pH 6.0. Substrate (80 μM, final concentration) was added and the mixture was incubated for 30 min at 20 °C in a stirred 20-mL reaction vessel closed with a septum. Headspace compounds were trapped by SPME (100 μm polydimethylsiloxane coated fiber, Supelco) and desorbed at 200 °C for 1 min in the injection port of a GC/FID (HP-Innowax column; 0.25 μm film thickness, 30 m × 0.32 mm, Hewlett-Packard). The column temperature was held at 35 °C for 2 min, increased to 200 °C, 10 °C·min^{-1}, and held at this temperature for 2 min. Nonvolatile compounds were extracted from the reaction mixture, previously acidified with HCl to pH 5, with an octadecyl solid-phase extraction column (J. T. Baker B.V., Deventer, the Netherlands) and eluted with 100% methanol. The compounds were reduced with an excess of $NaBH_4$ at 0 °C, esterified with ethereal diazomethane, silylated with silylating reagent (pyridine/1,1,1,3,3,3,-hexamethyldisilazane/chlorotrimethylsilane 5 : 1 : 1, v/v/v) and analyzed with GC/MS (Fisons GC 8000 series and Fisons Instruments MD 800 MassLab spectrometer, CP-Sil5 CB-MS column, 0.25-μm film thickness, 25 m × 0.25 mm, Chrompack). The column temperature was held at 140 °C for 2 min, increased to 280 °C, 6 °C·min^{-1}, and held at this temperature for 2 min. Electron impact mass spectra were recorded with an ionization energy of 70 eV.

Heme analyses

The heme staining procedure was adapted from Thomas *et al.* [23]. Three volumes of a 6.3-mM 3,3′,5,5′-tetramethylbenzidine (TMBZ) solution in methanol were mixed with 7 vol. 0.25 M sodium acetate buffer pH 5.0. One milliliter of this freshly prepared reagent was added to 50 μL protein solution and the color reaction was started by addition of 35 μL 3% (v/v) H_2O_2. Presence of heme was indicated by appearance of a blue color after 1 min, which further intensified during 30 min.

Spectrophotometric analyses of FPLC purified HPO lyase (CYP74B4v1 without N-terminal sequence, 0.2 mg·mL^{-1} in elution buffer) were carried out with native and reduced enzyme. HPO lyase was reduced by addition of sodium dithionite to a final concentration of 0.2% (w/v). Pyridine hemoferrochrome was prepared from the purified enzyme by addition of pyridine to a concentration of 20% and NaOH to 0.2 M.

RESULTS

Isolation and characterization of alfalfa HPO lyase genes

A cDNA library was constructed of alfalfa seedling mRNA. Two fragments originating from the bell pepper HPO lyase gene were used as hybridization probes to screen this cDNA library. With the 979-bp 3′ fragment, 23 positive clones were found, one of which also hybridized with the 482-bp 5′ bell pepper HPO lyase fragment. Nineteen clones contained putative full-length sequences. Restriction and sequence analyses revealed three different HPO lyase-like genes. They were named *CYP74B4v1*, *CYP74B4v2* and *CYP74B4v3* (D. R. Nelson, personal communication). The full-length cDNAs contained 1443 nucleotides, encoding proteins of 480 amino acids. All three contained an N-terminal methionine and a C-terminal lysine. The sequence of *CYP74B4v1* is shown in Fig. 2. *CYP74B4v2* contained the following differences: A13T, H32Y, E61D, V72I, N176K, K188Q, P207S, K257N, N273S, T312A, V394I, Q477R and *CYP74B4v3* contained the differences: E61D, N273S, T312A, S377F, Q477R. Their deduced masses were 53 877, 53 909 and 53 895 Da and their deduced pI values were 8.1, 8.1, and 8.4, respectively. Sequence database searches showed that the three genes shared homology with cytochrome P450s, as was also found for bell pepper and *Arabidopsis thaliana* HPO lyases [4,5]. The segments that are highly conserved in many cytochrome P450 families, i.e. the A, B, C and D domains, were present in *CYP74B4v1*, *v2* and *v3*, as well as the heme-binding cysteine (Cys-442) (Fig. 2). The amino-acid sequence of CYP74B4v1 showed 61% identity and 69% homology to bell pepper and

