Plants strike back

cucumber-like odor

old leather

freshly mowed grass

fatty green, fruity

leafy green, fruity

cucumber-like odor

traumatin (wound hormone)

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Fatty Acid Hydroperoxide Lyase: A Plant Cytochrome P450 Enzyme Involved in Wound Healing and Pest Resistance

Minke A. Noordermeer, Gerrit A. Veldink,* and Johannes F. G. Vliegenthart[a]

Plants continuously have to defend themselves against life-threatening events such as drought, mechanical damage, temperature stress, and potential pathogens. Nowadays, more and more similarities between the defense mechanism of plants and that of animals are being discovered. In both cases, the lipoxygenase pathway plays an important role. In plants, products of this pathway are involved in wound healing, pest resistance, and signaling, or they have antimicrobial and antifungal activity. The first step in the lipoxygenase pathway is the reaction of linoleic or linolenic acids with molecular oxygen, catalyzed by the enzyme lipoxygenase. The hydroperoxy fatty acids thus formed are highly reactive and dangerous for the plant and therefore further metabolized by other enzymes such as allene oxide synthase, hydroperoxide lyase, peroxygenase, or divinyl ether synthase. Recently, these enzymes have been characterized as a special class of cytochrome P450 enzymes. Hydroperoxide lyases cleave the lipoxygenase products, resulting in the formation of ω-oxo acids and volatile C_6- and C_9-aldehydes and -alcohols. These compounds are major contributors to the characteristic "fresh green" odor of fruit and vegetables. They are widely used as food flavors, for example, to restore the freshness of food after sterilization processes. The low abundance of these compounds in nature and the high demand make it necessary to synthesize them on a large scale. Lipoxygenase and hydroperoxide lyase are suitable biocatalysts for the production of "natural" food flavors. In contrast to lipoxygenase, which has been extensively studied, little is yet known about hydroperoxide lyase. Hydroperoxide lyases from different organisms have been isolated, and a few genes have been published lately. However, the structure and reaction mechanism of this enzyme are still unclear. The identification of this enzyme as a cytochrome P450 sheds new light on its structure and possible reaction mechanism, whereas recombinant expression brings a biocatalytic application into sight.

KEYWORDS:

cytochromes · enzyme catalysis · heme proteins · hydroperoxide lyase · lyases

1. Introduction

Volatile C_6- and C_9-aldehydes, and the corresponding alcohols, are important contributors to the characteristic flavors of fruits, vegetables, and green leaves. Because of their "fresh green" odor they are widely used as food additives, for example, to restore food freshness after sterilization processes. These short-chain aldehydes and alcohols are produced by higher plants in response to wounding and play an important role in wound healing and pest resistance.

The C_6- and C_9-aldehydes and alcohols are derived from linoleic and α-linolenic acids. Four enzymes are involved in the biosynthetic pathway leading to their formation: lipoxygenase, hydroperoxide lyase, (3*Z*,2*E*)-enal isomerase, and alcohol dehydrogenase. In contrast to lipoxygenase, which has been extensively studied, little is yet known about the enzyme hydroperoxide lyase. Hydroperoxide lyases from different organisms have been isolated, and a few genes have been published lately. However, the structure and reaction mechanism of this enzyme are still unclear. Recently, it has been discovered that hydroperoxide lyase belongs to a special class of cytochrome P450 enzymes.[1]

In this review we focus on the lipoxygenase pathway and discuss the recent developments in the characterization of hydroperoxide lyase. The identification of this enzyme as a cytochrome P450 sheds new light on its structure and possible reaction mechanism.

2. Wound responses in plants

Plants are continuously fighting against life-threatening events such as drought, mechanical damage, temperature stress, and potential pathogens. Like animals, plants are able to defend themselves by constitutive defense mechanisms and induced responses. Constitutive defenses can be physical barriers, such

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as the cuticle, toxic compounds, or enzymes capable of digesting essential components of pathogens. Induced responses are based on the recognition of a pathogen or damage, leading to reaction by the plant. The 18-amino acid peptide signal molecule systemin, which has been isolated from tomato leaves, is considered to be the main primary systemic response upon wounding.[2, 3] Systemin is readily transported from wound sites throughout the whole plant and propagates the signal by inducing the transcription of defense genes (Figure 1). The products of

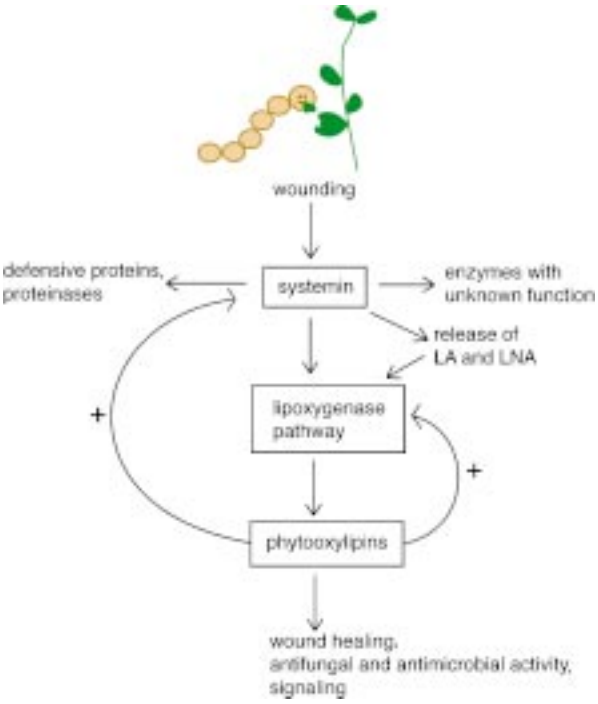


***Figure 1.** Induced wound responses in plants. The peptide signal molecule systemin is considered to be the main primary systemic response, and it propagates the signal by inducing the transcription of defense genes. Wounding and systemin induce the release of α-linolenic (LNA) and linoleic acids (LA),[6] which are then converted by enzymes of the lipoxygenase pathway into phytooxylipins.*

the upregulated genes can be classified into four categories: defensive proteins, signal pathway components, proteinases, and enzymes with unknown function. Defensive proteins are, for example, serine proteinase inhibitor I and II and polyphenol oxidase, which protect the plant against digestive proteases of herbivorous insects.[4] Proteolysis of the ingested food in the gut of herbivores is also affected, retarding their growth and development, and making the plant less attractive for animals.[5]