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GGCACGAGACCGAACTCTCTTCTCTTCTTCTCTACCATGTCACTCCCACCACCGATACCACCACCGTCCCTA    36
                                    M  S  L  P  P  P  I  P  P  P  S  L     12
GCAACTCCACCGAAAGCAAGGCCAACGGAGCTACCGATCCGGCAGATTCCGGGAAGCCACGGATGGCCATTG   108
 A  T  P  P  K  A  R  P  T  E  L  P  I  R  Q  I  P  G  S  H  G  W  P  L     36
CTAGGTCCCTTATCAGACAGGTTAGATTATTTCTGGTTCCAGAAGCCAGAAAACTTTTTCAGAACACGAATG   180
 L  G  P  L  S  D  R  L  D  Y  F  W  F  Q  K  P  E  N  F  F  R  T  R  M     60
GAAAAGTACAAGAGTACTGTATTCCGTACCAATGTTCCACCCACGTTCCCTTTCTTCACCAACGTGAATCCT   252
 E  K  Y  K  S  T  V  F  R  T  N  V  P  P  T  F  P  F  F  T  N  V  N  P     84
AATATTATTGCAGTTCTTGATTGTAAGTCTTTCTCTCACCTCTTTGATATGGACCTTGTTGACAAAAGAGAT   324
 N  I  I  A  V  L  D  C  K  S  F  S  H  L  F  D  M  D  L  V  D  K  R  D    108
GTTCTAGTTGGAGATTTTGTTCCTAGTGTTGAATTTACGGGAAATATTAGGGTTGGTGTTTATCAAGATGTT   396
 V  L  V  G  D  F  V  P  S  V  E  F  T  G  N  I  R  V  G  V  Y  Q  D  V    132
TCTGAACCTCAACATGCTAAGGCAAAGAATTTCTCCATGAACATTTTGAAGCAAAGTTCAAGCATATGGGTA   468
 S  E  P  Q  H  A  K  A  K  N  F  S  M  N  I  L  K  Q  S  S  S  I  W  V    156
CCAGAACTGATATCAAACCTAGACATATTTTTGGACCAAATTGAAGCAACACTGTCTAATTCTTCATCAGCA   540
 P  E  L  I  S  N  L  D  I  F  L  D  Q  I  E  A  T  L  S  N  S  S  S  A    180
AGTTACTTCTCCCCACTGCAGAAATTTCTGTTCACGTTCCTAAGCAAAGTACTTGCCAGAGCCGATCCTTCC   612
 S  Y  F  S  P  L  Q  K  F  L  F  T  F  L  S  K  V  L  A  R  A  D  P  S    204
CTTGATCCCAAAATCGCCGAATCAGGCTCCTCCATGCTCAACAAATGGCTCGCAGTTCAGCTTCTACCGACT   684
 L  D  P  K  I  A  E  S  G  S  S  M  L  N  K  W  L  A  V  Q  L  L  P  T    228
GTGAGTGTCGGCACCATCCAACCACTCGAAGAAATTTTCCTCCATTCCTTCTCTTACCCTTATGCTCTTGTC   756
 V  S  V  G  T  I  Q  P  L  E  E  I  F  L  H  S  F  S  Y  P  Y  A  L  V    252
AGTGGAGACTACAAAAACCTCTACAACTTCATTAAACAACATGGTAAGGAAGTGATAAAAAATGGAACGGAG   828
 S  G  D  Y  K  N  L  Y  N  F  I  K  Q  H  G  K  E  V  I  K  N  G  T  E    276
TTCGGGTTGAGTGAAGATGAAGCAATCCACAACTTGCTTTTTGTGTTGGGTTTCAACTCATATGGTGGGTTT   900
 F  G  L  S  E  D  E  A  I  H  N  L  L  F  V  L  G  F  N  S  Y  G  G  F    300
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 S  I  F  L  P  K  L  I  E  S  I  T  N  G  P  T  G  L  Q  E  K  L  R  K    324
GAAGCAAGAGAGAAAGGCGGTTCAACTCTTGGGTTTGACTCGTTAAAAGAGTTGGAACTAATAAATTCGGTG  1044
 E  A  R  E  K  G  G  S  T  L  G  F  D  S  L  K  E  L  E  L  I  N  S  V    348
GTTTATGAAACCCTGCGAATGAACCCACCGGTTCCACTTCAATTTGGTCGAGCCAGAAAAGATTTTCAACTC  1116
 V  Y  E  T  L  R  M  N  P  P  V  P  L  Q  F  G  R  A  R  K  D  F  Q  L    372
AGTTCATATGACTCCGCGTTCAATGTGAAGAAGGGTGAGTTATTATGTGGGTTTCAGAAGCTTGTAATGAGG  1188
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 D  P  V  V  F  D  E  P  E  Q  F  K  P  E  R  F  T  K  E  K  G  A  E  L    420
TTGAACTATTTGTATTGGTCTAATGGGCCACAAACTGGTTCGCCAACTGTGTCCAATAAACAGTGTGCAGGT  1332
 L  N  Y  L  Y  W  S  N  G  P  Q  T  G  S  P  T  V  S  N  K  Q  C  A  G    444
AAGGACATCGTGACCTTCACCGCTGCGTTGATTGTGGCACACTTGTTACGTCGGTATGATTTAATTAAAGGG  1404
 K  D  I  V  T  F  T  A  A  L  I  V  A  H  L  L  R  R  Y  D  L  I  K  G    468
GATGGTAGTTCCATTACTGCCTTACAGAAGGCCAAATGAAGAGTTAGGGTTGGTTTTCAATGTTTGTGATTA  1476
 D  G  S  S  I  T  A  L  Q  K  A  K  *                                     480
TCAAAGACATATTAATATATGTATATTTTCTTTATAAAAGAGAGCTTGTATTGTATTAATTCAAAGAGTACT  1548
TGCTTACAAAAATTATGATTAAAGTGAGCAGAGTTCAATTATGATTTTACAGTAGTTTACTCAAGTAAGTTA  1620
AGTAAGAAAAAGGGAAGAAATGAAAAAAAAAAAAAAAAAA
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Fig. 2. Alfalfa HPO lyase sequence (*CYP74B4v1*). The A, B, C and D domains which are characteristic for cytochrome P450s, are underlined. The heme-binding cysteine (Cys-442) is shown in bold. *CYP74B4v2* contained the following differences: A13T, H32Y, E61D, V72I, N176K, K188Q, P207S, K257N, N273S, T312A, V394I, Q477R and *CYP74B4v3* contained the differences: E61D, N273S, T312A, S377F, Q477R. The first 22 amino acids probably are an N-terminal extension.

A. thaliana HPO lyases. As the similarity starts at leucine 23, the first 22 amino acids are probably an N-terminal extension.

Cloning and expression of alfalfa HPO lyases in *E. coli*

The three genes were cloned into pQE30 and expressed in *E. coli* M15 cells. Because an N-terminal extension can reduce the enzyme activity, constructs lacking the first 22 amino acids were also cloned and expressed (using pQE32). Due to the vectors used, the expressed proteins contained the following N-terminal tag: Met-Arg-Gly-Ser-$(His)_6$-Gly-Ser(pQE30)/Ile(pQE32)-. If the cells were grown at 37 °C, all protein expressed was sequestered into insoluble inclusion bodies. By reducing the growth temperature following induction, it was possible to obtain active enzymes, as determined by their hydroperoxy fatty acid degrading capacity. In the cells, the degrading enzymes were present in both the soluble fraction and the membrane fraction. It was possible to solubilize the enzymes from the membrane fraction by addition of 0.2% (m/v) Triton X-100. The highest specific activity was found in the solubilized membrane fraction (Table 1). *E. coli* cells harboring the vector pQE without inserted gene did not contain any detectable hydroperoxy fatty acid degrading activity.

Table 1. Distribution of the hydroperoxy fatty acid degrading activity in the transformed *E. coli* cells. Three different alfalfa genes (*CYP74B4v1*, *v2*, *v3*) were cloned and expressed in *E. coli* with and without the 22 amino-acid N-terminal sequence. Enzyme activity in *E. coli* cell fractions was determined in $U \cdot mg^{-1}$, by measuring the decrease of A_{234} due to the decomposition of 13-HPOT.

Sample	CYP74B4v1- N-terminal sequence	CYP74B4v1 + N-terminal sequence	CYP74B4v2- N-terminal sequence	CYP74B4v3- N-terminal sequence
Soluble fraction	0.39	0.27	0.62	0.48
Solubilized membrane fraction	4.14	1.45	5.42	4.19

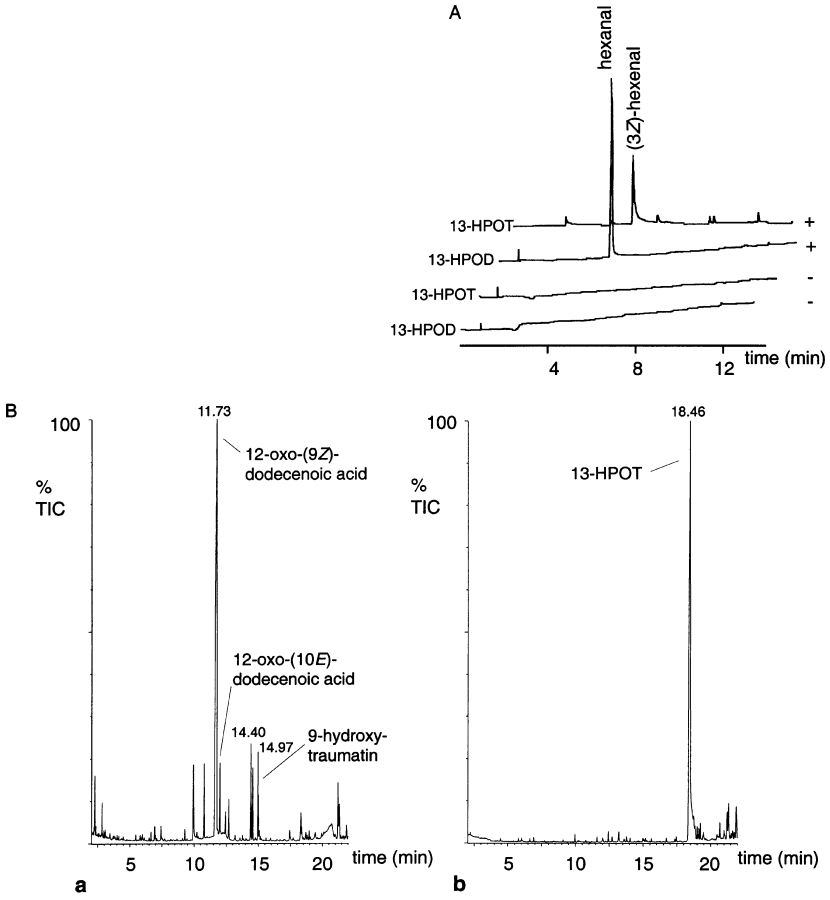

Fig. 3. Products of incubations solubilized membrane fractions of *E. coli* with various substrates. (A) Volatile products of incubations of 13-HPOD and 13-HPOT with the solubilized membrane fractions of *E. coli* containing the pQE32 plasmid with (+) or without (–) alfalfa HPO lyase gene. Volatile products were trapped by SPME and analyzed with GC/FID. (B) Nonvolatile products of incubations of 13-HPOT with the solubilized membrane fractions of *E. coli* containing the pQE32 plasmid with (a) or without alfalfa HPO lyase gene. (b) Nonvolatile products were extracted from the reaction mixture, reduced, esterified, trimethyl silylated and analyzed with GC/MS.

Enzyme specificity

To identify the reaction products, the solubilized membrane fractions of *E. coli* cells harboring the vector pQE32 with *CYP74B4v1*, *v2* or *v3* or without gene insert, were incubated with 13-HPOD, 13-HPOT, 9-HPOD and 9-HPOT (Fig. 3). The enzymes CYP74B4v1, v2 and v3 showed the same product specificity, whereas no products were found with the membrane fraction of *E. coli* cells harboring the pQE vector without inserted gene. The volatile products formed from 13-HPOD and 13-HPOT were identified as hexanal and (3*Z*)-hexenal, respectively. The nonvolatile product formed from both 13-HPOD and 13-HPOT showed peaks of the following characteristic ions in the electron impact spectrum: *m/z* [ion attribution; relative intensity], 300 [M^+(molecular ion); 0.13%], 285 [M^+-CH_3; 5.3%], 253 [M^+-CH_3O_2; 23.5%], 103 [CH_2OTMS^+ 97.8%], 73 [TMS^+ 100%] and was identified as 12-oxo-(9*Z*)-dodecenoic acid. With 9-HPOD or 9-HPOT as substrates, no products were found. As the products formed are all originating from the HPO lyase pathway, it can be concluded that the three genes are coding for three alfalfa 13-HPO lyase isoenzymes.

Table 2. Substrate specificity of three different alfalfa HPO lyases (CYP74B4v1, v2, v3) cloned and expressed in *E. coli* without 22 amino-acid N-terminal sequence. Enzyme activity in U·mg^{-1}, determined by measuring the decrease of A_{234}, due to the decomposition of substrate.