The most important signal pathway induced by wounding is probably the octadecanoid or lipoxygenase pathway. Wounding and systemin induce the release of α-linolenic acid (LNA)[*] and linoleic acid (LA),[6] which are then converted by enzymes of the lipoxygenase pathway

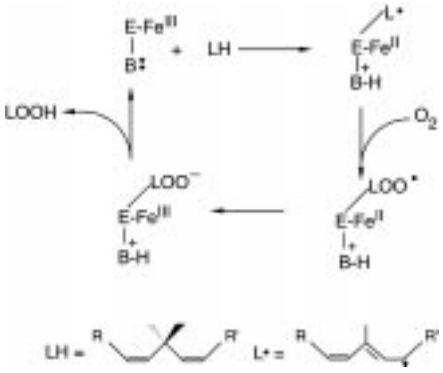
[*] *For abbreviations see ref. [130].*

into phytooxylipins. Important phytooxylipins formed in this pathway are hydroxy, epoxy and divinyl ether fatty acids, volatile aldehydes and alcohols, and the wound hormones jasmonic acid, traumatic acid, traumatin, and phytodienoic acid. The wound hormone jasmonic acid and its methyl ester can induce a wide range of secondary actions, such as further upregulation of the lipoxygenase pathway,[7–9] and the production of more defense proteins.[10–12] Furthermore, they are involved in developmental processes such as seedling growth, pollen formation, and flower development.[13, 14] Traumatin and traumatic acid also act as wound hormones and growth promoters.[15] The epoxy, hydroxy, and divinyl ether fatty acids are known to have antifungal activity.[16, 17] The volatile aldehydes and alcohols, which have a characteristic "green, fruity" smell, stimulate wound healing, have antifungal and antimicrobial activity, and induce the expression of the prosystemin gene, thus acting as a positive feedback and intercellular signal.[9, 18–24] Besides, they might be a signal for other plants in the surroundings to induce a defense response as well.[25] They can act as attractants or repellents of insects and might play a role in the production of floral scent.[26, 27] The lipoxygenase pathway is thus a major component of the plant defense system. The plant lipoxygenase pathway is highly analogous to the lipoxygenase pathway in animals. Both pathways are inhibited by salicylic acid and acetylsalicylic acid. In animals, salicylic acids inhibit the formation of prostaglandins, whereas in plants the formation of jasmonic acid is inhibited.[9, 28–30]

3. Lipoxygenase pathway

3.1. Lipoxygenase

The first step in the lipoxygenase pathway is the dioxygenation of LNA or LA by lipoxygenase (LOX, EC 1.13.11.12). Lipoxygenases are non-heme iron-containing dioxygenases catalyzing the addition of molecular oxygen to polyunsaturated fatty acids having a (1*Z*,4*Z*)-pentadiene moiety.[31] Lipoxygenase in its native form contains Fe^{II} and must first be oxidized to the Fe^{III} form by its own hydroperoxide product.[32] Upon binding the substrate, a hydrogen atom is abstracted and a fatty acid radical is formed (Scheme 1). Concomitantly, Fe^{III} is reduced to Fe^{II}. The fatty acid radical then binds dioxygen and is transformed into a peroxyl radical. Fe^{II} is oxidized to Fe^{III} and a peroxyl anion is formed. The anion subsequently reacts with a proton and the resulting hydroperoxy fatty acid is released from the Fe^{III} enzyme, which is then ready for another reaction cycle. Two different regiospecificities of plant lipoxygenases with LA and LNA as substrates are known: Some lipoxygenases form (13*S*)-hydroperoxy-LA and -LNA, others the 9*S* isomers or a mixture of both.



Scheme 1. *Reaction mechanism of lipoxygenase. Lipoxygenases are non-heme iron-containing dioxygenases catalyzing the addition of molecular oxygen to polyunsaturated fatty acids having a (1Z,4Z)-pentadiene moiety.[31] E-Fe^{II} = active enzyme; E-Fe^{II} = reduced enzyme; B = base.*

3.2. Secondary enzymes

Hydroperoxy fatty acids formed by LOX are reactive molecules that could do harm to a biological system and are thus further metabolized by other enzymes such as allene oxide synthase (AOS), peroxygenase (POX), divinyl ether synthase (DES), or fatty acid hydroperoxide lyase (HPL). Allene oxide synthase (EC 4.2.1.92) dehydrates the hydroperoxy fatty acids to unstable allene oxides, which are cyclized by allene oxide cyclase, or spontaneously hydrolyze into α- and γ-ketols (Scheme 2). 12-Oxo-(10,15*Z*)-phytodienoic acid, the cyclized product derived from α-linolenic acid, can be converted into jasmonic acid by 12-oxophytodienoic acid reductase followed by three β-oxidation steps.[33] Several allene oxide synthases have been cloned and the enzyme has been characterized as a cytochrome P450 enzyme (CYP74A).[34–38] Transcripts of AOS accumulate upon wounding, insect attack, or treatment with methyl jasmonate or ethylene.[9, 27, 37, 39] Overexpression of AOS increases the endogenous jasmonic acid level in potato plants, but not in tobacco.[40, 41] However, the jasmonic acid level in the transgenic tobacco plants, as well as in the potato plants, is drastically increased by wounding.