Substrate	CYP74B4v1	CYP74B4v2	CYP74B4v3
13-HPOD	105	89.5	72.1
13-HPOT	149	113	99.9
9-HPOD	1.07	0.77	2.73
9-HPOT	0.94	7.3×10^{-3}	1.63

Enzyme purification

Because the expressed HPO lyases contained a 6 × His-tag, it was possible to purify them by IMAC. HPO lyases were purified from the solubilized membrane fraction, as this

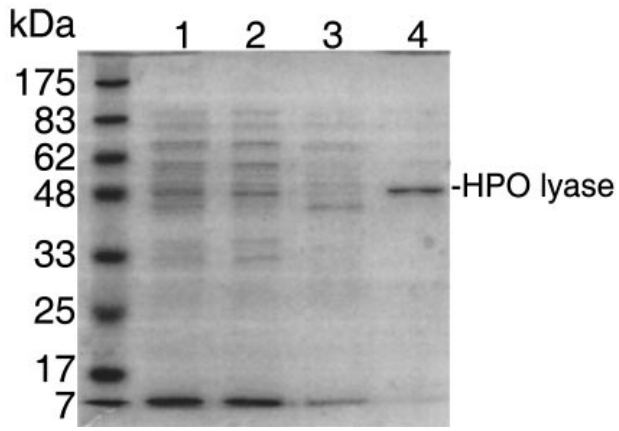

Fig. 4. Purification of expressed HPO lyase by IMAC. SDS/PAGE gel (10% polyacrylamide) of the purification fractions as detected by Coomassie Blue staining. Lane 1, solubilized membrane proteins; lane 2, flow through; lane 3, wash; lane 4, elution fraction.

Table 3. Kinetic parameters of three different alfalfa HPO lyases (CYP74B4v1, v2, v3) cloned and expressed in *E. coli* with or without 22 amino-acid N-terminal sequence. A Michaelis–Menten curve was fitted through enzyme activity values determined with concentrations of the substrate 13-HPOT ranging from 5 to 150 μM (values ± SD).

	CYP74B4v1-N-terminal sequence	CYP74B4v1 + N-terminal sequence	CYP74B4v2-N-terminal sequence	CYP74B4v3-N-terminal sequence
K_m (μM)	132 ± 17	54 ± 9	181 ± 29	63 ± 9
V_{max} (μmol·min^{-1}·mg^{-1})	632 ± 49	71 ± 5	691 ± 72	240 ± 16

fraction contained the highest specific activity. With this method pure enzyme could be obtained, as can be seen in Fig. 4. The yield was high, as 1 L of *E. coli* culture yielded up to 2.5 mg (500 U) of pure HPO lyase. The masses found by SDS/PAGE were in accordance with those calculated from the gene sequences. On native gel electrophoresis/PAGE tetramers were observed as well as a small fraction of dimers. Only in the presence of Triton X-100, HPO lyase bound to the Ni-nitriloacetic acid column. This suggests that Triton X-100 causes a change in the tertiary structure of the enzyme, leading to a better accessibility of the His-tag. The purified HPO lyases were very stable, i.e. no significant loss of activity was observed after 4 weeks at 4 °C, in contrast to previous samples of purified HPO lyases that were very unstable.

Enzyme kinetics

The substrate specificities of the three purified isoenzymes were determined and it appeared that these enzymes were highly specific for 13-hydroperoxy fatty acids, and did not use 9-hydroperoxy fatty acids as a substrate (Table 2). Furthermore, they showed a slight preference for 13-HPOT compared to 13-HPOD as was also found in a crude extract of alfalfa seedlings [17]. Their enzyme kinetics were determined and it appeared that the kinetics of CYP74B4v1 and v2 were quite similar, whereas CYP74B4v3 showed a much lower V_{max} and K_m (Table 3). CYP74B4v1 with the 22 amino-acid N-terminal sequence showed a strongly decreased V_{max} and K_m compared to the enzyme without this N-terminal sequence. Apparently, the enzymatic activity is strongly reduced by the N-terminal extension, whereas the binding of substrate to the enzyme is not affected.

Heme analyses

Heme-staining of the purified enzyme was performed with TMBZ and H_2O_2 and a strong blue color appeared, whereas solutions of nonheme proteins and elution buffer remained colorless. This indicated the presence of heme in the alfalfa HPO lyases, a feature of cytochrome P450s. In contrast to guayule allene oxide synthase (AOS), which lost its heme in the presence of 10 mM β-mercaptoethanol [24], alfalfa HPO lyases solubilized in membrane solubilization buffer containing 10 mM β-mercaptoethanol still contained heme after purification.

The absorption spectra of native and reduced alfalfa HPO lyase (Fig. 5A) are highly similar to the spectra of bell pepper HPO lyase [25] and flaxseed AOS [26]. Native alfalfa HPO lyase showed a Soret band at 390 nm, reduction led to a shift of this band to 416 nm. This indicates that the isolated HPO lyase is a cytochrome P450 enzyme in the ferric high-spin state [27]. The pyridine hemoferrochrome was prepared to ascertain the heme species in alfalfa HPO lyase. The absorption spectrum showed a sharp γ-band at 418 nm (Fig. 5B). The difference spectrum of the reduced hemochrome minus the nonreduced hemochrome showed an α-band around 556 nm and a β-band around 520 nm. These features are typical for heme *b* (protoheme IX) [28].

DISCUSSION

In contrast to other cytochrome P450 enzymes, HPO lyases and AOS do not need molecular oxygen nor an NADPH-dependent cytochrome P450 reductase to exert their activity. Instead, they utilize a hydroperoxide group. In accordance with this feature, they lack the typical P450 consensus sequence in the I-helix region that is thought to form the oxygen-binding pocket, and the highly conserved threonine in the GXXXT sequence, in which G and T establish close contact with oxygen [29]. Pro, replacing Phe as the first conserved residue of the heme-binding domain, is another common feature of HPO lyases and AOS. Furthermore, HPO lyases and AOS show a low affinity for carbon monoxide [30,31]. Because of these characteristics, they are considered to be a special subclass of the cytochrome P450 family named CYP74 [26].

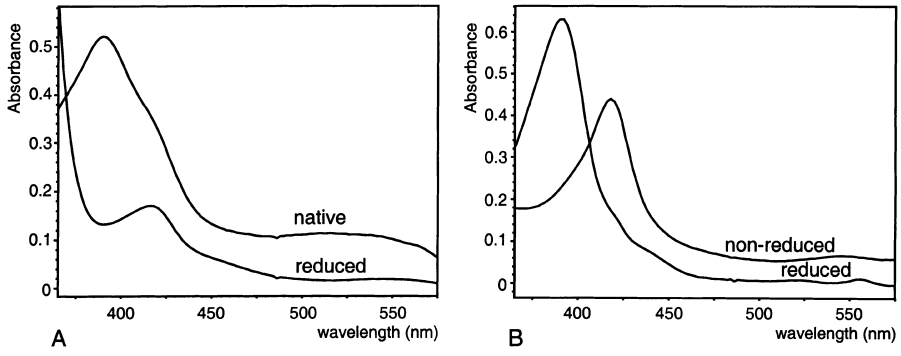

Fig. 5. Absorption spectra of purified alfalfa HPO lyase. (A) Absorption spectra of native and sodium dithionite reduced HPO lyase. (B) Absorption spectra of reduced and nonreduced pyridine hemoferrochrome of HPO lyase.

	Domain A	Domain B	Domain C	Domain D
AA-HPL	LLFVLGFNSYGGFS**I**FL	ELELINSVVYETLRMNPPV	RDPVVFDEPEQFKPERFTKEKGA	PTVSNKQ**C**AGKDIVTFTAALIV
AT-HPL	LLFVLGFNAYGGFS**V**FL	EMELVKSVVYETLRFNPPV	RDANVFDEPEEFKPDRYVGETGS	PSASNKQ**C**AAKDIVTLTASLLV
BP-HPL	LLFILGFNAFGGFT**I**FL	EMELVQSFVYESLRLSPPV	KDPKVFDEPEKFMLERFTKEKGK	PTESNKQ**C**AAKDAVTLTASLIV
AT-AOS	LLFATSFNTWGGMK**I**LF	KMELTKSVVYECLRFEPPV	RDPKIFDRADEFVPERFVGEEGE	PTVGNKQ**C**AGKDFVVLVARLFV

Fig. 6. Comparison of the highly conserved A, B, C and D domains of different cytochrome P450 proteins. The heme-binding cysteines and the isoleucines and valines which replace the highly conserved threonine in the I-helix region are shown in bold. AA-HPL, Alfalfa hydroperoxide lyases; AT-HPL, *A. thaliana* hydroperoxide lyase [5]; BP-HPL, bell pepper hydroperoxide lyase [4]; AT-AOS, *A. thaliana* allene oxide synthase [32].

Alfalfa HPO lyase

The characteristic cytochrome P450 A, B, C and D domains of several HPO lyases and AOS are aligned in Fig. 6. As can be seen, alfalfa HPO lyases show the characteristics of the CYP74 family and a high similarity to other HPO lyases and AOS. The hydropathy plot of alfalfa HPO lyase also shows a striking similarity to the two known HPO lyase sequences from other sources (Fig. 7). Comparison of the alfalfa HPO lyases with the other two HPO lyase sequences, indicates that 13-HPO lyases of different plants have similar structures. Phe303 and Leu304 in the A-domain and Thr449 in the D-domain, which are replaced by Leu, Phe and Val, respectively, in AOS sequences, might be conserved residues of HPO lyases. The last Ser in the D-domain of bell pepper and *A. thaliana* HPO lyases, which was previously considered as a diagnostic residue of HPO lyases [5], is not a conserved residue because it is replaced by Ala in alfalfa.

These alfalfa HPO lyase genes are the first full-length HPO lyase genes described besides the *A. thaliana* gene from the EST database [5]. The N-terminal sequences of the alfalfa HPO lyases are unusual, and different from the *A. thaliana* HPO lyase presequence and AOS presequences. The latter were considered to be transit sequences, targeting the enzyme to chloroplasts [5,32,33]. The alfalfa HPO lyase N-terminal sequences, however, are not enriched in serines and threonines like transit sequences. Furthermore, it is remarkable that the alfalfa HPO lyase N-terminal sequences contain numerous Pro residues, whereas a transit sequence usually lacks Pro [34]. Therefore, it is not likely that the alfalfa HPO lyase N-terminal sequences are transit sequences. The strongly decreased enzymatic activity of the enzyme with the 22 N-terminal amino acids present, suggests that the sequence is cleaved off to attain full activity. The fast release of volatile aldehydes in response to wounding of the plant, makes it reasonable to suppose that no *de novo* synthesis of HPO lyase occurs during a stress response. Instead, HPO lyase may already be present as pro-enzyme which is activated by cleaving off the N-terminal sequence.

In alfalfa seedlings, the largest part of HPO lyase activity was found in the soluble fraction, although some was present in the membrane fraction (Table 4). Expressed HPO lyase in *E. coli* was equally distributed over the membrane and soluble fractions. Using TMPRED (prediction of transmembrane regions and orientation; an algorithm based on statistical analysis of TMBase, a database of naturally occurring transmembrane proteins), a putative transmembrane region in the HPO lyases was found between positions 287 and 304. To what extent the enzyme is membrane bound, is subject of further investigations.

This is the first paper that describes the cloning of three different HPO lyase genes from one organism, which gives the opportunity to gain more insight into important residues. Only one single gene was found for bell pepper HPO lyase and *A. thaliana* AOS and HPO lyase [4,5,32]. Two of the three isoenzymes (i.e. CYP74B4v1 and v2) showed quite similar enzyme kinetics, whereas CYP74B4v3 showed a much lower V_{max} and K_m. CYP74B4v3 differs from v1 and v2 only by the substitution of Ser377 by Phe. This suggests that the amino acid at position 377 is important for the enzymatic action of HPO lyase, although it is not part of the conserved domains. The hydrophobic phenylgroup might lead to a higher affinity for the hydrophobic substrates and a slower release of the products, compared to the hydrophilic hydroxyl group. Bell pepper HPO lyase contains Ser at position 377 [4], whereas