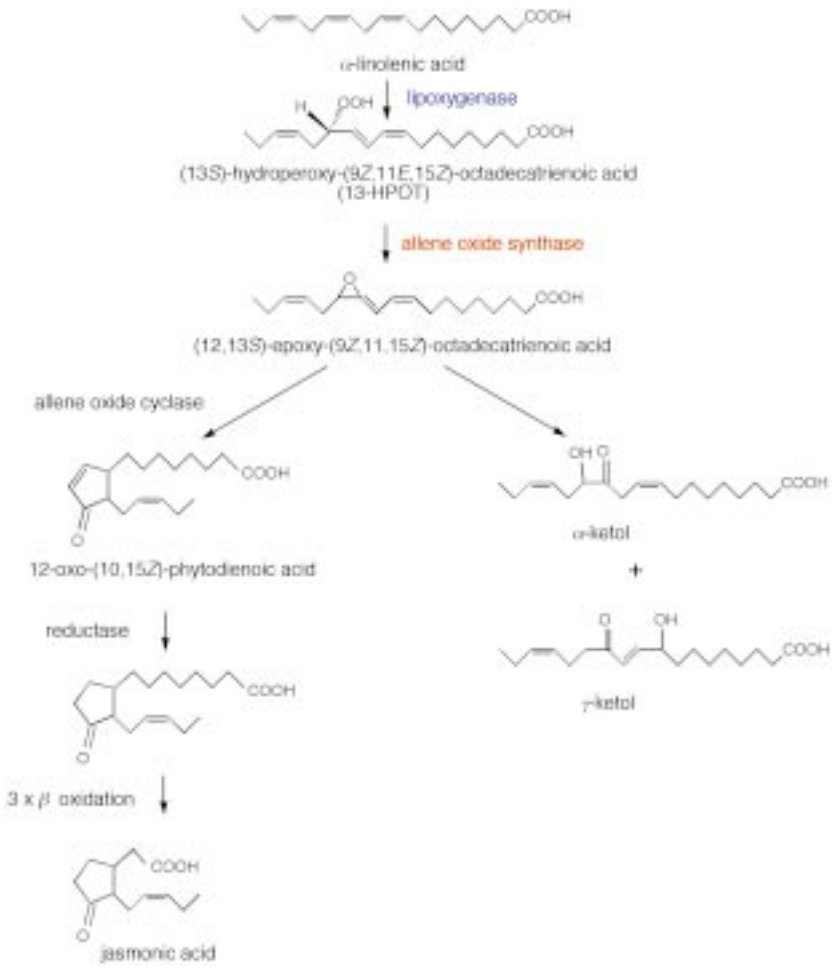
The action of peroxygenase on hydroperoxylinoleic and α-linolenic acids results in the formation of hydroxy and epoxyhydroxy fatty acids.[42] In the presence of hydroperoxy fatty acids peroxygenase can also act on LA and LNA, resulting in the formation of epoxy fatty acids. The epoxy groups can be further converted into two alcohol groups by the enzyme epoxide hydrolase, yielding di- and trihydroxy fatty acids.

Divinyl ether fatty acids are formed from the LOX products by an enzyme called divinyl ether synthase (DES).[43] It acts in a regio- and stereospecific way and is mainly found in the microsomal fraction.[44, 45] Recently, a DES from tomato has been cloned and characterized as a cytochrome P450 (CYP74D).[46] This DES converts 9-hydroperoxy fatty acids to colneleic acid and colnelenic acid and is mainly present in the plant roots.

Hydroperoxide lyases are enzymes that cleave the C—C bond adjacent to the hydroperoxy group in the LOX products, resulting in the formation of ω-oxo acids and volatile aldehydes (Scheme 3). The 3*Z* aldehydes can be isomerized to their 2*E* isomers and reduced by alcohol dehydrogenase to the corresponding alcohols.

4. Hydroperoxide lyase

In 1973 an "aldehyde lyase" was described, which was proposed to be responsible for the cleavage of fatty acid hydroperoxides in banana fruits.[47] Vick et al. demonstrated such lyase activity in



Scheme 2. *Lipoxygenase – allene oxide synthase pathway. Allene oxide synthase dehydrates the hydroperoxy fatty acids to unstable allene oxides, which are cyclized by allene oxide cyclase, or spontaneously hydrolyze into α- and γ-ketols. 12-Oxo-(10,15Z)-phytodienoic acid can be converted into jasmonic acid by 12-oxophytodienoic acid reductase followed by three β-oxidation steps.*[33]

watermelon seedlings and named it hydroperoxide lyase in 1976.[48] Since then, hydroperoxide lyases from a number of organisms have been purified and characterized. Recently, HPL from bell pepper, *Arabidopsis thaliana*, alfalfa, cucumber, tomato, and guava fruit have been cloned and expressed in *Escherichia coli*.[1, 27, 39, 49–51] Based on sequence homology and the EPR spectrum, HPL was identified as a cytochrome P450 enzyme (CYP74B).[1, 52]

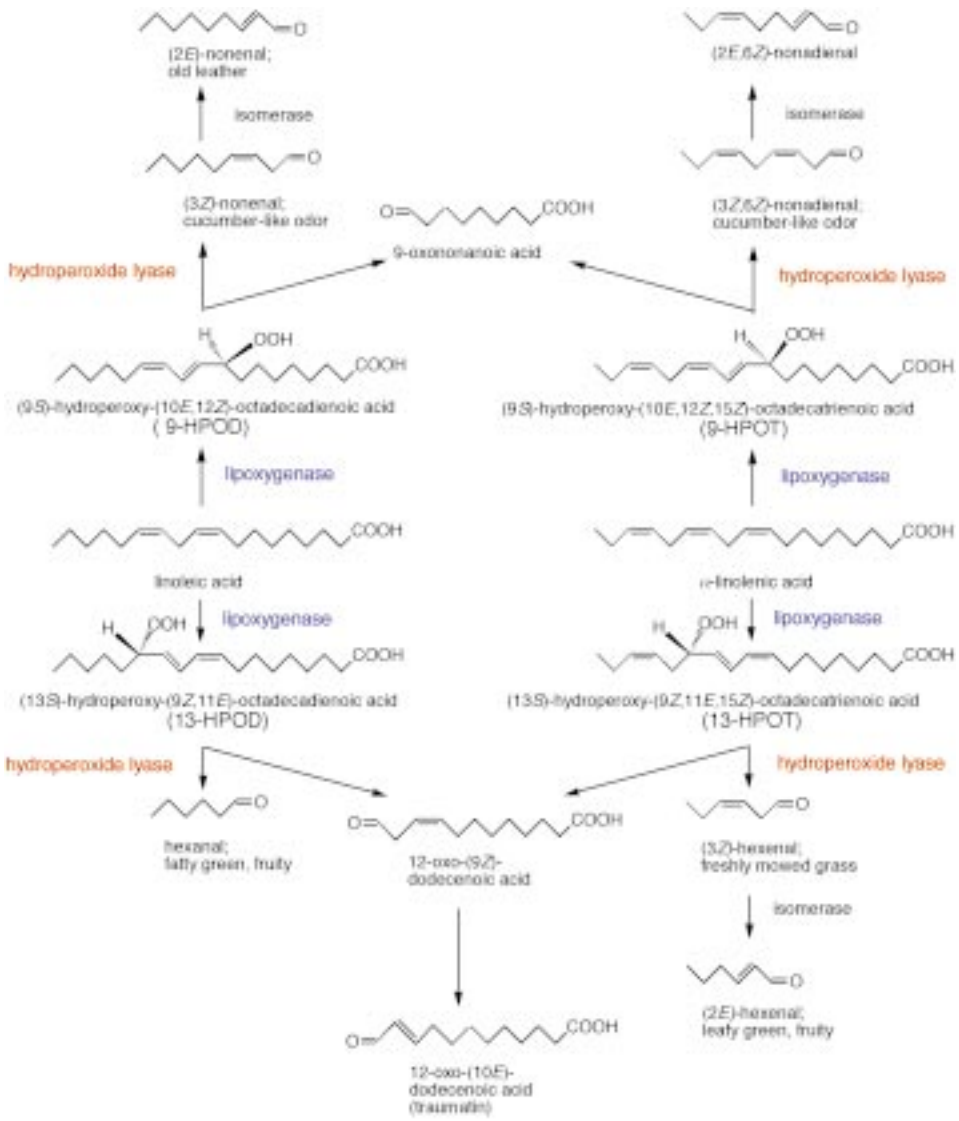
4.1. Substrate and product specificities of different hydroperoxide lyases

Plant HPL can be classified into two groups according to their substrate specificity. One group cleaves 13-hydroperoxylinoleic acid into 12-oxo-(9*Z*)-dodecenoic acid and hexanal, and 13-hydroperoxy-α-linolenic acid into 12-oxo-(9*Z*)-dodecenoic acid and (3*Z*)-hexenal. The other group cleaves the 9-hydroperoxy isomers of linoleic and α-linolenic acids into 9-oxononanoic acid and (3*Z*)-nonenal, or 9-oxononanoic acid and (3*Z*,6*Z*)-nonadienal, respectively. In watermelon seedlings, tea leaves, tomato fruits and leaves, apples, green bell peppers, and soybeans, only 13-HPL activity is present.[48, 53–57] Pears contain only 9-HPL activity.[58] Soybean and pea seeds/seedlings, cucumber fruits and seedlings, and alfalfa seedlings contain both HPL activities.[59–62] Matsui et al. succeeded in separating the 13- and 9-HPL activities from cucumber seedlings, indicating that different enzymes are specific for the different substrates.[63] However, recently a cucumber HPL was cloned that uses both substrates.[50] This HPL is remarkable because it shows a higher sequence similarity to AOS than to 13-HPL, and it has been grouped into its own subfamily, CYP74C. To obtain more knowledge about the differences betweeen 13- and 9-HPL, a specific 9-HPL should be isolated and cloned. Pears could be a good source as they only contain 9-HPL.

Conversion of the carboxyl group of the fatty acid hydroperoxide to a methyl ester or alcohol greatly reduces the activity of HPL from tea chloroplasts and soybeans,[56, 64, 65] indicating that the carboxyl group of the substrates is important for HPL activity. In contrast, hydroperoxy fatty acid ethanolamides are even better substrates for HPL than the corresponding fatty acids.[66] This suggests that the polarity of the headgroup of the substrates might be important for HPL activity and that the size of the headgroup is of minor importance.