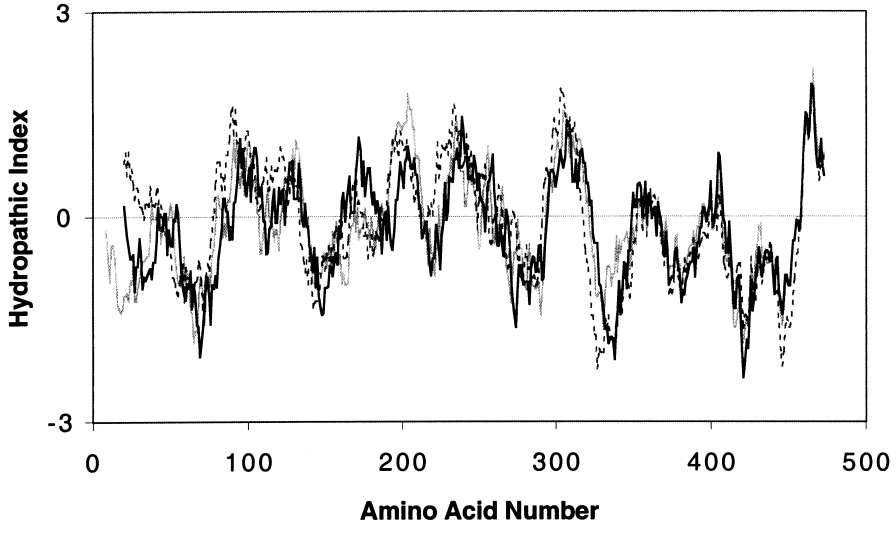

Fig. 7. Hydropathy plots of alfalfa, *A. thaliana* and bell pepper HPO lyases indicate that these enzymes have a similar structure. The hydrophobicity scale of Kyte *et al.* [43] and a 15-amino-acid window were used. black line, Alfalfa HPO lyase (CYP74B4v1); dashed line, bell pepper HPO lyase [4]; grey line, *A. thaliana* HPO lyase [5].

Table 4. Distribution of HPO lyase activity in alfalfa seedlings (%). HPO lyase activity was determined in different cell fractions of crude homogenates of alfalfa seedlings with 13-HPOD as a substrate, as was described in Noordermeer *et al*. [17]. The enzyme activity is expressed as percentage of the HPO lyase activity in the crude homogenate (mean values of two independent determinations). The largest part of alfalfa HPO lyase activity was found in the soluble fraction (123 000 *g* supernatant). The 10 000 *g* pellet contains chloroplasts and large membrane fragments, the 123 000 *g* pellet contains microsomes.

Crude homogenate	10 000 *g* supernatant	10 000 *g* pellet	123 000 *g* supernatant	123 000 *g* pellet
100	72	18	49	17

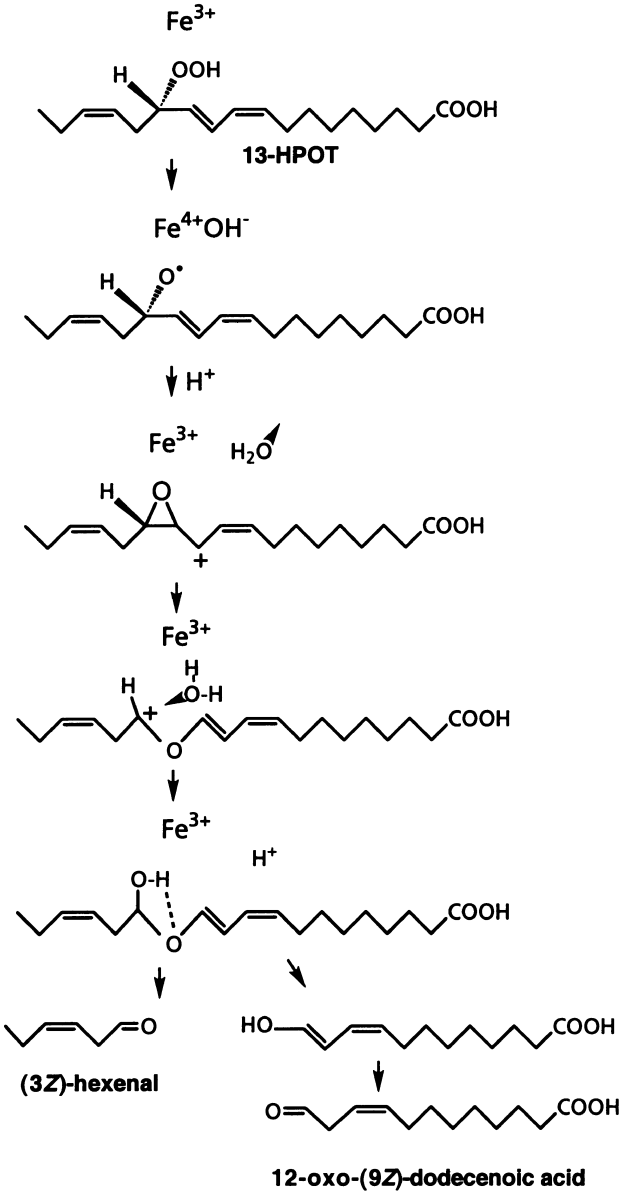

Fig. 8. Proposed reaction mechanism for HPO lyase. The mechanism is based on the known chemistry of cytochrome P450 enzymes. The first step comprises the homolytic cleavage of the oxygen–oxygen bond resulting in an alkoxy radical and a ferryl-hydroxo complex. The next step in the reaction mechanism is proton donation to the hydroxyl in the ferryl-hydroxo complex and abstraction of the electron of the alkoxy radical. This causes the formation of an allylic ether cation. Subsequently, addition of water to the carbocation occurs and a spontaneous rearrangement results in the formation of a C6-aldehyde and a C12-enol. An ω-oxo-acid is formed by keto-enol tautomerization of the C12-enol.

A. thaliana HPO lyase and AOS contain Ala at this position [5,32]. This residue is an obvious target for mutagenesis in future studies. The three isoenzymes appeared to be specific for 13-hydroperoxy fatty acids, although the HPO lyase fraction isolated from alfalfa seedlings also used 9-hydroperoxy fatty acids as substrate. This indicates the presence of HPO lyase(s) specific for 9-hydroperoxy fatty acids in alfalfa seedlings. So far, no gene has been reported encoding a sequence for 9-HPO lyase. As we found only 13-HPO lyases by screening a cDNA library with a 13-HPO lyase gene, the genes for 13- and 9-HPO lyases probably do not have a high homology.