The influence of the position and number of double bonds in the substrate on plant HPL activity was studied as well. HPL from tea leaves, tomatoes, and green bell pepper fruits show nearly ten times higher initial activity toward 13-hydroperoxy-α-linolenic acid than toward 13-hydroperoxylinoleic acid,[53, 55, 67, 68] whereas this introduction of a 15*Z* double bond in linoleic acid increases the activity of tea chloroplast HPL by 2% only.[64] Introduction of a 6*Z* double bond (13-hydroperoxy-(6*Z*,9*Z*,11*E*)-octadecatrienoic acid = 13-hydroperoxy-γ-linolenic acid) strongly decreases the activity of HPL.[53, 64, 68] Systematic investigation of the influence of the chain length by using an entire series of ω6-hydroperoxy-C_{14} – C_{24} dienoic and trienoic acids revealed that C_{22} fatty acids, and not the natural substrates (C_{18}), show the highest reactivities toward HPL.[69] 15-Hydroperoxyarachidonic acid (C20:4), however, is not a substrate for tea chloroplast HPL.[70] From a racemic mixture of (13*R*)-hydroperoxy-LA and (13*S*)-hydroperoxy-LA, only the *S* isomer is converted by HPL.[56] In conclusion, the structural requirements that substrates of plant HPL must meet are the presence of a *cis*,*trans*-conjugated diene system with the *trans* double bond adjacent to the (*S*)-hydroperoxide-bearing carbon atom.

Mushrooms contain a HPL which is specific for 10-hydroperoxy fatty acids.[71–74] The products of this HPL are 1-octen-3-ol and 10-oxo-(8*E*)-decenoic acid. Algae (*Chlorella pyrenoidosa* and *Oscillatoria*) cleave 13-hydroperoxy fatty acids into 13-oxo-(9*Z*,11*E*)-tridecadienoic acid and pentane or (2*Z*)-pentene (*Chlor-*



Scheme 3. *Lipoxygenase – hydroperoxide lyase pathway. Fatty acid hydroperoxide lyases are enzymes that cleave the C–C bond adjacent to the hydroperoxy group in the LOX products, resulting in the formation of ω-oxo acids and volatile aldehydes. The 3Z aldehydes can be isomerized to their 2*E *isomers and reduced by alcohol dehydrogenase to the corresponding alcohols.*

ella),[75] or pentanol (*Oscillatoria*).[76] However, this activity has recently been ascribed to lipoxygenase.[77] In mammals, only one HPL has been found (in rabbit leukocytes).[78] This HPL is proposed to cleave 15-hydroperoxyarachidonic acid (15-HPETE) into pentanol and 15-oxo-(5*Z*,8*Z*,11*Z*,13*E*)-pentadecatetraenoic acid.

4.2. Localization and regulation of hydroperoxide lyase

In watermelon seedlings the highest HPL activity is present in the hypocotyl – root junction;[48] in cucumber seedlings, roots are the richest source of HPL activity.[79] Tissue print immunoblot analysis of bell peppers showed that most HPL is located in the outer parenchymal cells of the pericarp.[55] This is in accordance with its primary role in protection against attacks from outside. Furthermore, the localization of HPL in plants seems to be linked to rapid cell growth and development.[80–82] Analogously, the activity of HPL in *Marchantia polymorpha* cells is highest when the cells are growing logarithmically and rapidly decreases when the cells enter the stationary phase.[83]

Hydroperoxide lyases are thought to be membrane proteins, although the genes of hydroperoxide lyases do not contain clearly identifiable transmembrane segments.[49] Detergents are needed for their solubilization. In some plant sources HPLs are localized in the chloroplasts[84–88] or in the microsomes,[53, 89, 90] whereas in others no specific localization in a particular organelle is observed.[49, 55, 91] The HPL gene from *Arabidopsis thaliana* contains a chloroplast-directing transit sequence,[39] but the genes from tomato and alfalfa do not.[27, 49, 92] The intracellular localization thus remains unclear and should be further studied by immunocytochemical methods.

HPL transcript levels increase in response to insect attack and mechanical wounding, but, unlike AOS, the enzyme is not induced by methyl jasmonate treatment.[27, 39] The rapid release of HPL products after wounding indicates that HPL is already present in the plant and that de novo synthesis is not necessary. The enzyme might be activated upon contact with its substrate, that becomes accessible by disruption of membranes during wounding. In this case, the enzyme should be present in the membrane or be transported to the wounding site. Another possibility is that the enzyme exists as a proenzyme, which is activated by cleaving off an N-terminal sequence. This was suggested because of the increased activity of alfalfa HPL upon removal of the first 22 N-terminal amino acids.[49]

4.3. Structure of hydroperoxide lyase

The HPL genes described so far code for proteins of 55 kDa.[1, 27, 39, 49, 51] Most isolated plant HPLs consist of subunits of 55 – 60 kDa,[53, 55, 76, 93, 94] but based on native polyacrylamide gel electrophoresis results it was suggested that the enzymes exist as trimers or tetramers of about 200 – 250 kDa.[48, 51, 54, 55, 57, 94]

5. Hydroperoxide lyase as a cytochrome P450 enzyme: implications for its structure and reaction mechanism

Cytochrome P450 enzymes are heme-containing monooxygenases that are present in all living organisms. This superfamily of enzymes originates from an ancestral gene which may have existed already 3.5×10^9 years ago. The name cytochrome P450 originates from the characteristic absorption maximum of the enzymes at 450 nm (Soret maximum) after reduction with sodium dithionite and treatment with carbon monoxide (the P stands for pigment).[95]

5.1. Structure

Six crystal structures of cytochrome P450 enzymes have been determined so far, five soluble prokaryotic cytochrome P450 enzymes and one eukaryotic microsomal P450 enzyme.[96–102] Analysis of the crystal structures revealed that these P450 enzymes have a triangular shape and are rich in secondary structure elements. They consist of an α-helix domain (40 – 70 %) and a β-sheet-rich domain (10 – 22 %) (Figure 2). The active-site heme is bound by a highly conserved cysteine residue and located inside the protein, sandwiched between two parallel helices. It is accessible through a hydrophobic channel. The B′, F, and G helices are supposed to be involved in substrate binding and specificity. The long I helix, and in particular its highly conserved threonine residue, is probably involved in the binding of oxygen and in proton delivery.[96, 103–105] The structure of the B′ helix and of the F/G loop are rather variable, which might account for the different substrate specificities.[101–103]

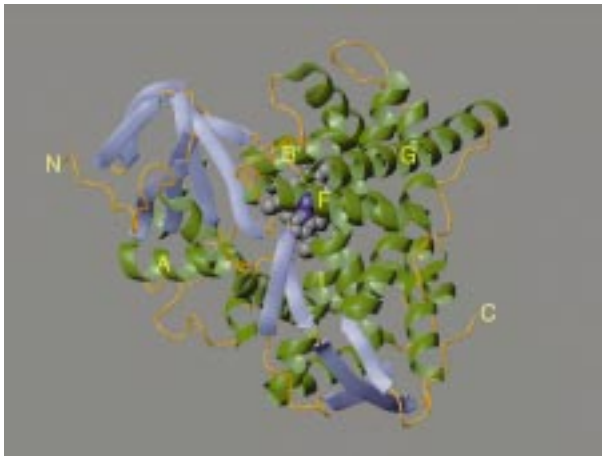


Figure 2. *A ribbon representation of the three-dimensional structure of P450BM-3 from* Bacillus megaterium. *Helices are labeled according to the P450cam nomenclature.[96] β-Sheets are indicated by arrows. (Reprinted from Julian A. Peterson's homepage with permission [http://p450terp.swmed.edu/Bills_folder/billhome.htm].)*

The question arises if these structures are useful as a general model for all cytochrome P450 enzymes and for HPL. The overall three-dimensional structures of the six crystallized cytochrome P450 enzymes are similar, despite their low sequence identity, indicating that all cytochrome P450 enzymes might have a comparable structure.[102, 104] The CD spectrum of alfalfa HPL shows that this enzyme contains a high percentage of α helices, similar to the crystallized P450 enzymes.[52] The molecular masses of the known HPLs are similar to those of other cytochrome P450 enzymes. The heme group in the active site was identified as heme *b*, another characteristic of cytochrome P450 enzymes.[106] HPLs also contain the highly conserved cysteine residue, which is supposed to bind the heme group.[1] Point mutants in which the conserved cysteine has been replaced by an alanine or serine residue are inactive and do not contain heme.[52] HPLs, however, have a low affinity for CO and most of them do not show an absorption maximum at 450 nm after reduction and treatment with CO.[92, 106] This behavior is similar to that of allene oxide synthase, which has a low affinity for CO too.[107] These enzymes are, therefore, unique within the cytochrome P450 family. HPLs, as well as allene oxide synthases, do not need molecular oxygen for their activity. These enzymes show little homology to other cytochrome P450 enzymes in the I helix region (oxygen-binding pocket) and lack the conserved threonine residue. The absence of the oxygen-binding pocket might explain their low affinity for CO. Crystallization and structure elucidation of HPLs is necessary to get more information about its structure and similarity to other cytochrome P450 enzymes.

5.2. Active site

The prosthetic group of cytochrome P450 enzymes is a protoporphyrin IX moiety (heme *b*). Therefore, it has been possible to use a variety of physical methods to study the structure of the active site.[108] The heme iron is located in the center of the protoporphyrin ring, bound by the four pyrrole nitrogen atoms. The heme-binding domain of most cytochrome P450 enzymes contains the characteristic sequence FxxGxxxCxxG, wherein the thiolate anion of the conserved cysteine serves as the fifth (axial) ligand to the heme iron.[96] The sixth (axial) ligand in cytochrome P450 enzymes is an OH group from a water molecule or hydroxide ion.[97, 109]

The Fe^{III} ion exists in a high-spin ($S = 5/2$) and a low-spin form ($S = 1/2$), depending on the configuration of the five 3d electrons. These forms are usually in equilibrium and possess different spectral properties.[110] Transitions between the spin states of cytochrome P450 enzymes are accompanied by slight energy alterations, and therefore the spin state is often affected by temperature, pressure, pH, and salt concentration.[111, 112] Changes in the spin state of hemoproteins are usually caused by the change in ligand state of the heme iron: The hexacoordinated state is low spin and the pentacoordinated state is high spin. Most of the cytochrome P450 enzymes are in the low-spin state. Interaction with a hydrophobic substrate or a ligand can lead to a shift from low-spin to high-spin state (type I interaction), but stabilization of the low-spin state has been observed too (type II interaction).[110, 113, 114] In a type I interaction, displacement of a water molecule by the substrate might lead to the movement of the iron ion out of the plane of the porphyrin ring, which makes the high-spin configuration favorable (Fig-

ure 3).[97, 115] It has also been shown that a single mutation of an amino acid in the active site can alter the spin equilibrium, depending on the hydrophobicity and size of the residue.[52]

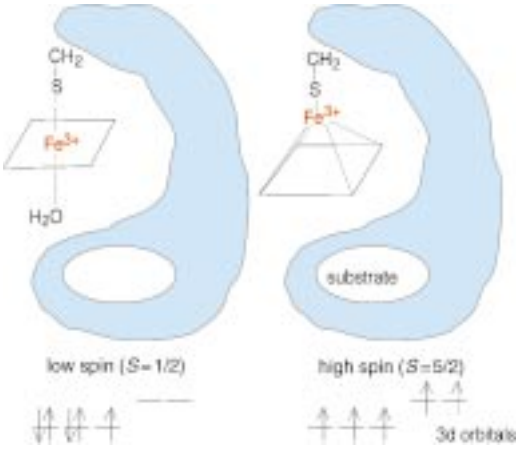


Figure 3. *Spin state shift in cytochrome P450 due to substrate binding (type I interaction). The Fe^{III} ion exists in high-spin ($S = 5/2$) and low-spin ($S = 1/2$) forms, depending on the configuration of the five 3d electrons. Changes in the spin state of hemoproteins are usually caused by the change in ligand state of the heme iron: The hexacoordinated state is low spin and the pentacoordinated state is high spin. Most of the cytochrome P450 enzymes are in the low-spin state. Interaction with a hydrophobic substrate or a ligand can lead to a shift from low-spin to high-spin state.*