Until now, the amounts of HPO lyase available were too low for characterization studies. HPO lyase preparations from plant extracts were unstable and difficult to purify. The cloned *A. thaliana* HPO lyase was partly degraded during purification [5] and the cloned bell pepper HPO lyase was not purified [4]. The high expression level, easy purification, good stability and high specificity make our cloned HPO lyases excellent tools for further studies on the reaction mechanism and structure, which is an important improvement in HPO lyase research. In addition, they may also be interesting for use in biocatalytic processes. Volatile C6- and C9-aldehydes and alcohols are synthesized industrially on a fairly large scale, and are widely used in perfumes and as food additives. Until now, fruit extracts are used as HPO lyase sources to obtain 'natural' flavors, but the presence of other hydroperoxy fatty acid degrading enzymes in these crude extracts leads to the production of unwanted side products.

Reaction mechanism

Relatively little is known about the reaction mechanism of HPO lyases. Based on a study with ^{18}O-labelled substrates, Hatanaka *et al*. [6] proposed a heterolytic mechanism similar to the acid-catalyzed cleavage of linoleate hydroperoxides in an aprotic solvent [7]. Matsui *et al*. [4] however, suggested that a radical process is involved because of the homology of HPO lyases with cytochrome P450 enzymes, but did not describe a possible mechanism. In support of a radical process, inhibition of the enzymatic activity by radical scavengers was observed, as well as a protective effect of radical scavengers against inactivation of HPO lyase by its substrate. In Fig. 8, we propose an integrated reaction mechanism for HPO lyases, based on the known chemistry of cytochrome P450 enzymes. We consider the homolytic cleavage of the oxygen-oxygen bond resulting in an alkoxy radical and a ferryl-hydroxo complex as the first step of the reaction mechanism. This direct formation of a ferryl-hydroxo complex as a result of the interaction between Fe(III)-porphyrin and a peroxide was also suggested in other systems [35–38]. AOS is also thought to act via a ferryl-hydroxo complex [39]. In contrast to the formation of an epoxyallylic cation by AOS, we propose that the next step in the HPO lyase reaction mechanism is proton donation to the hydroxyl in the ferryl-hydroxo complex and abstraction of the electron of the

alkoxy radical. This causes the formation of an allylic ether cation intermediate. Formation of such an allylic ether cation was also suggested by Hatanaka *et al*. in a heterolytic mechanism of HPO lyase [40] and in etheroleic acid biosynthesis [41]. Olias *et al*. [42] described an attempt to detect the allylic ether cation intermediate by using [1-^{14}C]13-HPOD. Subsequently, addition of water to the carbocation occurs and a spontaneous rearrangement results in the formation of a C6-aldehyde and a C12-enol. An ω-oxo-acid is formed by keto-enol tautomerization of the C12-enol. In contrast to the reaction mechanisms of O_2 using cytochrome P450 enzymes, the mechanism of HPO lyase is cyclic, i.e. no cofactor is used. The mechanism we describe here, is the first mechanism that unifies all observed features of HPO lyases.