In the case of HPL, the spin state is temperature-dependent.[52] Detergents induce a change in the spin equilibrium from low to high spin, similar to a type I interaction.[52] The influence of detergents on the spin state might be typical for enzymes of the CYP74 family, because the cytochrome P450 obtusifoliol 14α-demethylase (CYP51), which can be solubilized by Triton X-100, is in the low-spin state.[116] Because these detergents are too large to fit into the distal ligand pocket, they cannot directly interact with the iron ion. However, the EPR spectra of HPL in the presence or absence of Triton X-100 indicate that Triton X-100 does change the conformation of the active site (Figure 4).[52] Detergents probably induce the formation of high-spin complexes by indirectly perturbing the heme structure and expelling the original axial heme ligand (H_2O). In addition, they might change the bond length between the heme iron and the sulfur

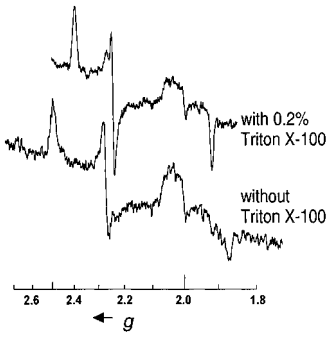


Figure 4. *EPR spectra of HPL in the presence or absence of 0.2% Triton X-100.[52] Triton X-100 changes the conformation of the active site.*

atom. A similar effect was observed when nitric oxide synthase was incubated with a number of large ligands.[114, 117] Because the CD spectra of HPL in the presence or absence of Triton X-100 are equal, the structure perturbation is apparently subtle.[52]

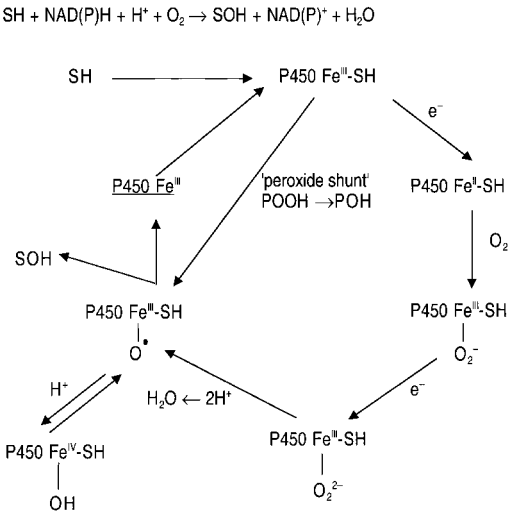
The functional importance of the spin state is still not understood. It has been suggested that the high-spin form is more rapidly reduced,[118] but, on the other hand, the reduction is mostly not the rate-determining step. The spin state of endothelial nitric oxide synthase, which appeared to be dependent on the source of the enzyme, shows no correlation with the enzyme activity.[114] In contrast, the removal of Triton X-100 and subsequent change in the spin equilibrium cause a twofold decrease in the enzymatic activity of HPL.[52] Similarly, the activities of HPL and AOS in crude extracts increase about twofold by adding detergents.[34, 53, 55, 59] It can thus be concluded that the spin state of HPL is important for the enzymatic activity.

It has been suggested that HPL activity is partly regulated by the organization of the membrane around the enzyme.[55] Detergents might mimic a membrane environment, causing a spin change from low to high spin, and thus converting the enzyme into a more active conformation. The ability of triglycerides to induce a change in the spin equilibrium from low to high spin confirms the essence of a membrane-like environment.[52] Because HPL is thought to be a membrane protein (see Section 4.2), it is likely that the more active, high-spin state is associated with the in vivo conformation of the enzyme.

5.3. Reaction mechanism

Cytochrome P450 enzymes catalyze many different reactions such as hydroxylations, epoxidations, *N*-demethylations, *O*-dealkylations, deaminations, sulfoxidations, desulfurations, and oxidative dehalogenations. The majority of the cytochrome P450 enzymes, however, catalyze NAD(P)H- and O_2-dependent hydroxylations. Instead of using NAD(P)H directly, most of these enzymes interact with a flavoprotein (cytochrome P450 reductase) that transfers electrons from the nicotinamide cofactor to the P450. However, HPL does not interact with cytochrome P450 reductase, nor does it use NAD(P)H or O_2. Cytochrome P450 enzymes can also utilize peroxides such as cumene hydroperoxide and *tert*-butylhydroperoxide as an oxygen donor. This bypass is usually referred to as the peroxide shunt.[119, 120] The general reaction mechanism of cytochrome P450 enzymes is shown in Scheme 4.

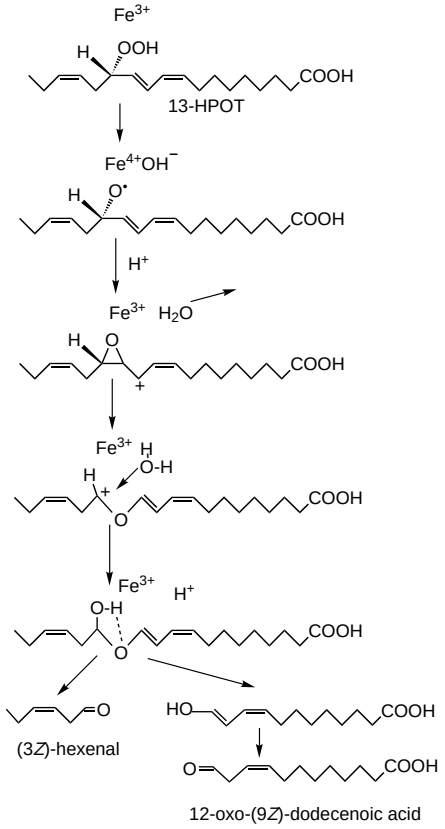
For a long time, a heterolytic reaction mechanism seemed to be most likely for plant HPL action, because the reaction products of the heterolytic reaction resemble the ones formed by hydroperoxide lyase, whereas the complex volatile product profile obtained by homolysis does not.[121] In a study with ^{18}O-labeled substrates, it was observed that the ^{18}O of the hydroperoxide is not transferred into hexanal but instead into 12-oxo-(9*Z*)-dodecenol.[122] Based on these results, Hatanaka et al. proposed a heterolytic mechanism.[68] However, the characterization of HPL as a cytochrome P450 enzyme makes a homolytic mechanism more likely. The HPL reaction resembles the peroxide shunt, and this reaction is supposed to occur through a



Scheme 4. *General reaction mechanism of cytochrome P450 enzymes.[129] The majority of the cytochrome P450 enzymes catalyze NAD(P)H- and O_2-dependent hydroxylations. Cytochrome P450 enzymes can also utilize peroxides such as cumene hydroperoxide and* tert*-butylhydroperoxide as an oxygen donor. This bypass is usually referred to as the peroxide shunt.[119, 120] POOH = organic peroxide; SH = substrate.*

homolytic scission of the hydroperoxy group.[119, 120] In support of a radical process, inhibition of the enzymatic activity by radical scavengers was observed. Furthermore, dithiothreitol and 13-hydroxylinoleic acid protect against inactivation of the enzyme by 13-hydroperoxylinoleic acid, which suggests that inactivation is caused by destruction of an essential SH group near the reaction center of HPL.[123] The observed protection by organic antioxidants and radical scavengers (α-tocopherol, nordihydroguaiaretic acid, butylated hydroxyanisole, and butylated hydroxytoluene) suggests that this SH group is destroyed by radical formation from fatty acid hydroperoxides near the reaction center of HPL.

In Scheme 5 we propose an integrated reaction mechanism for HPL, 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, including AOS.[120, 124–127] In contrast to the formation of an epoxyallylic cation by AOS, we propose that the next step in the HPL reaction mechanism is proton donation to the hydroxide ion in the ferryl-hydroxo complex and abstraction of one electron from 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 HPL[70] and in etheroleic acid biosynthesis.[45] Subsequently, addition of water to the carbocation occurs and a spontaneous rearrangement results in the formation of a C_6-aldehyde and a C_{12}-enol. An ω-oxo acid is formed by keto – enol tautomerization of the C_{12}-enol. In contrast to the reaction mechanisms of O_2-using cytochrome P450 enzymes, the mechanism of HPL is cyclic, that is, no cofactor is used.



Scheme 5. *Proposed reaction mechanism of HPL based on the known chemistry of cytochrome P450 enzymes.[49] The homolytic cleavage of the oxygen – oxygen bond resulting in an alkoxy radical and a ferryl-hydroxo complex is considered to be the first step.*

6. Summary and outlook

HPL is a very interesting enzyme for both fundamental research and biotechnological application as a biocatalyst for the production of natural food flavors. Since its discovery, it has been isolated from a number of organisms, and its substrate and product specificities have been extensively studied. Structural characterization of HPL and mechanistic studies could not be performed because of its instability and difficult purification. Furthermore, it is difficult to derive its structure and mechanism directly from the known structures and general reaction mechanism of cytochrome P450 enzymes because HPL is rather different from other cytochrome P450 enzymes. The recent cloning and expression of HPL in *E. coli* makes it possible to obtain larger quantities and stable enzyme preparations. This opens new ways to the characterization of this unique enzyme with methods that require high concentrations of protein.

Crystallization of HPL is a future challenge and will contribute to our knowledge of its structure and special status within the cytochrome P450 family. The recently developed transverse relaxation optimized spectroscopy (TROSY) gives the opportunity to characterize large proteins by NMR spectroscopy.[128] This is an excellent method to study the influence of detergents on the conformation of HPL in solution, as well as the position of the substrate in the active site. EXAFS and AXAFS spectroscopy may

provide more information on the conformation of the active site and on the perturbation of the heme group in the high-spin complex. Together with EPR studies on HPL in the presence of substrate, this will lead to a better understanding of the reaction mechanism.

The question if HPL is a membrane and chloroplastic protein can only be answered by studying its intracellular localization with immunocytochemical methods. However, there are no highly specific antibodies against HPL available yet, which might be due to a low immunogenity of the enzyme.

Overexpression or repression of HPL in plants will provide more information about its function in the defense mechanism and the flavor of plants. In addition, it may be possible to improve the flavor and resistance properties of crop plants with these genetic modifications.

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[130] Abbreviations: AOS = allene oxide synthase; AXAFS = atomic X-ray absorption fine structure; DES = divinyl ether synthase; EPR = electron paramagnetic resonance; EXAFS = extended X-ray absorption fine structure; HPL = hydroperoxide lyase; 15-HPETE = 15-hydroperoxyarachidonic acid; LA = linoleic acid; LNA = linolenic acid; LOX = lipoxygenase; POX = peroxygenase; TROSY = transverse relaxation-optimized spectroscopy.

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