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REFERENCES

1. Major, R.T., Marchini, P. & Sproston, T. (1960) Isolation from *Ginkgo biloba* L. of an inhibitor of fungus growth. *J. Biol. Chem.* **235**, 3298–3299.
2. Zeringue, H.J. (1992) Effects of C-6-C-10 Alkenals and alkanals on eliciting a defence response in the developing cotton boll. *Phytochemistry* **31**, 2305–2308.
3. Zimmerman, D.C. & Coudron, C.A. (1979) Identification of traumatin, a wound hormone, as 12-oxo-*trans*-10-dodecenoic acid. *Plant Physiol.* **63**, 536–541.
4. Matsui, K., Shibutani, M., Hase, T. & Kajiwara, T. (1996) Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P450 (CYP74B). *FEBS Lett.* **394**, 21–24.
5. Bate, N.J., Sivasankar, S., Moxon, C., Riley, J.M.C., Thompson, J.E. & Rothstein, S.J. (1998) Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. *Plant Physiol.* **117**, 1393–1400.
6. Hatanaka, A., Kajiwara, T., Sekiya, J. & Toyota, H. (1986) Oxygen incorporation in cleavage of ^{18}O-labeled 13-hydroperoxylinoleyl alcohol into 12-hydroxy-(3*Z*)-dodecenal in tea chloroplasts. *Z. Naturforsch.* **41c**, 359–362.
7. Gardner, H.W. & Plattner, R.D. (1984) Linoleate hydroperoxides are cleaved heterolytically into aldehydes by a Lewis acid in aprotic solvent. *Lipids* **19**, 294–299.
8. Vick, B.A. & Zimmerman, D.C. (1976) Lipoxygenase and hydroperoxide lyase in germinating watermelon seedlings. *Plant Physiol.* **57**, 780–788.
9. Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T. & Hatanaka, A. (1991) Fatty acid hydroperoxide cleaving enzyme, hydroperoxide lyase, from tea leaves. *Phytochemistry* **30**, 2109–2113.
10. Schreier, P. & Lorenz, G. (1982) Separation, partial purification and characterization of a fatty acid hydroperoxide cleaving enzyme from apple and tomato fruits. *Z. Naturforsch.* **37c**, 165–173.
11. Shibata, Y., Matsui, K., Kajiwara, T. & Hatanaka, A. (1995) Purification and properties of fatty acid hydroperoxide lyase from green bell pepper fruits. *Plant Cell Physiol.* **36**, 147–156.
12. Matoba, T., Hidaka, H., Kitamura, K., Kaizuma, N. & Kito, M. (1985) Contribution of hydroperoxide lyase to *n*-hexanal formation. *J. Agric. Food Chem.* **33**, 856–858.
13. Fauconnier, M.-L., Perez, A.G., Sanz, C. & Marlier, M. (1997) Purification and characterization of tomato leaf (*Lycopersicon esculentum* Mill.) hydroperoxide lyase. *J. Agric. Food Chem.* **45**, 4232–4236.
14. Kim, I.-S. & Grosch, W. (1981) Partial purification and properties of a hydroperoxide lyase from fruits of pear. *J. Agric. Food Chem.* **29**, 1220–1225.
15. Gardner, H.W., Weisleder, D. & Plattner, R.D. (1991) Hydroperoxide lyase and other hydroperoxide-metabolizing activity in tissues of soybean, glycine-max. *Plant Physiol.* **97**, 1059–1072.
16. Phillips, D.R. & Galliard, T. (1978) Flavour biogenesis. Partial purification and properties of a fatty acid hydroperoxide cleaving enzyme from fruits of cucumber. *Phytochemistry* **17**, 355–358.
17. Noordermeer, M.A., Veldink, G.A. & Vliegenthart, J.F.G. (1999) Alfalfa contains substantial 9-hydroperoxide lyase activity and a 3*Z*: 2E-enal isomerase. *FEBS Lett.* **443**, 201–204.
18. Matsui, K., Shibata, Y., Kajiwara, T. & Hatanaka, A. (1989) Separation of 13- and 9-hydroperoxide lyase activities in cotyledons of cucumber seedlings. *Z. Naturforsch.* **44c**, 883–885.
19. Logemann, J., Schell, J. & Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16–20.
20. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
21. Elshof, M.B.W., Janssen, M., Veldink, G.A. & Vliegenthart, J.F.G. (1996) Biocatalytic large-scale production of 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadeca-9,11-dienoic acid from hydrolysed safflower oil by a crude soybean-flour ectract as lipoxygenase source. *Rec. Trav. Chim. Pays-Bas* **115**, 499–504.
22. Suurmeijer, C.N.S.P., Pèrez-Gilabert, M., van der Hijden, H.T.W.M., Veldink, G.A. & Vliegenthart, J.F.G. (1998) Purification, product characterization and kinetic properties of soluble tomato lipoxygenase. *Plant Physiol. Biochem.* **36**, 657–663.
23. Thomas, P.E., Ryan, D. & Levin, W. (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.* **75**, 168–176.
24. Pan, Z., Camara, B., Gardner, H.W. & Backhaus, R.A. (1998) Aspirin inhibition and acetylation of the plant cytochrome P450, allene oxide synthase, resembles that of animal prostaglandin endoperoxide H synthase. *J. Biol. Chem.* **273**, 18139–18145.
25. Shibata, Y., Matsui, K., Kajiwara, T. & Hatanaka, A. (1995) Fatty acid hydroperoxide lyase is a heme protein. *Biochem. Biophys. Res. Commun.* **207**, 438–443.
26. Song, W.C. & Brash, A.R. (1991) Purification of an allene oxide synthase and identification of the enzyme as a cytochrome-P-450. *Science* **253**, 781–784.
27. Jefcoate, C.R.E. & Gaylor, J.L. (1969) Ligand interactions with hemoprotein P-450. II. Influence of phenobarbital and methylcholanthrene induction processes on P-450 spectra. *Biochemistry* **8**, 3463–3472.
28. Matsushita, K., Shinagawa, E., Adachi, O. & Ameyama, M. (1990) Cytochrome *a*1 of *Acetobacter aceti* is a cytochrome *ba* functioning as ubiquinol oxidase. *Proc. Natl Acad. Sci.* **87**, 9863–9867.
29. Porter, T.D. & Coon, M.J. (1991) Cytochrome P450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.* **266**, 13469–13472.
30. Itoh, A. & Vick, B.A. (1999) The purification and characterization of fatty acid hydroperoxide lyase in sunflower. *Biochim. Biophys. Acta* **1436**, 531–540.
31. Lau, S.M.C., Harder, P.A. & Okeefe, D.P. (1993) Low carbon monoxide affinity allene oxide synthase is the predominant Cytochrome-P450 in many plant tissues. *Biochemistry* **32**, 1945–1950.
32. Laudert, D., Pfannschmidt, U., Lottspeich, F., Hollander-Czytko, H. & Weiler, E.W. (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Mol. Biol.* **31**, 323–335.
33. Pan, Z., Durst, F., Werck-Reichhart, D., Gardner, H.W., Camara, B., Cornish, K. & Backhaus, R.A. (1995) The major protein of guayule rubber particles is a cytochrome P450. *J. Biol. Chem.* **270**, 8487–8494.

34. Von Heijne, G. & Nishikawa, K. (1991) Chloroplast transit peptides. The perfect random coil? *FEBS Lett.* **278**, 1–3.
35. Cadenas, E., Boveris, A. & Chance, B. (1980) Low-level chemiluminescence of hydroperoxide-supplemented cytochrome *c*. *Biochem. J.* **187**, 131–140.
36. White, R.E., Sligar, S.G. & Coon, M.J. (1980) Evidence for a homolytic mechanism of peroxide oxygen-oxygen bond cleavage during substrate hydroxylation by cytochrome P-450. *J. Biol. Chem.* **255**, 11108–11111.
37. Blake, R.C. & Coon, M.J. (1981) On the mechanism of action of cytochrome P-450. *J. Biol. Chem.* **256**, 12127–12133.
38. Dix, T.A. & Marnett, L.J. (1985) Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by hematin. *J. Biol. Chem.* **260**, 5351–5357.
39. Song, W.-C., Baertschi, S.W., Boeglin, W.E., Harris, T.M. & Brash, A.R. (1993) Formation of epoxyalcohols by a purified allene oxide synthase. *J. Biol. Chem.* **268**, 6293–6298.
40. Hatanaka, A., Kajiwara, T. & Sekiya, J. (1987) Biosynthetic pathway for C6-aldehydes formation from linolenic acid in green leaves. *Chem. Phys. Lipids* **44**, 341–361.
41. Grechkin, A.N., Ilyasov, A.V. & Hamberg, M. (1997) On the mechanism of biosynthesis of divinyl ether oxylipins by enzyme from garlic bulbs. *Eur. J. Biochem.* **245**, 137–142.
42. Olias, J.M., Rios, J.J., Valle, M., Zamora, R., Sanz, L.C. & Axelrod, B. (1990) Fatty acid hydroperoxide lyase in germinating soybean seedlings. *J. Agric. Food Chem.* **38**, 624–630.
43. Kyte, J. & Doolittle, R.F. (1982) Amino acid scale: hydropathicity. *J. Mol. Biol.* **157**, 105–132.